Analysis of Xenopus cyclin A

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by

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Abstract

Maturation Promoting factor is the enzyme that catalyses the entry of cells into mitosis or meiosis. It consists of at least two subunits: a protein kinase encoded by a member of the cyclin dependent kinase (cdk) family and a regulatory cyclin subunit. This thesis describes studies on the functional domains of *Xenopus* cyclin A1. I have made a series of deletion and point mutants of *Xenopus* cyclin A1 and developed assays to test the properties of these altered proteins.

I have shown that deletion of as few as 14 residues from the C-terminus of cyclin A, or mutation of highly conserved residues within its 'cyclin box', gives proteins that are severely impaired in their ability to bind to p34\(^{cdk2}\) or p33\(^{cdk2}\).

An important property of mitotic cyclins is their rapid destruction at the end of M phase, I have demonstrated that those mutant cyclin A1 subunits that cannot bind efficiently to p34\(^{cdc2}\) or p33\(^{cdk2}\) cannot be destroyed at the end of M-phase. Some mutants of cyclin A1 that contained deletions within the N-terminus of the protein also resisted normal destruction. After showing that the deletion of two conserved motifs from these cyclin A mutants was not responsible for their stability, I concluded that it was due to conformational changes within their N-termini. Mutation of the sequence motif in cyclin A1 that shows homology to the 'destruction box' of cyclin B, also gives a protein that is unable to be proteolysed.

Wild-type cyclin A1 is normally a nuclear protein in interphase. I have shown that those cyclin A1 mutants that cannot bind to p34\(^{cdc2}\) are excluded from the nucleus in tissue culture cells, and that the N-terminus of cyclin A is not required for the correct subcellular localisation of this protein.
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<td>Glutathione-S-transferase</td>
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<td>N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]</td>
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<td>Horseradish peroxidase</td>
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<td>ICRF</td>
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<td>NLS</td>
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<td>N-tris[hydroxymethyl]-methyl-2-aminoethane-sulfonic acid</td>
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Chapter 1

Introduction

The cell cycle

Cell growth and division, the process by which two daughter cells are produced from a single mother cell, is fundamental to life. While cell growth is a continuous process, both the replication of its chromosomes and cellular division are discontinuous, and the correct control of these processes is crucial to ensure that genetic material is not lost or damaged. For unicellular organisms the worst consequence of genetic damage is cell death. For multicellular organisms, on the other hand, genetic damage leading to the death of odd cells does not generally cause adverse consequences for that organism. On the other hand, genetic damage that gives rise to a growth advantage for a cell may be the first step in the pathway leading to cancer. Thus it is vital for both unicellular and multicellular organisms alike to regulate their cell cycles.

Simple visual study of cells distinguishes two ‘states’ during cell division cycles. In Mitosis (M phase), the chromosomes condense, the nuclear envelope breaks down, mitotic spindles form and separate the sister chromatids, and finally the cell divides. Interphase is the time between cell divisions, and during this time the DNA remains decondensed and very little can be seen to occur by eye. Interphase can, however, be divided into three phases (Howard and Pelc, 1953). After dividing, a cell passes into the G1 phase (Gap 1), where it grows and synthesises cellular components, including proteins and RNA, but not DNA. This is followed by S phase (DNA Synthesis) when the cell replicates its chromosomes, and then comes a second ‘gap’ phase, G2, during which the cell prepares for mitosis. Cells may withdraw from the cell cycle during G1 into a ‘resting’ state known as G0. This may occur for a number of reasons, for example, it could be due to lack of growth factors or because the cell is terminally differentiated. G0 cells re-enter the cell cycle during G1.

The length of a cell cycle can vary from a few minutes for the cells of some rapidly dividing early embryos, to about 24 hours for rapidly dividing somatic cells. The difference in length of the cell cycle is partly because S phase in embryonic cells is much shorter than that in somatic cells, and partly due to the difference in the length of time that cells take to pass through G1 and G2; in the
early embryonic cell cycles of many organisms cells do not have significant ‘Gap’
phases and alternate between S and M phases with little time in between. This is
probably because the eggs of organisms such as *Xenopus* and clams, unlike
somatic cells, already contain most of the nutrients and cellular components
required to produce many cells and thus early embryos do not grow between cell
divisions. Indeed, these embryos are unable to grow until they have developed a
means of feeding. Despite these differences, however, at a molecular level, the
regulation of these seemingly diverse cell cycles show remarkable similarity.

**Maturation Promoting Factor**
The molecular difference between cells at different stages of the cell cycle has
been studied for many years.

Analysis of cells at different stages of the cell cycle showed that the
cytoplasm of M phase cells contained a dominant ‘factor’ that could drive
interphase nuclei into M phase (Gurdon, 1968; Johnson and Rao, 1970; Rao and
Johnson, 1970). Moreover, the microinjection of cytoplasm from metaphase
arrested frog eggs into prophase arrested frog oocytes caused them to mature into
eggs faster than if they had been stimulated to mature with progesterone (Masui
and Markert, 1971; Smith and Ecker, 1971). This ‘dominant factor’, present in M
phase cells, was named **Maturation Promoting Factor** (MPF).

Closer analysis of *Xenopus* early embryonic development revealed that
MPF activity oscillates in the cell cycle, peaking at mitosis (Wasserman and
Smith, 1978; Gerhart *et al.*, 1984). In general, the activation of MPF requires
protein synthesis, and inhibitors of this process block MPF activation and the
mitotic cycles in *Xenopus* embryos (Brachet *et al.*, 1975; Wasserman and Masui,
1975; Miao-Lye *et al.*, 1983). The first meiotic cycle of clams and starfish is,
however, independent of protein synthesis (Dorée, 1982; Hunt *et al.*, 1992) and
although the first meiotic cycle of frog oocytes cannot take place in the absence
of protein synthesis when induced by progesterone, it can occur in the presence of
protein synthesis inhibitors when oocyte maturation is induced by the
microinjection of MPF (Brachet *et al.*, 1975; Wasserman and Masui, 1975;
Gerhart *et al.*, 1984). This suggests that in these oocytes MPF is present but not
active, and that the microinjection of small doses of active MPF catalyses the
activation of the pre-MPF into active MPF by post-translational modification
(Cyert and Kirschner, 1988; Dunphy and Newport, 1988). Some 15 years after its
initial identification, MPF was finally purified (Lohka *et al.*, 1988). It was shown
to contain the *Xenopus* homologue of yeast p34CDC28/cdc2 (Arion *et al.*, 1988;
Dunphy *et al.*, 1988; Gautier *et al.*, 1988; Labbé *et al.*, 1988) and a B-type cyclin
Both p34\textsuperscript{cdc2} and cyclin B had been previously identified; cyclins as proteins in marine invertebrate embryos whose levels oscillated during the cell cycle (see later) and p34\textsuperscript{CDC28/cdc2} as a protein that was essential for cell cycle progression in both fission and budding yeast (see below).

Identification of \textit{CDC28/cdc2} as a key regulator in the cell cycle

Cell division cycle, or \textit{CDC}, mutants of the budding yeast, \textit{Saccharomyces cerevisiae}, were identified as mutants that arrested cell cycle progression at a unique stage of the cycle (Hartwell, 1967; Hartwell \textit{et al}., 1970). One such mutant, \textit{CDC28}, blocked the cell cycle at a point in G1 called \textit{START} (Hereford and Hartwell, 1974). \textit{START} (Hartwell \textit{et al}., 1974), which in higher eukaryotes may be analogous to the restriction point (Pardee, 1974), is the point beyond which cells are committed to DNA synthesis and completion of the cell cycle.

\textit{CDC} mutants were also isolated in the fission yeast, \textit{Schizosaccharomyces pombe} (Nurse \textit{et al}., 1976). One of the genes identified in this way, \textit{cdc2}, was singled out as an important regulator of the cell cycle. Its gene product was essential for cells to progress into mitosis (Nurse \textit{et al}., 1976; Simanis and Nurse, 1986), but dominant mutants of this gene could give strains of \textit{S. pombe} that divided prematurely (Thuriaux \textit{et al}., 1978; Nurse and Thuriaux, 1980). This suggested that the \textit{cdc2} gene product was involved in the control of the cell cycle.

More detailed analysis of \textit{CDC28} and \textit{cdc2} showed that although most alleles of \textit{CDC28} affected \textit{START} in \textit{S. cerevisiae}, one allele did arrest cells in G2 (Piggott \textit{et al}., 1982; Reed and Wittenberg, 1990). Similarly, although \textit{cdc2} had its major effect at mitosis, it was also required at S phase when cells were emerging from G0 (Nurse and Bissett, 1981). The \textit{CDC28} and \textit{cdc2} genes were then shown to be functionally homologous, as \textit{CDC28} was isolated from an \textit{S. cerevisiae} gene bank as a gene that complemented a \textit{cdc2} mutation in \textit{S. pombe} (Beach \textit{et al}., 1982). Once both genes had been sequenced, the proteins were shown to be 62% identical at the amino acid level (Hayles and Nurse, 1986).

Indication that the elements of the mechanism by which the cell cycle is controlled were likely to be conserved between all eukaryotic cells came when the human homologue of the \textit{S. pombe cdc2} gene was cloned (Lee and Nurse, 1987). Human \textit{cdc2} was isolated as a cDNA from a human library that, when expressed in \textit{S. pombe}, could complement the \textit{cdc2-33} mutation. The gene was sequenced and shown to display 63% amino acid identity to \textit{S. pombe cdc2} and 58% amino acid identity to \textit{S. cerevisiae CDC28}.

The p34\textsuperscript{cdc2} kinase subunit has now been identified in many different organisms, and a number of more distantly related p34\textsuperscript{cdc2}-like proteins have also
been identified. Since p34^{cd}c2 is the member of the family about which most is
known, and is the one whose regulation by phosphorylation is most studied,
however, I will largely confine the discussion to p34^{cd}c2 in the following sections.
The other p34^{cd}c2-like proteins may or may not be regulated in a similar manner.

**Regulation of the activity of cyclin dependent protein kinases**

**p34^{cd}c2 activity is regulated by phosphorylation**

In the next few sections I will use the term MPF to mean active p34^{cd}c2 kinase. This
definition is not wholly satisfactory for two reasons, both of which will be
discussed later; firstly, MPF is an activity that is also possessed by proteins other
than cyclin and p34^{cd}c2, and secondly, several cyclin/p34^{cd}c2 complexes can act
as MPF. However, I hope that using the term MPF will make the argument in the
next few sections easier to follow.

Although the accumulation of cyclins through the cell cycle is continuous,
and is required for MPF activity, the activation of MPF does not follow the same
pattern as cyclin accumulation, being activated rapidly and being active for only a
short period during each cell cycle (Minshull et al., 1990), see figure 1.1. Moreover,
frog oocytes, which contain inactive cyclin B/p34^{cd}c2 complexes
(Kobayashi et al., 1991), can be induced to mature in the absence of new protein
synthesis by the microinjection of catalytic doses of MPF (Brachet et al., 1975;
Wasserman and Masui, 1975; Gerhart et al., 1984). This suggests that the activity

![Figure 1.1](image.png)

**Figure 1.1** Cyclin accumulation is gradual, while MPF activation is not
The figure shows the difference between the pattern of accumulation of cyclin
proteins and the activation of MPF. Cyclin accumulation is represented by the
solid line, while MPF activity is represented by the shaded area. The difference
in patterns suggests that MPF activity is not regulated solely by cyclin binding.
of MPF is controlled post-translationally. In these cells MPF activity is controlled by reversible phosphorylation, although recently it has emerged that inhibitors of p34<sup>cdc2</sup> and p34<sup>cdc2</sup>-related kinases exist, which could also be involved in the control of MPF activity (see later).

The regulation of MPF by phosphorylation was first shown by Gould and Nurse (1989). They showed that in <i>S. pombe</i>, p34<sup>cdc2</sup> was phosphorylated on tyrosine 15, and that phosphorylation at this site dramatically reduced as cells entered mitosis. Moreover, substitution of tyrosine 15 of p34<sup>cdc2</sup> with a phenylalanine residue gave a strain of <i>S. pombe</i> in which a high proportion of cells were inviable because of premature entry into mitosis. Phosphorylation of p34<sup>cdc2</sup> on tyrosine 15 in <i>S. pombe</i> thus appeared to be vital in order to prevent cells from entering mitosis before all other necessary steps in the cell cycle had been completed. This result was confirmed when it was shown that dephosphorylation of tyrosine 15 of <i>S. pombe</i> p34<sup>cdc2</sup> by a purified human protein-tyrosine phosphatase <i>in vitro</i> was sufficient to trigger p34<sup>cdc2</sup> activation (Gould <i>et al.</i>, 1990).

Tyrosine phosphorylation was also implicated in the regulation of MPF activity in other organisms, as tyrosine dephosphorylation of p34<sup>cdc2</sup> accompanied its activation during the entry into M phase in <i>Xenopus</i> egg extracts, starfish oocytes and mouse fibroblasts (Dunphy and Newport, 1989; Morla <i>et al.</i>, 1989; Pondaven <i>et al.</i>, 1990; Solomon <i>et al.</i>, 1990). It was not clear, however, that MPF could be activated simply by dephosphorylation of tyrosine 15 of p34<sup>cdc2</sup> in these higher eukaryotes. Exposure of inactive mouse MPF to purified tyrosine phosphatase removed more than 75% of p34<sup>cdc2</sup> phosphotyrosine, but the complex remained inactive (Morla <i>et al.</i>, 1989). This discrepancy can probably be accounted for by the phosphorylation of threonine 14 of p34<sup>cdc2</sup> in higher eukaryotes (Krek and Nigg, 1991a; Norbury <i>et al.</i>, 1991) and the demonstration that dephosphorylation of both threonine 14 and tyrosine 15 is required for MPF activation in these organisms (Krek and Nigg, 1991b; Norbury <i>et al.</i>, 1991). In contrast to p34<sup>cdc2</sup> bound to B-type cyclins, p34<sup>cdc2</sup> bound to cyclin A is only weakly tyrosine phosphorylated and cyclin A protein added to <i>Xenopus</i> egg extracts is activated rapidly with no apparent lag phase (Clarke <i>et al.</i>, 1992). This suggests that a given cdk can be regulated in a different manner depending on the cyclin to which it binds.

Threonine 14 and tyrosine 15 are part of the ATP binding site of p34<sup>cdc2</sup> (Hanks <i>et al.</i>, 1988; De Bondt <i>et al.</i>, 1993; Marcote <i>et al.</i>, 1993), although the phosphorylation of these residues does not appear to prevent ATP binding (Atherton-Fessler <i>et al.</i>, 1993). One suggestion (De Bondt <i>et al.</i>, 1993) is that threonine 14 and tyrosine 15 phosphorylation mediate their inactivation of p34<sup>cdc2</sup> kinase by interfering with substrate binding. Alternatively, they could
affect ATP orientation by changing the conformation of the GxGTYG loop (De Bondt et al., 1993).

Surprisingly, tyrosine 15 phosphorylation does not appear to play a significant regulatory role in the budding yeast, *S. cerevisiae*. Dephosphorylation of tyrosine 19 of p34CDC28 (which is equivalent to tyrosine 15 of p34cdc2) is cell-cycle regulated, but, unlike in *S. pombe*, replacing tyrosine 19 of p34CDC28 with a phenylalanine residue, so that the protein can no longer be tyrosine phosphorylated, does not lead to premature mitosis of the yeast cells (Amon et al., 1992; Sorger and Murray, 1992). Thus, although the phosphorylation of tyrosine 15 appears to be regulatory in most organisms, it is not an entirely universal control mechanism.

p34cdc2 is also phosphorylated on threonine 161 (Ducommun et al., 1991; Gould et al., 1991; Desai et al., 1992; Krek and Nigg, 1992; Solomon et al., 1992). The presence of a threonine or serine at this site has been shown to be required for MPF activity in *S. pombe* (Booher and Beach, 1986; Ducommun et al., 1991; Gould et al., 1991; Krek et al., 1992) and for the activation of reticulocyte lysate-synthesised p34cdc2 in *Xenopus* egg extracts (Solomon et al., 1992). Phosphorylation of a residue equivalent to threonine 161 in p34cdc2 is required for the activation of many members of the serine/threonine kinase family, including cAMP-dependent protein kinase and the MAP kinases, see De Bondt et al. (1993).

The exact relationship between cyclin binding and phosphorylation of threonine 161 is not completely clear. Some studies indicate that phosphorylation of threonine 161 is required to enable cyclin to bind to p34cdc2 (Ducommun et al., 1991), and that the binding of cyclin to a p34cdc2 mutant that cannot be phosphorylated on threonine 161 is severely reduced (Gould et al., 1991). In contrast, others have found that the phosphorylation of threonine 161 is not required for cyclin binding (Desai et al., 1992), and that cyclin binding may even be necessary for threonine 161 phosphorylation (Solomon et al., 1992). A recent report from Lee et al. suggests an explanation for these discrepancies. They show that although threonine 161 phosphorylation stabilises cyclin/p34cdc2 complexes, the initial rate of association between cyclin and p34cdc2 is not affected by the phosphorylation state of the p34cdc2 subunit (Lee et al., 1994).

The crystal structure of the p34cdc2-related protein, p33cdc2, suggests that phosphorylation of threonine 160 (equivalent to threonine 161 in p34cdc2) may change the location of a protein loop in p33cdc2 such that it no longer blocks the substrate binding site (De Bondt et al., 1993). As p34cdc2 and p33cdc2 are highly homologous, it is likely that phosphorylation of threonine 161 in p34cdc2 has the same effect in this protein.
Regulation of threonine 14 and tyrosine 15 phosphorylation

Even before the biochemical details were clear, the *weel* gene product, p107\(^{\text{weel}}\), which was predicted to be a protein kinase, had been shown to be a dose dependent regulator of entry into mitosis (Russell and Nurse, 1987b). On the basis of amino acid sequence homology, p107\(^{\text{weel}}\) was predicted to be a serine/threonine protein kinase (Hanks *et al*., 1988). However, Featherstone and Russell showed that p107\(^{\text{weel}}\) was in fact capable of tyrosine phosphorylation, both on itself and exogenous substrates (Featherstone and Russell, 1991). Using baculovirus expressed proteins it was demonstrated that tyrosine phosphorylation of p34\(^{\text{cdc2}}\) was dependent on the presence of p107\(^{\text{weel}}\) (Parker *et al*., 1991). The first demonstration that p107\(^{\text{weel}}\) was itself able to phosphorylate p34\(^{\text{cdc2}}\) came from Parker *et al.* (1992), who showed that the *S. pombe* p107\(^{\text{weel}}\) could phosphorylate human p34\(^{\text{cdc2}}\) on tyrosine 15, but not threonine 14. This was confirmed by reports showing that the human homologue of p107\(^{\text{weel}}\), which could block cell division when overexpressed in HeLa cells, was able to phosphorylate human p34\(^{\text{cdc2}}\) exclusively on tyrosine 15, and inactivate it (Parker and Piwnica-Worms, 1992; McGowan and Russell, 1993). Free p34\(^{\text{cdc2}}\) is not a substrate for the p107\(^{\text{weel}}\) kinase (Solomon *et al*., 1990; Parker *et al*., 1992), and in *S. cerevisiae* phosphorylation of p34\(^{\text{CDC28}}\) by the p107\(^{\text{weel}}\) homologue occurs only on p34\(^{\text{CDC28}}\) bound to B-type cyclins and not on p34\(^{\text{CDC28}}\) bound to G1-type cyclins (Booher *et al*., 1993). In *S. pombe* a second kinase, the product of the *mikl* gene, co-operates with p107\(^{\text{weel}}\) in the negative regulation of p34\(^{\text{cdc2}}\) (Lundgren *et al*., 1991), probably by also phosphorylating tyrosine 15. The identity of the kinase that phosphorylates p34\(^{\text{cdc2}}\) on threonine 14 is not yet known, but a recent report has shown that a kinase that can phosphorylate both tyrosine 15 and threonine 14 of p34\(^{\text{cdc2}}\) is associated with membranes in *Xenopus* egg extracts (Kornbluth *et al*., 1994).

The phosphorylation state of p107\(^{\text{weel}}\) oscillates between an underphosphorylated form in interphase and a hyperphosphorylated version during mitosis. The mitosis-specific hyperphosphorylation of p107\(^{\text{weel}}\), in the N-terminal region of the protein, results in a substantial reduction in its activity as a p34\(^{\text{cdc2}}\)-specific tyrosine kinase (Tang *et al*., 1993). In *S. pombe* the protein kinase activity of p107\(^{\text{weel}}\) is inhibited by phosphorylation by the protein kinase product of the *niml* gene (Russell and Nurse, 1987a; Parker *et al*., 1993; Wu and Russell, 1993). However, Nim1 phosphorylation occurs on the C-terminal catalytic domain of p107\(^{\text{weel}}\) (Coleman *et al*., 1993). It thus appears that there are at least two different protein kinases that regulate the activity of p107\(^{\text{weel}}\). The identity of the other kinase is not yet known.

The product of the *cdc25* gene from *S. pombe* had long been known to be a dose dependent activator of mitosis, and to be required for the entry of cells into
mitosis in normal *S. pombe* cells, although not in *weel* mutants (Fantes, 1979; Russell and Nurse, 1986). This effect was mediated by MPF activation (Booher *et al.*, 1989; Moreno *et al.*, 1989), more specifically, by promoting the dephosphorylation of tyrosine 15 of p34^cdc2^ (Gould *et al.*, 1990; Kumagai and Dunphy, 1991; Strausfeld *et al.*, 1991). Although sequence analysis of cdc25 had not shown it to be a protein phosphatase (Russell and Nurse, 1986), Dunphy and Kumagai showed that purified cdc25 could catalyse the dephosphorylation of several model phosphatase substrates, including p-nitrophenyl phosphate and two distinct tyrosine-phosphorylated peptides (Dunphy and Kumagai, 1991). It was subsequently shown that cdc25 could directly dephosphorylate tyrosine 15 of p34^cdc2^ (Gautier *et al.*, 1991; Kumagai and Dunphy, 1991; Millar *et al.*, 1991; Strausfeld *et al.*, 1991; Lee *et al.*, 1992). In fission yeast, a second protein tyrosine phosphatase, encoded by *pyp3*, appears to act co-operatively with cdc25 to dephosphorylate tyrosine 15 of p34^cdc2^ (Millar *et al.*, 1992).

In *Xenopus* extracts cdc25 becomes phosphorylated in its N-terminal region and active near the onset of mitosis. Artificial dephosphorylation of the protein leads to its inactivation, strongly suggesting that phosphorylation of cdc25 is required for its activity (Izumi *et al.*, 1992; Kumagai and Dunphy, 1992). There is now much evidence that active p34^cdc2^ kinase can itself phosphorylate and activate cdc25 (Clarke *et al.*, 1993; Hoffmann *et al.*, 1993; Izumi and Maller, 1993; Strausfeld *et al.*, 1994), but there is some disagreement as to whether only the cyclin B/p34^cdc2^ complex can carry out this function (Hoffmann *et al.*, 1993), or whether both cyclin B/p34^cdc2^ and cyclin A/p34^cdc2^ are able to phosphorylate cdc25 (Izumi and Maller, 1993). The activation of cdc25 by cyclin B/p34^cdc2^ kinase may require a specific region of the cyclin B protein, termed the 'P' box (Zheng and Ruderman, 1993). This region, which is highly conserved between B-type cyclins, shows homology to a small region usually present in protein tyrosine kinases, but absent from cdc25 (Galaktionov and Beach, 1991).

The protein phosphatase that inactivates cdc25 by dephosphorylation has not been identified, but there is evidence to suggest that it may be a type-2A protein phosphatase (Clarke *et al.*, 1993).

**Regulation of threonine 161 phosphorylation**

Many protein kinases, such as cAMP-dependent kinase, are activated by autophosphorylation. From comparisons of the crystal structure of p33^cdk2^ and other protein kinases, it is clear that threonine 160 of p33^cdk2^ is in the region of the protein in which autophosphorylations can occur (De Bondt *et al.*, 1993). To date, however, no autophosphorylation has been observed on threonine 161 of p34^cdc2^ (Desai *et al.*, 1992; Solomon *et al.*, 1992). The serine/threonine kinase that is responsible for the phosphorylation of threonine 161, Cdc2 Activating
Kinase (CAK), was partially purified from Xenopus egg extract and was shown to be able to phosphorylate and activate p34cdc2 in the presence of cyclin B (Solomon et al., 1992). CAK has subsequently been found to contain the p34cdc2-related protein kinase p40MO15, which is able to activate cyclin-bound p34cdc2 and the p34cdc2-related kinase p33cdk2 (Fesquet et al., 1993; Poon et al., 1993; Solomon et al., 1993). When p40MO15 was identified as a subunit of CAK, there was evidence that it required both association with an activator subunit and phosphorylation to be active (Fesquet et al., 1993; Poon et al., 1993; Solomon et al., 1993). Very recently the activator subunit of p40MO15 has been cloned and shown to be a cyclin-like protein, with homology to cyclin C and the msc2 and cell gene products from yeast. It has been named cyclin H (Fisher and Morgan, 1994). CAK activity requires both p40MO15 (now re-named cdk7) and cyclin H, and the presence of threonine 170, which is equivalent to threonine 161 in p34cdc2, in the p40MO15 subunit (Fisher and Morgan, 1994).

**Protein inhibitors of cyclin dependent kinases**

During the last year, several novel protein inhibitors of cyclin dependent kinases have been discovered, and much work has been published on them. The identification of these inhibitors sheds new light on the negative regulation of cdk activity, which had previously been thought to occur entirely through regulation of cyclin levels and through the regulation of phosphorylation/dephosphorylation on threonine 14 and tyrosine 15 (or their equivalents), although in S. cerevisiae it had been clear for some time that tyrosine 15 phosphorylation was not the only negative control on the activity of cyclin/p34CDC28 complexes.

The first report of a protein that could bind to p34cdc2 and inhibit its kinase activity was from Mendenhall (1993), who reported the purification of a protein (p40) from S. cerevisiae, first identified as a substrate of p34CDC28 kinase, which bound tightly to p34CDC28 and inhibited its activity. Soon afterwards Peter et al. showed that the α-factor-dependent phosphorylation of FAR1 by FUS3 in S. cerevisiae allowed it to bind to the CLN2/p34CDC28 complex. Cells containing mutants of FAR1 that could not bind to CLN2/p34CDC28, or cells in which FAR1 was improperly phosphorylated and could not associate with CLN2/p34CDC28, were unable to arrest the cell cycle in response to α factor, suggesting that the formation of this complex is required for cell cycle arrest in response to external signals (Peter et al., 1993).

Several cyclin dependent kinase inhibitors in organisms other than budding yeast have now been identified. One such inhibitor was identified independently by several different groups as a 21 kDa protein, called variously cip1, p21 and CAP20, that could bind to certain cyclin/cdk complexes (Xiong et al., 1992; Gu et al., 1993; Harper et al., 1993). It was shown to bind to and
inhibit the kinase activity of a number of cyclin complexes, including cyclin A/cdk2, cyclin D1/cdk2, cyclin D2/cdk2, cyclin D1/cdk4 and cyclin E/cdk2 (Gu et al., 1993; Harper et al., 1993; Xiong et al., 1993). Its affinity for cyclin B/cdc2 complexes is much lower (Gu et al., 1993; Xiong et al., 1993). However, it was shown that even in normal proliferating fibroblasts the majority of cyclin dependent kinases exist in a quaternary complex of a cyclin, a cdk, PCNA and p21 (Zhang et al., 1993). This paradox was resolved when it was demonstrated that, although most cdks did exist in quaternary complexes with p21, only those cyclin/cdk complexes that were bound to multiple subunits of p21 showed inhibited kinase activity (Zhang et al., 1994). The link between p21 and cell cycle arrest came in a number of ways. Firstly, one of the groups that cloned p21 (as sdil), identified it as a cDNA that, when overexpressed in proliferating cells, blocked DNA synthesis (Noda et al., 1994). They showed that the expression of sdil was increased 10-20 fold in senescent compared with young cells, and that the increase in sdil mRNA level closely paralleled the onset of the senescent phenotype and loss of cell proliferation. Furthermore, sdil expression was also increased in young cells made non-dividing by deprivation of growth factors or contact inhibition. The cdk inhibitor p21 was also isolated as a gene, named WAF1, whose induction was associated with wild-type but not mutant p53 expression in a human brain tumour cell line (El-Deiry et al., 1993). Introduction of WAF1 cDNA suppressed the growth of human brain, lung and colon tumour cells in culture and it was shown that the WAF1 promoter included a p53 site. It has since been shown that the arrest of γ-irradiated cells prior to S phase is correlated with an inhibition of cyclin E/p33^cdk2^ kinase, and that this inhibition is mediated by p21 (Xiong et al., 1993; Dulic et al., 1994). Since cells lacking p53 express very low levels of p21 (El-Deiry et al., 1993; Xiong et al., 1993), this data suggests that in γ-irradiated cells p53 induces expression of p21 mRNA. The increased levels of p21 then inhibit cyclin E/p33^cdk2^, and maybe other G1 cyclin dependent kinase complexes, and causes arrest of the cell cycle in G1. p21 has also been suggested to inhibit PCNA-dependent DNA replication in the absence of cyclin/CDK complexes, possibly by blocking the ability of PCNA to activate DNA polymerase δ (Waga et al., 1994).

A cyclin dependent kinase inhibitor that has been directly implicated in tumourigenesis was isolated as a protein that could bind to cdk4. p16^INK^ can bind to cdk4, but not cdk2 and inhibits the kinase activity of cyclin D1, cyclin D2 and cyclin D3/cdk4 kinases (Serrano et al., 1993). p16^INK^ is deleted or mutated in a wide variety of tumours, and up to 75% of all cancer cell lines showed mutation of this gene, although mutation of p16^INK^ is less common in uncultured tumours (Kamb et al., 1994; Nobori et al., 1994; Spruck III et al., 1994). Thus
the activity of p16\textsuperscript{INK} appears to be of vital importance in controlling the cell cycle.

The cyclin dependent kinase inhibitor p27\textsuperscript{Kip1} was identified by two groups as a protein involved in the arrest of cells in G1 by transforming growth factor β (and by contact inhibition (Polyak et al., 1994a; Slingerland et al., 1994). It was also recognised as an inhibitor that allowed the arrest of HeLa cells in G1 by lovastatin with high concentrations of cyclin A and E/p33\textsuperscript{cdk2} complexes, but low kinase activity (Hengst et al., 1994); as an inhibitor of cyclin/cdk2 complexes that was inactivated during interleukin 2-induced proliferation of human T lymphocytes (Firpo et al., 1994), and as a protein that interacted with cyclin D1/cdk4 complexes (Toyoshima and Hunter, 1994). It can bind to and inhibit cyclin A/cdk2, cyclin E/cdk2, cyclin B/cdc2 and cyclin D/cdk4 kinase activities (Firpo et al., 1994; Hengst et al., 1994; Polyak et al., 1994a; Polyak et al., 1994b; Slingerland et al., 1994; Toyoshima and Hunter, 1994). It appears that the binding of p27\textsuperscript{Kip1} to cyclin D/cdk4 complexes may actually mediate release from G1 arrest by sequestering the inhibitor and thereby allowing the activation of cyclin E/cdk2 kinase (Firpo et al., 1994; Polyak et al., 1994a). Although the activity of p27\textsuperscript{Kip1} varies through the cell cycle and is more active in growth-arrested cells than in proliferating cells (Firpo et al., 1994; Hengst et al., 1994; Polyak et al., 1994a; Slingerland et al., 1994), its mRNA and protein levels remain constant (Polyak et al., 1994b; Toyoshima and Hunter, 1994), suggesting that its activity may be regulated in a post-translational manner. The sequence of p27\textsuperscript{Kip1} shows it to have a region of strong homology with the cyclin dependent kinase inhibitor, p21, and overexpression of p27\textsuperscript{Kip1} in mammalian cells, like p21, causes them to arrest in G1 (Polyak et al., 1994b; Toyoshima and Hunter, 1994).

A cyclin dependent kinase inhibitor that appears to have enzymatic activity has been isolated as a cdc2-binding protein (Gyuris et al., 1993). Cdi1 is a 21 kDa protein that can bind to cdc2, cdk2 and cdk3, but not cdk4. Its expression peaks late in G1 and early S phase, and in vivo it is found associated with cdk2. Overexpression of the protein causes cells to be delayed in G1, and increased levels of cdc2 actually accentuated this effect, suggesting that cdi1 does not simply sequester cdc2. Cdi1 has tyrosine phosphatase activity against model substrates and, although it has yet to be shown to act on any real protein, the ability of the inhibitor to delay cells in G1 depends on it being an active phosphatase, indicating that its tyrosine phosphatase activity is important for its function. Its role in the cell cycle has yet to be determined.

Finally, a mention must go to the fission yeast gene, rum1, which was isolated as an S. pombe gene which, when overexpressed, caused cells to undergo repeated rounds of DNA replication without carrying out mitosis. Deletion of the
gene gave cells that were unable to arrest in the G1 interval pre START when starved, and in ruml− cells in which START was blocked with the cdc10Δ mutation, cells still entered mitosis despite failure to replicate their DNA (Moreno and Nurse, 1994). This data suggests that there could be a direct link between cyclin dependent kinase inhibitors and checkpoint control, although it should be stressed that a direct interaction between rum1 and p34cdc2 has yet to be shown.

Was the original MPF really p34cdc2 and cyclin?
Although MPF was purified and shown to consist of p34cdc2 and cyclin (see above), only 1% of the original MPF activity was left at the end of the purification procedure (Lohka et al., 1988). Despite active p34cdc2/cyclin B kinase being ultimately responsible for oocyte maturation, our current understanding of the proteins that regulate cell cycle progression suggests that MPF activity, assayed by the ability of injected cytoplasm to cause maturation of frog oocytes, could be caused by a number of different proteins, or a mixture of these proteins. When crude M phase cytoplasm is injected into oocytes its MPF activity could due to the presence of active cyclin/p34cdc2 kinase, which activates the pre-MPF present in the oocyte, probably by activating cdc25. Alternatively, maturation of the oocytes could be caused by active cdc25 in the injected extract, which dephosphorylates the pre-MPF in the extract and thus activates it. A third source of MPF activity could be the protein kinases and phosphatases that indirectly regulate the phosphorylation state of p34cdc2 by keeping cdc25 active while inactivating wee1. Thus, although active cyclin/p34cdc2 complex can act as MPF, and activation of p34cdc2 kinase is required for oocyte maturation, in crude egg cytoplasm MPF activity is probably a mixture of proteins which together form an efficient trigger for the activation of the latent p34cdc2 kinase present in the oocyte cytoplasm. Such a description could also explain why the ATP analogue ATPγS stabilises maturation promoting factor (Gerhart et al., 1984), and why the inclusion of ATPγS in the Xenopus egg extract was found to be essential for the stability of MPF during purification (Lohka et al., 1988). If the ATPγS in these extracts stably thio-phosphorylates either cdc25 (which is activated by phosphorylation) or wee1 (which is inactivated by phosphorylation - see above), this would help to maintain p34cdc2 kinase activity. In support of this suggestion, it has been shown that the microinjection of thio-phosphorylated cdc25-C into stage VI Xenopus oocytes causes them to mature into eggs (Hoffmann et al., 1993). Alternatively, ATPγS could be stabilising MPF by preventing cyclin degradation (see Chapter 6), or by some other means.
The cyclin dependent protein kinase family

In fission and budding yeast, a single p34\(^{CDC28}/cdc2\) protein kinase subunit regulates both START and the G2→M transition. The different activities of this subunit comes from its association with different cyclins; so in \textit{S. cerevisiae} at START p34\(^{CDC28}\) is associated with the G1 cyclins and at the G2→M transition it is associated with the B-type cyclins (see later). In higher organisms, however, the regulation of passage through START and G2→M appears to be more complicated. In addition to a variety of cyclins (see later) there exist at least two different p34\(^{cdc2}\)-related proteins, which have different functions through the cell cycle. These kinases are known as cyclin dependent kinases, or cdks, and, as their name suggests must (in most cases) have been shown to bind to and be activated by a cyclin subunit to be defined as such. As many cyclins can bind to more than one different cdk, and cdks can bind to more than one different cyclin subunit, defining the role of the individual cyclins and cdks in the cell cycle is very difficult. Not all cdks are directly involved in the regulation of the cell cycle, however. Indeed, in many cases their roles have yet to be clearly defined.

cdc2

p34\(^{cdc2}\) was the first cdk to be isolated and for that reason is the most studied (see above). Despite being re-named cdk1, it is still usually called p34\(^{cdc2}\). As discussed above, p34\(^{cdc2}\), when bound to a B-type cyclin and in the correct phosphorylation state, catalyses the entry into M phase. In higher eukaryotes p34\(^{cdc2}\) is associated with both A- and B-type cyclins (Minshull \textit{et al}., 1990; Clarke \textit{et al}., 1992; Hamaguchi \textit{et al}., 1992) and the activity of cyclin A/p34\(^{cdc2}\) peaks before that of cyclin B/p34\(^{cdc2}\) (Minshull \textit{et al}., 1990; Whitfield \textit{et al}., 1990). Although in yeast, p34\(^{cdc2}\) is required both to enter mitosis and to pass START (Nurse and Bissett, 1981; Piggott \textit{et al}., 1982), in higher eukaryotes it appears only to be required in mitosis. Microinjection of antibodies against p34\(^{cdc2}\) was found to be ineffective in blocking the G1→S transition in human tissue culture cells, but caused and arrest before entry into mitosis (Riabowol \textit{et al}., 1989), and immunodepletion of p34\(^{cdc2}\) from \textit{Xenopus} eggs did not prevent entry of the extracts into S phase (Fang and Newport, 1991). In addition, the cell cycle arrest in the mouse FT210 cell line, which has a temperature-sensitive mutation in \textit{cdc2}, occurred exclusively during G2 at the restrictive temperature (Th'ng \textit{et al}., 1990; Hamaguchi \textit{et al}., 1992), and cells in \textit{Drosophila} embryos containing a mutant \textit{cdc2} gene arrest in G2 after the maternal p34\(^{cdc2}\) has run out (Stern \textit{et al}., 1993). No evidence for a G1 arrest was found in these cells. Finally, a dominant negative mutant of human p34\(^{cdc2}\) arrested human cells at the G2→M transition (van den Heuvel and Harlow, 1993).
The *cdk2* gene was originally isolated from *Xenopus* eggs and accordingly was called Eg1. It was at first thought to correspond to the *Xenopus* version of *cdc2*, which had not been cloned at the time (Paris *et al.*, 1991). The human homologue of *cdk2* was isolated by complementation of *S. cerevisiae* *CDC28*, and by the polymerase chain reaction (PCR) (Elledge and Spottswood, 1991; Ninomiya-Tsuji *et al.*, 1991; Tsai *et al.*, 1991). It encodes a 33 kDa protein with over 60% amino acid identity to p34^cdc2_. The *cdk2* gene product, p33^cdk2_, is thought to be required for the G1 to S transition and for DNA synthesis. Microinjection of anti-p33^cdk2_ antibodies into mammalian cells prevents entry into S phase (Pagano *et al.*, 1993; Tsai *et al.*, 1993) and depletion of p33^cdk2_ from a *Xenopus* egg extract blocks DNA replication (Fang and Newport, 1991). In addition, expression of a dominant negative mutant of p33^cdk2_ in human cells causes a G1 block (van den Heuvel and Harlow, 1993) and p33^cdk2_ specifically localises at subnuclear sites of DNA replication in terminally differentiated myotubes that have been forced into S phase by the expression of SV40 large T antigen (Cardoso *et al.*, 1993). p33^cdk2_ binds to both cyclin A (Pines and Hunter, 1990; Tsai *et al.*, 1991; Elledge *et al.*, 1992; Rosenblatt *et al.*, 1992) and cyclin E (Koff *et al.*, 1991; Dulic *et al.*, 1992; Koff *et al.*, 1992). There is also evidence the D-type cyclins can bind to p33^cdk2_ (Dulic *et al.*, 1993; Ewen *et al.*, 1993; Bates *et al.*, 1994).

The human *cdk3* gene was isolated by PCR during a search for p34^cdc2_-related clones (Meyerson *et al.*, 1992). It encodes a 35 kDa protein with 76% amino acid identity to human p33^cdk2_. It has not been demonstrated to bind to or be activated by a cyclin subunit, but has been classified as a cyclin dependent kinase on the basis of its high homology to p33^cdk2_ and on its ability to complement a *CDC28* mutation (Meyerson *et al.*, 1992). A dominant negative form of cdk3 can block human cells in G1 (van den Heuvel and Harlow, 1993). The levels of cdk3 mRNA in normal tissues are very low in comparison to other cdks, however, suggesting that it may not be important in cell cycle regulation (Meyerson *et al.*, 1992).

cdk4, which was discovered at around the same time as human p34^cdc2_, was originally called PSK-J3. The gene encodes a 34 kDa protein kinase with 45% amino acid identity to p34^cdc2_. It was first identified in a screen with mixed oligonucleotide probes derived from conserved regions of serine/threonine protein kinases (Hanks, 1987). It has since been shown to bind to D-type cyclins (Matsushime *et al.*, 1992; Ewen *et al.*, 1993; Kato *et al.*, 1993; Bates *et al.*, 1994;
Kato et al., 1994). Cyclin D/cdk4 complexes can bind to and phosphorylate the retinoblastoma gene product and the retinoblastoma-like protein p107 (Matsushime et al., 1992; Dowdy et al., 1993; Kato et al., 1993). The role of this kinase in the cell cycle is not yet clear.

**cdk5**

The human cdk5 gene was originally identified during a PCR screen for p34cdc2-related clones (Meyerson et al., 1992). It encodes a 33 kDa protein with about 60% amino acid identity to p34cdc2 and p33^cdk2, and is found associated with cyclins D1 and D3 in human diploid lung fibroblast WI38 cells (Xiong et al., 1992). It was also isolated from bovine brain (Lew et al., 1992) and shown to be part of the tau protein kinase II (Kobayashi et al., 1993). In bovine brain it is found associated with a 23 kDa protein (Ishiguro et al., 1994) which has essentially no similarity to the rest of the cyclin family. It is noteworthy that this kinase is found at high levels in neurones, which do not divide, suggesting that it is not involved in the regulation of the cell cycle.

**cdk6**

cdk6 was also isolated by Meyerson et al., during a PCR screen for p34cdc2-related clones (Meyerson et al., 1992). The cdk6 gene encodes a 40 kDa protein which shows 71% amino acid identity with cdk4. cdk6 is found associated with cyclin D1 in the human squamous carcinoma cell line, UMSCC2 (Bates et al., 1994).

**cdk7**

The kinase subunit of the cdk activating kinase (CAK), p40MO15, has recently been re-named cdk7, as it has been shown to bind to a novel cyclin subunit, cyclin H (Fisher and Morgan, 1994). CAK is responsible for the activating phosphorylations on threonine 161 of p34cdc2 and threonine 160 of p33^cdk2 (see above). To be active as a CAK, p40MO15 must be bound to cyclin H. The presence of threonine 170, the residue equivalent to threonine 161 in p34cdc2, is also required for CAK activity, and the p40MO15/cyclin H complex may autophosphorylate at this site (Fisher and Morgan, 1994).

**PHO85**

The PHO5 gene in *S. cerevisiae* encodes a secreted acid phosphatase whose transcription is repressed when the yeast are grown in high concentrations of inorganic phosphate. PHO4 is required for the activation of PHO5 and PHO80 and PHO85 are required for the negative regulation of PHO5. PHO85 has been shown to be a p34CDC28 homologue; the proteins show 51% homology at the
amino acid level (Toh-e et al., 1988). Recently it has been shown that PHO80, which interacts with PHO85, has homology to cyclins, showing 33% identity within the cyclin box to the ORFD and HCS26 cyclins (Kaffman et al., 1994). The complex of PHO80 and PHO85 phosphorylates PHO4 and this phosphorylation is correlated with negative regulation of PHO5 (Kaffman et al., 1994). Thus at least in S. cerevisiae, cyclins and cyclin dependent kinases are not just involved in direct regulation of the cell cycle.

Other p34cdc2-related proteins have been isolated, but I will not discuss them in this thesis as they have not been shown to bind to cyclin subunits and their functions are unknown. As suggested by the case of the PHO85 kinase, it is possible that many of them do not play a direct role in the regulation of the cell cycle.

**The cyclin family**

In unfertilised clam eggs the level of protein synthesis is very low until fertilisation, when stored maternal mRNA starts being synthesised and the pattern of newly translated proteins changes (Rosenthal et al., 1980). While studying this change in the pattern of protein synthesis on fertilisation of sea urchin and clam eggs, Evans et al. noticed newly synthesised proteins that accumulated rapidly through the cell cycle, but were abruptly destroyed in mitosis (Evans et al., 1983). This pattern, of accumulation followed by specific destruction, was in contrast to the majority of newly synthesised proteins, whose levels accumulate progressively in the early embryonic cycles. The sea urchin *Arbacia punctulata* contained one protein whose level oscillated with the cell cycle; the sea urchin *Lytechinus pictus* and the surf clam, *Spisula solidissima*, contained two. In view of their periodic accumulation and destruction, these proteins were called cyclin A and cyclin B.

The pattern of accumulation and destruction of cyclin proteins suggested that they might be of importance in the regulation of the cell cycle. Evidence that this was so came when clam cyclin A and sea urchin cyclin B were cloned. It was shown that microinjection of the mRNA encoding either of them caused the maturation of *Xenopus* oocytes into eggs (Swenson et al., 1986; Pines and Hunt, 1987). This indicated that both A and B-type cyclins were able to drive the G2→M transition in *Xenopus* oocytes.

Further evidence that cyclins were important in the cell cycle came from experiments carried out in cell free extracts made from *Xenopus* eggs. These extracts are able to undergo multiple cell cycles *in vitro*, but when the mRNA encoding cyclins B1 and B2 was ablated, the extracts were no longer able to enter
mitosis and became blocked in a G2-like state (Minshull et al., 1989). This showed that the synthesis of cyclins was necessary for mitotic cell cycles in cleaving *Xenopus* embryos. That cyclin translation was sufficient for the induction of mitosis in *Xenopus* egg extracts was shown by Murray and Kirschner (1989a). They used egg extracts in which the endogenous mRNA had been destroyed using RNase treatment, but which, after the addition of RNase inhibitor, were able to translate added mRNA. In the absence of added mRNA, these extracts did not synthesis any proteins and were arrested in interphase. The addition of mRNA encoding either sea urchin cyclin B, *Xenopus* cyclin B1 or *Xenopus* cyclin B2, however, was sufficient to drive multiple cell cycles in these extracts. This result was confirmed when it was shown that the addition of bacterially produced cyclin B or baculovirus produced cyclin A or cyclin B to a *Xenopus* eggs extract arrested in interphase, was able to drive RNase treated extracts into mitosis (Solomon et al., 1990; Roy et al., 1991).

Since the discovery of cyclins in marine invertebrates, cyclins have been identified in eukaryotes from yeast to man, (see Hunt, 1991). In addition, the number of different types of cyclin has risen from two to over ten, and they are now implicated in the G1→S transition, passage through START and entry into S phase, as well as more diverse functions such as the regulation of p40^MO15^ kinase (cdk7), and regulation of phosphate metabolism in *S. cerevisiae*.

Probably the set of cyclins with the best defined roles in the cell cycle are those in the budding yeast, *S. cerevisiae*.

**Regulation of the *S. cerevisiae* cell cycle by one cdk but multiple cyclins**

**CDC28**

In the budding yeast, *S. cerevisiae*, only one type of cyclin dependent kinase that acts to regulate the cell cycle has been isolated. As described above, p34^CDC28^ was initially isolated as a mutant that blocked the cell cycle at START (Hartwell et al., 1970; Hereford and Hartwell, 1974) and has subsequently been shown to be required for both START and the entry into M phase (Piggott et al., 1982; Reed and Wittenberg, 1990; Surana et al., 1991). It can associate with, and is active in conjunction with, a wide variety of cyclin subunits, and the distinct kinases thus formed carry out different roles in the cell cycle.

**CLN1, CLN2 and CLN3**

The first cyclin to be identified in *S. cerevisiae* was originally called WHI1 or DAF1, but is now known as CLN3. *WHI1-1* and *DAF1-1* were dominant mutations that allowed cells to divide at abnormally small cell sizes by shortening
the G1 phase of the cell cycle. \textit{WHI1-1} and \textit{DAFI-1} cells were also unable to arrest the cell cycle in response to \(\alpha\)-factor (Cross, 1988; Nash et al., 1988). Deletion of the \textit{DAFI} gene showed that it was not essential, although cells harbouring the deletion were larger than their wild-type counterparts (Cross, 1988).

Two more cyclin-like proteins involved in G1 control in \textit{S. cerevisiae} were isolated by their ability to rescue a temperature sensitive mutation of p34\textit{CDC28} (Hadwiger et al., 1989). The proteins encoded by \textit{CLN1} and \textit{CLN2} were closely related to each other, and a region of about 100 amino acids near their N-termini showed small, but significant homology to the region of similarity between cyclin proteins, known as the cyclin box. Deletion of either \textit{CLN1} or \textit{CLN2} on its own gave no observable phenotype, but deletion of both genes at once produced cells that grew very slowly and were large and misshapen. Moreover, a dominant mutation of \textit{CLN2}, gave rise to cells that were very small, and advanced the G1\(\rightarrow\)S transition. These cells were also impaired in their ability to arrest the cell cycle in G1 in response to external signals (Hadwiger et al., 1989). These results suggested that \textit{CLN1} and \textit{CLN2} were involved in G1 control of the cell cycle in \textit{S. cerevisiae}. This was confirmed when it was shown that deletion of \textit{CLN1}, \textit{CLN2} and \textit{CLN3} (\textit{WHI1/DAFI}) at the same time was lethal for the budding yeast, and caused the cells to arrest in G1 (Richardson et al., 1989; Cross, 1990).

The abundance of \textit{CLN1} and \textit{CLN2} mRNA varies through the cell cycle, peaking as cells pass through START and declining dramatically as cells enter S phase. The transcripts also decrease in abundance in response to mating pheromone (Wittenberg et al., 1990). The cell cycle regulated accumulation of \textit{CLN1} and \textit{CLN2} mRNA is mirrored by the accumulation of the proteins, whose concentrations peak in G1 and decrease rapidly thereafter. \textit{CLN1} and \textit{CLN2} transcripts are also rapidly lost following exposure of cells to mating pheromone (Wittenberg et al., 1990; Tyers et al., 1993). In contrast, the levels of both the \textit{CLN3} transcript and protein are constant through the cell cycle (Nash et al., 1988; Tyers et al., 1992; Tyers et al., 1993). All three CLN cyclins can bind to and activate p34\textit{CDC28} (Wittenberg et al., 1990; Tyers et al., 1992; Tyers et al., 1993), although the kinase activity of CLN3/p34\textit{CDC28} is normally much lower than the kinase activity of CLN1/p34\textit{CDC28} and CLN2/p34\textit{CDC28}, due to the lower concentration of CLN3 found in wild-type \textit{S. cerevisiae} cells (Tyers et al., 1993). The activity of the CLN/p34\textit{CDC28} complexes mirror the availability of the proteins; CLN1 and CLN2 associated activities oscillate through the cell cycle, whereas the CLN3 associated activity does not (Wittenberg et al., 1990; Tyers et al., 1992; Tyers et al., 1993).
The abundance of CLN mRNA has a rapid effect on CLN protein concentration because the proteins are very unstable (Richardson et al., 1989; Wittenberg et al., 1990; Tyers et al., 1992). Thus the cell cycle regulation of CLN1 and CLN2 protein concentration is controlled by the availability of the CLN1 and CLN2 transcripts. The instability of the CLN3 has been shown to be mediated by the C-terminal third of the protein, as deletion of this region lead to accumulation of the protein (Cross, 1990; Tyers et al., 1992; Cross and Blake, 1993). This explains the dominant nature of the WHI1-1 and DAF1-1 mutations, both of which encoded C-terminally truncated proteins (Cross et al., 1988; Nash et al., 1988). The C-terminal third of CLN3 is rich in PEST sequences, which have been implicated in protein instability (Rogers et al., 1986). The C-termini of CLN1 and CLN2 also contain PEST sequences, and this region is almost certainly important in the instability of both of these proteins too. In support of this are experiments showing that cells carrying a truncated form of CLN2, CLN2-1, which is missing the final 30% of its coding region, show a similar phenotype to cells harbouring the WHI1-1 and DAF1-1 mutations (Hadwiger et al., 1989).

CLN1 and CLN2 expression appears to be regulated by a positive feedback loop, as the appearance of CLN1 and CLN2 mRNAs is dependent on active p34CdC28 and at least one functional CLN gene (Cross and Tinkelenberg, 1991; Dirick and Nasmyth, 1991). It has been suggested that CLN/p34CdC28-dependent activation of CLN1 and CLN2 transcription is mediated by the transcription factors SWI4 and SWI6 (Nasmyth and Dirick, 1991; Ogas et al., 1991; Cross et al., 1994; Stuart and Wittenberg, 1994), possibly by the induction of SWI4 transcription by CLN3, as an artificial burst of CLN3 expression early in G1 has been shown to induce SWI4 mRNA production (Tyers et al., 1993).

**CLB1, CLB2, CLB3 and CLB4**

In addition to these G1 cyclins, six B-type cyclins have been identified in *S. cerevisiae*. CLB1, CLB2 and CLB4 were isolated as genes that could suppress the growth defect of cells containing an allele of *cdc28* that was defective in mitosis, but that could not suppress the growth defect of a START-defective allele of *cdc28* (Surana et al., 1991). CLB3 was isolated by PCR (Surana et al., 1991). CLB1, CLB3 and CLB4 were also cloned using degenerate oligonucleotides in PCR reactions (Ghiara et al., 1991; Fitch et al., 1992). CLB1 and CLB2 mRNA and protein are absent in G1 and early S phase, peak just before anaphase and disappear as the cells enter anaphase (Ghiara et al., 1991; Surana et al., 1991; Fitch et al., 1992; Richardson et al., 1992; Grandin and Reed, 1993). In contrast, CLB3 and CLB4 mRNA and protein begin to accumulate earlier; at the end of G1 or the beginning of S phase, rising before CLB1 and CLB2 mRNA levels, but remaining high and decreasing at the same time as that of CLB1 and CLB2 (Fitch
et al., 1992; Richardson et al., 1992; Grandin and Reed, 1993). The transcriptional activation of CLB1 and CLB2 is involved in a positive feedback loop analogous to that of the CLN1 and CLN2 cyclins in G1; CLB2/p34CDC28 kinase activates the expression of the CLB1 and CLB2 genes (Amon et al., 1993). In addition, CLB1 and CLB2 kinases inhibit transcription of the CLN1 and CLN2 genes, and this inhibition is dominant over activation by CLN kinases. This negative regulation may occur via inhibition of the SWI4 gene product, which associates with CLB2 and is a substrate for CLB2-associated p34CDC28 kinase in vitro (Amon et al., 1993). Inhibition of the activity of the CLB1-4 cyclins at the end of mitosis is likely to occur by proteolysis of the proteins, since all four contain the consensus sequence shown to be required for the destruction of cyclins A and B, the destruction box (Murray et al., 1989; Glotzer et al., 1991; Kobayashi et al., 1992; Lorca et al., 1992). This has indeed shown to be the case for CLB2 (Amon et al., 1994); whereas in embryonic systems, cyclin destruction has been shown to be confined to a short window at the end of mitosis (Hunt et al., 1992), in S. cerevisiae destruction of CLB2 continues for the duration of G1, until cyclin proteolysis is inactivated by the accumulation of CLN cyclins (Amon et al., 1994). In support of this idea, Amon et al. showed that a CLB2 protein with a mutation in its destruction box is able to accumulate in G1. CLB1-4 appear to have overlapping functions, but of the four genes, CLB2 appears to be most important, as a CLB2 deletion mutant causes a delay at the G2→M boundary, whereas all the other single deletions shown no phenotype. Double or triple CLB deletions that contain a CLB2 deletion are inviable, while other double and triple deletions are not (Fitch et al., 1992; Richardson et al., 1992). There is disagreement as to the arrest point of cells simultaneously deleted for all four CLB genes; Fitch et al. maintain that such cells arrest at the entry to M phase but with no mitotic spindle, while Richardson et al. show an arrest during S phase (Fitch et al., 1992; Richardson et al., 1992).

CLB5 and CLB6

The other two B-type cyclins that have been isolated from S. cerevisiae are CLB5 and CLB6. CLB5 was isolated as a gene that was able to rescue a triple cln deletion mutant on a low copy number plasmid (Epstein and Cross, 1992). CLB5 was independently isolated as a neighbouring gene to CLB2, and CLB6 was isolated as a neighbour of CLB1 (Schwob and Nasmyth, 1993). The concentration of both CLB5 and CLB6 mRNA peaks in late G1, at the same time as CLN2 mRNA, and disappears soon after the end of S phase. Deletion of either CLB5 or CLB6 gives viable cells, although cells deleted for CLB5 were slow to complete S phase (Epstein and Cross, 1992; Schwob and Nasmyth, 1993). A double CLB5CLB6 deletion delays the onset of S phase relative to bud
emergence, but does not show a delay in S phase (Schwob and Nasmyth, 1993). In the absence of CLN1 and CLN2 gene function, CLB5 and CLB6 become essential for entry into S-phase, as quadruple CLN1CLN2CLB5CLB6 deletion mutants arrest as large cells with unreplicated DNA. In addition, in the absence of CLB3 and CLB4, CLB5 is required for spindle formation, as cells deleted for all three genes arrest in G2 with no mitotic spindle (Schwob and Nasmyth, 1993). Thus it appears that CLB5 and CLB6 have a role in S phase, and that CLB5 has an additional role in the formation of the mitotic spindle.

**Regulation of the cell cycle of S. cerevisiae**

Regulation of the cell cycle in *S. cerevisiae*, as it is currently understood, can thus be summarised as follows, and as shown in figure 1.2:

CLN1 and CLN2 proteins accumulate in G1 as their expression is no longer repressed by CLB2/p34CDC28, and once their accumulation is initiated by CLN3/p34CDC28, CLN1/p34CDC28 and CLN2/p34CDC28 can activate their own transcription. At the same time CLB5 and CLB6 accumulate, and between them CLN1, CLN2, CLN3, CLB5 and CLB6, probably all associated with p34CDC28, initiate and carry out S-phase, possibly with help from CLB3/p34CDC28 and CLB4/p34CDC28, which accumulate slightly later in the cell cycle. Once CLB1/p34CDC28, CLB2/p34CDC28, CLB3/p34CDC28 and CLB4/p34CDC28 start to accumulate, the expression of CLN1 and CLN2 is inactivated and the expression of CLB1 and CLB2 is activated further by CLB2/p34CDC28. CLB1/p34CDC28 and CLB2/p34CDC28 catalyse the entry into and progression through M-phase, with help from CLB3, CLB4 and CLB5, which are required to form the mitotic spindle. At the end of mitosis, CLB destruction is triggered and continues into G1, until the proteolytic machinery is inactivated by the accumulation of CLN kinases, which are now synthesised as CLB2/p34CDC28, which represses their expression, is inactive. And so the cycle begins again.

**Other cyclin-like proteins in S. cerevisiae**

Ccl1

Kin28 is an essential serine-threonine kinase in *S. cerevisiae* that shows 37% homology to p34CDC28. The *ccl1* gene was isolated as a high copy number suppressor of a kin28ts mutation (Valay *et al.*, 1993). It encodes a protein of 393 amino acids that shows 22% identity with human and *Drosophila* cyclin C and 34% identity with the fission yeast msc2 gene product. CCL1 mRNA levels remain constant through the cell cycle. Deletion of the *ccl1* gene from *S. cerevisiae* gives inviable cells, indicating that it is an essential gene. As yet its function is unknown.
Figure 1.2 Control of the cell cycle in *S. cerevisiae* by many different cyclins
A schematic representation of the functions of the cyclins with known roles in the regulation of the cell cycle in the budding yeast, *S. cerevisiae*. For details, see text.
HCS26 and ORFD

Two other cyclin-like proteins have been identified in *S. cerevisiae*, HCS26 and ORFD. *HCS26* was identified, with *CLN1* and *CLN2*, as a high copy number suppressor of a *SWI4* deficient cell (Ogas *et al.*, 1991), and *ORFD* was cloned as a neighbour of the cell cycle gene *CDC48* (Fröhlich *et al.*, 1991). *HCS26* and *ORFD* transcription, like that of *CLN1* and *CLN2*, is dependent on *CLN* cyclin activation (Tyers *et al.*, 1993), but the function of these two cyclin-like proteins is as yet unknown.

Cyclins in other organisms

Cyclin A

Despite being one of the first cyclins to be discovered, the role of cyclin A in the cell cycle is still far from clear. The injection of cyclin A mRNA is able to promote the maturation of *Xenopus* oocytes to eggs (Swenson *et al.*, 1986; Kobayashi *et al.*, 1992; this thesis, Chapter 3). *Xenopus* oocytes already contain B-type cyclins which are bound to p34^cdc2^, although the complexes are inactive (Gautier and Maller, 1991; Kobayashi *et al.*, 1991; Ruderman *et al.*, 1991). Thus newly synthesised cyclin A is able to bind to p34^cdc2^, and as it is not inactivated by phosphorylation on tyrosine 15 (Clarke *et al.*, 1992), can trigger activation of the endogenous cyclin/p34^cdc2^, probably by activating cdc25. It has been suggested, however, that cyclin A/p34^cdc2^ kinase is unable to activate cdc25 (Hoffmann *et al.*, 1993). If this is the case, then the mechanism of activation of pre-MPF by cyclin A is less clear.

In cleaving frog and clam embryos cyclin A appears to be mainly associated with p34^cdc2^, In these cells cyclin A kinase activity peaks at mitosis, slightly earlier than the kinase associated with cyclin B (Minshull *et al.*, 1990; Hunt *et al.*, 1992), probably because cyclin A-bound p34^cdc2^ is not subject to the same regulatory phosphorylations as cyclin B-bound p34^cdc2^ (see above). Cyclin A is able induce clam or *Xenopus* egg extracts to enter mitosis in the absence of B-type cyclins (Luca *et al.*, 1991; Lorca *et al.*, 1992) and the synthesis of cyclin A is sufficient to overcome a G2→M block in cyclin A and B deficient *Drosophila* embryos (Knoblich and Lehner, 1993). Furthermore, the synthesis of cyclin A alone is sufficient to maintain the metaphase state of *Patella* oocytes (van Loon *et al.*, 1991). Thus cyclin A clearly has a role in M phase, at least in embryos. Cyclin A has also been shown to play a part in mitosis in somatic human cells, as entry into mitosis can be blocked by microinjection of anti-cyclin A antibodies into cells progressing through S phase (Pagano *et al.*, 1992). It is not entirely clear, however, whether in this situation cyclin A was bound to p34^cdc2^ or p33^cdk2^.  

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In somatic cells cyclin A is associated with p33^{cdk2} (Pines and Hunter, 1990; Tsai et al., 1991; Elledge et al., 1992; Rosenblatt et al., 1992), although it can also be found bound to p34^{cdc2} (Pagano et al., 1992). It is not entirely clear whether the association of cyclin A with these two kinase subunits changes through the cell cycle. In somatic cells cyclin A is thought to be involved in DNA replication, since the microinjection of anti-cyclin A antibodies into mammalian cells in G1 inhibited DNA synthesis (Girard et al., 1991; Pagano et al., 1992), as did the microinjection of anti-sense cDNA constructs for cyclin A (Girard et al., 1991; Zindy et al., 1992). In addition, cyclin A and p33^{cdk2}, but not cyclin B or p34^{cdc2}, were found to localise specifically at subnuclear sites of DNA replication (Cardoso et al., 1993). Cyclin A thus appears to play a role in both S and M phase. Probably cyclin A/p34^{cdc2} complexes are involved in M phase while cyclin A/p33^{cdk2} complexes are involved in S phase, but it is not clear whether cyclin A changes cdk partner during the cell cycle, or whether there are distinct subsets of cyclin A through the cell cycle. The second scenario seems more likely in light of results showing that cyclin A and p34^{cdc2} form extremely stable complexes at all phases of the cell cycle (Kobayashi et al., 1994) and since cyclin A and p33^{cdk2} probably also form tight complexes (Hideki Kobayashi, pers. comm.).

The cyclin A/p33^{cdk2} complex has been found associated with the S phase-specific transcription factor E2F (Bandara et al., 1991; Mudryj et al., 1991; Bandara et al., 1992; Devoto et al., 1992; Lees et al., 1992; Pagano et al., 1992), although it is possible that their binding is indirect, through the retinoblastoma protein, or the retinoblastoma-related protein, p107 (Bandara et al., 1991; Ewen et al., 1992; Faha et al., 1992; Lees et al., 1992; Shirodkar et al., 1992). The function of these complexes is not clear, although a recent report showed that complex formation between cyclin A-kinase and E2F-1 resulted in the shut-off of E2F-1-dependent DNA binding function during late S phase and G2 (Krek et al., 1994). There is also evidence that cyclin A kinase can phosphorylate the retinoblastoma protein and inhibit its function (Hinds et al., 1992; Dynlacht et al., 1994).

Cyclin A localises to the nucleus in interphase and may associate with condensing chromosomes in prophase (Pines and Hunter, 1991). The nuclear localisation of cyclin A is dependent on its ability to bind to a cdk subunit (Maridor et al., 1993; this thesis, Chapter 9).

A second cyclin A protein has recently been identified in *Xenopus* (Michael Howell, pers. comm.). This cyclin A protein (cyclin A2) is more similar to the cyclin As isolated from somatic cells (such as human cyclin A) than it is to the previously identified cyclin A in *Xenopus* (cyclin A1). Cyclin A2 protein is found in *Xenopus* somatic cells, where cyclin A1 protein is not detected,
suggesting that there are different embryonic and somatic forms of cyclin A. However, both cyclin A1 and cyclin A2 proteins are present in *Xenopus* eggs and embryos up to early gastrula, during which time they are both complexed with p34^cdc2. In contrast to cyclin A2, which is present throughout embryogenesis, cyclin A1 protein is only detectable in embryos up to the early gastrula stage, when it disappears. During embryogenesis cyclin A2 becomes progressively bound to p33^cdk2, and in *Xenopus* somatic cells is mainly found associated with p33^cdk2 (Michael Howell, pers. comm.). The existence of two forms of cyclin A is not unique to *Xenopus*, as both cyclin A1 and cyclin A2 have been identified in mice (Mark Carrington, pers. comm.), suggesting that other organisms may also have two cyclin A proteins. Thus, when comparing the function of cyclin A in early embryonic cells and the function of cyclin A in somatic cells, we are neither comparing exactly the same proteins, nor the same kinds of complexes.

A-type cyclins have not been identified in either fission or budding yeast, although as A- and B-type cyclins show high homology to each other and the yeast cyclins are much more distantly related to both, it is possible that one of the budding yeast B-type cyclins is really an A-type cyclin.

**B-type cyclins**

As discussed above, B-type cyclins bind to p34^cdc2 and are required for mitosis. The deletion of the gene encoding the *S. pombe* B-type cyclin, *cdc13*, results in arrest of the cells before mitosis (Booher and Beach, 1987), and antisense ablation of the mRNAs encoding cyclins B1 and B2 in *Xenopus* egg extracts also prevents entry into mitosis (Minshull et al., 1989). Furthermore, cyclin B deficient *Drosophila* embryos show delays and abnormalities in mitosis, but only a double deficiency of cyclins A and B leads to arrest in the G2 before mitosis 15, when the cyclin protein made from maternal mRNA has run out (Knoblich and Lehner, 1993).

Despite having been known to be involved in the catalysis of mitosis for many years, the substrates of cyclin B/p34^cdc2 kinase are still not well defined. It is thought to play a role in nuclear envelope break-down in mitosis as the lamins, the main component of the nuclear lamina, are *in vitro* substrates for cyclin B/p34^cdc2 kinase, and phosphorylation of the lamins by this kinase causes lamin disassembly *in vitro* (Peter et al., 1990; Dessev et al., 1991; Peter et al., 1991). Moreover, when chicken lamin was expressed in *S. pombe*, where no endogenous lamins have been found, they could be broken down by the M phase but not the S phase activity of p34^cdc2 kinase (Enoch et al., 1991).

Mitosis is accompanied by an inhibition of intracellular transport processes, and purified MPF and recombinant cyclin B, but not A, were shown to inhibit endocytotic vesicle fusion in *Xenopus* egg extracts *in vitro* (Tuomikoski et
In mammalian cells, however, cyclin A was almost as potent an inhibitor of vesicle fusion as the endogenous H1 kinase of mitotic cytosol, which is predominantly cyclin B/p34\(^{\text{cdc2}}\) (Woodman et al., 1993). Mitotic cytosol also inhibited intra Golgi transport in vitro (Stuart et al., 1993).

The main kinase responsible for mitotic histone phosphorylation is cyclin B/p34\(^{\text{cdc2}}\) (Arion et al., 1988; Labbé et al., 1989; Langan et al., 1989). Very little is known about the mechanism of chromosome condensation, however, and it is not clear whether it is mediated by histone phosphorylation.

Both cyclin B1 and cyclin B2 are cytoplasmic proteins during interphase and undergo abrupt translocation to the nucleus at the onset of mitosis (Pines and Hunter, 1991; Gallant and Nigg, 1992). The cytoplasmic localisation of these proteins through most of the cell cycle is mediated by a cytoplasmic retention signal, and does not require p34\(^{\text{cdc2}}\) binding (Pines and Hunter, 1994).

Recently a third B-type cyclin, designated cyclin B3, has been isolated from chicken (Gallant and Nigg, 1994). Although designated a B-type cyclin on its amino acid sequence homology to other B-type cyclins and because of its pattern of expression in synchronised cells, it shows distinct similarities with cyclin A; being localised to the nucleus throughout the cell cycle and being able to bind to both p34\(^{\text{cdc2}}\) and p33\(^{\text{cdk2}}\), unlike cyclins B1 and B2. Another B-type cyclin has also been isolated from Xenopus. This cyclin is more homologous to cyclins B1 and B2 than chicken B3 is, and thus appears to be yet another B-type cyclin, cyclin B4 (Michael Howell, pers. comm.).

**Cyclin C**

Cyclin C was first isolated as a human cDNA that was able to substitute for G1 cyclin genes in *S. cerevisiae* (Lew et al., 1991). In HeLa cells cyclin C mRNA is present throughout the cell cycle, but showed 2-fold elevated levels during G1 (Lew et al., 1991). *Drosophila* cyclin C has also been identified and can rescue *cln* deficient *S. cerevisiae*. Yeast cells rescued by a plasmid constitutively expressing *Drosophila* cyclin C are unusually small, consistent with an unregulated high level of G1 cyclin function (Léopold and O’Farrell, 1991). Very little is known about cyclin C, however, and its kinase partner has yet to be identified.

**D-type cyclins**

Cyclin D1 was originally isolated as a putative oncogene, PRAD1, which was clonally rearranged with the parathyroid hormone locus in a subset of benign parathyroid tumours (Motokura et al., 1991). It was subsequently isolated as a gene that rescued a strain of *S. cerevisiae* lacking the G1 cyclins (Lew et al., 1991; Xiong et al., 1991). Cyclin D1 was also isolated in a screen for genes that
were induced by the growth factor, colony stimulating factor 1, in mouse macrophages, and was initially called CYC1. Subsequent screens using probes made from conserved fragments of the gene isolated two more related genes, CYC2 and CYC3, now known as cyclins D2 and D3 (Matsushime et al., 1991). D-type cyclins appear to bind to a number of cdk subunits, including cdk2, cdk4 (PSK-J3), cdk5 and cdk6 (PLSTIRE) (Matsushime et al., 1992; Xiong et al., 1992; Dulic et al., 1993; Bates et al., 1994; Meyerson and Harlow, 1994), although the interactions between D-type cyclins and cdks may have different specificities in different cells (Bates et al., 1994).

In human lung fibroblasts cyclin D1 protein is nuclear and its levels increase as quiescent cells progress into G1. In S phase, the accumulation of the protein decreases and it disappears from the nucleus. Microinjection of antisense plasmids or antibodies against cyclin D1 in early G1 caused the cells to arrest prior to S phase, but injection into cells in late G1 did not cause arrest, in contrast to anti-cyclin A antibodies (Baldin et al., 1993; Quelle et al., 1993). Furthermore, overexpression of mouse cyclin D1 in rodent fibroblasts increased their rates of G0→S and G1→S transit by several hours, leading to an equivalent contraction of their mean cell generation times. Such cells manifested a reduced serum requirement for growth and were smaller in size than their normal counterparts (Quelle et al., 1993). Thus cyclin D1 appears to be required for progression through G1, and acts at an earlier point in the cell cycle than cyclin A. Overexpression of cyclin D2 also shortened the G0→S interval in rodent fibroblasts and reduced their serum dependency, but did not alter cell size significantly (Quelle et al., 1993).

All three D-type cyclins have been reported to be able to bind to and phosphorylate the retinoblastoma gene product (Matsushime et al., 1992; Dowdy et al., 1993; Ewen et al., 1993; Kato et al., 1993; Meyerson and Harlow, 1994), although cyclins D2 and D3 may bind with more affinity than cyclin D1. This may be because cyclin D1 appears to bind better to underphosphorylated retinoblastoma protein than to the hyperphosphorylated form (Dowdy et al., 1993). Whether phosphorylation of the retinoblastoma protein by D-type cyclins is physiologically important is not yet clear, but it has been demonstrated cyclin D2 expression is able to reverse growth suppression in cells expressing the retinoblastoma protein, apparently by inducing overt retinoblastoma phosphorylation (Ewen et al., 1993). There is also evidence that cyclin D1 expression is regulated by the retinoblastoma protein, raising the possibility of a regulatory loop between cyclin D1 and the retinoblastoma protein (Müller et al., 1994).
**Cyclin E**

The isolation of cyclin E was reported simultaneously by two groups, who had isolated it as a gene that could substitute for the G1 cyclins in *S. cerevisiae* (Koff *et al.*, 1991; Lew *et al.*, 1991). Cyclin E has been shown to be able to bind to both p34^cd2^ and p33^cdk2^, but p33^cdk2^ seems to be its main kinase subunit (Koff *et al.*, 1991; Dulic *et al.*, 1992; Koff *et al.*, 1992; Knoblich *et al.*, 1994). The activity of cyclin E-associated kinase, the abundance of cyclin E itself and the abundance of the cyclin E/p33^cdk2^ complex all increase during G1, are maximal in late G1, close to the G1→S border, and decline through S phase and G2 (Dulic *et al.*, 1992; Koff *et al.*, 1992). This suggests that the cyclin E/p33^cdk2^ kinase functions during the G1 phase of the cell cycle. Consistent with this are experiments carried out by Tsai *et al.*, who showed that pre-clearing lysates that had been made from fibroblasts 15 hours after serum stimulation with anti-cyclin E antibodies depleted the extract of 90% of its p33^cdk2^ associated kinase. They then showed that p33^cdk2^ antibodies were able to block entry into S phase (Tsai *et al.*, 1993). In addition, overexpression of human cyclin E in mammalian fibroblasts was found to shorten the duration of G1, to decrease cell size and to diminish the serum requirement for the G1→S transition (Ohtsubo and Roberts, 1993). Moreover, *Drosophila* embryos lacking the cyclin E gene arrest either before or during S phase after division 16, when protein made from the maternal cyclin E mRNA runs out. Ectopic expression of cyclin E after division 16 in wild-type dorsal epidermal cells of *Drosophila* embryos also caused them to enter S phase and progress through another cell cycle. These cells usually exit from the mitotic cycle at this stage (Knoblich *et al.*, 1994). Thus cyclin E appears to be required during G1 for the entry into S phase.

The cyclin E/p33^cdk2^ complex has been shown to bind to the transcription factor E2F (Lees *et al.*, 1992; Dynlacht *et al.*, 1994), although it does not appear to phosphorylate it (Dynlacht *et al.*, 1994). In contrast, cyclin E/p33^cdk2^ kinase can phosphorylate the retinoblastoma protein, and this activity overcomes retinoblastoma protein-mediated suppression of cell proliferation (Hinds *et al.*, 1992; Dynlacht *et al.*, 1994).

**Cyclin F**

The isolation of cyclin F has not yet been published, but it has been isolated from several organisms, including humans and *Xenopus* (Steve Elledge and Michael Howell, pers. comm.). The kinase subunit to which it binds is not known.

**Cyclin G**

Cyclin G was isolated during a screen for new src kinase family members from rat (Tamura *et al.*, 1993). It shows greatest homology to the fission yeast cyclin,
Cigl, and at its C-terminus contains a sequence similar to the tyrosine phosphorylation site of avian erbB and human EGF-receptor, suggesting that it might undergo modification by a tyrosine kinase. Cyclin G mRNA does not show any cell cycle dependency (Tamura et al., 1993).

**Cyclin H**

Cyclin H has recently been identified as the activating subunit of the p40<sub>MO15</sub> (cdk7) kinase subunit (Fisher and Morgan, 1994). The cyclin H gene encodes a protein of 323 amino acids with a predicted molecular weight of 37.6 kDa, with closest homology to the fission yeast cyclin mcs2 and the budding yeast cyclin CCL1 (identities of 24% and 23% respectively). Its closest mammalian homologue is human cyclin C, with an identity of 19%.

cdc13

The cdc13 gene was first identified as a gene required for cell cycle progression in fission yeast. At the non-permissive temperature, cdc13-117 cells arrested during mitosis (Nurse et al., 1976; Nasmyth and Nurse, 1981). This gene was later identified as an allele-specific suppressor of cdc2 that was required for the G2, but not the G1, function of cdc2 (Booher and Beach, 1987). Once it had been cloned (Booher and Beach, 1988; Hagan et al., 1988), the cdc13 gene product was recognised to be a homologue of B-type cyclins (Goebel and Byers, 1988; Solomon et al., 1988) and was shown to interact with p34<sup>cdc2</sup> to form an active kinase, whose function was required to initiate M phase in S. pombe (Booher et al., 1989; Moreno et al., 1989).

cig1

The fission yeast cyclin homologue, cig1, was identified during a search for B-type cyclins in S. pombe (Bueno et al., 1991). It was identified by PCR, using oligonucleotides with sequences deduced from two highly conserved regions in the cyclin box of B-type cyclins. The predicted protein sequence of cig1 does indeed show homology to B-type cyclins. Constitutive overexpression of cig1<sup>+</sup> in S. pombe was lethal, causing cessation of growth and arrest in G1 (Bueno et al., 1991), however, the deletion of cig1 had no observable effect on cell viability or progression through the cell cycle (Bueno et al., 1991; Bueno et al., 1993; Forsburg and Nurse, 1994). cig1<sup>+</sup> expression was unable to rescue a strain of S. cerevisiae that lack the G1 (CLN) cyclins (Bueno et al., 1991). The role of this cyclin in the cell cycle is thus unclear.
**cig2/cycl7**

A third B-type cyclin from fission yeast was identified as a gene function that could rescue G1 cyclin-deficient *S. cerevisiae* (Bueno and Russell, 1993). The disruption of *cig2* in *S. pombe* delays the onset of mitosis, and a *cig2* null allele exhibits synthetic lethal interactions with *cdc25* and *cdc2* mutations. However, the mitotic phenotypes caused by the disruption of *cig2* are not reversed by increased production of *cdc13*, and a *cdc13* mutation is not rescued by increased production of *cig2*. This suggests that *cig2*, like *cdc13*, interacts with p34cdc2, but that the two proteins have distinct, non-overlapping functions.

Obara-Ishihara and Okayama recently identified a B-type cyclin an extragenic suppressor of a *patl* mutation in *S. pombe* (Obara-Ishihara and Okayama, 1994). Inactivation of the *patl* kinase causes the onset of meiosis, regardless of ploidy, nutritional conditions and availability of mating partners (Nurse, 1985). They called this cyclin *cycl17*. Despite a high homology to *cdc13*, *cycl17* had no detectable G1 or G2 function. However, the *cycl17* null mutation was greatly enhanced in conjugation, and overexpression of *cycl17* inhibited conjugation. Consistent with a role in conjugation, *cycl17* mRNA concentration was low in the mitotic cycle and markedly induced during conjugation.

Obara-Ishihara and Okayama report that a sequence comparison between *cycl17*+ and *cig2*+ show that they are very likely to be identical. However, they were unable to confirm previous claims that *cycl17/cig2* was synthetically lethal with *cdc2-3w* or that there was a G2→M delay in *cycl17/cig2* disrupted mutants. Thus the function of *cig2/cycl17* remains unclear.

**puc1**

*puc1* was isolated as an *S. pombe* gene that conferred increased α-factor resistance on an *S. cerevisiae* strain deficient in *CLN3* function (Forsburg and Nurse, 1991). Although the predicted protein sequence of the *puc1* gene product shows homology to the G1 family of cyclins, neither deletion of *puc1* nor overexpression of the *puc1* protein in *S. pombe* had any effect on the length of G1 or at the G1→S transition (Forsburg and Nurse, 1994). In contrast, *S. pombe* cells constitutively overexpressing the *puc1* protein were delayed in G2, displaying an elongated phenotype. This phenotype was accentuated in cells carrying the *cdc13-117* mutation (Forsburg and Nurse, 1991). In addition, diploid strains of *S. pombe* that were deleted for *puc1* showed a small acceleration of meiosis, and modest overexpression of *puc1*+ on a high copy plasmid delayed both conjugation and meiosis (Forsburg and Nurse, 1994). Thus *puc1*+ may contribute to negative regulation of the timing of sexual development in fission yeast, and function at the transition between cycling and non-cycling cells.
**mcs2**

The *mcs2* gene was identified as an extragenic suppressor of mitotic catastrophe and allele-specific interactions with *cdc2-3w* mutations (Molz *et al.*, 1989). The *mcs2* gene has now been cloned and shown to encode a new cyclin that shows the greatest homology to human cyclin C, (Molz and Beach, 1993), *S. cerevisiae* CCL1 (Valay *et al.*, 1993) and cyclin H (Fisher and Morgan, 1994). It is an essential gene and although *mcs2* cells did not arrest at a uniform point in the cell cycle, they displayed characteristics of cells arrested in mitosis. Mcs2 protein levels are constant through the cell cycle and the protein is localised to the nucleus at all points. Although *mcs2* is not associated with histone H1 kinase activity, myelin basic protein kinase activity was detected in *mcs2* immunoprecipitates, and this activity is constant through the cell cycle. A kinase with which *mcs2* interacts was isolated by suppressor analysis. This kinase, designated *csk1*, shows 30-33% homology to the cdk family across its kinase domain, and accounts for a significant proportion of the kinase activity associated with *mcs2*. The *csk1* gene is not essential, and may encode either an *mcs2*-associated protein kinase or an upstream activator of the *mcs2*-associated kinase (Molz and Beach, 1993). Its similarity to cyclin H, the activating subunit of p40<sub>MO15</sub>, the kinase that phosphorylates p34<sub>cdc2</sub> and p33<sub>cdk2</sub> on threonine 161/160, suggests that it may play a similar role in *S. cerevisiae*.

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**Regions of homology within cyclin proteins**

The sequence homology between different members of the cyclin family is not extensive and is confined to a region of about 100 amino acids, known as the cyclin box. Outside this region there is little overall homology, although related cyclins, such as cyclins A and B, are conserved through more of their length. Mitotic cyclins also have a small region of homology within their N-termini which is known as the destruction box (see below), although the spacing of the sequence with respect to the start of the cyclin box is somewhat variable. Some G1 cyclins have PEST-rich sequences in their C-termini which are thought to confer instability on the proteins (Rogers *et al.*, 1986). As previously discussed, this has been shown to be the case for CLN2 and CLN3 from *S. cerevisiae*. Schematic representations of all the cyclins known to be involved in the cell cycle, and cyclins F and G, are shown in figure 1.3. The cyclin box is shown in white, the destruction box in pale grey and PEST rich sequences are shown in dark grey.
Figure 1.3  The family of cyclin proteins
Schematic representations of the cyclins known to be involved in regulation of the cell cycle, and cyclins F and G, are shown. The cyclin boxes (□) are aligned. Also shown are the positions of the destruction box (■) and PEST rich sequences (■). The diagrams are to scale.
Mitotic cyclin destruction

The cyclin destruction box

Mitotic cyclins were first identified in cleaving eggs because of their abrupt destruction at the end of mitosis, just before the metaphase to anaphase transition (Evans et al., 1983). Mitotic cyclin destruction requires the presence of a short conserved region of about 10 amino acids in the N-terminus of the protein. This region is known as the destruction box. The sequence of A- and B-type destruction boxes is discussed in Chapter 7 (see figure 7.1). All A- and B-type cyclins have a destruction box, although it should be noted that in CLB6 from S. cerevisiae, the arginine residue that is absolutely conserved in the destruction boxes of all other mitotic cyclins, is replaced by a lysine residue (Schwob and Nasmyth, 1993). Mutation of conserved residues within the destruction box, or deletion of the box, causes A- and B-type cyclins to evade proteolysis (Murray et al., 1989; Glotzer et al., 1991; Luca et al., 1991; Kobayashi et al., 1992; Lorca et al., 1992; Amon et al., 1994; this thesis, Chapter 7).

Properties of cyclin destruction

There is persuasive evidence that cyclin destruction is necessary for the inactivation of MPF and the progression of cells into interphase. Protease resistant forms of A- and B-type cyclins can prevent the inactivation of MPF and exit from mitosis in frog and clam egg extracts (Murray et al., 1989; Luca et al., 1991), and the microinjection of indestructible cyclin B also arrested fertilised Xenopus eggs in mitosis (Murray et al., 1989). Moreover, expression of a protease resistant form of chicken cyclin B2 in HeLa cells caused them to arrest in a pseudomitotic state (Gallant and Nigg, 1992) and the expression of an indestructible form of CLB1 in S. cerevisiae caused a mitotic arrest phenotype (Ghiara et al., 1991). Cyclin destruction may be necessary for the exit from mitosis, but it is probably not required for the metaphase to anaphase transition, as in both budding yeast and frog egg extract, anaphase can occur in the presence of active cyclin B/p34CDC28/cdc2 kinase (Holloway et al., 1993; Surana et al., 1993). However, the separation of sister chromatids requires the cyclin proteolytic machinery to be active (Holloway et al., 1993), suggesting that the proteolysis of a protein other than cyclin B is needed for anaphase to occur, and that degradation of this protein takes place via the same pathway as cyclin destruction. It is possible that this ‘other’ protein is cyclin A, as BHK cells in which the degradation of both cyclin A and cyclin B is inhibited are arrested in metaphase, whereas cells in which the degradation of only cyclin B is inhibited are arrested after anaphase (P. R. Andreassen, F. Lacroix, R. Fotedar and Margolis, R. L., pers. comm.).
There is evidence that the activation of p34\(^{cd}c2\) is necessary to trigger cyclin proteolysis in *Xenopus* egg extracts (Félix *et al.*, 1990b); this requirement may reflect the need for p34\(^{cd}c2\) kinase activation of the system that ligates ubiquitin to cyclin (Hershko *et al.*, 1994). Cyclin B-associated kinase, but not cyclin A-associated kinase, is able to trigger cyclin proteolysis (Luca *et al.*, 1991), indeed, cyclin A kinase may actually delay or even inhibit cyclin destruction (Luca *et al.*, 1991; Lorca *et al.*, 1992; this thesis, Chapter 6).

Cyclin destruction can also be inhibited by addition of the kinase inhibitor 6-dimethyl aminopurine (Félix *et al.*, 1989; Luca and Ruderman, 1989), EDTA and the depletion of ATP (Luca and Ruderman, 1989), suggesting that there is at least one ATP-dependent step needed for cyclin destruction. Conversely, ATP\(_{\gamma}S\) can inhibit cyclin destruction (see Chapter 6), suggesting that protein dephosphorylation is also important for cyclin proteolysis. The involvement of a cysteine protease in cyclin destruction has also been suggested, as the neutral cysteine protease inhibitor, N-acetylleucylleucylnorleucinal, can inhibit both cyclin A and cyclin B degradation (Sherwood *et al.*, 1993; P. R. Andreassen, F. Lacroix, R. Fotedar and R. L. Margolis, pers. comm.) This is supported by evidence showing that reagents which interfere with sulphydryl groups, such as N-ethylmaleimide, CuCl\(_2\), ZnCl\(_2\) and p-hydroxymercuribenzoic can inhibit cyclin destruction, as can the trypsin inhibitor, tosyl-lysine chloromethyl ketone, which has been suggested to block cyclin destruction by virtue of its ability to block the activity of certain cysteine proteases (Luca and Ruderman, 1989).

Cyclin destruction can be triggered by the addition of okadaic acid, a potent inhibitor of type 1 and type 2A phosphatases, to CSF-arrested *Xenopus* egg extracts or to *Xenopus* interphase egg extracts (Lorca *et al.*, 1991a; this thesis, Chapter 6). Thus the cyclin destruction machinery appears to require both protein phosphorylation and protein dephosphorylation in order to be activated.

The presence of an intact destruction box is necessary, but not sufficient for cyclin A destruction. As shown in Chapter 7 of this thesis and Stewart *et al.* (1994), cyclin A proteins must also be able to bind to p34\(^{cd}c2\) in order to be destroyed. Cyclin B2 must also be able to bind to p34\(^{cd}c2\) in order to be destroyed (van der Velden and Lohka, 1993; Stewart *et al.*, 1994), but the requirement for cyclin B1 is less clear. Some cyclin B1 proteins that cannot bind to p34\(^{cd}c2\) are destroyed with almost normal kinetics, whereas other (more subtle) mutants, that are unable to bind to p34\(^{cd}c2\) are indestructible (Stewart *et al.*, 1994). In addition, a construct known as 13-66protein A, containing residues 13-66 of sea urchin cyclin B (including the destruction box) fused to protein A, which is presumably unable to bind to p34\(^{cd}c2\), can be destroyed by the cyclin proteolytic machinery (Glotzer *et al.*, 1991; Stewart *et al.*, 1994), albeit more slowly than wild-type cyclin B (Dolores Harrison, pers. comm.). As shown in
this thesis, Chapter 8, and Stewart et al. (Stewart et al., 1994), it also appears that the conformation of its N-terminus is important for cyclin A destruction.

Although both A- and B-type cyclins require an intact destruction box for proteolysis, and mutation or deletion of this in either protein is sufficient to prevent the exit from M phase, the destruction of cyclin A normally occurs slightly earlier in the cell cycle than that of cyclin B (Luca and Ruderman, 1989; Minshull et al., 1990; Whitfield et al., 1990; Hunt et al., 1992). In addition, disruption of mitotic spindle formation with colchicine strongly retarded cyclin B destruction but had no effect on cyclin A proteolysis (Whitfield et al., 1990; Hunt et al., 1992). This, in combination with the data that shows that while cyclin A and B2 may need to be able to bind to p34cdc2 in order to be destroyed, the requirement is less stringent for cyclin B1, suggests that although the destruction of each of the mitotic cyclins may occur by the same pathway, the recognition of the proteins and the triggers for their destruction are not the same.

The involvement of ubiquitination in cyclin destruction

The enzyme(s) responsible for the recognition of cyclin and initiation of its proteolysis have not been identified, and the mechanism of their control is unknown. Two lines of evidence, however, implicate the ubiquitin system in cyclin degradation. Firstly, ubiquitinated intermediates of sea urchin cyclin B constructs were detected in Xenopus egg extracts in which cyclin proteolysis was permanently activated, but not in interphase extracts. Efficient ubiquitination was dependent on the presence of an intact destruction box, and the flux through the ubiquitinated intermediates for cyclin B constructs with a wild-type destruction box was adequate to account for the total cyclin degradation (Glotzer et al., 1991). Secondly, the addition of methylated ubiquitin, which is unable to form polyubiquitin chains, inhibited cyclin degradation in clam embryo extracts. Furthermore, the addition of excess wild-type ubiquitin to an such extract completely overcame the inhibitory effects of methylated ubiquitin on cyclin destruction (Hershko et al., 1991).

The components that ligate ubiquitin to cyclin were partially purified recently (see Hershko and Ciechanover (1992) for review on the ubiquitin pathway), and it was shown that one of them was only active in M phase extracts, and was dependent on activation by p34cdc2 (Hershko et al., 1994). These data certainly suggest that ubiquitination is necessary for cyclin degradation, but is it the trigger? Data from Andrea Klotzbücher in the laboratory, and that shown in Chapter 7 of this thesis, suggests that cyclins can be ubiquitinated in M phase before cyclin destruction is triggered, and that some cyclin A mutants that are stable when cyclin destruction is triggered are nevertheless ubiquitinated to the same level as wild-type cyclins. Thus, although cyclins may be degraded via the
ubiquitin pathway, ubiquitination is not necessarily the signal that triggers cyclin destruction.

The degradation of ubiquitin-conjugated proteins is thought to occur via the 26S proteasome complex, see Hershko and Ciechanover (1992). In both fission and budding yeast, mutations in proteins that show homology to subunits of the human and Drosophila 26S proteasome complex arrest the cell cycle in mitosis (Ghislain et al., 1993; Gordon et al., 1993). In addition, the 26S proteasome is activated at the metaphase to anaphase transition in ascidian cells (Kawahara et al., 1992). This data is consistent with a role for the 26S proteasome in cyclin destruction. However, it is not certain that the arrest of cell cycle progression in the yeast 26S proteasome mutants is due to the inability of the cells to degrade their cyclins. While budding yeast 26S proteasome mutants accumulate CLB2 and CLB3 protein (Ghislain et al., 1993), there does not appear to be such an accumulation of cdc13 in fission yeast with a defective 26S proteasome (Gordon et al., 1993). Thus, although the 26S proteasome is implicated in cyclin degradation, the evidence is far from conclusive.

Cyclin destruction in embryonic and somatic cells
In embryonic systems the cyclin destruction pathway is only active for a very brief interval at the end of mitosis; in clam embryos cyclin proteolysis is active for about 5 minutes, by which time most of the cyclin has been degraded and MPF turned off (Hunt et al., 1992). In contrast, the destruction of the CLB (B-type) cyclins S. cerevisiae continues into G1, until the accumulation of the CLN (G1 type) cyclins turns the proteolysis pathway off. Moreover, CLN cyclins are required to maintain the protease in its inactive state in G1 (Amon et al., 1994). It may be that cyclin destruction in the somatic cells of higher eukaryotes follows the same pattern.

CSF-arrest in Xenopus eggs
In vertebrates, unfertilised eggs are arrested at the metaphase of meiosis II. This appears to be due to a factor called cytostatic factor (CSF) (Masui and Markert, 1971). In amphibian eggs CSF activity appears at the time of germinal vesicle breakdown (GVBD) during oocyte maturation, remains high in the unfertilised egg, and disappears soon after fertilisation, when second meiosis is completed (Masui and Markert, 1971; Meyerhof and Masui, 1977; Meyerhof and Masui, 1979). In 1989, evidence was discovered that the c-mos oncogene is one of the essential components of CSF. Microinjection of synthetic mos mRNA into two-cell Xenopus embryos induced cleavage arrest at metaphase, and immunodepletion of endogenous p39mos caused the loss of cleavage-arresting
activity from egg cytosol (Sagata et al., 1989). Very recently it has been shown that c-mos deficient female mice show reduced fertility, apparently because their mature eggs are unable to arrest at metaphase of meiosis II (Colledge et al., 1994; Hashimoto et al., 1994). The ability of p39mos to cause CSF arrest requires its kinase activity (Okazaki et al., 1991). It has also been reported that p33cdk2 is required for CSF arrest. In Xenopus oocytes the ablation of the mRNA encoding p33cdk2 by the microinjection of antisense oligonucleotides, resulted in the absence of the metaphase block, which could be restored by the introduction of purified p33cdk2 to the oocytes (Gabrielli et al., 1993).

The fertilisation of Xenopus eggs causes a large increase in intracellular Ca2+ levels (Busa and Nuccitelli, 1985), which triggers release of the cell from CSF arrest and resumption of the cell cycle (Newport and Kirschner, 1984; Murray et al., 1989; Lorca et al., 1991b). This Ca2+ transient inactivates CSF and triggers p39mos inactivation and degradation (Watanabe et al., 1989). The inactivation of p39mos and its degradation is apparently not required for cyclin proteolysis and exit from meiotic metaphase, however, for when the kinetics of cyclin and p39mos degradation were compared, it was shown that cyclin was consistently degraded several minutes before p39mos, and the MPF activity decreased before that of the CSF (Watanabe et al., 1991). In addition, when the Ca2+ concentration is raised to the micromolar range for only 30 seconds in Xenopus egg extracts, cyclin degradation is induced but p39mos is not degraded (Lorca et al., 1991b). Thus, although p39mos is required for CSF arrest and an increase in intracellular Ca2+ causes both cyclin and p39mos proteolysis in Xenopus eggs, its degradation is not the trigger that releases cells from meiotic arrest.

Evidence has recently been found that cyclin degradation, inactivation of MPF activity and the consequent progression of cells into interphase is mediated by calmodulin-dependent protein kinase II (CaMKII). A constitutively active version of CaMKII caused cyclin degradation and MPF inactivation in Xenopus CSF-arrested egg extract in the absence of Ca2+, and the microinjection of this form of CaMKII into unfertilised eggs caused MPF inactivation and p39mos degradation (Lorca et al., 1993). Furthermore, a specific inhibitor of CaMKII was able to prevent cyclin degradation and MPF inactivation after Ca2+ addition to a Xenopus CSF-arrested egg extract, and after activation of Xenopus eggs. It is not yet clear how active CaMKII causes cyclin destruction.

**Cyclins as oncogenes**

Cancerous cells show abnormal proliferative regulation when compared to non-cancer cells. Since cyclins are intimately involved in control of the cell cycle, it
would seem possible that cyclins could be involved in cancer. Cyclins A, D and E have indeed been implicated in oncogenesis.

The integration of the hepatitis B virus into one of the introns of cyclin A in a human hepatocellular carcinoma lead to the identification of the human homologue of cyclin A (Wang et al., 1990). The integration caused the formation of a fusion protein between viral PreS2/S sequences and human cyclin A, which showed strong expression from the viral PreS2/S promoter and was stable, due to the deletion of the cyclin A N-terminus (Wang et al., 1992). Although epidemiological evidence suggests a link between the hepatitis B virus and liver carcinomas, the molecular mechanism(s) are not clear. Evidence from the case of cyclin A and others, suggests that it could act by integrating close to a gene involved in cellular proliferation and modifying its expression. However, at present the evidence is only circumstantial. It seems likely, given that there is only one report of cyclin A being involved in oncogenesis, that cyclin A was not, in fact, the cause of this human hepatocellular carcinoma.

Evidence for the involvement of cyclin D1 in oncogenesis is far stronger. Cyclin D1 was actually first identified as a candidate oncogene that was rearranged with the parathyroid hormone locus in a subset of benign parathyroid tumours (Motokura et al., 1991), and the proximity of the overexpressed cyclin D1 gene to 11q13 translocation breakpoints in B-cell tumours also strongly suggests its identity with the bcl-1 oncogene (Motokura et al., 1991; Rosenberg et al., 1991; Withers et al., 1991; Schuuring et al., 1992; Seto et al., 1992). Cyclin D1 overexpression has also been implicated in other cancers, including oesophageal cancer (Jiang et al., 1992), and breast cancer (Buckley et al., 1993; Musgrove et al., 1993; Musgrove et al., 1994; Wang et al., 1994). The coding sequence of cyclin D1 in at least some of these tumours appears to be normal, implying that cyclin D1 may be oncogenic when overexpressed, or when expressed at the wrong time (Rosenberg et al., 1993). This idea is supported by experiments showing that overexpression of cyclin D1 can contribute to cell transformation by complementing a defective adenovirus E1A oncogene in cultured primary rodent cells (Hinds et al., 1994). The roles of cyclins D2 and D3 in oncogenesis have been less well studied, but cyclin D2 has been identified as a gene whose expression is altered by the integration of proviruses in rodent T-cell leukaemias (Hanna et al., 1993), supporting a role for cyclin D2 in oncogenesis.

The argument that cyclin D can be intimately involved in tumourigenesis is further strengthened by the evidence presented above, that the cyclin D/cdk4 kinase inhibitor, p16INK is likely to be a major tumour suppressor gene, whose deletion or mutation is evident in many tumours. The involvement of p16INK in tumourigenesis suggests that other cyclin dependent kinase inhibitors may also
act as tumour suppressors, and indicates an important role for the regulation of cyclin dependent kinases in the prevention of cancer.

Cyclin E has also been implicated in oncogenesis. Human cyclin E normally migrates at around 50 kDa on polyacrylamide gels, but in all ten of the breast cancer tumour lines studied by Keyomarsi and Pardee (1993) the cyclin E protein showed deranged expression, with the cyclin E antibody recognising one, two or three cyclin E proteins running at 50, 42 and 35 kDa on Western blots. In addition, one of the lines showed an 8 fold amplification of the cyclin E gene and a 64 fold overexpression of the cyclin E mRNA (Keyomarsi and Pardee, 1993). Furthermore, using surgical material obtained from patients with various malignancies, Keyomarsi et al. showed that breast cancers, other solid tumours and malignant lymphocytes from patients with lymphatic leukaemia, showed severe quantitative and qualitative alteration in cyclin E protein production, independent of the S phase fraction of the samples (Keyomarsi et al., 1994).

This thesis
This thesis describes studies on the functional domains of cyclin A. I have investigated the regions of cyclin A that are required for the protein to bind to p34cdc2 and p33cdk2, to be destroyed efficiently at the end of M phase and to be localised to the nucleus. Unexpectedly, the three functions have turned out to be highly related; cyclin A destruction and the nuclear localisation of the protein require that it be able to bind to p34cdc2 and p33cdk2.
Chapter 2

Materials and Methods

Reagents and donated plasmid constructs

Reagents and enzymes
Reagents were purchased from BDH or Sigma, unless otherwise stated, and chemicals were of AnalR or the highest grade available.

   Enzymes for use in molecular biology were purchased from New England Biolabs, unless otherwise stated.

Oligonucleotides
Oligonucleotides were synthesised by the ICRF oligonucleotide unit.

Donated Reagents
Bovine serum albumin (BSA) and p13suc1 coupled to cyanogen bromide activated Sepharose beads were supplied by Dr Jörg Adamczewski (now CNRS, Strasbourg). Bacterially expressed protein A-bovine cyclin A (pAcycA) and protein A-bovine cyclin A missing its last 16 residues (pAcycACA16) fusion proteins were also supplied by Dr Jörg Adamczewski (Kobayashi et al., 1992), as was a bacterially expressed fusion protein between glutathione-S-transferase and human p33cdk2 (GST-cdk2). A bacterially expressed fusion protein between glutathione-S-transferase and Xenopus cyclin A2 was supplied by Dr Michael Howell (ICRF) and a bacterially expressed fusion protein between glutathione-S-transferase and Xenopus cyclin B1 was a gift from Dr Katsumi Yamashita (ICRF). Bacterially expressed, full length, non-tagged p34cdc2 was purified from inclusion bodies by Dr Jeremy Minshull (now University of California, San Francisco) and bacterially expressed Xenopus cyclin A which was missing its first 56 amino acids was provided by Dr Randy Poon (now Salk Institute, San Diego).

Donated Constructs
Frog cyclins A1 in pGEM1, B1 in pGEM1 and B2 in pGEM2 were from Dr J. Minshull (now University of California, San Francisco)(Minshull et al., 1990). The clone of cyclin B2 in pET16b was kindly provided by Dr Katsumi Yamashita.
The construct encoding NA133 (construct A, see above and figure 2.2) was constructed by Dr Mary Dasso from *Xenopus* cyclin A (see below). The Δ88-144, Δ102-158, Δ109-161 and Δ101-169 cyclin A constructs were supplied by Dr Hideki Kobayashi (Kyushu University, Japan)(Kobayashi *et al.*, 1992). The construct encoding glutathione-S-transferase fused to the N-terminus of the cdc2 gene with a C-terminal hexa-histidine tag (GST-cdc2H6) in pGEX-KG was supplied by Dr Randy Poon (now Salk Institute, San Diego), see (Poon *et al.*, 1993 and his PhD thesis). The vector containing rat CD2 under the control of the SV40 early promoter and mouse cyclin B1 under the control of the human cytomegalovirus promoter (pCD2/CMV-mouse cyclin B1, see figure 2.5), was a gift from Dr Chris Norbury (IMM, Oxford).

**Antibodies**

Protein A-purified monoclonal anti-c-myc monoclonal antibody, 9E10 (Evan *et al.*, 1986), was supplied by the ICRF hybridoma unit. The anti-p34cdc2 monoclonal antibody, A17, was raised against the C-terminal two-thirds of *Xenopus* p34cdc2 and was supplied by Dr Julian Gannon (ICRF). In general, hybridoma culture supernatant was utilised as a source of this antibody, but on occasions protein A purified antibody was used. The anti-human retinoblastoma protein monoclonal antibody, IF8 (Bartek *et al.*, 1992), was also supplied by Dr Julian Gannon (ICRF). Hybridoma culture supernatant was utilised as a source of this antibody. The polyclonal anti-cyclin A antibody was raised in rabbits by Dr Jeremy Minshull (now University of California, San Francisco) against the largest Sau3A fragment of *Xenopus* cyclin A1 (Minshull *et al.*, 1990). The anti-*Xenopus* -cyclin A monoclonal antibody, XLA1-3, was prepared by Dolores Harrison (ICRF) and recognises an epitope lying between residues 88 and 106 of *Xenopus* cyclin A1 (D. Harrison and T. Hunt, unpublished data). Hybridoma culture supernatant was utilised as a source of this antibody. Protein A purified monoclonal anti-rat CD2 antibody, OX34, was supplied by the ICRF hybridoma unit and FITC-conjugated OX34 was provided by Dr Chris Norbury (now IMM, Oxford).

**Buffers, solutions and media**

Commonly used buffers and media are detailed here, more specialised ones will be described when their use is described.
<table>
<thead>
<tr>
<th>Name</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer A</td>
<td>20 mM Tris-Cl, pH 8.0; 20 mM NaCl; 0.1 mM EDTA; 1 mM DTT</td>
</tr>
<tr>
<td>Bead Buffer</td>
<td>50 mM Tris-Cl, pH 7.4; 5 mM NaF; 250 mM NaCl; 5 mM EDTA; 5 mM EGTA; 0.1% Nonidet P-40; 10 µg/ml leupeptin; 10 µg/ml soya bean trypsin inhibitor; 10 µM benzamidine</td>
</tr>
<tr>
<td>Cicirelli Buffer</td>
<td>50 mM β-glycerophosphate; 7 mM NaF; 0.3 mM EDTA; 15 mM MgCl₂; 2 mM DTT; pH 7.3</td>
</tr>
<tr>
<td>PBSA</td>
<td>170 mM NaCl; 3 mM KCl; 10 mM Na₂HPO₄; 2 mM KH₂PO₄</td>
</tr>
<tr>
<td>pH 1.9 buffer</td>
<td>2.2% (v/v) formic acid; 7.8% (v/v) acetic acid</td>
</tr>
<tr>
<td>pH 3.5 buffer</td>
<td>5% (v/v) acetic acid 0.5% (v/v) pyridine</td>
</tr>
<tr>
<td>pH 4.72 buffer</td>
<td>10% (v/v) n-butanol; 5% (v/v) pyridine; 5% (v/v) acetic acid</td>
</tr>
<tr>
<td>Phospho-chromatography buffer</td>
<td>37.5% (v/v) n-butanol; 25% (v/v) pyridine; 7.5% (v/v) acetic acid</td>
</tr>
<tr>
<td>SDS sample buffer</td>
<td>2% (w/v) SDS; 80 mM Tris-Cl, pH 6.8; 10 % (v/v) glycerol; 0.002% (w/v) bromophenol blue. 50 µl/ml 2-mercaptoethanol added just before use.</td>
</tr>
<tr>
<td>TE buffer</td>
<td>10 mM Tris-Cl, pH 8.0; 1 mM EDTA</td>
</tr>
<tr>
<td>TAE buffer</td>
<td>40 mM Tris Base; 40 mM acetic acid; 2 mM EDTA</td>
</tr>
<tr>
<td>GTE</td>
<td>50 mM glucose; 25 mM Tris-Cl, pH 8.0; 10 mM EDTA</td>
</tr>
<tr>
<td>SDS/NaOH</td>
<td>1% (w/v) SDS; 0.2 M NaOH</td>
</tr>
<tr>
<td>KOAc</td>
<td>3 M potassium acetate; 2 M acetic acid</td>
</tr>
<tr>
<td>IMAC5</td>
<td>20 mM Tris-Cl, pH 8.0; 0.5 M NaCl; 5 mM imidazole</td>
</tr>
<tr>
<td>IMAC25</td>
<td>20 mM Tris-Cl, pH 8.0; 0.5 M NaCl; 25 mM imidazole</td>
</tr>
<tr>
<td>IMAC50</td>
<td>20 mM Tris-Cl, pH 8.0; 0.5 M NaCl; 50 mM imidazole</td>
</tr>
<tr>
<td>IMAC150</td>
<td>20 mM Tris-Cl, pH 8.0; 0.5 M NaCl; 150 mM imidazole</td>
</tr>
<tr>
<td>IMAC25/Tx100</td>
<td>20 mM Tris-Cl, pH 8.0; 1 M NaCl; 25 mM imidazole; 0.5% (v/v) TritonX-100; 0.5% (v/v) Tween-20</td>
</tr>
<tr>
<td>2xTY</td>
<td>10 g Bactotryptone; 10 g Yeast Extract; 5 g NaCl per litre</td>
</tr>
<tr>
<td>L-broth</td>
<td>10 g Bactotryptone; 5 g Yeast Extract; 10 g NaCl per litre</td>
</tr>
</tbody>
</table>
Figure 2.1  The sequence of wild-type *Xenopus* cyclin A in pGEMl
The complete sequence of wild-type *Xenopus* cyclin A, including the entire 3' untranslated region, in pGEMl. The amino acid sequence of cyclin A, in one letter code, is shown above the nucleotide sequence. The recognition sites for four of the restriction enzymes mentioned in the text are shown in bold type and underlined.
Construction of Xenopus cyclin A mutants

c-myc tagged cyclin A

An NcoI restriction enzyme site was added to a full-length clone of Xenopus cyclin A1 in pGEM1 (clone XL4) (Minshull et al., 1990) using the polymerase chain reaction (PCR). A mutagenic 5' primer (oligonucleotide 1, see Appendix 1) was designed to introduce an NcoI restriction enzyme site just before the second methionine residue of cyclin A, the fifth residue of the protein (see figure 2.1). Oligonucleotide 1 was used to amplify the cyclin A DNA by PCR, in conjunction with a non-mutagenic 3' primer (oligonucleotide 2, see Appendix 1) that was complementary to the DNA sequence just downstream of the end of the coding region of cyclin A1. The fragment of DNA thus generated was digested with NcoI and NsiI (for the position of the NsiI site in wild-type cyclin A, see figure 2.1) and inserted between the NcoI and NsiI sites of construct A (for a map of the unique restriction enzyme sites in this construct, see figure 2.2A). Construct A, from Mary Dasso, is the HindIII/EcoRI DNA fragment of cyclin A1 in a pGEM2 based plasmid which contains the translational leader of the influenza virus NS protein (Dasso and Jackson, 1989). In order to maintain the correct reading frame, a synthetic oligonucleotide had been ligated between the NS transcriptional leader and the start of the cyclin fragment in this construct. The ligation of the cyclin A NcoI/NsiI fragment into construct A re-created the full-length cyclin A1 coding sequence and 3' untranslated region (UTR), except at the extreme N-terminus of the construct, where the DNA encoding for the first four residues of the cyclin A protein had been deleted by digesting the PCR-amplified cyclin A DNA fragment with NcoI (see above). This deleted fragment of DNA was replaced by DNA encoding for three different residues (M D P), which were 5' to the NcoI restriction enzyme site in construct A. This construct was digested with NcoI and BsmI and in place of the small fragment of DNA that this cuts out from the N-terminus of the clone (the DNA encoding residues 5-21 of wild-type cyclin A, see figure 2.1) was ligated a double stranded oligonucleotide (made by annealing oligonucleotides 3 and 4 together, see Appendix 1) that encoded the 12 amino acid epitope recognised by the anti-c-myc antibody, 9E10. Unfortunately, this construct (construct B) contains two HindIII restriction enzyme sites, one in the cyclin A clone and one in the cloning cassette (see figure 2.2B). In order to remove the HindIII restriction enzyme site from the cloning cassette, construct A was digested with BamHI and NsiI (see figure 2.2A) to remove one HindIII restriction enzyme site, the underhanging DNA at the restriction enzyme sites was filled in using deoxynucleotides and the large fragment of E. coli DNA polymerase 1 (Klenow fragment) and the two ends ligated back together again. The resulting DNA plasmid was digested with HindIII, to cut at the remaining HindIII site, (in the cloning cassette), the underhanging DNA on each side of the
Construct A
4232 base pairs

Construct B
4601 base pairs
Figure 2.2  Plasmid maps of constructs A, B, C and D
The plasmid maps of constructs A, B, C and D, discussed in the text, are shown. For details of each construct, see text. Only unique restriction enzyme sites are shown, with the exception of the two HindIII sites in construct B. These sites are indicated with asterisks. The numbering of the restriction enzyme sites refers to the number of nucleotides (in a clockwise direction) from the start of the T7 RNA polymerase promoter. The open reading frames (ORF) and 3' untranslated regions (3'UTR) for 3 of the constructs are indicated.
restriction enzyme site were filled in using deoxynucleotides and Klenow, and the
two ends were ligated back together again (construct C, see figure 2.2C). The
BstEII/EcoRI fragment of construct B (the entire coding sequence of cyclin A,
plus most of the cloning cassette) was then placed into BstEII/EcoRI digested
construct C, to form construct D (figure 2.2D). To add a T7 RNA polymerase
terminator sequence to construct D, it was digested with EcoRI and NaeI (see
figure 2.2D) and in place of the fragment of DNA that this removed, was ligated
the EcoRI/EcoRV DNA fragment from the construct pET8/pGEX which
contained the T7 RNA polymerase terminator sequence. This construct was
named c-myc cyclin A (see figure 2.3).

Figure 2.3  Plasmid map of c-myc cyclin A
The plasmid map of c-myc cyclin A is shown. For details of its construction see
text. Unique restriction enzyme sites are shown. The numbering of the
restriction enzyme sites refers to the number of nucleotides (in a clockwise
direction) from the start of the T7 RNA polymerase promoter. The open reading
frame (ORF) and 3' untranslated region (3'UTR) for this construct are indicated.
C-terminal deletions of cyclin A

The C-terminal deletions of cyclin A were constructed using c-myc cyclin A. Mutagenic oligonucleotides were designed such that when they were used as 3' primers in a PCR they amplified cyclin A DNA with stop codons in the desired places in the sequence, to produce constructs that encoded proteins with deletions of 14, 24, 50, 79, 97 and 139 amino acids from the C-terminus (CA14, CA24, CA50, CA79, CA97 and CA139 respectively). These oligonucleotides (numbers 5 - 10; for a list of the sequences of all oligonucleotides used and a diagram of where they bind to cyclin A, see Appendix 1) contained a BamHI site downstream of the stop codon and were used for PCR in conjunction with a non-mutagenic 5' primer (oligonucleotide 11, see Appendix 1) that spanned the HindIII restriction enzyme site (for position of this site on c-myc cyclin A, see figures 2.3 and 3.1). The DNA fragments amplified by PCR using oligonucleotides 5 - 10 as the 3' primers and oligonucleotide 11 as the 5' primer were digested with BamHI and HindIII restriction enzymes and ligated into HindIII and BclI digested c-myc cyclin A. The positions of the stop codons introduced into c-myc cyclin A in these constructs are shown in detail in figure 3.1, and schematically in Appendix 1, figure A1.1.

R197A

Mutagenesis of individual residues within cyclin A was carried out by PCR, as described by Horton and Pease (1991). I will briefly describe the use of this method for the substitution of arginine 197 with an alanine residue. See figure 2.4 for a diagrammatic explanation. Two mutagenic oligonucleotides (oligonucleotides 12 and 13, see Appendix 1) that were complementary to each other were designed such that when they were used as primers in a PCR they introduced point mutations into the cyclin A sequence to change arginine 197 to an alanine residue. Two separate PCR reactions were set up using c-myc cyclin A as the template DNA; one using oligonucleotides 12 and 2, the other using oligonucleotides 13 and 11. The DNA fragments thus amplified (DNAs A and B respectively) were separated from shorter fragments and unused oligonucleotides by agarose gel electrophoresis on a 1% (w/v) agarose gel in TAE buffer containing 0.5 μg/ml ethidium bromide, visualised briefly with UV illumination and cut out from the gel. The DNA was purified using acid washed silica (see below). The two purified DNA fragments were mixed, denatured and used for PCR with the ‘outside’ primers oligonucleotides 2 and 11. As the 3' end of DNA B will be complementary to the 5' end of DNA A and the product of the top strand of DNA B and the bottom strand of DNA A can be extended in a PCR reaction, to give a product that extends from the site of annealing of oligonucleotide 11 to that of oligonucleotide 2 (see figure 2.4). The DNA
Figure 2.4 Making point mutations by PCR
The method of making point mutations in DNA is shown schematically. The oligonucleotides used to make R197A are indicated. Asterisks in the oligonucleotides show mismatches with the DNA sequence, asterisks in the DNA sequence indicate the introduction of a point mutation due to the mismatches in the oligonucleotides.

fragment thus generated was digested with HindIII and NsiI and inserted into HindIII and NsiI digested c-myc cyclin A.

R197K, D226A and D226E
R197K was constructed using oligonucleotides 14 and 15 as the mutagenic primers; D226A was constructed using oligonucleotides 16 and 17 as the
mutagenic primers and D226E was constructed using oligonucleotides 18 and 19 as the mutagenic primers. In all three cases, oligonucleotides 2 and 11 were used as the ‘outside’ primers. The DNA fragments were digested with HindIII and NsiI and inserted into HindIII and NsiI cut c-myc cyclin A.

**ATVA cyclin A, Δ109-161ATVA and Δ101-169ATVA**

The two absolutely conserved residues of the cyclin destruction box, arginine 41 and leucine 44, were mutated in the cyclin A constructs c-myc cyclin A, Δ109-161, and Δ101-169 using oligonucleotides 20 and 21. The ‘outside’ primers used were oligonucleotides 13 and 22 for ATVA cyclin A and oligonucleotides 22 and 23 for Δ109-161ATVA and Δ101-169ATVA. The ATVA cyclin A DNA fragment was digested with NcoI and HindIII and inserted into NcoI and HindIII cut c-myc cyclin A. The Δ109-161ATVA and Δ101-169ATVA DNA fragments were digested with BsmI and NsiI and inserted into BsmI and NsiI digested Δ109-161 and Δ101-169 (Kobayashi et al., 1992), respectively.

**‘FVD mutant’ and S136A cyclin A**

The ‘FVD mutant’ of cyclin A was made using oligonucleotides 24 and 25 as the mutagenic primers and oligonucleotides 22 and 13 as the ‘outside’ primers. The DNA fragment was digested with NcoI and HindIII and inserted into NcoI and HindIII cut c-myc cyclin A. S136A cyclin A was made using oligonucleotides 26 and 27 as the mutagenic primers and oligonucleotides 20 and 23 as the ‘outside’ primers. The DNA fragment was digested with EcoRV and NsiI and inserted into EcoRV and NsiI digested c-myc cyclin A.

**c-myc NA133, c-myc NA129, c-myc NA114 and c-myc NA107**

c-myc cyclin A was made by PCR using construct A (NA133) cyclin A as the template. A mutagenic 5’ primer (oligonucleotide 28) was designed to insert a BsmI restriction enzyme site at the very beginning of the open reading frame of NA133. This was used to amplify DNA by PCR from NA133 in conjunction with oligonucleotide 2 as the 3’ primer. The DNA fragment thus generated was digested with BsmI and NsiI and inserted into BsmI and NsiI cut c-myc cyclin A. c-myc NA129, c-myc NA114 and c-myc NA107 cyclin As were made by PCR using c-myc cyclin A as the template. Mutagenic 5’ primers (oligonucleotides 29, 30 and 31 for c-myc NA129, c-myc NA114 and c-myc NA107 respectively) were used to insert BsmI restriction enzyme sites at the desired starts of the constructs. These were used to amplify DNA by PCR from c-myc cyclin A in conjunction with oligonucleotide 2 as the 3’ primer. The DNA fragments thus generated were digested with BsmI and NsiI and inserted into BsmI and NsiI cut c-myc cyclin A.
c-myc Δ88-144

c-myc Δ88-144 was constructed by digesting non-tagged Δ88-144 (Kobayashi et al., 1992) with BsmI and EcoRI and inserting it into BsmI and EcoRI cut c-myc cyclin A.

pCD2/CMV cyclin A mutants

A vector containing rat CD2 under the control of the SV40 early promoter and mouse cyclin B1 under the control of the human cytomegalovirus promoter (pCD2/CMV-mouse cyclin B1, see figure 2.5A), kindly provided by Dr Chris Norbury (IMM, Oxford), was digested with SalI and NotI to remove the mouse cyclin B1 gene. In place of the mouse cyclin B1 gene was ligated a double stranded oligonucleotide (made by annealing oligonucleotides 35 and 36, see Appendix 1 and figure 2.5B) which had ends compatible with the SalI and NotI digested plasmid and contained restriction enzyme sites for NheI, PacI, EcoRV and SpeI. This plasmid (pCD2/CMV) was digested with NheI and SpeI and into it was ligated NheI/SpeI fragments from the following constructs: c-myc cyclin A, CΔ14, R197K, D226E, ‘FVD mutant’, S136A, ATVA, c-myc NΔ133, and c-myc Δ88-144 cyclin As. These were named pCD2/CMV-CΔ14 cyclin A, pCD2/CMV-R197K cyclin A, pCD2/CMV-D226E cyclin A, pCD2/CMV-FVD cyclin A, pCD2/CMV-S136A cyclin A, pCD2/CMV-ATVA cyclin A, pCD2/CMV-c-myc NΔ133, and pCD2/CMV-c-myc Δ88-144 cyclin A respectively.

C-terminal ‘swap’ constructs between cyclins A and B1

A NotI site was introduced into c-myc cyclin A 77 amino acids from the end of the protein, at a run of 4 alanine residues. The introduction of this site did not change the amino acid sequence of the protein. The NotI site was introduced by PCR mutagenesis, using the mutagenic oligonucleotides 32 and 33 (see Appendix 1) and the ‘outside’ oligonucleotides 2 and 34. The DNA fragment was digested with NsiI and BclI and inserted into NsiI and BclI digested c-myc cyclin A. A NotI site was introduced into full-length cyclin B1 in pGEM1 (Minshull et al., 1989) 86 amino acids from the end of the protein, at a run of 3 alanine residues. The introduction of this site did not change the amino acid sequence of the protein. The NotI site was introduced by PCR mutagenesis using the mutagenic oligonucleotides 37 and 38 (see Appendix 1) and the ‘outside’ oligonucleotides 39 and 40, which span restriction enzyme sites BclI and BsmI respectively. The DNA fragment was digested with NsiI and BclI and inserted into NsiI and BclI digested c-myc cyclin A.
Figure 2.5  The mammalian expression vector pCD2/CMV-mouse cyclin B1 and the added cloning cassette

(A) The structure of the mammalian expression vector, pCD2/CMV-mouse cyclin B1, provided by Dr Chris Norbury. (B) The sequence of the oligonucleotide cassette that was ligated into the above vector in place of the mouse cyclin B1 gene. The vector thus created was called pCD2/CMV.

Cyclin A/B1Δ2

In order to be able to use the BglIII site that was close to the desired site of mutagenesis in cyclin A/B1, it was necessary to delete the BglII restriction enzyme site that is present in the c-myc tag of cyclin A/B1. This was achieved by digesting cyclin A/B1 with Nsil and SphI, which cut in the cyclin box of cyclin A and in the plasmid sequence downstream of the end of the 3' UTR of cyclin B1, respectively, and placing this DNA fragment into Nsil and SphI digested cyclin A
in pGEM1 (the original cyclin A clone, without a c-myc tag). This procedure reconstructs cyclin A/B1 without the c-myc tag. This construct was then digested with BglII and NdeI, which cuts out a fragment of 23 base pairs, and in its place was ligated a double stranded oligonucleotide (made by annealing oligonucleotides 41 and 42) which changed valine 343 to alanine, valine 345 to cysteine, methionine 346 to leucine and deletes glutamine 347 and histidine 348 (all numbering refers to the amino acid number of wild-type cyclin B1). This construct was called cyclin A/B1Δ2.

Construction of other mutants

c-myc p33cdk2

The DNA encoding Xenopus p33cdk2 in a plasmid containing the β globin leader behind a T7 RNA polymerase promoter was used as the template for PCR with the oligonucleotides 43 and 44. These oligonucleotides amplify the whole of the coding region of the cdk2 gene; the 5' primer, oligonucleotide 43, is non mutagenic and spans the NcoI restriction enzyme site that is present at the initiating methionine; the 3' primer, oligonucleotide 44, adds an NcoI site in place of the stop codon at the end of the coding region of the cdk2 gene. The DNA fragment thus amplified was digested with NcoI and ligated into NcoI digested pT7-βTAG (kindly provided by Dr Richard Treisman). This vector contained the DNA encoding the c-myc epitope, recognised by the monoclonal antibody 9E10, with an NcoI restriction enzyme site at the initiating methionine, preceded by a T7 RNA polymerase promoter and the 5' UTR of human β-globin. This gives full-length, C-terminally c-myc tagged p33cdk2. This construct was digested with EcoRI, which cuts just 3' to the end of the c-myc epitope, the underhanging DNA on each side of the restriction enzyme site were filled in using deoxynucleotides and Klenow, and the two ends were ligated back together again. This allowed the creation of a stop codon just downstream of the DNA encoding the c-myc epitope.

H10cyclin B2

The clone of cyclin B2 in pET16b was kindly provided by Dr Katsumi Yamashita. This clone was digested with NcoI and BamHI, with cuts out the entire coding region and 3' UTR of the clone, and placed in NcoI and BclII digested c-myc cyclin A. This gives the H10cyclin B2 construct a 5' UTR that allows efficient translation or the synthetic mRNA made from this construct. The clone was linearised with EcoRI before being used for in vitro transcription by the T7 RNA polymerase, as the presence of two 3' UTRs appears to inhibit translation of the mRNA transcribed in Xenopus egg extracts (data not shown).
Molecular biology techniques

Most molecular biology techniques were performed as described in Sambrook et al. (1989) and Ausubel et al. (1991). I will only describe less standard methods in this section.

Polymerase Chain Reaction (PCR)

Most PCR reactions were carried out in 100 μl volumes, but occasionally smaller volumes were employed. The components of all the PCR reactions described in this thesis, whether mutagenic or not, were as follows (concentrations are final concentrations):

- 100 ng/ml template DNA;
- 200 nM each oligonucleotide primer;
- 20 μM each dNTP;
- 20 U/ml Taq DNA polymerase (Perkin Elmer Cetus)

in 10 mM Tris-Cl, pH 8.3; 50 mM KCl; 1.5 mM MgCl2; 0.001% gelatin (Perkin Elmer Cetus PCR buffer)

In general, the DNA template, primers and buffer were mixed and incubated in a boiling water bath for 1 minute before the addition of the dNTPs and the Taq DNA polymerase. The reactions were then placed in a Perkin Elmer Cetus thermal cycler and the following temperature profile was generally used:

- 94°C for 30 seconds (denaturing);
- 50-60°C for 30 seconds (annealing - see below);
- 72°C for 90 seconds (extending)

Sometimes the final step was lengthened by between 1 and 3 seconds each cycle. This did not appear to be necessary, however, as there was no noticeable difference between PCRs carried out with and without this lengthening. At the end of the last cycle, the reactions were incubated at 72°C for a further 10 minutes before being incubated at 4°C until removed from the machine. The temperature of annealing (°C, second step) was calculated using the simple formula (number of T or A residues in the oligonucleotide x2) + (number of C or G residues in the oligonucleotide x4) - 5. The temperature of annealing was matched as far as possible when ordering pairs of oligonucleotides, but in the case of differing annealing temperatures for two oligonucleotides that were to be used in the same reaction, the lower temperature was used. In the case of mutagenic oligonucleotides, the residues that did not match the DNA template were not included in the calculation.
Screening transformed *E. coli* colonies for the presence of the correct plasmid by PCR

A very simple method for determining whether *E. coli* colonies that had been transformed with a ligation mixture contained the desired plasmid construct was by amplification of that plasmid by PCR. The precise method employed varied depending on the circumstances. If a deletion or addition to the original plasmid had been made then primers were employed that annealed to the plasmid either side of the site of deletion or addition; the size of the amplified product then indicated whether the desired deletion/addition was present in the plasmid contained in each bacterial colony. Sometimes one or both of the oligonucleotides used for the PCR would only anneal to the desired construct. When making point mutations, very often a restriction enzyme site was created or destroyed by the mutation. If PCR primers were chosen so that this restriction enzyme site was unique in the portion of DNA that they amplified, then a simple restriction enzyme digest of the amplified DNA indicated the presence or absence of the point mutation in the plasmid contained in each bacterial colony.

The PCR reactions for this method of determining which colonies of bacteria contained the desired plasmid were carried out in 25 μl volumes. Apart from this, the PCRs were carried out essentially as described above, except that no template DNA was added. Instead a toothpick containing some *E. coli* from the colony to be tested was ‘washed’ in the mix of buffer and oligonucleotide primers and then the toothpick was placed in a sterile culture tube containing bacterial growth medium. Careful labelling was very important! The PCR tubes containing the bacteria were incubated in a boiling water bath for 2 minutes, the rest of the components for the PCR reaction were added and the DNA contained within the bacteria in the tube was subjected to amplification by PCR, as normal. Towards the end of my PhD research, Dr Michael Howell in the laboratory purified some Taq DNA polymerase, and I used 0.2 μl of this enzyme per PCR reaction for screening *E. coli* colonies. This makes this method of screening colonies much cheaper, as the Taq DNA polymerase is by far the most expensive component of PCR reactions.

**DNA preparation**

Small-scale DNA preparation by alkaline lysis

Colonies of bacteria were picked into 1.5 ml of media and grown in 2xTY for 5 or 6 hours, or in L-broth overnight, with the appropriate selection (typically 50 μg/ml ampicillin). Bacteria from 1.5 ml of culture were pelleted by centrifugation (5 minutes in a microcentrifuge) and the pellet was resuspended in 90 μl GTE. The bacteria were lysed by the addition of 180 μl SDS/NaOH and incubated at room temperature for 5 minutes. The solution was neutralised by the addition of
135 μl of KOAc and the debris removed by centrifugation in a microcentrifuge for 10 minutes. The supernatant was transferred to a new tube and the nucleic acids pelleted by the addition of 1 ml of ethanol and incubation at -80°C for 10 minutes. After spinning to pellet the nucleic acid, it was dried and resuspended in 40 μl TE buffer. The rRNA was then precipitated from this solution by the addition of 20 μl of 10 M LiCl and incubation on ice for 15 minutes, pelleted by centrifugation (5 minutes in a microcentrifuge) and the DNA precipitated from the resulting supernatant by the addition of 200 μl of ethanol and incubation at -80°C for 5 minutes. The DNA was harvested by centrifugation (20 minutes in a microcentrifuge), washed with 80% (v/v) ethanol, air dried and resuspended in 20 μl TE buffer. This DNA was used for restriction enzyme digest (digestion could not be for longer than two hours, as the DNA degraded after this time) or, at the beginning of my PhD, for double stranded sequencing after further precipitation with polyethyleneglycol (PEG) (see below).

PEG precipitation of DNA
The DNA prepared as described above was resuspended in 50 μl of TE buffer and to it was added 25 μl of 20% (v/v) PEG in 2.5 M NaCl. This was allowed to stand at room temperature for 20 minutes and then the DNA was precipitated by centrifugation (20 minutes in a microcentrifuge). The DNA pellet was resuspended in TE buffer and extracted once with phenol followed by two extractions with chloroform. The DNA was spun hard to ensure that any chloroform that was left was at the bottom of the tube. Half of this DNA was then used for each sequencing reaction.

Small-scale DNA preparation by boiling
Colonies of bacteria were picked into 1.5 ml of media and grown in 2xTY for 5 or 6 hours or in L-broth overnight with the appropriate selection. Bacteria from 1.5 ml of culture were pelleted by centrifugation (5 minutes in a microcentrifuge) and all but 100 μl of the media was removed. The bacteria were resuspended in this media and were lysed by the addition of 300 μl of STET (8% (w/v) sucrose; 5% (v/v) triton X-100; 50 mM Tris-Cl, pH 8.0; 50 mM EDTA; 1 mg/ml freshly added lysozyme). These reactions were immediately heated at 100°C in a boiling water bath for exactly 90 seconds and then centrifuged in a microfuge in the cold room for 10 minutes without delay. The resulting supernatant was transferred to a new tube and the DNA was precipitated by the addition of an equal volume of propan-2-ol. The DNA was pelleted by centrifugation (10 minutes in a microcentrifuge in the cold-room), washed with 80% (v/v) ethanol and air-dried. The DNA pellet was resuspended in 40 μl TE buffer. Restriction enzyme digests on DNA prepared in this way were as successful as digests carried out on DNA prepared
by the alkaline lysis method and double stranded sequencing of this DNA, using 
the second method described below (8 μl of the resuspended DNA per reaction), 
worked extremely well without further purification.

Large-scale DNA preparation by alkaline lysis

An overnight culture of *E. coli* containing the plasmid (400 ml of 2xTY 
containing the appropriate antibiotic selection) was pelleted by centrifugation at 
3000 rpm for 5 minutes in a Sorvall GSA rotor and resuspended in 20 ml GTE. 
The bacteria were lysed with 40 ml SDS/NaOH and incubation at room 
temperature for 10 minutes, followed by neutralisation of the solution with 30 ml 
KOAc. The debris was pelleted by centrifugation at 5 000 rpm in a Sorvall GSA 
rotor for 10 minutes and the supernatant filtered through four layers of 
cheesecloth. The nucleic acid was precipitated by the addition of 56 ml of 
propan-2-ol and pelleted by centrifugation at 8 000 rpm in a Sorvall GSA rotor 
for 10 minutes. The pellet was resuspended in 10 ml of TE buffer and the rRNA 
was precipitated from this by the addition of 4 ml 10 M LiCl and incubation on 
ice for 10 minutes. The rRNA was pelleted by centrifugation at 10 000 rpm in a 
Sorvall SS34 rotor for 10 minutes and the DNA in the supernatant precipitated by 
the addition of 8.6 ml propan-2-ol. The DNA was precipitated by centrifugation at 
10 000 rpm in a Sorvall SS34 rotor for 10 minutes, washed with 80% (v/v) 
ethanol, and dried in a vacuum dessicator for 5 minutes. The pellet was 
resuspended in 750 μl TE buffer and to it was added 40 μl of a 10 mg/ml solution 
of boiled RNase A. This was incubated at 37°C for 15 minutes and then 263 μl of 
a solution of 200 μg/ml proteinase K in 2% (w/v) SDS; 40 mM Tris-Cl, pH 7.5; 
20 mM EDTA was added and the DNA solution was incubated at 37°C for a 
further 30 minutes. This was then extracted three times with a 1:1 (v/v) mixture 
of phenol and chloroform and twice with chloroform alone and the DNA was 
precipitated with 0.1 volumes of 4 M ammonium acetate and 0.6 volumes of 
propan-2-ol. The DNA was pelleted by centrifugation at room temperature in a 
microcentrifuge for 15 minutes, washed with 80% (v/v) ethanol, dried in a 
vacuum dessicator for 2 minutes and resuspended in 400 μl TE buffer. DNA 
prepared in this way was used without further purification for all purposes except 
in transient transfection experiments, when it was further purified by PEG 
precipitation (see below).

Further purification of DNA for transient transfection experiments

DNA to be used for transient transfection experiments was further purified by 
PEG precipitation. To the DNA was added 0.5 volumes of 40% (w/v) PEG-6000 
in 30 mM MgCl₂, they were mixed well and incubated at room temperature for at 
least 10 minutes. The DNA was pelleted by centrifugation in a microcentrifuge
for 20 minutes at room temperature and washed twice with 80 % (v/v) ethanol. The pellet was air dried and resuspended in TE buffer.

**Purification of DNA fragments from agarose gels**

DNA fragments were separated by electrophoresis on 1% agarose gels (SeaKem ME agarose, FMC) in TAE buffer (40 mM Tris base; 40 mM acetic acid; 2 mM EDTA) containing 0.5 μg/ml ethidium bromide. For DNA fragments smaller than 500 base pairs, 5% NuSieve 3:1 agarose (FMC) gels were used. The DNA fragments were visualised briefly with UV illumination and the agarose containing them was excised. The agarose was dissolved by incubation in a saturated NaI solution (90.8 g NaI and 1.5 g Na₂SO₃ in 100 ml) at 55°C for about 10 minutes. To the dissolved agarose solution was added 5 μl of acid washed silica and this was allowed to stand at room temperature for 5 - 10 minutes, with occasional mixing. The silica was pelleted by centrifugation in a microcentrifuge for 15 seconds and washed 3 times with 1 ml EtOH/NET (50 % (v/v) ethanol; 50 mM NaCl; 5 mM Tris-Cl, pH 7.5; 0.5 mM EDTA) each time. The silica was then washed once in 80 % (v/v) ethanol and allowed to air dry for 5 minutes. The DNA was eluted from the silica by incubation with 20 μl TE buffer at 55°C for 10 minutes. The buffer was removed and the elution procedure was repeated to extract the final traces of DNA. Recovery of the DNA fragment was checked by agarose gel electrophoresis of a small fraction of the eluate, usually 1 μl.

**Sequencing**

All sequencing in this thesis was done using double stranded DNA. About 5 μg DNA per sequencing reaction was denatured using one of two methods. In the first method the DNA was incubated with 4 μl 2 M NaOH and 4 μl 5 mM EDTA in a volume of 40 μl for 5 minutes at room temperature. To this incubation was then added 60 μl 1.5 M ammonium acetate, pH 4.5 and 300 μl of ethanol. The reaction was mixed and incubated at -80°C for 5 minutes and the DNA was precipitated by spinning in a microcentrifuge at room temperature for 20 minutes. The DNA pellet was washed with 80% (v/v) ethanol and resuspended in 7 μl sterile, distilled H₂O. Annealing of the primer and sequencing then took place as described in Sequenase manual, using Sequenase enzyme (version 2.0, United States Biochemical). The second method, which was employed during the second half of my PhD, was a much more convenient method of denaturation. To the DNA was added 50 ng sequencing primer and 1 μl 1 M NaOH in a volume of 10 μl. This was incubated at 68°C for 10 minutes and immediately 4 μl of TDMN buffer (280 mM TES; 120 mM HCl; 80 mM MgCl₂; 200 mM NaCl; 50 mM DTT) was added. This was allowed to stand at room temperature for 10 minutes.
and then used immediately for sequencing with Sequenase enzyme, following the Sequenase manual, or stored on ice until required.

**T7 RNA polymerase transcription to produce capped mRNA**

The protocol for T7 RNA polymerase transcription of plasmid constructs was based on that by Nielsen and Shapiro (1986). Plasmid DNA encoding the gene to be transcribed was linearised by restriction enzyme digest at, or downstream of, the 3' end of the 3' UTR. Although many of the plasmid constructs used for *in vitro* transcription contained T7 RNA polymerase terminator sequences, it was found that these were not 100% efficient at terminating transcription and as a consequence the mRNA made from non-linearised plasmids was translated extremely poorly in *Xenopus* egg extracts. For this reason plasmid DNA was always linearised before transcription, whether or not it contained a T7 RNA polymerase terminator sequence. After linearisation of the DNA, the restriction enzyme was removed by extraction of the digestion reaction with a 1:1 (v/v) mixture of phenol and chloroform, followed by two extraction with chloroform alone and ethanol precipitation of the DNA. The DNA was washed with 80% (v/v) ethanol, air dried and resuspended in TE buffer at a concentration of approximately 1 mg/ml. The following reaction mixture was then set up to synthesise capped mRNA: 40 μg/ml linearised DNA; 1 mM rATP, 1 mM rUTP; 1 mM rCTP; 100 μM rGTP; 5 mM DTT; 15 mM MgCl₂; 10 mM Tris-Cl, pH 8.0; 250 U/ml either RNasin (Boehringer) or RNAguard (Pharmacia); 0.5 mM RNA Cap Structure Analogue 7mG(5')ppp(5')G sodium salt (New England Biolabs, #1404) and 40 μl/ml T7 RNA polymerase (purified by Shaun Mackie or Anne McBride). The reaction was incubated at 37°C for 30 minutes, after which time 1 mM rGTP was added and the incubation continued at 37°C for a further 60 minutes. The reaction was extracted twice with a 1:1 (v/v) mixture of phenol and chloroform, once with chloroform alone and the nucleic acid was precipitated with an equal volume of 4 M ammonium acetate and 5 volumes of ethanol. The nucleic acid was precipitated by centrifugation in a microcentrifuge for 20 minutes and washed with 80% (v/v) ethanol. The pellet was air dried and resuspended in 0.2 mM EDTA. I typically carried out transcription reactions in a volume of 100 μl (i.e. using 4 μg of DNA) and resuspended the resulting mRNA in a volume of 50 μl, which gave mRNA at a concentration of between 1 and 2 mg/ml. The mRNA was stored at -80°C.
Use of Xenopus oocytes and eggs

Microinjection of mRNA into Xenopus oocytes

Stage VI oocytes were obtained from the ovaries of female *Xenopus laevis*, with the use of mild digestion with collagenase, as described by Colman (1984). About 50 nl of a 1 mg/ml solution of *in vitro* transcribed mRNA was injected into each oocyte, which were kept at 22°C in modified Barth’s medium (88 mM NaCl; 1 mM KCl; 2.4 mM NaHCO₃; 15 mM HEPES, pH 7.6; 0.3 mM Ca(NO₃)₂·4H₂O; 0.41 mM CaCl₂·6H₂O; 0.82 mM MgSO₄·7H₂O; 10 μg/ml streptomycin; 10 μg/ml penicillin G). Maturation was assessed by the appearance of the characteristic white spot on the animal pole of the oocyte, and checked in doubtful cases by dissection. The ability of a selection of oocytes to mature in response to progesterone was also checked.

Xenopus egg extracts

Cytostatic factor (CSF) arrested and interphase Xenopus egg extracts were prepared as described by Murray (1991). Briefly, female *Xenopus* were induced to ovulate by injection of Pregnant Mare Serum Gonadotrophin and Human Chorionic Gonadotrophin by Gary Martin (ICRF animal unit). The laid eggs were collected and others were manually expelled from the frogs. For CSF-arrested egg extracts, the eggs were dejellied by incubation in 2% (w/v) cysteine in 0.1 mM CaC₂; 1 mM MgC₂; 10 mM KCl; 4.5 ml 1 M NaOH per 100 ml for about 5 minutes. The eggs were washed four times in XB (100 mM KCl; 0.1 mM CaCl₂; 2 mM MgCl₂; 5 mM EGTA; 50 mM sucrose; 10 mM HEPES, pH 7.7 (with KOH)) at 16°C (or as near as possible) and twice more in XB containing the protease inhibitors leupeptin, pepstatin and chymostatin at 10 μg/ml. The eggs were transferred, in a minimal volume of buffer, to an SW50 centrifuge tube containing 0.5 ml of versilube (Andpak-EMA) below 1 ml of XB containing protease inhibitors and 100 μg/ml cytocholasin B, and spun in a bench-top eppendorf centrifuge at 16°C for about 1 minute, until the speed reached about 1.4 rpm. This packed the eggs tightly (but did not crush them) and allowed the versilube to rise to the surface of the tube through the eggs, thus displacing any buffer that was surrounding the eggs and replacing it with versilube. Buffer and versilube that had risen to the top of the tube were removed and the eggs crushed at 16°C by spinning at 10 rpm in a Sorvall swing out, HB4, rotor for 10 minutes. The resulting cytoplasmic layer was removed to a tube at 4°C, using a syringe and needle through the side of the tube, and to it was added 10 μg/ml leupeptin, pepstatin, chymostatin and cytocholasin B. The extract was centrifuged at 4°C at 10 rpm in a Sorvall swing out, HB4, rotor for 10 minutes, the cytoplasmic layer again removed and this time supplemented with 75 mM creatine phosphate, 1 mM MgCl₂, 0.1 mM EGTA, 1 mM rATP and 200 mM sucrose. The extract was
aliquoted, frozen in liquid nitrogen, and stored at -80°C. Interphase egg extract
was made by first activating the eggs by two washed in distilled water before
dejellying and then electrical activation, see Murray (1991).

Translation of mRNA in *Xenopus* egg extracts

**Pure egg extract**

An aliquot of CSF-arrested or interphase egg extract was thawed on ice and to it
was added a 1/10th volume of *in vitro* transcribed mRNA (stock at 1-2 mg/ml)
and a 1/20th volume of $[^{35}S]$methionine. This reaction mix was incubated at
23°C for 2 hours. For analysis by SDS PAGE and autoradiography, 1 μl of this
translation reaction was added to 24 μl SDS sample buffer, mixed and boiled for
2 minutes and between 5 and 10 μl of this was loaded on the polyacrylamide gel.
No more could be loaded because of the protein concentration in the extracts. For
most of the duration of my PhD I used $[^{35}S]$methionine purchased from
Amersham (1000 Ci/mmol, 10 mCi/ ml, #SJ1515), but towards the end we
changed to a mixture of $[^{35}S]$methionine and $[^{35}S]$cysteine (approx. 70%
methionine and 15% cysteine) from ICN (Trans $[^{35}S]$-label, 10 mCi/ml, #51006).
I directly compared these two $[^{35}S]$methionine preparations for use in translation
and destruction assays using CSF-arrested egg extracts, and could see no
difference between them (not shown). When 2 mRNAs were to be translated at
the same time, a 1/10th volume of each mRNA was added to the extract. No
more than 2 mRNAs could be used at once because the extract became too dilute
and translation efficiency was severely reduced. When a translation reaction
containing no exogenous mRNA was to be compared to reactions containing
added mRNA, a 1/10th volume of 0.2 mM EDTA was added to the ‘no mRNA’
extract to keep the dilution factor the same in each case.

10% (v/v) added reticulocyte lysate

Translation reactions carried out in CSF-arrested egg extract with 10% (v/v)
added reticulocyte lysate were set up in an similar manner to those carried out in
pure CSF-arrested egg extract. The only difference was that, in addition to
adding mRNA and $[^{35}S]$methionine, a 1/10th volume of rabbit reticulocyte lysate
(made in the laboratory by Dr Tim Hunt, and others) was added to the extract.
This addition significantly enhanced the translation of added mRNA in the extract
(see Appendix 2).

50% (v/v) added reticulocyte lysate

Rabbit reticulocyte lysate (40 μl) was supplemented with 5 μl of 0.2 M creatine
phosphate, 5 μl of KM (2 mM KCl; 11 mM MgCl$_2$; 1 mM EDTA), 5 μl of amino
acid mix (3 mM L-leucine; 3 mM L-valine and 2 mM all the other amino acids,
with the exception of L-methionine and L-cysteine, pH 7.2) and 5 μl
[35S]methionine (see above). An equal volume of this mix was added to freshly-
thawed CSF-arrested egg extract, to which a 1/20th volume of [35S]methionine
had already been added. To the mixture of rabbit reticulocyte lysate and CSF-
arrested egg extract was added a 1/10th volume of synthetic mRNA and the
translation reaction was incubated at 23°C for 2 hours.

**Cyclin destruction assays in Xenopus egg extracts**

Cyclin destruction assays were carried out as follows. The mRNA encoding the
cyclin to be tested was translated in freshly-thawed CSF-arrested egg extract, in
the presence of [35S]methionine, as described above. Occasionally the translation
was carried out in CSF-arrested egg extract containing 10% (v/v) added
reticulocyte lysate (see above), which had little effect on the destruction assay
(see Chapter 5), but improved cyclin translation (see Appendix 2). At the end of
the translation reaction, cycloheximide was added to the reaction mix to a final
concentration of 100 μg/ml (a stock of either 1 or 2 mg/ml was used) and the
extract was incubated at 23°C for a further 10 minutes. The zero time point was
then taken (before the addition of Ca2+ to the extract, as cyclin destruction was, at
times, very rapid once triggered). A 1/20th volume of 8 mM CaCl2 was then
added to the extract, mixed (by pipetting up and down) and placed back at 23°C.
In ‘-Ca2+’ destruction assays, a 1/20th volume of H2O was added to the extract in
place of the Ca2+. Aliquots were taken from the assay at intervals thereafter,
usually 10, 20 30, 60 and 90 minutes after Ca2+ addition. The volume of sample
taken at each time point depended on the way in which it was to be analysed
subsequently, but I always ensured that an equal volume of extract was removed
at each time point; thus the volume of the zero time point was slightly smaller
than the subsequent ones, as it had not been diluted with Ca2+.

If the samples were simply to be analysed by SDS PAGE and
autoradiography, 1 μl aliquots were removed from the reaction at each time point
and added to 24 μl of SDS sample buffer. These were mixed and stored on dry
ice until the end of the experiment, when they were boiled for 2 minutes and
between 5 and 10 μl of each (typically 7.5 μl) was loaded on the polyacrylamide
gel.

If samples were to be used in a histone H1 kinase assay, 1 μl aliquots were
taken into clean eppendorf tubes and stored on dry ice until use in the kinase
assay.

If samples were to be affinity purified aliquots of between 5 and 10 μl
were removed at each time point. These were diluted into 400 μl of ice-cold bead
buffer and stored on ice until all the samples had been collected.
**Immunological Methods**

**Western Blotting**

The protein samples to be analysed were separated by SDS PAGE, the gel was soaked in transfer buffer (20 mM Tris base; 150 mM glycine; 0.1% (w/v) SDS; 20% (v/v) methanol) for 30 minutes and the proteins were transferred to a nitrocellulose membrane (Hybond C-super) using a semi-dry transfer apparatus (Hoefer) and transfer buffer, according to the instructions. Selected lanes on the blot were labelled by running 2 μl of a 0.5 mg/ml solution of the pink dye Pyronin Y (Aldrich) in SDS sample buffer (without 2-mercaptoethanol) on the polyacrylamide gel. This dye transfers from the gel to the nitrocellulose with the proteins. The membrane was stained with 0.2% (w/v) Ponceau S in 3% (w/v) trichloroacetic acid and destained with distilled water. The positions of the molecular weight markers and the positions of the lanes were marked on the membrane with a charcoal pencil and the protein staining was washed off using PBSA. The membrane was blocked with TBST (10 mM Tris-Cl, pH 8.0; 150 mM NaCl; 0.05% (v/v) Tween-20) containing 4% (w/v) skimmed milk powder at 4°C overnight (typically 12-16 hours) and then incubated with neat cell culture supernatant containing the primary antibody at room temperature for between 1 and 2 hours. The membrane was washed twice with TBST containing 4% (w/v) skimmed milk powder and then three times with TBST containing 2% (w/v) skimmed milk powder; each wash was for at least 10 minutes and carried out at room temperature. The primary antibody was detected by probing the membrane with a rabbit anti-mouse antibody conjugated to horseradish peroxidase (Dakopatts) diluted 5 000 fold in TBST containing 2% (w/v) skimmed milk powder for 1 hour at room temperature. The membrane was washed twice with TBST containing 2% (w/v) skimmed milk powder and then three times with TBST alone; each wash was for at least 10 minutes and carried out at room temperature. The membrane was developed using the Amersham enhanced chemiluminescence system (ECL), according to the manufacturer’s instructions.

**Immunoprecipitation**

If the sample that was to be subjected to immunoprecipitation was a translation reaction and had taken place in the presence of [³⁵S]methionine, an aliquot of it was generally removed for analysis by SDS PAGE and autoradiography before immunoprecipitation. Antibody was added to the (remainder of the) sample (in general 5 μl of sample was used for each immunoprecipitation reaction), and the reactions were incubated on ice for 1 hour. When using cell culture supernatant as a source of antibody, 100 μl of supernatant was added to 5 μl of translation reaction; when using polyclonal serum, 1 μl was added per reaction; when using protein A purified monoclonal antibody about 15 μg of antibody was added per
reaction. When using purified antibody or serum, the volume of the reaction was made up to 20 µl with wash buffer (see below). At the end of the incubation on ice, the reaction mixes were spun for 1 minute in a microcentrifuge at room temperature to precipitate any solid particles that might be present (this was especially important when carrying out immunoprecipitations from CSF-arrested egg extract, as this extract often contained particulate matter that could cause a serious background problem if not removed). The immunocomplexes present in the supernatant were then recovered by incubation of the samples with protein A Sepharose in a volume of 200 µl (the reactions were made up to this volume by the addition of wash buffer - see below) for 1 hour at 4°C or 20 minutes at room temperature, with rotation. For the first half of my PhD, I used 10-20 µl of Pharmacia protein A Sepharose per standard reaction; during the second half of my PhD I used 5-10 µl of Biorad ‘affiprep’ protein A per standard reaction. There did not appear to be much difference in these two protein A matrices, except that the affiprep protein A was, in my opinion, much easier to use. The protein A-bound complexes were washed 5 times with wash buffer (see below) and during the final wash the beads were transferred to a clean eppendorf tube. The bead-bound proteins were then used in kinase assays or simply eluted in SDS sample buffer and analysed by SDS PAGE and autoradiography. For the standard immunoprecipitation assays (starting with 5 µl of extract), half of the immunoprecipitated proteins were analysed by SDS PAGE and autoradiography.

At the beginning of my PhD, the wash buffer that I used was 80 mM Sodium-β-glycerophosphate; 20 mM EGTA; 50 mM NaF; 1 mM DTT; 1 mM PMSF; 10 µg/ml leupeptin; 10 µg/ml soya bean trypsin inhibitor; 100 µM benzamidine, but after about one year I began to use bead buffer instead, as it seemed to remove more of the non-specific interactions without significantly affecting the specific protein-protein interactions that I was investigating.

**Affinity purification**

**p13sup1 Sepharose affinity purification**

Samples were spun for one minute in a microcentrifuge at room temperature and the supernatant was incubated with 10-20 µl of p13sup1 Sepharose in 200 µl bead buffer per standard reaction (in general, 5 µl of a translation reaction) for 1 hour at 4°C. The beads were then washed 5 times with bead buffer and during the final wash they were transferred to a clean eppendorf tube. The proteins were then eluted from the beads by boiling in SDS sample buffer for 5 minutes before analysis by SDS PAGE and autoradiography. In general, half of the bead-bound proteins were analysed on the gel.
Glutathione Sepharose affinity purification
Samples were spun for one minute in a microcentrifuge at room temperature and the supernatant was incubated with 10-20 µl of Glutathione Sepharose in 200 µl bead buffer per standard reaction (in general, 5 µl of a translation reaction containing a GST fusion protein) for 1 hour at 4°C. The beads were then washed 5 times with bead buffer and during the final wash they were transferred to a clean eppendorf tube. The proteins were then eluted from the beads by boiling in SDS sample buffer for 5 minutes before analysis by SDS PAGE and autoradiography. In general, half of the bead-bound proteins were analysed on the gel.

Small-scale Ni²⁺-NTA agarose affinity purification
Samples were spun for one minute in a microcentrifuge at room temperature and the supernatant was incubated with 20 µl of Ni²⁺-NTA agarose in 200 µl IMAC5 per sample (10 µl samples) for 1 hour at 4°C. The agarose-bound proteins were washed once with IMAC5, twice with IMAC25/Tx100 and once more with IMAC5, during which they were transferred to a clean eppendorf tube. The proteins were then eluted from the beads by boiling in SDS sample buffer for 5 minutes, before analysis by SDS PAGE and autoradiography. Half of each sample was run on the gel.

Bacterial expression and purification of GST-cdc2H6
The E. coli bacterial strain BL21(DE3) was transformed with the plasmid encoding the hexa-histidine tagged glutathione-S-transferase-p34cdc2 fusion protein, GST-cdc2H6. Bacteria containing the plasmid were grown in 400 ml of 2xTY containing 50 µg/ml ampicillin at 37°C to an A₆₀₀ of approximately 1.0. At this point the culture was diluted with 400 ml of fresh 2xTY media containing 50 µg/ml ampicillin, and 40 µM (final concentration) isopropyl-β-D-thiogalactopyranoside (IPTG) was added to induce the expression of GST-cdc2H6. After incubation at room temperature for approximately 16 hours, the cells were harvested by centrifugation and resuspended in 60 ml of ice-cold IMAC5. All subsequent steps were carried out at 4°C. The cells were lysed by the addition of 1 mg/ml lysozyme to the suspension for 15 minutes, followed by the addition of 0.5% (v/v) Nonidet-P40 for 5 minutes. After sonication, the lysate was centrifuged at 18,000 g for 20 minutes and passed through a 0.45 µM filter (Millipore). The supernatant was loaded onto a 5 ml Ni²⁺-NTA agarose (Qiagen) column over at least 2 hours. The column was washed with 5 bed volumes of IMAC5, followed by two washes with 5 bed volumes each of IMAC25/Tx100, finally it was washed with 5 bed volumes of IMAC25 containing 1µg/ml each leupeptin, pepstatin A and chymostatin. The GST-cdc2H6 protein was eluted off
in 10 half-bed volumes of IMAC150, and samples of each fraction were analysed by SDS PAGE to determine in which fractions the protein had eluted. Those fractions containing the GST-cdc2H6 protein were dialysed against buffer A overnight and the protein was concentrated with a Microsep concentrator (Flowgen) according to the manufacturer’s instructions.

**Assays employing protein phosphorylation**

**Histone H1 kinase assay**
The histone H1 kinase activity of proteins in an extract was measured by incubating 1 µl of extract with 6.5 µl of Cicirelli buffer and 2.5 µl of reaction mix (2 mg/ml histone H1; 1 mM DTT; 15 mM Mg acetate; 300 µM rATP and 1.25 µCi [γ-32P]ATP (Amersham, #PB10168)) at 23°C for 10 minutes. Bead-bound proteins were washed in Cicirelli buffer before the addition of 7.5 µl of Cicirelli buffer and 2.5 µl of reaction mix. Reactions were stopped by the addition of SDS sample buffer and boiling. Typically, 25 µl of SDS sample buffer was added to the 10 µl reaction and 10 µl of this was loaded on the gel.

**Cyclin phosphorylation**
Non-[35S]methionine labelled bead-bound proteins were washed with Cicirelli buffer and resuspended in 10 µl of Cicirelli buffer containing between 5 and 10 µCi of [γ-32P]ATP (Amersham, #PB10168). The reaction was incubated at room temperature for 30 minutes and stopped by the addition of SDS sample buffer. If the cyclin phosphorylation assay was to investigate whether certain cyclin proteins were able to be phosphorylated, 25 µl of SDS sample buffer was added to the 10 µl reaction mix and 10 µl of this was loaded on the gel. If the cyclin was being phosphorylated for phosphoamino acid or phosphopeptide analysis, 10 µl of a 5x stock of SDS sample buffer was added to the 10 µl reaction mix and half of this was loaded on the gel.

**The separation and detection of proteins**

**SDS polyacrylamide gel electrophoresis (SDS PAGE)**
The method of SDS PAGE was modified from Anderson *et al.* (1973), with the main modification being that neither the stacking or resolving gels contained SDS. All of the polyacrylamide gels that are described in this thesis were run using 15% resolving gels. Mixes of the resolving and stacking gels were prepared frequently from stock solutions and stored at 4°C. The composition of the gel mixes were as follows:
<table>
<thead>
<tr>
<th>Component</th>
<th>Resolving gel (15%)</th>
<th>Stacking gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% (w/v) acrylamide</td>
<td>50.0 ml</td>
<td>25.0 ml</td>
</tr>
<tr>
<td>1% (w/v) bisacrylamide</td>
<td>8.6 ml</td>
<td>20.0 ml</td>
</tr>
<tr>
<td>1.5 M Tris-Cl, pH 8.8</td>
<td>25.0 ml</td>
<td>-</td>
</tr>
<tr>
<td>1.0 M Tris-Cl, pH 6.8</td>
<td>-</td>
<td>18.8 ml</td>
</tr>
<tr>
<td>H₂O</td>
<td>16.4 ml</td>
<td>86.2 ml</td>
</tr>
<tr>
<td></td>
<td>100 ml</td>
<td>150 ml</td>
</tr>
</tbody>
</table>

In general, acrylamide was of electron grade (BDH), but lower grades also worked well so long as they were deionised and filtered before use. Stocks of 30% (w/v) acrylamide and 1% (w/v) bisacrylamide were deionised with MB5113 mixed bed ion exchange resin (BDH) and filtered through a 0.22 μm filter (Millipore).

Resolving gel mixes were polymerised with a final concentration of 0.05% (v/v) TEMED (N,N,N',N'-tetramethyl-ethylenediamine) and 0.05% (w/v) ammonium persulphate; stacking gel mixes with a final concentration of 0.1% (v/v) TEMED and 0.1% (w/v) ammonium persulphate. Both mini (plates 12x8 cm) and standard size (plates 20x13 cm) gels were cast and run using apparatus purchased from Cambridge Electrophoresis. Samples to be analysed by SDS PAGE were resuspended in SDS sample buffer (80 mM Tris-Cl, pH 6.8; 2% (w/v) SDS, 10% (v/v) glycerol; 0.002% (w/v) bromophenol blue and 50 μl/ml 2-mercaptoethanol added just before use) and incubated in a boiling water bath for 2 minutes. Bead-bound proteins were eluted in SDS sample buffer by boiling for 5 minutes.

SDS PAGE was carried out in SDS PAGE running buffer (25 mM Tris base; 192 mM glycine; 0.1% (w/v) SDS). Mini gels were typically run with a limiting voltage of 200 V at the beginning of the run with current limiting at 20 milliamperes during the second half of the run; standard gels were typically run with a limiting voltage of 250 V at the beginning of the run with current limiting at 30 milliamperes during the second half of the run. Electrophoresis was continued until the bromophenol blue dye front had just run off the bottom of the gel.

**Gel filtration**

The extracts to be analysed by gel filtration were clarified by centrifugation in a microcentrifuge at 4°C for 30 minutes. The supernatant was diluted 1:1 with buffer B (100 mM KCl, 10 mM NaCl, 1.1 mM MgCl₂, 0.1 mM EDTA, 10 mM HEPES, pH 7.2 with KOH) and spun twice more for 5 minutes each time at room temperature. The samples were loaded onto a Superdex 200 HR 10/30 FPLC gel filtration column equilibrated with buffer B at 0.5 ml/min at room temperature.
Fractions of 0.5 ml were collected and incubated for 30 minutes with acid washed silica (Sigma, S5631) to concentrate the protein. The silica was harvested by spinning and the supernatant removed. 20 µl of SDS sample buffer was added to the silica and boiled for 5 minutes. 10 µl of the supernatant from each sample was analysed by SDS-PAGE followed by immunoblotting. The column was calibrated using the following proteins: catalase (250 kDa), alcohol dehydrogenase (141 kDa), bovine serum albumin (67 kDa), ovalbumin (44 kDa), carbonic anhydrase (30 kDa) and cytochrome C (12.4 kDa). Dextran blue was used to determine the void volume of the column.

**Coomassie blue staining**

Gels for autoradiography were stained for 5 minutes with Coomassie blue (5 g/l Coomassie blue R250; 45% (v/v) methanol; 45% (v/v) glacial acetic acid), gels for analysis by scanning densitometry were stained for 20 minutes. Gels were destained in hot destain (25% (v/v) methanol and 7% (v/v) glacial acetic acid) for as long as was required to see the protein bands.

**Autoradiography**

Radioactively labelled proteins on polyacrylamide gels were detected by autoradiography. Gels that had been stained with Coomassie blue were dried under vacuum onto Whatman paper (3MM). \(^{35}\text{S}\)-labelled proteins were detected by exposure to hyperfilm β-max (Amersham) at room temperature; \(^{32}\text{P}\)-labelled proteins were detected by exposure to Fuji X-ray or Kodak X-omat film. Radiolabelled proteins in gels that contained both \(^{35}\text{S}\)- and \(^{32}\text{P}\)-labelled proteins were usually detected by exposure to hyperfilm β-max as this film was more sensitive to \(^{35}\text{S}\) than to \(^{32}\text{P}\) and so gave bands of more equal intensity. Weakly \(^{32}\text{P}\)-labelled proteins were detected on Fuji X-ray or Kodak X-omat film that had been pre-flashed and was put in front of an intensifying screen and stored at -80°C. In general gels were exposed to film overnight (12-16 hours), exceptions that differ greatly from this norm will be stated in the text.

**Analysis of phosphorylated proteins**

**Phosphoamino acid analysis**

The mRNAs encoding the cyclin A constructs to be analysed were translated in CSF-arrested egg extract with 50% (v/v) added reticulocyte lysate, in the absence of \(^{35}\text{S}\)methionine, and immunoprecipitated using the anti-c-\text{myc} antibody, 9E10. The immunoprecipitates were incubated with \([γ-^{32}\text{P}]\text{ATP}\), the proteins separated by SDS PAGE and transferred to nitrocellulose. The membrane was washed in water, wrapped in Saran wrap, and exposed to pre-flashed Kodak X-omat film for
between 0.5 and 1 hours. The positions of the $^{32}$PO$_4$-labelled cyclin A bands on the membrane were identified using the autoradiograph, and they were excised from the nitrocellulose. The fragments of nitrocellulose were blocked in 0.5% (w/v) polyvinyl-Pyrrolidone (PVP) in 100 mM acetic acid for 30 minutes at 37°C and then washed five times with large quantities of H$_2$O, followed by 2 washes in freshly made 50 mM NH$_4$HCO$_3$ with 5% (v/v) added acetonitrile. The pieces of nitrocellulose were then cut up into very small fragments and incubated with 6 µg of V8 endopeptidase in 100 µl of 50 mM NH$_4$HCO$_3$ with 5% (v/v) added acetonitrile at 37°C overnight. The supernatant (now containing peptides resulting from the digestion of the cyclin protein with the V8 endopeptidase) was removed from the pieces of nitrocellulose to a fresh tube, the fragments were washed once with 100 µl of H$_2$O and this was added to the digest supernatant. The peptides were then lyophilised in a speed-vac, resuspended in 200 µl of H$_2$O and dried again. This procedure was repeated 4 or 5 times until no salt crystals were visible in the bottom of the tube when it was dried. Finally the peptides were resuspended in 10 µl 100 mM acetic acid. Half of the peptides were analysed at a time by thin layer electrophoresis in pH 4.72 buffer (10% (v/v) n-butanol; 5% (v/v) pyridine; 5% (v/v) acetic acid) on polygram TLC (thin layer chromatography) plates (Macherey-Nagel) followed by chromatography using phospho-chromatography buffer (37.5% (v/v) n-butanol; 25% (v/v) pyridine; 7.5% (v/v) acetic acid) according to Boyle et al. (1991). The positions of the phosphorylated peptides on the TLC plates were visualised by exposing the plates to autoradiography film.

**Edman degradation of phosphorylated peptides**
The silica containing the phosphorylated peptides on the TLC plates was scraped off and the peptides extracted by incubation in 100 µl of 7% ammonia; 50% (v/v) methanol for 2 hours. The supernatant was then filtered through a 0.45 µm ultra-free unit and freeze dried. The freeze dried material was dissolved in 30 µl of 50% (v/v) acetonitrile and covalently attached to an arylamine coated poly(vinylidene difluoride) membrane (Coull et al., 1991). Edman chemistry was performed for 20 cycles, with the resulting phenyl thiohydantoin residue being diverted for collection in a fraction collector, ready for subsequent Cerenkov counting, for 30 minutes each. This entire procedure was carried out by the ICRF Protein Sequencing Laboratory.

**Phosphoamino acid analysis of proteins**
The mRNAs encoding the cyclin A constructs to be analysed were translated in CSF-arrested egg extract with 50% (v/v) added reticulocyte lysate, in the absence of [$^{35}$S]methionine, and immunoprecipitated using the anti-c-myc antibody, 9E10.
The immunoprecipitates were incubated with [γ-32P]ATP, the proteins separated by SDS PAGE and transferred to immobilon (Millipore). The membrane was dried and exposed to pre-flashed Kodak X-omat film for between 0.5 and 1 hours. The positions of the 32PO4-labelled cyclin A bands on the membrane were identified using the autoradiograph, and they were excised from the immobilon. The membrane was re-wetted by incubation in methanol for 30 seconds followed by incubation in H2O for 30 seconds, cut into small pieces and placed in a screw-capped eppendorf tube. To it was added 200 μl of 5.7 M HCl and all the air was removed by freezing the HCl in liquid nitrogen, filling the rest of the tube with liquid nitrogen and replacing the lid of the tube just as the last of the nitrogen was boiling off. The tube was then heated at about 110°C for 75 minutes. When it had cooled, the supernatant was removed and lyophilised in a speed-vac. The amino acid mix was resuspended in 10 μl of 100 mM acetic acid and half of this was analysed at a time by thin layer electrophoresis in pH 3.5 buffer (5% (v/v) acetic acid 0.5% (v/v) pyridine) on polygram TLC (thin layer chromatography) plates (Macherey-Nagel). The positions of phosphoserine, phosphothreonine and phosphotyrosine on the plate when electrophoresed in this manner were detected by running approximately 15 nmoles of each of the three phosphoamino acids mixed in with the sample being analysed. The three phosphoamino acid standards were also run individually. These standard phosphoamino acids were detected by staining with 0.25% (w/v) ninhydrin in acetone followed by heating with a hair dryer. The positions of the phosphorylated amino acids from the proteins were visualised by autoradiography and the amino acid type determined by comparison with the positions of the ninhydrin-stained standard phosphoamino acids.

**Phosphoamino acid analysis of peptides**

The silica containing the phosphorylated peptides on the TLC plates was scraped off and the peptides extracted by washing twice with pH 1.9 buffer (2.2% (v/v) formic acid; 7.8% (v/v) acetic acid) and once with 50% (v/v) pyridine. All the washes were combined and the peptides lyophilised in a speed-vac and resuspended in 100 μl 5.7 M HCl. The procedure then continued as described above.

**Cell culture, transient transfections and immunofluorescence**

**Cell culture**

All cell culture work was carried out using Cos1 cells (SV40 transformed African Green Monkey Kidney cells, derived from CV1 cells). Cells were grown in E4
medium (Dulbecco’s modified Eagle’s medium) supplemented with 10% (v/v) foetal calf serum at 37°C in 7.5% (v/v) CO₂. Cells were split when confluent by rinsing them three times with versene (0.02% (w/v) EDTA in PBSA) containing 0.05% (w/v) trypsin and leaving them at 37°C until the cells became detached from the dishes (usually about 5 minutes). They were then diluted between 10 and 25 fold into fresh medium and transferred to new tissue culture dishes.

**Transient calcium phosphate transfections**

Cells for transient transfection experiments were seeded at a concentration of 6x10⁴/ml onto 19 mm (diameter) coverslips that had been prepared by boiling in 8.8 g/l Hexametaphosphate; 79.12 g/l Sodium Metasilicate for 20 minutes, followed by 2 or 3 rinses in distilled water, boiling for 10 minutes in distilled water, rinsing in ethanol and sterilisation by baking.

DNA for transfection was prepared by alkaline lysis and PEG precipitation. The following stock solutions were used:

- 2x HBS: 10 g/l HEPES; 16 g/l NaCl
- 100x PO₄: 35 mM Na₂HPO₄; 35 mM NaH₂PO₄
- CaCl₂: 2 M, tissue culture grade

All solutions were filter sterilised.

The procedure for preparing the DNA precipitate for a 9 cm (diameter) plate was as follows:

550 μl of 2xHBS was mixed with 11 μl of 100x PO₄. To 22 μg of DNA was added enough H₂O to make the volume of the final mix to 1.1 ml, and 68.75 μl of CaCl₂. The DNA/CaCl₂ mixture was immediately added dropwise to the HBS/PO₄ mixture whilst bubbling air through it with an automatic pipettor and a Pasteur pipette. The final composition of the transfection solution was therefore 5 g/l HEPES, pH 7.05 (exactly) with NaOH; 8 g/l NaCl; 3.5 mM Na₂HPO₄; 3.5 mM NaH₂PO₄; 125 mM CaCl₂; 20 μg/ml DNA. The mixture was allowed to stand at room temperature for about 30 minutes, was mixed by vortexing and 1 ml of it was added dropwise over the surface of a 9 cm (diameter) dish containing cells that had been seeded 24 hour previously in the standard medium (E4 supplemented with 10% foetal calf serum). The transfection solution was gently mixed with the media by 'swirling' the plates. The cells were returned to the incubator and left overnight. The next morning the media was removed, the cells were washed once with versene (0.02% (w/v) EDTA in PBSA) to remove excess Ca²⁺, and fresh media was added. The cells were taken for analysis between 26 and 30 hours later.
Immunofluorescence
FITC-conjugated anti-rat CD2 antibody, OX34, was provided by Dr Chris Norbury (IMM, Oxford). The anti-c-myc antibody, 9E10, was conjugated to TRITC exactly as described in Harlow and Lane (1988).

Coverslips containing transfected cells were transferred to a new dish and washed twice with PBSA. The cells were then fixed by immersion in freshly made 50% (v/v) methanol; 50% (v/v) acetone for 2 minutes and then washed once more in PBSA. An antibody incubation chamber was set up in which a circle of Whatman 3MM paper was wetted with PBSA and placed in the lid of a petri dish. A piece of parafilm was placed on this paper and onto this were placed 30 µl drops of antibody solution. The coverslips containing the fixed cells to be stained were inverted onto the drops of antibody solution, the other half of the petri dish was placed on top and the chamber was incubated in the dark at 4°C overnight. The antibody solution contained FITC-conjugated OX34 and TRITC-conjugated 9E10, diluted in PBSA containing 3% (w/v) bovine serum albumin (BSA). After the antibody incubation the coverslips were washed twice with PBSA, for five minutes at room temperature each time, once with PBSA containing Hoescht (33258) at 1 µg/ml, rinsed briefly with distilled water and mounted on slides in glycerol containing 2.5% DABCO (1,4-Diazabicyclo[2.2.2]octane; Aldrich). The coverslips were sealed onto the slides with nail varnish and viewed using a fluorescence microscope.
Chapter 3

Cyclin A mutants

This thesis describes studies aimed to elucidate the role of cyclin A in the cell cycle. In this chapter I describe the construction and assay of cyclin A mutants designed to study the domain structure of *Xenopus* cyclin A. I hoped that it might be possible to make a 'dominant negative' mutant of cyclin A, which retained the ability to make its normal protein-protein interactions, including the ability to bind to, but not activate, p34cdc2.

Construction of c-myc tagged cyclin A

Since the systems to be used in the investigation of cyclin A function, *Xenopus* oocytes and *Xenopus* egg extracts, contain endogenous cyclin A protein (between 0.5 and 1 nM, see (Kobayashi et al., 1991; this thesis, Appendix 2), an epitope-tagged version of *Xenopus* cyclin A was constructed in order to allow the mutant cyclin A constructs to be distinguished from the endogenous wild-type protein.

Before the construction of the epitope-tagged cyclin A described below, I made C-terminal deletion mutants of cyclin A. In these constructs the entire 3' untranslated region (UTR) of cyclin A was removed as a consequence of adding the epitope tag to the C-terminus of the protein. Although synthetic mRNA transcribed from these mutants was translated well in rabbit reticulocyte lysate, the same mRNA translated extremely poorly in *Xenopus* egg extracts (data not shown). Subsequently, Carl Peto in the laboratory showed that progressive deletion of the 3' UTR of cyclin A gave constructs whose translation efficiency in *Xenopus* egg extract progressively decreased. It was therefore very important that in the construction of the new epitope-tagged cyclin A, the 3' UTR of the construct was preserved.

The epitope tagged cyclin A that was ultimately used was constructed by mutating a wild-type *Xenopus* cyclin A clone, using the polymerase chain reaction (PCR), so that it contained an *NcoI* site at its second methionine residue, the fifth amino acid of the protein. This plasmid was then digested with *NcoI* and *BsmI* (see figure 3.1), which removes the DNA sequence coding for residues 5-21 of the protein. This DNA fragment was replaced with a synthetic oligonucleotide encoding a 12 residue peptide that contained an epitope from the *c-myc* gene,
**Figure 3.1  Nucleotide and amino acid sequence of c-myc tagged cyclin A**

Epitope tagged Xenopus cyclin A was constructed by replacing the DNA encoding for the first 21 amino acids of wild-type cyclin A with an oligonucleotide encoding a peptide containing an epitope from the c-myc gene, recognised by the monoclonal antibody, 9E10. The amino acid sequence of c-myc cyclin A, in one letter code, is shown above the nucleotide sequence. The epitope recognised by 9E10 is shown in bold type and underlined. The amino acid sequence of wild-type cyclin A is shown above that of the c-myc tagged version where the two sequences differ. The positions of the engineered stop codons for the cyclin A C-terminal deletions are shown as asterisks above the amino acid sequence, along with the name of the mutant. The numbers refer to the distance (in nucleotides) from the T7 RNA polymerase promoter.
recognised by the monoclonal antibody 9E10 (Evan et al., 1986). The whole of the coding region and the entire 3' UTR of this c-myc tagged version of cyclin A was then placed in a pGEM2 based vector (constructed by Mary Dasso) downstream of a T7 RNA polymerase promoter and a translational leader derived from the influenza virus NS protein (Dasso and Jackson, 1989). This added three unrelated amino acids to the N-terminus of the protein. A T7 RNA polymerase terminator sequence was placed downstream of the 3' UTR of cyclin A. For further details of the construction of c-myc cyclin A, see Chapter 2.

The amino acid and nucleotide sequence of c-myc tagged *Xenopus* cyclin A is shown in figure 3.1. The epitope recognised by the monoclonal antibody, 9E10 is in bold and underlined; the first 21 amino acids of wild-type cyclin A are shown above the amino acid sequence of c-myc tagged cyclin A for comparison.

It had previously been demonstrated that a sea urchin cyclin B protein that was missing its first 13 amino acids was able to bind to and activate p34$^\text{cdc2}$ and was destroyed normally. In addition, a sea urchin cyclin B construct from which the first 90 amino acids had been removed was able to bind to and activate p34$^\text{cdc2}$, although it was not destroyed (Murray et al., 1989). It was therefore likely that c-myc tagged cyclin A (c-myc cyclin A) would be able to bind to and activate p34$^\text{cdc2}$, and get destroyed in a manner identical to wild-type cyclin A. In this and subsequent chapters this will be shown to be the case. Nevertheless, c-myc cyclin A was used as a positive control when using c-myc tagged cyclin A mutants and non-tagged cyclin A was used when investigating the properties of non-tagged cyclin A mutants.

**C-terminal deletions of cyclin A**

Since Hideki Kobayashi in the laboratory was making N-terminal deletions of cyclin A, I began by making C-terminal deletions.

**Construction of C-terminal deletions of cyclin A**

Deletions of 14, 24, 50, 79, 97 and 139 amino acids were made from the C-terminus of c-myc cyclin A using PCR. Fragments were amplified between specially designed 3' oligonucleotides which introduced internal stop codons, and a non-mutagenic 5' oligonucleotide spanning the internal *HindIII* site (shown on figure 3.1 in bold type). The 3' primers contained a *BamHI* site downstream of the stop codon. The PCR fragments were digested with *HindIII* and *BamHI* and inserted between the *HindIII* and *BclII* sites of c-myc cyclin A (see Chapter 2 and Appendix 1 for further details). The *BclII* site (shown on figure 3.1 in bold type) is at the start of the 3' UTR of cyclin A and thus the 3' UTR of these cyclin A
mutants was preserved. The positions of the introduced stop codons are indicated by asterisks on figure 3.1.

**C-terminal deletions of cyclin A cannot bind to p34\(\text{cdc2}\)**

In order to test whether the deletion mutants of cyclin A could bind to p34\(\text{cdc2}\), *in vitro* transcribed mRNAs (see Chapter 2) encoding c-myc cyclin A and the cyclin A mutants with deletions of 14, 24 and 50 amino acids from their C-termini (CA14, CA24 and CA50 respectively) were translated in CSF-arrested egg extract in the presence of 0.6 mCi/ml \[^{35}\text{S}]\text{methionine}\) at 23°C for 2 hours (see Chapter 2). The translation reactions were split into two aliquots, one of which was incubated with \(\text{p13}^{\text{Suc1}}\) Sepharose. \(\text{p13}^{\text{Suc1}}\) binds to p34\(\text{cdc2}\) and related proteins and can be used as an affinity resin for p34\(\text{cdc2}\) and associated proteins (Brizuela et al., 1987; Draetta et al., 1989; Labbé et al., 1989; Pondaven et al., 1990). The other aliquot was incubated with Sepharose coupled to bovine serum albumin (BSA) as a negative control. The radioactive proteins that bound to the affinity beads were analysed by SDS PAGE and autoradiography. Newly translated cyclin proteins can associate with the p34\(\text{cdc2}\) available in CSF-arrested egg extract (between 450 and 600 nM, see Appendix 2) and are therefore retained by

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**Figure 3.2 The C-terminally deleted cyclin A mutants cannot be precipitated on p13\(\text{Suc1}\) Sepharose**

The mRNAs encoding the cyclin A constructs indicated above the figure were translated in CSF-arrested egg extract in the presence of \[^{35}\text{S}]\text{methionine}\). Lanes 1 - 5, the complete translation reaction; lanes 6 - 10, precipitation with p13\(\text{Suc1}\) Sepharose; lanes 11 - 15, control precipitation with BSA Sepharose. Visualised by autoradiography. Asterisk on right indicates position of the endogenous B-type cyclins.
p13\textsuperscript{suc1} Sepharose. As figure 3.2 shows, while wild-type cyclin A bound to p13\textsuperscript{suc1} Sepharose, (lane 7), the cyclin A mutants CA14, CA24 and CA50 did not (lanes 8 - 10). This implies that these mutants cannot bind to the p34\textsuperscript{cdc2} or related proteins in the egg extract. During the translation reaction B-type cyclins are translated from the endogenous mRNA present in the CSF-arrested egg extract. The labelled B-type cyclins thus generated can bind to p34\textsuperscript{cdc2} and are therefore precipitated on the p13\textsuperscript{suc1} Sepharose. They served as internal controls. The position of the B-type cyclins is marked with an asterisk on the right hand side of figure 3.2.

Since it is still not entirely clear how p13\textsuperscript{suc1} binds to p34\textsuperscript{cdc2}, and because it can also bind to a large number of other proteins in \textit{Xenopus} egg extract, I tested the binding of the mutant cyclin A constructs to p34\textsuperscript{cdc2} by immunoprecipitation with an anti-p34\textsuperscript{cdc2} monoclonal antibody, A17. The mRNAs encoding c-myc cyclin A or the C-terminal deletions of cyclin A were translated in CSF-arrested egg extract and the translation reactions were split into two aliquots. One was incubated with the anti-p34\textsuperscript{cdc2} monoclonal antibody, A17, and the other with a control antibody against human retinoblastoma protein, IF8. IF8 does not cross react with \textit{Xenopus} retinoblastoma protein or any other \textit{Xenopus} proteins in egg extracts. Any labelled protein that is present in immunoprecipitates using this antibody, therefore, is binding non-specifically to the antibody or the protein A Sepharose. The immune complexes were collected on protein A Sepharose and the products analysed by SDS PAGE and autoradiography (see Chapter 2). Figure 3.3A shows that while wild-type cyclin A bound strongly to p34\textsuperscript{cdc2} (lane 7), CA14, CA24 and CA50 did not (lanes 8 - 10). The faint bands corresponding to CA14, CA24 and CA50 seen in lanes 7 - 10 (figure 3.3A) are probably due to non specific binding, since similar bands can be seen in the control immunoprecipitation (lanes 13 - 15).

A similar test was carried out on the translation products of the other C-terminal deletions of cyclin A, CA79, CA97 and CA139. These constructs were also unable to bind to p34\textsuperscript{cdc2} (figure 3.3B, lanes 8 - 10).

**The C-terminal deletions of cyclin A cannot activate histone H1 kinase activity**

The previous experiments indicated that p34\textsuperscript{cdc2} could not associate with the C-terminally deleted cyclin A constructs. However, it was possible that a small amount of p34\textsuperscript{cdc2} associated with some or all of these cyclin A mutants, but that this association was below the limit of detection on the autoradiograph. Histone H1 is phosphorylated by activated p34\textsuperscript{cdc2} kinase \textit{in vitro}, and its phosphorylation is easier to detect than the co-precipitation of cyclin A with p34\textsuperscript{cdc2} (compare the intensity of the [\textsuperscript{35}S]-labelled cyclin band in lane 7 of figure 3.3A with that of the
Figure 3.3  The C-terminal deletion mutants of cyclin A cannot be immunoprecipitated with an anti p34<sup>cdc2</sup> antibody
The mRNAs encoding the cyclin A constructs indicated above the figure were translated in CSF-arrested egg extract in the presence of [<sup>35</sup>S]methionine. Lanes 1 - 5, the complete translation reaction; lanes 6 - 10, immunoprecipitation with the anti p34<sup>cdc2</sup> monoclonal antibody, A17; lanes 11 - 15, control immunoprecipitation with a monoclonal antibody against human retinoblastoma protein, IF8. Visualised by autoradiography. Asterisks on right indicate positions of the endogenous B-type cyclins.
[\textsuperscript{32}P]-labelled histone H1 in lane 7 of figure 3.4). The phosphorylation of histone H1 therefore provides a more sensitive assay for detecting the binding of the cyclin A deletion mutants to p34\textsuperscript{cdc2}. To test whether CA14, CA24 or CA50 cyclin A could activate p34\textsuperscript{cdc2}, the mRNAs encoding each construct and that encoding c-myc cyclin A were translated in CSF-arrested frog egg extract and immunoprecipitated with the anti-c-myc monoclonal antibody, 9E10 and protein A Sepharose. The bead-bound complexes were incubated with purified histone H1 and [\textsuperscript{\gamma}\textsuperscript{32}P]ATP and the products analysed by SDS PAGE and autoradiography (see Chapter 2). Figure 3.4 shows that wild-type cyclin A activated p34\textsuperscript{cdc2} histone H1 kinase (lane 7). In contrast, CA14, CA24 and CA50 had no p34\textsuperscript{cdc2} histone H1 kinase activity associated with them (lanes 8 - 10). Under these conditions, then, CA14, CA24 and CA50 cannot activate p34\textsuperscript{cdc2} kinase at all, presumably because they cannot bind to p34\textsuperscript{cdc2}, as suggested by the co-immunoprecipitation results.

**Figure 3.4** The C-terminally deleted cyclin A mutants do not have associated histone H1 kinase activity
The mRNAs encoding the cyclin A constructs indicated above the figure were translated in CSF-arrested egg extract in the presence of \textsuperscript{35}S]methionine. Lanes 1 - 5, the complete translation reaction; lanes 6 - 10, immunoprecipitation with the anti-c-myc monoclonal antibody, 9E10, followed by incubation of the bead-bound proteins with purified histone H1 and [\textsuperscript{\gamma}\textsuperscript{32}P]ATP. Visualised by autoradiography.
The C-terminal deletions of cyclin A are unable to promote significant Xenopus oocyte maturation

Stage VI Xenopus oocytes are arrested late in the G2 phase of the cell cycle and are induced to enter meiosis and mature into eggs in vivo by progesterone. The injection of mRNA encoding A- or B-type cyclins into stage VI Xenopus oocytes has been shown to cause the artificial maturation of the oocyte into an egg (Swenson et al., 1986; Pines and Hunt, 1987).

The oocyte maturation activity of c-myc tagged wild-type, CA14, CA24 and CA50 cyclin A protein was tested by the microinjection of approximately 50 ng of the mRNA encoding each construct into stage VI Xenopus oocytes. The microinjection was performed by Hideki Kobayashi. The oocytes were scored for meiotic maturation by the presence of a white spot on the animal pole in matured eggs (see Chapter 2). While 96% of the oocytes injected with wild-type cyclin A matured, none of those injected with the mRNA encoding CA50 cyclin A did (Table 3.1). CA14 and CA24 cyclin A showed a slight activity, since 6% of those oocytes injected with CA14 mRNA, and 15% of those oocytes injected with CA24 mRNA matured. This suggests that the oocyte maturation assay is capable of detecting weak interactions that are disrupted when cyclin A/p34^cdc2 complexes are harvested on beads. Nevertheless, this assay confirms that the CA14, CA24 and CA50 cyclin A deletion mutants are seriously impaired in activity. Moreover, the ability of cyclin A to bind to p34^cdc2 is likely to be required for it to promote

<table>
<thead>
<tr>
<th>Construct</th>
<th>Number oocytes injected</th>
<th>Number oocytes matured</th>
<th>% maturation</th>
</tr>
</thead>
<tbody>
<tr>
<td>c-myc cyclin A</td>
<td>24</td>
<td>23</td>
<td>96</td>
</tr>
<tr>
<td>CA14</td>
<td>32</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>CA24</td>
<td>26</td>
<td>4</td>
<td>15</td>
</tr>
<tr>
<td>CA50</td>
<td>36</td>
<td>0</td>
<td>0</td>
</tr>
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Table 3.1 Oocyte maturation by C-terminally deleted cyclin A constructs

Stage VI Xenopus oocytes were each injected with 50 ng mRNA encoding the construct to be tested, and scored for maturation (see Chapter 2). Microinjection was performed by Hideki Kobayashi.
oocyte maturation. Thus the p34\textsuperscript{cdc2} binding assay is a good indicator of whether a particular cyclin A mutant is active or not.

It was surprising that deletion of as few as 14 amino acids from the C-terminus of cyclin A rendered the protein unable to bind to or activate p34\textsuperscript{cdc2}, and therefore unable to promote oocyte maturation. Results obtained by Hideki Kobayashi in the laboratory showed that residues up to and including valine 161 could be deleted from the N-terminus of \textit{Xenopus} cyclin A without adversely affecting the binding to or activation of p34\textsuperscript{cdc2}, or induction of meiotic maturation in oocytes (Kobayashi \textit{et al.}, 1992).

\textbf{Point mutations of cyclin A}

I next chose to mutate conserved residues within the cyclin box of cyclin A and to see whether the resulting mutants could still bind to and activate p34\textsuperscript{cdc2}. Within the cyclin box there are five residues that are conserved in nearly all cyclins, including the \textit{S. cerevisiae} CLN cyclins and cdc13 and cig1 from \textit{S. pombe}. In \textit{Xenopus} cyclin A they are R197, D226, L241, K252 and E281 (see bold residues on figure 3.5). Since these residues are conserved in all cyclins that are known to bind to and activate a cdk subunit, except cyclin H, it is likely that they play a part in this binding and activation.

\textbf{Construction of cyclin A point mutants}

Four point mutants of cyclin A were constructed from \textit{c-myc} cyclin A using PCR (see Chapter 2 and Appendix 1); arginine 197 was changed either to an alanine or a lysine residue, to give R197A and R197K respectively, and aspartic acid 226 was changed either to an alanine or a glutamic acid residue to give D226A and D226E respectively (see figure 3.5 for position of these two residues).

\textbf{R197A and D226A cyclin A cannot bind to p34\textsuperscript{cdc2}}

The mRNAs encoding either \textit{c-myc} cyclin A, R197A, D226A or CA14 (as a negative control) were translated in CSF-arrested egg extract, and the translation reactions were split into two aliquots. One was immunoprecipitated using the anti-p34\textsuperscript{cdc2} monoclonal antibody, A17, the other using a control antibody against human retinoblastoma protein, IF8. The products were analysed by SDS PAGE and autoradiography. As shown in figure 3.6, while wild-type cyclin A can bind to p34\textsuperscript{cdc2} (lane 7), R197A, D226A and CA14 cannot (lanes 8 and 9). The faint bands corresponding to R197A, D226A and CA14 seen in lanes 8 - 10 are probably due to non specific binding, since similar bands can be seen in the control immunoprecipitations (lanes 13 - 15).
Figure 3.5  Conservation within the cyclin box of 10 different cyclins
The cyclin boxes of a selection of cyclins are shown, to indicate the conservation that exists within this region. The boxed residues are those that are conserved throughout the cyclins shown in this figure. A dot in the cyclin sequence indicates that the residue is the same as that shown in the consensus sequence at the bottom of the figure; a dot in the consensus sequence indicates that there is no consensus for this residue. The dash in the consensus sequence indicates that, to keep the spacing, a space has been inserted in all the sequences apart from human cyclin E. The five residues that are conserved in nearly all cyclins are shown in bold type in the consensus sequence.
R197K and D226E cyclin A can bind to p34<sup>cdc2</sup> only very weakly

Although it is unlikely that the single point mutations R197A and D226A cause the whole structure of the cyclin A protein to be distorted, thereby making it unable to bind to p34<sup>cdc2</sup>, these substitutions do not conserve the charges of the amino acids or replace them with residues of a similar structure. Misfolding of the protein is, therefore, a possibility that cannot be ruled out in these two mutants. The two conservative substitution mutants, R197K and D226E, that are even less likely to distort the structure of the protein, were therefore tested for their ability to bind to p34<sup>cdc2</sup>.

The mRNAs encoding c-myc cyclin A, R197K, D226E or R197A (as a negative control) were translated in CSF-arrested egg extract and the translation reactions were split into two aliquots. One was immunoprecipitated using the anti-p34<sup>cdc2</sup> monoclonal antibody, A17, and the other using a control antibody against human retinoblastoma protein, IF8. The products were analysed by SDS PAGE and autoradiography. As shown in figure 3.7, even the conservative point mutations, R197K and D226E, appear to render cyclin A unable to bind to p34<sup>cdc2</sup> (lanes 8 and 9).

The binding of c-myc cyclin A, CA14, CA24, CA50, R197A, D226A, R197K and D226E was also tested in a different way. The mRNAs encoding these cyclin A constructs were translated in a 1:1 (v/v) mixture of CSF-arrested egg extract and reticulocyte lysate, in the presence of <sup>35</sup>S-methionine and 1.25 μM of a bacterially expressed, hexa-histidine tagged, fusion protein between glutathione-S-transferase and p34<sup>cdc2</sup> (GST-cdc2H<sub>6</sub>). At the end of the translation reaction, the GST-cdc2H<sub>6</sub> bound proteins were affinity purified on glutathione Sepharose, and analysed by SDS PAGE and autoradiography; the data was also quantitated using a phosphorimager. Figure 3.8 shows that while c-myc cyclin A could bind to GST-cdc2H<sub>6</sub>, CA14, CA24, CA50, R197A and D226A could not (lanes 12 - 16). The faint bands that can be seen in these lanes are probably due to precipitation of <sup>35</sup>S-labelled cyclin A protein translated from the endogenous cyclin A mRNA in the extract, as this band was also present in the reaction in which no exogenous mRNA was translated (see lane 10). R197K and D226E could, however, bind weakly to GST-cdc2H<sub>6</sub> (see lanes 17 and 18), although the affinity of c-myc cyclin A for GST-cdc2H<sub>6</sub> was about 6 and 4 times greater than the affinities of R197K and D226E for GST-cdc2H<sub>6</sub>, respectively (quantitative data not shown).

Thus making conservative substitutions of two of the most highly conserved residues within the cyclin box severely impairs the ability of the cyclin A protein to bind to p34<sup>cdc2</sup>, but does not abolish it entirely.
Figure 3.6  R197A and D226A cyclin A cannot bind to p34<sup>cdc2</sup>

The mRNAs encoding the cyclin A constructs indicated above the figure were translated in CSF-arrested egg extract in the presence of <sup>35</sup>S-methionine. Lanes 1 - 5, the complete translation reaction; lanes 6 - 10, immunoprecipitation with the anti p34<sup>cdc2</sup> monoclonal antibody, A17; lanes 11 - 15, control immunoprecipitation with a monoclonal antibody against human retinoblastoma protein, IF8. Visualised by autoradiography. Asterisk on right indicates position of the endogenous B-type cyclins.

Figure 3.7  R197K and D226E cyclin A cannot bind to p34<sup>cdc2</sup>

The mRNAs encoding the cyclin A constructs indicated above the figure were translated in CSF-arrested egg extract in the presence of <sup>35</sup>S-methionine. Lanes 1 - 5, the complete translation reaction; lanes 6 - 10, immunoprecipitation with the anti p34<sup>cdc2</sup> monoclonal antibody, A17; lanes 11 - 15, control immunoprecipitation with a monoclonal antibody against human retinoblastoma protein, IF8. Visualised by autoradiography. Asterisk on right indicates position of the endogenous B-type cyclins.
The mRNAs encoding the cyclin A constructs indicated above the figure were translated in a 1:1 (v/v) mixture of CSF-arrested egg extract and rabbit reticulocyte lysate in the presence of [35S]methionine and 1.25 µM GST-cdc2H6 protein. Lanes 1 - 9, the complete translation reaction; lanes 10 - 18, precipitation with glutathione Sepharose. Visualised by autoradiography.

The binding of cyclin A to p34cdc2 is a very tightly controlled process, requiring specific residues within the cyclin box of cyclin A. The results described above endorse the view that the cyclin box, and the five conserved residues in particular, is intimately involved with cdk binding.

Discussion

C-terminal deletions of cyclin A

Within the family of A-type cyclins there is less conservation in the C-termini of the protein than within the cyclin boxes. This conservation decreases further when other families of cyclins are included in the analysis (see figures 3.9 and 3.10). It was therefore surprising that deleting as few as 14 residues from the C-terminus of Xenopus cyclin A rendered the protein unable to bind to p34cdc2. Maridor et al. (1993) have confirmed this result, showing that deletion of 15 amino acids from the C-terminus of chicken cyclin A leaves the protein unable to bind to p34cdc2. They found that mutants which have C-terminal deletions of 4 or 8 residues can still bind to p34cdc2. Lees and Harlow (1993) have also shown that although a human cyclin A construct containing a deletion of 10 amino acids its C-terminus of was still able to bind to p34cdc2, deletion of a further 21 amino acids from this construct abolished association of the protein with p34cdc2. Thus
Figure 3.9 Alignment of the cyclin boxes and C-termini of several A- and B-type cyclins

The cyclin boxes and entire C-termini of a selection of A- and B-type cyclins are shown. The shaded residues are those that are conserved sequence at the bottom of the figure; a dot in the consensus sequence indicates that there is no consensus for this residue. The dash in the cyclin sequence indicates that, to keep the alignment, a space has been inserted into that sequence.
Figure 3.10  Alignment of the cyclin boxes and C-termini of several A-type cyclins

The cyclin boxes and entire C-termini of a selection of A-type cyclins are shown. The shaded residues are those that are conserved throughout the cyclins shown in this figure. A dot in the cyclin sequence indicates that that residue is the same as that shown in the consensus sequence at the bottom of the figure; a dot in the consensus sequence indicates that there is no consensus for this residue. The dash in the cyclin sequence indicates that, to keep the alignment, a space has been inserted into that sequence.
only very small deletions from the C-terminus of cyclin A can be tolerated before the protein becomes unable to bind to or activate p34cdc2.

The importance of the C-terminus in p34cdc2 binding could be explained by one of two reasons. Firstly, a residue or residues required for the binding of cyclin A to p34cdc2 could be present in the extreme carboxy-terminus of the protein and deletion of this/these amino acids would render the protein unable to bind to p34cdc2. Alternatively, the conformation of cyclin A could be such that deletions from the C-terminus of the protein disrupt the structure of the whole protein, so that the improperly folded cyclin A is no longer able to bind to p34cdc2. Evidence from two sources suggest that the second explanation is likely to be the correct one. Firstly, Jörg Adamczewski in the laboratory carried out partial proteolytic digests of a protein A-bovine cyclin A fusion protein (pAcycA) and a C-terminally deleted version of this construct (pAcycACΔ16), from which the last 16 residues of the cyclin A protein had been deleted (Kobayashi et al., 1992). Partial digestion of the full length construct with trypsin or proteinase K left a fragment of the protein that corresponded to the cyclin box and most, or all, of the C-terminus of the protein. This suggests that this region is tightly folded in pAcycA. In contrast, partial digestion of the C-terminally deleted construct to the same degree as the wild-type gave no protected fragment, suggesting that the tightly folded domain of pAcycA is unfolded in pAcycACΔ16. It thus appears that the C-terminus of cyclin A is required for the correct folding of bovine cyclin A. The second piece of evidence comes from Lees and Harlow (1993). They show that while C-terminal deletions of human cyclin A are unable to bind to p34cdc2 in a wild-type cyclin A background, when these deletions are made in combination with deletion of residues 241-275 and an N-terminal truncation of 173 amino acids, the construct can bind to p34cdc2. This implies that residues in the C-terminus of cyclin A are not necessary for p34cdc2 binding, although in the absence of deletion of the N-terminus and residues 241-275, the C-terminus is clearly required. This suggests that deletion of the N-terminus and residues 241-275 somehow stabilises the cyclin A protein such that it is able to tolerate C-terminal deletions.

**Oocyte maturation by the C-terminal deletions of cyclin A**

Despite their inability to bind to or activate p34cdc2 under immunoprecipitation conditions, CAΔ14 and CAΔ24 cyclin As retain a low level of oocyte maturation activity. This is probably due to a very weak association of CAΔ14 and CAΔ24 with p34cdc2, which is disrupted during the lengthy process of immunoprecipitation. Oocyte maturation may therefore be a slightly more sensitive assay for the association of cyclin A with p34cdc2 than the immunoprecipitation assay, but, with the exception of constructs that may bind very weakly to p34cdc2, this assay
and the co-immunoprecipitation assay give identical results. This indicates that oocyte maturation by cyclin A requires that the cyclin A protein be able to bind to and activate p34cdc2, a result supported by the oocyte maturation ability of internally deleted Xenopus cyclin A proteins (Kobayashi et al., 1992) and various human cyclin A deletion mutants (Lees and Harlow, 1993).

Point mutants of cyclin A

Mutation of arginine 197 or aspartic acid 226 to alanine residues also produced cyclin A proteins that were unable to bind to p34cdc2. These residues are two of five that are conserved within the cyclin box of nearly all cyclins, with the notable exception of cyclins C and H. Since R197 and D226 were not replaced with residues of a similar shape or charge in these constructs, it is possible that the mutant cyclin A proteins were unable to bind to p34cdc2 purely as a result of distortion of their conformation, and not because these residues are important in p34cdc2 binding. Until the structure of cyclin A is determined, it is not possible to predict with great accuracy what effect particular substitutions will have on the conformation of the protein, but substitutions where one residue is replaced with another of equal charge and similar shape are unlikely to cause gross misfolding. When R197 and D226 were replaced with residues that conserved their charges and were of similar shape, the resulting proteins (R197K and D226E) were able to bind to p34cdc2, but with severely impaired ability. As R197K and D226E can bind to p34cdc2, albeit weakly, this suggests that these substitutions are not causing serious misfolding of the protein. Given that all cyclins, except cyclin H, that are known to bind to a cdk subunit conserve these two residues as arginine and aspartic acid, these results suggest that R197 and D226 (and their equivalents in other cyclins) are intimately involved in cdk binding. I do not believe that the apparent discrepancy between the results obtained from co-immunoprecipitating R197K and D226E with endogenous p34cdc2 and those obtained by precipitating R197K and D226E bound to GST-cdc2H6 identifies a true difference between the ability of these mutants to bind to p34cdc2 and the ability to bind to GST-cdc2H6. In the experiment shown in figure 3.7, translation was carried out in pure CSF-arrested egg extract, whereas in the experiment shown in figure 3.8, translation was carried out in a 1:1 (v/v) mixture of CSF-arrested egg extract and reticulocyte lysate. In addition, precipitation on glutathione Sepharose is a more efficient procedure than immunoprecipitation using the monoclonal antibody, A17 (data not shown). Thus the concentration of cyclin A protein precipitated on the glutathione Sepharose was much higher than that precipitated on the protein A Sepharose. The cyclin A bands on the autoradiograph shown in figure 3.8 were therefore much darker than those shown in figure 3.7, and so even weak binding of the cyclin A constructs to the GST-cdc2H6 was visible. If the autoradiograph
shown in figure 3.7 had been exposed for a greater length of time, then it is likely that the weak binding of R197K and D226E would have been identified in this experiment also.

**What next?**

A 'dominant negative' form of cyclin A, that retained the ability to bind to, but not activate, p34\(^{cdc2}\) would be a very powerful aid to understanding the specific role of cyclin A. One could look at the effects that the mutant had both on *in vivo* (e.g. *Xenopus* oocytes) and *in vitro* (e.g. *Xenopus* egg extract) systems. Such a form of cyclin A might also make the identification of cyclin A kinase substrates easier. If the phosphorylation of substrates by cyclin A kinase is a rapid process, and they are released immediately after phosphorylation, then there is only a very small 'window' in which to 'catch' the substrate complexed with the cyclin A kinase. The inability of a cyclin A/p34\(^{cdc2}\) complex containing a dominant negative cyclin A mutant to phosphorylate the substrate to which it was bound may delay the release process and therefore give a larger chance of isolating the complex. Dominant negative forms of p34\(^{cdc2}\) and p33\(^{cdk2}\) which have lost the ability to act as kinases due to a single point mutation within their ATP binding site have been constructed, and can give some indications as to the roles of cdk complexes in the cell cycle. Since both p34\(^{cdc2}\) and p33\(^{cdk2}\) are able to bind to more than one cyclin subunit, however, such experiments do not dissect the roles of specific cyclins within the cell cycle.

Of all the cyclin A mutants that have been made to look at p34\(^{cdc2}\) binding and activation (over 25 in total) however, none has been able to bind to p34\(^{cdc2}\) but not activate it. There are two possibilities, either dominant negative mutants of cyclin A can exist and the cyclin A mutants that Hideki Kobayashi and I have made have just not identified them, or mutants of cyclin A that can bind to but not activate p34\(^{cdc2}\) do not exist. This would be the case if the cyclin protein was unable to bind to p34\(^{cdc2}\) without activating it.

As the structure of cyclin proteins is not known, the generation of cyclin A mutants takes an 'informed guess' approach - one can look to see which regions and residues are conserved and which are not, but one never really knows which are important. Even when one has results, it is not always clear what their significance is. Hideki Kobayashi and I made many of the 'obvious' mutants (C-terminal deletions, N-terminal deletions, point mutations of conserved residues and small deletions within the cyclin box) and gathered much data, but this information did not bring us any closer to understanding the role that cyclin A plays in the cell cycle, or how it performs that role. In retrospect, therefore, the method employed to study the domain structure of cyclin A, that of making specific mutations, was probably not the best approach. If I were doing this
project again, I would probably randomly mutagenise the cyclin A plasmid and screen for those cyclin A mutants that no longer killed yeast at high copy number. In this way I would probably to identify mutants of cyclin A that could not bind to p34cdc2. This would be an unprejudiced method of identifying those residues and regions of the cyclin A protein that are important in p34cdc2. Other screens could possibly be devised that may identify cyclin A mutants that could bind to, but not activate, p34cdc2.

At this point in my PhD, then, I had a choice; to make more cyclin A mutants (by whatever method) in the hope of discovering more about the domain structure of cyclin A and with the possibility still remaining of making a dominant negative cyclin A mutant, or to use the cyclin A mutants already constructed to look at other cyclin A properties. I decided to use the mutants already generated to look at other properties of cyclin A.
Chapter 4

The binding of A- and B-type cyclins to p33cdk2

At the start of my PhD two closely related Xenopus cdks, p34^cd2 and p33^cdk2, had been identified. The latter was originally known as Eg1, as it was initially identified as a protein whose mRNA was polyadenylated and translated only from the time of progesterone-induced maturation until the time of fertilisation in Xenopus eggs (Paris et al., 1991). It was, at first, thought to correspond to Xenopus p34^cd2.

The binding of p33^cdk2 to Xenopus cyclin A

It had previously been shown that immunoprecipitates of cyclins A, B1 and B2 from frog egg extract did not contain any p33^cdk2 protein (Minshull et al., 1990), and when the experiments described below were started, there had been no reports that cyclin A could bind to p33^cdk2.

Xenopus cyclin A protein can bind to p33^cdk2 when they are co-translated in a mixture of egg extract and rabbit reticulocyte lysate

In order to test whether Xenopus cyclin A was able to bind to Xenopus p33^cdk2, the mRNAs encoding non-tagged cyclin A and either p34^cd2, or p33^cdk2, or both, were co-translated in a 1:1 (v/v) mixture of CSF-arrested egg extract and rabbit reticulocyte lysate. This mixed translation system translates added mRNA to a higher level than pure CSF-arrested egg extract (see Appendix 2). The translation reactions were immunoprecipitated using the anti-cyclin A monoclonal antibody, XLA1-3, and the products analysed by SDS PAGE and autoradiography. Figure 4.1A shows that in this system Xenopus cyclin A could bind to both p34^cd2 (lanes 4 and 6) and p33^cdk2 (lanes 5 and 6).

The cyclin A/p33^cdk2 complex possesses histone H1 kinase activity

In order to test whether the cyclin A/p33^cdk2 complex had histone H1 kinase activity, a tagged version of p33^cdk2 was constructed. This construct was made
because most of the anti-p34\textsuperscript{cyc2} antibodies that had been raised recently in the laboratory were found to be unable to precipitate p34\textsuperscript{cyc2} complexed with cyclins. I was therefore worried that the anti-p33\textsuperscript{cdk2} antibody that we were using in the laboratory (Minshull et al., 1990) may show the same characteristic.

A c-myc tag was added to the C-terminus of cdk2 using PCR (see Chapter 2). The resulting construct encoded a protein that was slightly larger than p34\textsuperscript{cyc2} when analysed by SDS-PAGE (figure 4.2, lanes 9 and 10) and which could be specifically immunoprecipitated using the anti-c-myc monoclonal antibody, 9E10 (figure 4.1B, lanes 6 and 7).

The mRNA encoding c-myc p33\textsuperscript{cdk2} was translated, either alone or in combination with that encoding non-tagged cyclin A, in a 1:1 (v/v) mixture of egg extract and reticulocyte lysate. In addition, a translation reaction containing c-myc cyclin A mRNA was set up as a positive control. The translation reactions were immunoprecipitated using the anti-c-myc monoclonal antibody, 9E10, and the bead-bound proteins incubated with purified histone H1 and [\gamma-\textsuperscript{32P}]ATP (see Chapter 2). The products were analysed by SDS-PAGE and autoradiography. Figure 4.1B, lane 11, shows that the cyclin A/c-myc p33\textsuperscript{cdk2} complex had histone H1 kinase activity (note that the [\textsuperscript{35S}]-labelled c-myc p33\textsuperscript{cdk2} band is obscured by the [\gamma-\textsuperscript{32P}]-labelled histone H1). The histone H1 kinase activity associated with c-myc cyclin A (due to the co-precipitation of endogenous p34\textsuperscript{cyc2} from the egg extract) was similar in level to that generated by the cyclin A/p33\textsuperscript{cdk2} complex (compare lanes 11 and 12). While this gives an indication that the cyclin A/p33\textsuperscript{cdk2} complex had a histone H1 kinase activity similar to that of the cyclin A/p34\textsuperscript{cyc2} complex, this experiment does not give quantitative data, as the concentration of complex precipitated in each case was not determined.

**Cyclin A cannot bind to p33\textsuperscript{cdk2} when they are co-translated in pure egg extract**

The binding of cyclin A to co-translated p33\textsuperscript{cdk2} was tested in CSF-arrested egg extract in the absence of reticulocyte lysate. The mRNAs encoding either c-myc p33\textsuperscript{cdk2} or non-tagged p34\textsuperscript{cyc2} were translated in CSF-arrested egg extract in the presence or absence of non-tagged cyclin A mRNA. The translation reactions were immunoprecipitated using either the anti-c-myc monoclonal antibody, 9E10 (those reactions containing c-myc p33\textsuperscript{cdk2}), or the anti-p34\textsuperscript{cyc2} monoclonal antibody, A17 (those reactions containing translated p34\textsuperscript{cyc2}), and the products analysed by SDS-PAGE and autoradiography. Figure 4.2 shows that while the cyclin A co-translated with p34\textsuperscript{cyc2} was precipitated with the anti-p34\textsuperscript{cyc2} antibody (lane 12), the cyclin A co-translated with c-myc p33\textsuperscript{cdk2} was not precipitated by the anti-c-myc antibody at a level above that of background (compare lanes 8 and 10). Note that the cyclin A precipitated with the anti-
Figure 4.1  Cyclin A can bind to p33cdc2 in a mixture of frog egg extract and reticulocyte lysate and the complex has histone H1 kinase activity

The mRNAs encoding the constructs indicated above the figures were translated in a 1:1 (v/v) mixture of CSF-arrested egg extract and rabbit reticulocyte lysate in the presence of $[^{35}\text{S}]$methionine. (A) Lanes 1 - 3, the complete translation reaction; lanes 4 - 6, immunoprecipitation with the anti cyclin A monoclonal antibody, XLAl-3. (B) Lanes 1 - 4, the complete translation reaction; lanes 5 - 8, immunoprecipitation with the anti c-myc monoclonal antibody, 9E10; lanes 9 - 12, immunoprecipitation with the anti c-myc monoclonal antibody, 9E10, followed by incubation of the bead-bound proteins with purified histone H1 and $[\gamma-^{32}\text{P}]$ATP. Visualised by autoradiography.
Figure 4.2 Co-translated cyclin A and p33\textsuperscript{cdk2} cannot bind to each other in pure egg extract

The mRNAs encoding the constructs indicated above the figure were translated in CSF-arrested egg extract in the presence of \textsuperscript{35}Smethionine. Lanes 1 - 6, the complete translation reaction; lanes 7 - 10, immunoprecipitation with the anti-c-myc monoclonal antibody, 9E10; lanes 11 and 12, immunoprecipitation with the anti-p34\textsuperscript{cdc2} monoclonal antibody, A17. Visualised by autoradiography.

p34\textsuperscript{cdc2} antibody was bound to both newly translated (and therefore labelled) p34\textsuperscript{cdc2} and endogenous p34\textsuperscript{cdc2}, as the antibody does not distinguish between the two.

It was possible that the reason for the discrepancy between those experiments carried out in a mixture of egg extract and reticulocyte lysate and those carried out in pure egg extract was the presence or absence of reticulocyte lysate. To check whether this was the case, c-myc cyclin A and p33\textsuperscript{cdk2} mRNAs were co-translated in CSF-arrested egg extract, RNase A was added to prevent further translation, and the reaction was split into two aliquots. To one was added an equal volume of reticulocyte lysate, to the other, an equal volume of bead buffer. The reactions were left at 23°C for a further 30 minutes, followed by immunoprecipitation using the anti-c-myc monoclonal antibody, 9E10. The products were analysed by SDS PAGE and autoradiography (see Chapter 2). As figure 4.3 shows, the addition of reticulocyte lysate to the reaction before...
Figure 4.3 Cyclin A cannot bind to p33cdk2 when reticulocyte lysate is added after translation has taken place in pure frog egg extract

The mRNAs encoding c-myc cyclin A and p33cdk2 were translated in CSF-arrested egg extract in the presence of [35S]methionine. Translation was stopped with RNase A and the reaction split into two aliquots. To one was added an equal volume of reticulocyte lysate, to the other an equal volume of bead buffer, and the reactions were incubated at 23°C for 30 minutes. Lane 1, the complete translation reaction; lanes 2 and 3, immunoprecipitation of the reactions indicated above the figure with the anti-c-myc monoclonal antibody, 9E10. Visualised by autoradiography.

immunoprecipitation had no effect on the binding of cyclin A to p33cdk2 when translation had taken place in CSF-arrested egg extract (compare lanes 2 and 3).

It is most likely that the difference in binding between cyclin A and p33cdk2 under different translation conditions is due to the differing relative concentrations of translated p33cdk2 compared with endogenous p34cdc2 (see Discussion).

The C-terminal deletions of cyclin A cannot bind to p33cdk2

Xenopus p34cdc2 and p33cdk2 show over 60% identity to each other at the amino acid level. It is therefore likely that the region(s) of cyclin A required for binding to p34cdc2 would be similar to those required for binding to p33cdk2. To test this hypothesis, the C-terminal deletions of cyclin A, which are unable to bind to p34cdc2, were tested for their p33cdk2 binding ability. The mRNAs encoding c-
Figure 4.4 The C-terminally deleted cyclin A mutants cannot bind to p33\textsubscript{cdk2}

The mRNAs encoding the constructs indicated above the figures were translated in a 1:1 (v/v) mixture of CSF-arrested egg extract and rabbit reticulocyte lysate in the presence of [\textsuperscript{35}S]methionine. (A) Lanes 1 - 6, the complete translation reaction; lanes 7 - 12, immunoprecipitation with the anti cyclin A polyclonal antibody. (B) Lanes 1 - 11, the complete translation reaction; lanes 12 - 22, immunoprecipitation with the anti c-\textit{myc} monoclonal antibody, 9E10. Visualised by autoradiography.
B

Translation

Immunoprecipitation with α-c-myc antibody

kDa

66.5
65.6
57.6
55.6
43.0
35.7
29.0

No mRNA
cdc2
wild-type+cdc2
Ca79+cdc2
Ca79+cdc2
Ca97+cdc2
Ca113+cdc2
Ca139+cdc2
No mRNA
cdc2
wild-type+cdc2
Ca79+cdc2
Ca79+cdc2
Ca97+cdc2
Ca97+cdc2
Ca113+cdc2
Ca139+cdc2

Translation

Immunoprecipitation with α-c-myc antibody

p34cdc2
p33cdc2

cyclin A constructs
myc cyclin A and the C-terminal cyclin A deletion mutants were co-translated with either p34cdc2 or p33cdk2 mRNA in a 1:1 (v/v) mixture of egg extract and reticulocyte lysate. The translation reactions were immunoprecipitated using either a polyclonal anti-cyclin A antibody (Kobayashi et al., 1991) (in the case of CA14) or the anti-c-myc antibody, 9E10 (in the case of CA79, CA97 and CA139), and the products were analysed by SDS PAGE and autoradiography. Figure 4.4 shows that while wild-type cyclin A could bind to both p34cdc2 and p33cdk2 (figure 4.4A, lanes 9 and 10 and figure 4.4B, lanes 15 and 16), the C-terminal deletions of cyclin A could bind to neither (figure 4.4A, lanes 11 and 12 and figure 4.4B, lanes 17 - 22). Thus those C-terminal deletion mutants that cannot bind to p34cdc2 are also unable to bind to p33cdk2.

The point mutants of cyclin A, R197A, R197K, D226A and D226E, can bind only very weakly to p33cdk2

The binding of c-myc cyclin A, CA14, CA24, CA50, R197A, D226A, R197K and D226E to p33cdk2 was tested using a different method from the one described above. The mRNAs encoding these cyclin A constructs were translated in a 1:1 (v/v) mixture of CSF-arrested egg extract and reticulocyte lysate in the presence of [35S]methionine and 2.5 μM of a bacterially expressed fusion protein between glutathione-S-transferase and human p33cdk2 (GST-cdk2). At the end of the translation reaction, the GST-cdk2 bound proteins were affinity purified on glutathione Sepharose and analysed by SDS PAGE and autoradiography; the data was also quantitated using a phosphorimager. Figure 4.5 shows that while c-myc cyclin A was able to bind to GST-cdk2 (lane 11), CA14, CA24, CA50 and R197A were not (lanes 12 - 15). The faint bands that can be seen in these lanes are probably due to precipitation of [35S]-labelled cyclin A protein translated from the endogenous cyclin A mRNA in the extract, as this band was also present in the reaction in which no exogenous mRNA was translated (see lane 10). D226A appeared to be able to bind extremely weakly to GST-cdk2 (lane 16), with an affinity that was approximately 7 fold weaker than that of c-myc cyclin A for GST-cdk2. R197K and D226E could also bind weakly to GST-cdk2 (lanes 17 and 18), their affinities were approximately 3.5 and 3 fold weaker than c-myc cyclin A, respectively. A similar experiment carried out using GST-cdc2H6 instead of GST-cdk2 (see Chapter 3) showed that R197K and D226E could bind to GST-cdc2H6 with affinities that were 6 and 4 fold less than that of c-myc cyclin A for GST-cdc2H6. In that experiment, however, D226A did not appear to be able to bind to GST-cdc2H6 at all. Thus, apart from the D226A mutant, there is a very good correlation between the binding of cyclin A mutants to p34cdc2 and p33cdk2; those that can bind to p34cdc2 can also bind to p33cdk2, and those that cannot bind to p34cdc2 cannot bind to p33cdk2 either.
Figure 4.5  R197A cannot bind to p33cdk2, but D226A, R197K and D226E can bind weakly
The mRNAs encoding the cyclin A constructs indicated above the figure were translated in a 1:1 (v/v) mixture of CSF-arrested egg extract and rabbit reticulocyte lysate in the presence of [35S]methionine and 2.5 μM GST-cdk2 protein. Lanes 1 - 9, the complete translation reaction; lanes 10 - 18, precipitation with glutathione Sepharose. Visualised by autoradiography.

Δ109-161 cyclin A can bind to both p34cdc2 and p33cdk2, while Δ101-169 cyclin A can bind to neither
In order to investigate further whether the regions of cyclin A required for binding to p34cdc2 were the same as those required for p33cdk2 binding, I tested the p33cdk2 binding of two of the N-terminal deletions of cyclin A made by Hideki Kobayashi in the laboratory. He had already shown that Δ109-161 could bind to p34cdc2 while NΔ101-169 could not (Kobayashi et al., 1992). The mRNAs encoding either these two mutants or c-myc cyclin A were incubated with the mRNAs encoding either p34cdc2 or p33cdk2 in a 1:1 (v/v) mixture of egg extract and reticulocyte lysate. The translation reactions were immunoprecipitated using the polyclonal anti-cyclin A antibody and the products analysed by SDS PAGE and autoradiography. Figure 4.6 shows that while wild-type and Δ109-161 cyclin A could bind to both p34cdc2 and p33cdk2 (lanes 11 - 14), Δ101-169 could bind to neither (lanes 15 and 16). Thus the region(s) of cyclin A required for binding to p34cdc2 and p33cdk2 must be very similar.
The binding of Xenopus cyclins B1 and B2 to p33^cdk2

There is some disagreement as to whether B-type cyclins bind to p33^cdk2 under normal circumstances. That B-type cyclins can associate with p33^cdk2 under conditions in which in vitro translated proteins, bacterially expressed or baculovirus expressed proteins are mixed together has been demonstrated (see, for example, Desai et al., 1992; Connell-Crowley et al., 1993; Peeper et al., 1993; Solomon et al., 1993), but it is not clear whether this interaction occurs in vivo. Most studies in mammalian tissue culture cells have shown that p33^cdk2 is mainly associated with cyclin A and cyclin E (Tsai et al., 1991; Koff et al., 1992; Rosenblatt et al., 1992), but Tsai et al. have identified cyclin B-associated p33^cdk2 in human myeloid leukaemia cells (Tsai et al., 1993).
Cyclins B1 and B2 do not associate with p33cdk2 in a mixture of egg extract and reticulocyte lysate

To test whether cyclins B1 and B2 can associate with p33cdk2 under conditions which allow cyclin A-p33cdk2 binding, the mRNAs encoding non-tagged cyclin A, B1 or B2 were co-translated with c-myc p33cdk2 mRNA in a 1:1 (v/v) mixture of CSF-arrested egg extract and reticulocyte lysate. The translation reactions were immunoprecipitated using the anti-c-myc monoclonal antibody, 9E10 and the products analysed by SDS PAGE and autoradiography. Figure 4.7 shows that while cyclin A could bind to p33cdk2 in this system (lane 4) cyclins B1 and B2 were not co-precipitated with the 9E10 antibody (lanes 5 and 6).

Cyclins B1 and B2 do not associate with bacterially expressed p33cdk2 in CSF-arrested egg extract

Figure 4.8 shows that p33cdk2 also cannot bind to B-type cyclins under alternative conditions. In this experiment, 1.2 μM either GST-cdc2H6 or GST-cdk2 were added to CSF-arrested egg extract in which c-myc cyclin A mRNA was then translated in the presence of [35S]methionine. Those proteins bound to GST-cdc2H6 or GST-cdk2 at the end of the translation reaction were affinity purified on glutathione Sepharose, and the products analysed by SDS PAGE and autoradiography. Figure 4.8 shows that both c-myc cyclin A and the endogenous B-type cyclins could bind to GST-cdc2H6 (lane 3). In contrast, only c-myc cyclin A and not the B-type cyclins could bind to GST-cdk2 (lane 4).

At least under the conditions described here then, p33cdk2 does not appear to bind to B-type cyclins. There must, therefore, be a difference between cyclin A and cyclins B1 and B2 that allows cyclin A to bind to both p34cdc2 and p33cdk2, but cyclins B1 and B2 to bind only to p34cdc2.

Swapping the C-termini of cyclins A and B1

The C-terminus is not highly conserved between different cyclins, but this region contains several residues that are conserved between A- and B-type cyclins, and within the cyclin A family there is more conservation (see figures 3.9, 3.10 and 4.11). Since deletions of as few as 14 amino acids from the C-terminus of cyclin A made the protein unable to bind to p34cdc2 or p33cdk2, the C-terminus of cyclin A is obviously important in cdk binding.
Figure 4.7  Cyclin A can bind to p33cdk2, but cyclins B1 and B2 cannot
The mRNAs encoding the constructs indicated above the figure were translated in a 1:1 (v/v) mixture of CSF-arrested egg extract and reticulocyte lysate in the presence of [35S]methionine. Lanes 1 - 3, the complete translation reaction; lanes 4 - 6, immunoprecipitation with the anti c-myc monoclonal antibody, 9E10. Visualised by autoradiography.

Figure 4.8  GST-cdk2 can bind to cyclin A but not to the endogenous B-type cyclins
The mRNA encoding c-myc cyclin A was translated in CSF-arrested egg extract in the presence of [35S]methionine and 1.2 μM bacterially expressed GST-cdc2H6 or GST-cdk2 protein. Lanes 1 and 2, the complete translation reactions; lanes 3 and 4, precipitation of the GST-cdc2H6 or GST-cdk2 bound proteins on glutathione Sepharose. Visualised by autoradiography. Asterisk on right indicates position of the endogenous B-type cyclins.
Construction of C-terminal ‘exchange’ constructs between cyclins A and B1

In order to test whether the C-terminus is the region that determines the specificity of cdk partner in cyclin proteins, the C-termini of cyclins A and B1 were ‘exchanged’.

Cyclin A and B1 mutants were made in which NotI sites were engineered into the constructs 77 (cyclin A) and 86 (cyclin B1) amino acids from their C-termini, at a run of 4 (cyclin A) or 3 (cyclin B1) alanine residues (see Chapter 2, and figure 4.11). The presence of the NotI sites did not change the amino acid sequences of the cyclins. These constructs were then digested with NotI and their C-terminal portions exchanged, so that the mutant proteins produced were c-myc cyclin A with its last 77 residues replaced by the last 86 residues of cyclin B1 (cyclin A/B1) and cyclin B1 with its last 86 residues replaced by the last 77 residues of cyclin A (cyclin B1/A), see figures 4.9 and 4.11.

The C-terminal ‘exchange’ constructs cannot bind to p34cdc2

In order to test whether these constructs could bind to p34cdc2, the mRNAs encoding c-myc cyclin A, cyclin A/B1 or cyclin B1/A were translated in CSF-arrested egg extract, the translation reactions immunoprecipitated with either the

![Diagram of cyclin constructs](image)

**Figure 4.9** The C-terminal ‘exchange’ constructs of cyclins A and B1

Representations of wild-type cyclins A and B1, and of the C-terminal ‘exchange’ constructs, cyclin A/B1 and cyclin B1/A. Cyclin A is shown in white and cyclin B1 is shown in black. The numbers at the ends of the constructs indicate the total number of amino acids in the protein and the numbers at the exchange sites of the hybrid cyclins indicate the amino acid position of the exchange. The diagram is to scale.
Figure 4.10  The C-terminal ‘exchange’ constructs of cyclins A and B1 cannot bind to p34\textsuperscript{cdc2}

The mRNAs encoding the cyclin constructs indicated above the figure were translated in CSF-arrested egg extract in the presence of \textsuperscript{[\textit{35}S]}methionine. Lanes 1 - 4, the complete translation reaction; lanes 5 - 8, immunoprecipitation with the anti-p34\textsuperscript{cdc2} monoclonal antibody, A17; lanes 9 - 12, control immunoprecipitation with a monoclonal antibody against human retinoblastoma protein, IF8. Visualised by autoradiography. Asterisk on right indicates position of the endogenous B-type cyclins.

anti-p34\textsuperscript{cdc2} monoclonal antibody, A17 or a control antibody against the human retinoblastoma protein, IF8. The products were analysed by SDS PAGE and autoradiography. Figure 4.10 shows that while wild-type cyclin A could bind to p34\textsuperscript{cdc2} (lane 6), neither cyclin A/B1 or cyclin B1/A could (lanes 7 and 8). The faint bands corresponding to cyclin A/B1 and cyclin B/A1 seen in lanes 7 and 8 are probably due to non-specific binding, since similar bands can be seen in the control immunoprecipitation (lanes 11 and 12).

Deletion of 2 residues from cyclin A/B1

This result was unexpected as no residues have been deleted from the constructs. However, although the C-termini of cyclins A and B do not show a great deal of conservation, there are six conserved hydrophobic residues (see figure 4.11). One residue in particular, tyrosine 398 in \textit{Xenopus} cyclin A, marked with an asterisk on figure 4.11, is not correctly aligned in cyclins A/B1 and B1/A. If the positioning of the hydrophobic residues is important, then this might explain the inability of cyclins A/B1 and B1/A to bind to p34\textsuperscript{cdc2}.
Figure 4.11  Alignment of the C-termini of cyclins A, B1 and B2 from various organisms and *Xenopus* cyclin A/B1, cyclin B1/A and cyclin A/B1Δ2 constructs

Conserved hydrophobic residues are shown in bold type. The ‘swap’ mutant C-termini are shown in between the sequences of B- and A-type cyclins; the sites of the ‘swaps’ are shown above and below the sequences. The point mutations in cyclin A/B1Δ2 are shown by arrows above the sequence and the two residues deleted are indicated below the sequence of cyclin A/B1 by a Δ sign. The tyrosine residue that cyclin A/B1Δ2 was constructed to move back into alignment is indicated by an asterisk above the sequences.
In order to test this hypothesis, another construct was made in which two residues were deleted from cyclin A/B1 to bring tyrosine 398 (numbering according to wild-type cyclin A) back into alignment with the tyrosine residues of the other cyclin As (see figure 4.11 for position of deletion). In addition, three residues just upstream of the deletion site were changed to make the region more like cyclin A (see figure 4.11). The construct containing this deletion and amino acid changes was made by digesting the cyclin A/B1 construct with BglII and NdeI which cut out a 24 base pair section of DNA and replacing it with a 17 base pair oligonucleotide which encoded the desired changes to the sequence (see Chapter 2). This construct was called cyclin A/B1A2. For this to be possible, the c-myc tag on the cyclin A/B1 construct had first to be removed by swapping the very N-terminus of c-myc cyclin A with that of wild-type cyclin A at convenient restriction enzyme sites. This was necessary the DNA encoding the c-myc tag contains a BglII site. Cyclin A/B1A2 cannot bind to p34cdc2 or p33cdk2

To test the binding of cyclin A/B1A2 to p34cdc2 and p33cdk2, mRNAs encoding non-tagged cyclin A, cyclin A/B1 or cyclin A/B1A2 were co-translated with either p34cdc2 or p33cdk2 in a 1:1 (v/v) mixture of CSF-arrested egg extract and reticulocyte lysate. The translation reactions were immunoprecipitated using the anti-cyclin A monoclonal antibody, XLA1-3, and the products analysed by SDS PAGE and autoradiography. While wild-type cyclin A could bind to both p34cdc2 and p33cdk2 (figure 4.12, lanes 11 and 12), neither cyclin A/B1 nor cyclin A/B1A2 could bind to either (figure 4.12, lanes 13 - 16). It is possible that the reason that no p34cdc2 binding by cyclin A/B1A2 can be seen is because the mRNA encoding p34cdc2 did not translated very well in this experiment (see lanes 3, 5 and 7) and the band is just too faint to see. This seems unlikely, however, as the p34cdc2 band is clearly visible in the immunoprecipitation reaction containing wild-type cyclin A (lane 11).

These results show that exchanging the C-termini of cyclins A and B1 makes chimera proteins that are unable to bind to either p34cdc2 or p33cdk2. The possibility remains that the C-terminus of the protein is the region that confers the ability (or inability) of A- and B-type cyclins to bind to p33cdk2. In order to test this hypothesis further it would be necessary to make more subtle mutations to the C-termini of A- and B-type cyclins, as it appears that the C-terminus of cyclin A at least is very sensitive to conformational change.
The mRNAs encoding the constructs indicated above the figure were translated in a 1:1 (v/v) mixture of CSF-arrested egg extract and rabbit reticulocyte lysate in the presence of $[^{35}\text{S}]$methionine. Lanes 1 - 8 the complete translation reaction; lanes 9 - 16, immunoprecipitation with the anti-cyclin A monoclonal antibody, XLA1-3. Visualised by autoradiography.

**Discussion**

*Xenopus* cyclin A can bind to p33$^\text{cdk2}$ in CSF-arrested egg extract when the concentration of both partners is increased by the translation of added mRNA. However, the situations described above are artificial ones, and although cyclin A is able to bind to p33$^\text{cdk2}$ in egg extract this does not mean that under normal circumstances they are found associated. The concentration of p33$^\text{cdk2}$ in *Xenopus* egg extracts is at least 10 times lower than that of p34$^\text{cdc2}$ (Gabrielli et al., 1992; Kobayashi et al., 1992) and probably most, if not all of it, is associated with cyclin E. Indeed, recent work by Michael Howell in the laboratory has indicated that cyclin A1 (the cyclin A described in this thesis) is probably never associated with p33$^\text{cdk2}$ to a significant extent. He has also shown that the levels of cyclin A1 decline to a very low level at around stage 11 in *Xenopus* tadpole development and that cyclin A1 is not detectable at all in the *Xenopus* somatic cell lines WAK and XTC, in contrast to the *Xenopus* cyclin A2 that he has isolated. Thus it appears that the function of *Xenopus* cyclin A1 may be very
different to the function of the A-type cyclins that have been found associated with p33cdk2 in mammalian somatic cells. See Chapters 1 and 10.

The binding of cyclin A to p33cdk2 in egg extracts and mixtures of egg extract and reticulocyte lysate

Why can cyclin A only bind to p33cdk2 in co-translation reactions that are carried out in a 1:1 (v/v) mixture of CSF-arrested egg extract and reticulocyte lysate? It is most likely that these difference are due to the differing relative concentrations of translated p33cdk2 compared with endogenous p34cdc2. When p33cdk2 is translated in pure egg extract, the concentration of protein synthesised from the added mRNA by the end of the translation reaction is probably between 1 and 8 nM (Appendix 2) while the concentration of endogenous p34cdc2 is between 450 and 600 nM (Appendix 2). When translation reactions are carried out in 1:1 (v/v) mixtures of egg extract and reticulocyte lysate, the concentration of synthesised protein is probably increased to between 25 and 60 nM (Appendix 2) because of the higher translation efficiency of this system compared with pure CSF-arrested egg extract. The concentration of endogenous p34cdc2, however, will be halved, to between 225 and 300 nM. Thus in translation reactions carried out in pure egg extract there is as much as a 600 fold excess of endogenous p34cdc2 over translated p33cdk2 for the cyclin A to bind to, whereas in translation reactions carried out in a mixture of egg extract and reticulocyte lysate there is as little as 11 fold more p34cdc2 than p33cdk2. In addition, in egg extract/reticulocyte lysate translation reactions, because of the higher translation efficiency of the system, there is more translated cyclin A protein available for binding to p33cdk2.

The hypothesis, that cyclin A does not bind to p33cdk2 in pure egg extract because of the high level of p34cdc2 compared to p33cdk2, is supported by the experiment shown in figure 4.8. In this experiment bacterially expressed GST-cdc2H6 or GST-cdk2 was added to CSF-arrested egg extract in which cyclin A mRNA was then translated. Those proteins bound to GST-cdc2H6 or GST-cdk2 at the end of the translation reaction were affinity purifed on glutathione Sepharose. Under such conditions of relatively high p34cdc2 and p33cdk2 concentrations (around 1.2 µM for each; at least twice that of the endogenous p34cdc2 protein) the translated cyclin A can bind to both GST-cdc2H6 and GST-cdk2.

The binding of the cyclin A deletion and point mutants to p33cdk2

With the exception of the cyclin A point mutant D226A, the ability of a cyclin A mutant protein to bind to p33cdk2 correlates with the ability of that mutant to bind to p34cdc2. Those deletion mutants that cannot bind to p34cdc2 cannot bind to p33cdk2 and those that can bind to p34cdc2 can also bind to p33cdk2. The point
mutant, R197A, cannot bind to either p34cdc2 or p33cdk2. The two conservative substitution mutants of cyclin A, R197K and D226E, can bind only weakly to GST-cdc2H6 and they can also bind only weakly to GST-cdk2. The binding of these mutants to GST-cdk2 is a little stronger than their binding to GST-cdc2H6, (calculated by measuring the absorbances of the precipitated bands corresponding to R197K and D226E on the phosphorimager and comparing them to the absorbance of the band corresponding to precipitated c-myc cyclin A. The efficiency of translation of the added mRNA for each construct was also taken into consideration). This may reflect a true difference, or it may simply be due to slight differences in the two experiments, for example, the concentration of GST-cdc2H6 added to the experiment shown in figure 3.8 was 1.25 µM whereas the concentration of GST-cdk2 added to the experiment shown in figure 4.5 was 2.5 µM. The slight difference between the experiments may explain why the cyclin A point mutant, D226A, appears to be able to bind to GST-cdk2, albeit very weakly, but not to GST-cdc2H6. If these results are to be taken on face value, however, it appears that the conserved residues R197 and particularly D226 are slightly less important for p34cdc2 binding than for p34cd2 binding in cyclin A. It should be noted, however, that the other cyclins that have been shown to be able to bind to p33cdk2, cyclin D and cyclin E, do conserve arginine and aspartic acid residues in the equivalent positions to R197 and D226 in Xenopus cyclin A, so it would seem that these are likely to be important in binding to this cdk subunit, especially since cyclin E appears to bind mainly to p33cdk2 in vivo (Dulic et al., 1992; Koff et al., 1992; Knoblich et al., 1994; Michael Howell, pers. comm.)

**The C-terminal exchange mutants**

Since the cyclin box is the region that all cyclins have in common and almost all cyclins are known to bind to cdk subunits, it seems probable that the cyclin box is the region of cyclins that allows cdk binding. The feature(s) that distinguish one cyclin from another in terms of which cdk subunit they can bind to could lie within the less conserved regions of the cyclin box, or they could lie outside of this region all together. I reasoned that since the C-termini of cyclins are not as highly conserved as the cyclin boxes, and that the C-termini of A-type cyclins show more homology amongst themselves than to other cyclins, and since the C-terminus of Xenopus cyclin A is required for p34cdc2 and p33cdk2 binding, this could be the region important in distinguishing to which cdk subunit the cyclin binds. For this reason I exchanged the C-termini of cyclins A and B1. Neither of these exchange constructs could even bind to p34cdc2, however, the cdk that both parent constructs could bind to. As the exchanges were engineered so as not to change the amino acid sequence of the protein at all, the C-termini of cyclins A
and B1 that were exchanged were not of exactly equal length (77 amino acids from the C-terminus of cyclin A and 86 amino acids from the C-terminus of cyclin B1). This led to the misalignment of a tyrosine residue that was almost conserved in position between cyclins A and B (this tyrosine is one residue nearer the N-terminus in A-type cyclins as compared with B-type cyclins in our alignment, see figure 4.11). Realignment of this tyrosine residue, however, did not ‘rescue’ the p34^{cdc2} binding ability of the cyclin A/B1 construct. These results do not allow any firm conclusions to be drawn about the function of the C-termini of cyclins A and B1. It could be that these regions are required to act in conjunction with other parts of the cyclin protein for distinguishing between different cdk subunits. If the other parts of the cyclin protein involved are different in cyclins A and B1, then swapping the C-termini could make ‘hybrid’ recognition domains and cause the proteins to be unable to bind to any cdk. However, I think it much more likely that the reason for the inability of cyclin A/B1, cyclin A/B1Δ2 and cyclin B1/A to bind to p34^{cdc2} is simply distortion of the cyclin conformation due to the exchange of the C-termini. We already know that cyclin A is sensitive to deletions from its C-terminus (see previous chapter) and a deletion of 24 amino acids from the C-termini of cyclins B1 and B2 makes them unable to bind to p34^{cdc2} (Stewart et al., 1994), probably due to distortion of the protein conformation, so this would seem the most obvious explanation.

As more cyclins are discovered a good way to predict which regions of the protein/residues are important in determining the specificity of the cdk partner would be to compare their sequences. For example, A-type cyclins can bind to both p34^{cdc2} and p33^{cdk2} and they should therefore have sequences that allow them to bind to both of these proteins, whereas cyclin E should have mainly p33^{cdk2} binding sequences and B-type cyclins mainly p34^{cdc2} binding sequences. Of course, sequence analysis will not give useful results if the difference between the cyclins in terms of which partners they can bind to is determined solely by the conformation of the protein and has no sequence basis. In that case we will have to wait until the structure of the cyclins bound to their partners is determined to identify the differences.
Chapter 5

The cyclin destruction assay

One of the most remarkable properties of mitotic cyclins is their rapid proteolysis at the end of mitosis (Evans et al., 1983). This degradation is required for progression of the cell cycle, since indestructible cyclin mutants arrest cells in mitosis (Murray et al., 1989; Ghiara et al., 1991; Glotzer et al., 1991; Luca et al., 1991; Gallant and Nigg, 1992). Although cyclin destruction is a key event in the cell cycle, the enzymes responsible for the recognition of cyclin and the initiation of its proteolysis have not been identified, and the mechanism of their control are not known. Two lines of evidence, however, suggest that the ubiquitin system may play a major part in cyclin degradation. Firstly, high molecular weight ubiquitinated intermediates of sea urchin cyclin B were detected in Xenopus egg extracts undergoing cyclin destruction, and the flux through these intermediates was adequate to account for the total loss of cyclin (Glotzer et al., 1991). Secondly, the addition of carboxymethylated ubiquitin to clam embryo extracts inhibited cyclin proteolysis (Hershko et al., 1991).

To study the destruction of the cyclin A mutants described in the previous chapters, it was necessary to develop an assay system that allowed the analysis of individual constructs in a reproducible manner.

Unfertilised Xenopus eggs are arrested in the metaphase of meiosis II with high cyclin kinase activity (Masui and Markert, 1971; Lohka and Masui, 1983). This arrest is due to an activity known as cytostatic factor (CSF), which involves the c-mos kinase (Sagata et al., 1989; Okazaki et al., 1991; Colledge et al., 1994; Hashimoto et al., 1994) and possibly p33c^cdk2 (Gabrielli et al., 1993). CSF arrest is overcome by fertilisation of the egg, which triggers an increase in intracellular calcium levels (Busa and Nuccitelli, 1985), leading to the proteolysis of the mitotic cyclins, inactivation of MPF and the consequent resumption of the cell cycle (Newport and Kirschner, 1984; Murray et al., 1989; Lorca et al., 1991b). Ca^2+ triggered inactivation of MPF and CSF appears to be mediated by calmodulin-dependent protein kinase II (Lorca et al., 1993). In extracts made from unactivated Xenopus eggs, known as CSF-arrested egg extracts (Murray, 1991), the effects of fertilisation can be mimicked by the addition of 0.4 mM Ca^2+ (Lohka and Maller, 1985; Murray et al., 1989; Lorca et al., 1992; van der
Velden and Lohka, 1993). In the absence of Ca$^{2+}$, however, the cyclins are stable (see figure 5.2, lanes 7 - 12 and 19 - 24) and cyclin kinase activity remains high.

**The Assay**

The cyclin destruction assay used throughout this and the following chapters was as described below. The mRNA encoding the cyclin A construct to be tested was translated in freshly-thawed CSF-arrested egg extract in the presence of $[^{35}\text{S}]$methionine, for 2 hours at 23°C. Cycloheximide was added to a final concentration of 100 µg/ml and the extract was incubated at 23°C for a further 10 minutes to stop the translation reaction. 0.4 mM CaCl$_2$ was then added to the reaction to trigger cyclin destruction, and samples were taken at intervals, added to SDS sample buffer and stored on dry ice until the end of the experiment. The zero time point was always taken before Ca$^{2+}$ was added to the translation reaction. The extent of cyclin degradation at each time point was analysed by SDS PAGE and autoradiography (see, for example, figure 5.1). If necessary, the intensity of the $[^{35}\text{S}]$-labelled cyclin band at each time point was quantified by scanning densitometry, in which case the data was shown in the form of a graph.

**Variability of the assay system**

CSF-arrested egg extracts prepared from different *Xenopus* females vary considerably. A typical series of cyclin destruction assays, in all of which cyclin A mRNA was translated in the CSF-arrested extract before cyclin destruction was triggered, is shown in figure 5.1. Extracts A and B were made at the same time as each other but at a different time from extracts D and E, which were also made at the same time as each other. Extract C was made at a different time from all four of the other extracts. Each of the extracts was made from a different *Xenopus* female, but all were prepared using exactly the same protocol. The test destruction assays of $[^{35}\text{S}]$-labelled proteins in extracts A, B and C were carried out at the same time as each other, but at a different time from those carried out in extracts D and E, which were tested at the same time as each other. The protocol followed for the destruction assays (as detailed in Chapter 2 and above) was identical in each case. It can be seen from figure 5.1 that the ability of the extract to translate both the exogenous and endogenous mRNA can vary considerably between different extracts (compare the intensities of $[^{35}\text{S}]$-labelling between extract A and extract C). In addition, the speed of cyclin destruction can vary from 10 minutes (for example, see extract E) to over 30 minutes (for example, see extract D). In some extracts cyclin destruction was not triggered at all by the addition of 0.4 mM Ca$^{2+}$ (data not shown). For this reason, wild-type cyclin A was included in every assay as a control when studying the destruction of cyclin A mutants, and comparisons between the rate and extent of cyclin destruction
Figure 5.1 Variations in translation and cyclin destruction using different CSF-arrested egg extracts
The mRNA encoding c-myc cyclin A was translated in the CSF-arrested egg extracts indicated below the figure in the presence of $[^{35}\text{S}]$methionine. Extracts A and B were made at the same time as each other but at a different time from extracts C, D and E. Extracts D and E were also made at the same time as each other. Each of the extracts was made from the eggs of a different *Xenopus* female. The standard destruction assay was used (see Chapter 2) and samples were taken at the times indicated above the figure. Cyclin destruction was tested at the same time in extracts A, B and C, but at a different time to extracts D and E, which were also tested at the same time as each other. Visualised by autoradiography. Asterisk on right indicates position of the endogenous B-type cyclins.
under various conditions were always performed at the same time and in the same batch of extract. Sometimes rabbit reticulocyte lysate was added to those CSF-arrested egg extracts which did not translate added mRNA very well. The addition of up to a 1/10th volume of reticulocyte lysate at the beginning of the translation reaction usually had little effect on cyclin proteolysis, but greatly enhanced the ability of the CSF-arrested egg extract to translate added mRNA (see Appendix 2). Occasionally the added reticulocyte lysate caused gradual cyclin destruction even in the absence of Ca^{2+}, but this was distinguishable from Ca^{2+}-triggered cyclin proteolysis. Cyclin destruction triggered by the addition of Ca^{2+} to CSF-arrested egg extract occurs with distinct kinetics; there is a lag phase during which little destruction takes place, there is then rapid cyclin destruction for 10 - 20 minutes after which time the cyclin destruction machinery becomes inactive. Cyclin proteolysis in the extracts to which reticulocyte lysate but no Ca^{2+} had been added was gradual and occurred with the same kinetics throughout the length of the assay. This non-Ca^{2+}-triggered destruction is probably due to the highly active ubiquitin ligating system that is present in rabbit reticulocyte lysate (Hershko and Ciechanover, 1992). This gradual destruction did not cause any problems in the interpretation of results.

Despite the variability of different CSF-arrested egg extracts, the cyclin destruction assay described above gave highly reproducible results; within the same batch of egg extract cyclin proteolysis always occurred with similar kinetics, and even using different batches of extract indestructible cyclin mutants were always indestructible and destructible mutants were always destructible, even though the length of time taken for that destruction may have varied. It was also a very convenient and easy assay to use.

**Advantages of the assay**

This method of analysing cyclin destruction offered a number of advantages over the other assay systems that have been used, particularly as it did not require the use of bacterially-expressed proteins. It is difficult to know what fraction of bacterially produced protein is folded correctly, and incorrectly folded protein may not be destroyed in the same manner as correctly folded cyclin protein. In addition, the length of the translation reaction that occurred before cyclin destruction was triggered gave the newly-translated cyclins ample time to associate with proteins in the CSF-arrested egg extract, such as p34^{cd2}, with which they are normally found complexed. It was also an advantage not to have to express and purify all the mutant cyclin A proteins to be tested, which would have been very time consuming. This assay system also had the advantage of a 'built-in' positive control. The endogenous mRNA encoding the B-type cyclins was translated at the same time as the added mRNA encoding the cyclin A mutant
to be tested. These $[^{35}\text{S}]$-labelled B-type cyclins were therefore visible on the autoradiographs along with the mutant cyclin A protein, and were proteolysed when cyclin destruction was triggered by the addition of Ca$^{2+}$ to the reaction (see, for example, figure 5.1). The proteolysis of the endogenous B-type cyclins served as a good internal control to show that cyclin destruction had been triggered in each reaction, even when the mutant cyclin A protein was not destroyed.

c-myc tagged and non-tagged cyclin A behave the same in the cyclin destruction assay
To check that c-myc cyclin A did not behave differently from non-tagged cyclin A in the cyclin destruction assay, the mRNAs encoding both constructs were translated in CSF-arrested egg extract and cyclin destruction triggered by the addition of Ca$^{2+}$. The products were analysed by SDS PAGE and autoradiography. Figure 5.2 shows that while both c-myc tagged and non-tagged cyclin A were stable if no Ca$^{2+}$ is added to the extract (lanes 7 - 12 and 19 - 24) both were destroyed by 30 minutes in the reaction to which Ca$^{2+}$ has been added (lanes 1 - 6 and 13 - 18). Thus there is no difference in the destruction of c-myc and non-tagged cyclin A. Nevertheless, c-myc cyclin A was always used as the positive control when c-myc tagged cyclin A mutants were tested for their destructibility, and non-tagged cyclin A as a control in assays of non-tagged cyclin A mutants.

How long does the cyclin destruction machinery remain active in CSF-arrested egg extract once proteolysis is triggered by Ca$^{2+}$?
In fertilised clam eggs the period during which cyclin destruction occurs has been shown to be about 5 minutes (Hunt et al., 1992). Outside of this ‘window’ of destruction, the cyclin proteins are stable. In S. cerevisiae, in contrast, cyclin destruction continues throughout the G1 phase of the cell cycle until the activation of the G1 cyclins towards the end of G1 (Amon et al., 1994). This probably reflects the difference between the embryonic cell cycle, which is very short, and the somatic cell cycle, which is much longer (see figure 5.3).

To study how long the cyclin destruction machinery stays active in Xenopus CSF-arrested egg extract, a cyclin A ‘substrate’ was prepared by the translation of c-myc cyclin A mRNA in a 50:50 (v/v) mixture of CSF-arrested egg extract and rabbit reticulocyte lysate. At the end of the translation reaction, 100 μg/ml cycloheximide was added to prevent further translation. To freshly thawed CSF-arrested egg extract (‘test’ extract), 100 μg/ml cycloheximide was added and an aliquot was removed. Cyclin destruction was triggered in the rest of the ‘test’ extract by the addition of Ca$^{2+}$, and samples of the cyclin A ‘substrate’ were
Figure 5.2 The destruction of c-myc and non-tagged cyclin A occurs with similar kinetics
The mRNA encoding either c-myc or non-tagged cyclin A was translated in CSF-arrested egg extract in the presence of [35S]methionine. The standard destruction assay was used (see Chapter 2) and samples were taken at the times indicated above the figure. Lanes 1 - 12, c-myc cyclin A; lanes 13 - 24, non-tagged cyclin A; lanes 1 - 6 and 13 - 18, Ca2+ added to the destruction assay; lanes 7 - 12 and 19 - 24, H2O added to the assay in place of Ca2+ (i.e. no cyclin destruction was triggered). Visualised by autoradiography. Asterisk on right indicates position of the endogenous B-type cyclins.
Figure 5.3  Cyclin accumulation and destruction in embryonic and somatic cell cycles
The level of mitotic cyclins in cells is represented by the line. The shaded area represents the time during which the cyclin destruction machinery is active. The phases of the cell cycle are shown below the figures. The top panel represents mitotic cyclin accumulation and destruction in embryonic cells, and the bottom panel the accumulation and destruction of mitotic cyclins in somatic cells.

added to aliquots of the ‘test’ extract at 0, 10, 20, 30, 45, 60 and 90 minutes after Ca²⁺ addition. Cyclin A ‘substrate’ was also added to the aliquot of ‘test’ extract to which no Ca²⁺ had been added. Samples were taken from each of these destruction assays at 0, 30 and 90 minutes after the addition of the ‘substrate’ to the ‘test’ extract. Thus the final time point was taken 180 minutes after Ca²⁺ was added to the ‘test’ extract; 90 minutes after cyclin A ‘substrate’ was added to the
extract. The extent of cyclin A destruction in each reaction was assessed by SDS PAGE and autoradiography, and quantitated using the phosphorimager. If the cyclin destruction machinery in the 'test' extract is still active when the cyclin A 'substrate' is added to it, then the cyclin A protein will be destroyed. If the destruction machinery has turned off by the time the cyclin A 'substrate' is added to the extract, then the cyclin A protein will remain stable. Figure 5.4 shows that the cyclin destruction machinery was slowing down by 30 minutes after the addition of Ca\(^{2+}\) to the CSF-arrested egg extract and that by 45 minutes after the addition of Ca\(^{2+}\) very little cyclin A destruction occurred. Although the cyclin destruction machinery is clearly not highly active in the reaction where the 'substrate' was added to the 'test' extract 45 or 60 minutes after the Ca\(^{2+}\), a low level of cyclin destruction does appear to be occurring. It is not clear whether this is specific cyclin destruction or whether it is due to non-specific protein degradation. At the very last time point, a substantial amount of cyclin degradation has occurred (most right-hand lane/block in figure 5.4). This is unlikely to be the result of the 'test' extract re-entering M-phase, as this extract had cycloheximide added to it before cyclin destruction was triggered, and should therefore have contained no cyclin protein to 'drive' it into M-phase until the 'test' extract was added. It is more probable that this cyclin destruction is due to non-specific protein degradation by the extract, which has been incubated at 23°C for three hours. Since the speed of cyclin destruction can vary between different egg extracts then the exact length of time that the cyclin destruction machinery remains active will probably also vary. However, from this result it is possible to say that in CSF-arrested egg extract the cyclin destruction machinery is active for about 30-45 minutes after the addition of Ca\(^{2+}\). In vivo this activation period must be shorter, as embryonic *Xenopus* cell cycles only take about 30 minutes each.
Figure 5.4  Duration of activity of the cyclin destruction machinery in CSF-arrested egg extract

(A) The mRNA encoding c-myc cyclin A was translated in a 50:50 (v/v) mixture of CSF-arrested egg extract and rabbit reticulocyte lysate in the presence of [35S]methionine (cyclin A ‘substrate’). Freshly thawed CSF-arrested egg extract (‘test’ extract) was taken and an aliquot was removed. To the rest was added Ca\(^{2+}\) to trigger cyclin destruction. At the times indicated above the figure (Ca\(^{2+}\) addition to the extract being time 0) an aliquot of the cyclin A ‘substrate’ was added to an aliquot of the ‘test’ extract. Samples were then removed from these destruction reactions at the times indicated below the figure (the time of addition of the ‘substrate’ to the ‘test’ extract being time 0). Visualised by autoradiography.  

(B) The absorbance of the cyclin A bands in the experiment shown in (A) was quantitated using the phosphorimager and expressed as proportions of the absorbance of the bands when the cyclin A ‘substrate’ was added to the ‘test’ extract.
The ATP analogue ATPγS can inhibit cyclin destruction. In order to investigate whether ATPγS can inhibit cyclin destruction, CSF-arrested egg extract was incubated with [3H]thymidine to allow the endogenous B-type cyclins to become labelled. Cyclin destruction was triggered with Ca^{2+} in the presence or absence of 1 mM ATPγS. The ATPγS was added either at the same time as the Ca^{2+} or 10 minutes before, at the same time as the cycloheximide. The samples collected subsequently were stored on dry ice until they were analyzed for their histone H1 kinase activity using purified histone H1 and [$\gamma$-32P]ATP, followed by SDS-PAGE and autoradiography. Figure 6.1 shows that in the assay in which no ATPγS was added the B-type cyclins and histone H1 kinase activity had disappeared by 30 minutes after Ca^{2+} addition (lane 17). In the reactions to which ATPγS was added, however, cyclin destruction and the consequent disappearance of histone H1 kinase activity was not complete even by the end of the destruction reaction, 90 minutes after Ca^{2+} addition to the extract (lanes 8 and 18). When the ATPγS was added 10 minutes before destruction is triggered, rather than with the Ca^{2+}, the inhibition of cyclin destruction was much more marked (compare lanes 4 to 6 with 9 to 11). The addition of 1 mM ATP to a destruction assay has no effect on cyclin proteolysis (see Figure 6.1). ATPγS added to a CSF-arrested egg extract to 1 mM concentration inhibits cyclin destruction and the consequent histone H1 kinase inactivation considerably, although the inhibition is not complete.

ATPγS inhibits cyclin destruction at a concentration of 1 mM
To check at what concentration the ATPγS could inhibit cyclin destruction, assays were carried out in the presence of different concentrations of this ATP analogue. The mRNA encoding oocyte cyclin A was translated in CSF-arrested egg extract and either 0, 1, 10, 100 or 1000 pM of ATPγS was added to the translation.
Chapter 6

Properties of cyclin destruction

The effects of ATPγS, AMP-PNP, GTPγS, okadaic acid and high levels of cyclin A1, A2 and B1 kinase on cyclin destruction were briefly investigated, using the destruction assay described in Chapter 5.

The ATP analogue ATPγS can inhibit cyclin destruction

In order to investigate whether ATPγS can inhibit cyclin destruction, CSF-arrested egg extract was incubated with [35S]methionine to allow the endogenous B-type cyclins to become labelled. Cyclin destruction was triggered with Ca2+ in the presence or absence of 1 mM ATPγS. The ATPγS was added either at the same time as the Ca2+ or 10 minutes before, at the same time as the cycloheximide. The samples collected subsequently were stored on dry ice until they were analysed for their histone H1 kinase activity using purified histone H1 and [γ-32P]ATP, followed by SDS PAGE and autoradiography. Figure 6.1 shows that in the assay to which no ATPγS was added the B-type cyclins and histone H1 kinase activity had disappeared by 20 minutes after Ca2+ addition (lane 19). In the reactions to which ATPγS was added, however, cyclin destruction and the consequent disappearance of histone H1 kinase activity was not complete even by the end of the destruction reaction, 90 minutes after Ca2+ addition to the extract (lanes 8 and 16). When the ATPγS was added 10 minutes before destruction is triggered, rather than with the Ca2+, the inhibition of cyclin destruction was much more marked (compare lanes 1 - 8 with 9 - 16). The addition of 1 mM rATP to a destruction assay has no effect on cyclin proteolysis (see figure 6.3). ATPγS added to a CSF-arrested egg extract to 1 mM thus inhibits cyclin destruction and the consequent histone H1 kinase inactivation considerably, although the inhibition is not complete.

ATPγS inhibits cyclin destruction at a concentration of 1 mM

To check at what concentration the ATPγS could inhibit cyclin destruction, assays were carried out in the presence of different concentrations of this ATP analogue. The mRNA encoding c-myc cyclin A was translated in CSF-arrested egg extract and either 0, 1, 10, 100 or 1000 μM of ATPγS was added to the translation
Figure 6.1 ATPγS can inhibit cyclin destruction in CSF-arrested egg extract
CSF-arrested egg extract was incubated with [35S]methionine in the absence of any exogenous mRNA. The standard destruction assay was used. Samples were taken at the times indicated above the figure and incubated with purified histone H1 and [γ-32P]ATP (see Chapter 2). Lanes 1 - 8, 1 mM ATPγS added to the destruction reaction with the cycloheximide (i.e. 10 minutes before Ca^{2+} addition); lanes 9 - 16, 1 mM ATPγS added to the destruction reaction with the Ca^{2+}; lanes 17 - 24, no ATPγS added to the destruction reaction. Visualised by autoradiography.
Figure 6.2  Concentration of ATPγS required to inhibit cyclin destruction
The mRNA encoding c-myc cyclin A was translated in CSF-arrested egg extract in the presence of [35S]methionine. The standard destruction assay was used (see Chapter 2. The ATPγS was added with the cycloheximide and samples taken at the times indicated above the figure. Lanes 1 - 6, no ATPγS added; lanes 7 - 12, 1 μM ATPγS added; lanes 13 - 18, 10 μM ATPγS added; lanes 19 - 24, 100 μM ATPγS added; lanes 25 - 30 1 mM ATPγS added. Visualised by autoradiography. Asterisk on right indicates position of the endogenous B-type cyclins.
reaction with the cycloheximide, and incubated at 23°C for 10 minutes. Cyclin destruction was triggered by the addition of Ca²⁺ to the extract and samples were collected for analysis by SDS PAGE and autoradiography. Figure 6.2 shows that although 1 mM ATPγS can inhibit cyclin destruction (lanes 25 - 30), concentrations below this have no effect (lanes 7 - 18). Thus a concentration of between 100 μM and 1 mM ATPγS was needed to inhibit cyclin destruction.

**Another ATP analogue, AMP-PNP, cannot inhibit cyclin destruction**

To investigate whether another ATP analogue, AMP-PNP could also inhibit cyclin destruction, another destruction assay was carried out. CSF-arrested egg extract was incubated with [³⁵S]methionine to allow the endogenous B-type cyclins to become labelled, 1 mM ATPγS or 1 mM AMP-PNP was added to the extract with the cycloheximide and incubated for a further 10 minutes, before cyclin destruction was triggered with Ca²⁺. The samples collected subsequently were stored on dry ice until they were analysed for their histone H1 kinase activity using purified histone H1 and [γ-³²P]ATP, followed by SDS PAGE and autoradiography. As expected, in the absence of Ca²⁺ histone H1 kinase activity remained high in the extract to which ATPγS was added (figure 6.3, lanes 7 - 12). In the presence of Ca²⁺ the ATPγS markedly inhibited cyclin destruction and therefore prevented the inactivation of histone H1 kinase activity (compare lanes 1 - 6 with lanes 19 - 24), although histone H1 phosphorylation did decline much more in this experiment than in the experiment shown in figure 6.1 (compare figure 6.1, lanes 1 - 8 with figure 6.3 lanes 1 - 6). AMP-PNP, in contrast, had no effect on cyclin destruction and the consequent decrease in histone H1 kinase activity, at all (compare lanes 13 - 18 in figure 6.3 with lanes 19 - 24).

**GTPγS also inhibits cyclin destruction**

It was possible that the effect that ATPγS was having on cyclin destruction was not actually due to the ATPγS itself, but because small amounts of GTPγS could be synthesised when the thiophosphate group from the ATPγS was transferred to GDP. To test whether GTPγS could inhibit cyclin destruction, a reaction was set up in which c-myc cyclin A mRNA was translated in CSF-arrested egg extract and then 1 mM of either rATP, ATPγS, rGTP or GTPγS was added to the reaction. After a 10 minute incubation, Ca²⁺ as added to trigger cyclin destruction and samples were collected for analysis by SDS PAGE and autoradiography. Neither 1 mM rATP or rGTP had any effect on cyclin destruction (figure 6.4, lanes 7 - 12 and 25 - 30), but adding either 1 mM ATPγS or GTPγS to the extract inhibited cyclin destruction to a significant extent.
Figure 6.3 AMP-PNP cannot inhibit cyclin destruction in CSF-arrested egg extract
CSF-arrested egg extract was incubated with [35S]methionine in the absence of any exogenous mRNA. The standard destruction assay was used. Samples were taken at the times indicated above the figure and incubated with purified histone H1 and [γ-32P]ATP (see Chapter 2). Lanes 1 - 12, 1 mM ATPγS added to the destruction reaction with the cycloheximide; lanes 13 - 18, 1 mM AMP-PNP added to the destruction reaction with the cycloheximide; lanes 19 - 24, H₂O added to the destruction reaction with the cycloheximide; lanes 1 - 6 and 13 - 24, Ca²⁺ added to the destruction assay; lanes 7 - 12, H₂O added to the assay in place of Ca²⁺ (i.e. no cyclin destruction was triggered). Visualised by autoradiography.
Figure 6.4 GTPγS can also inhibit cyclin destruction

The mRNA encoding c-myc cyclin A was translated in CSF-arrested egg extract in the presence of [35S]methionine. The standard destruction assay was used (see Chapter 2) and samples were taken at the times indicated above the figure. Lanes 1 - 6, H2O added to the destruction reaction with the cycloheximide; lanes 7 - 12, 1 mM ATPγS added to the destruction reaction with the cycloheximide; lanes 13 - 18, 1 mM rATP added to the destruction reaction with the cycloheximide; lanes 19 - 24 1 mM GTPγS added to the destruction reaction with the cycloheximide; lanes 25 - 30, 1 mM rGTP added to the destruction reaction. Visualised by autoradiography. Asterisk on right indicates position of the endogenous B-type cyclins.
If ATPγS was inhibiting cyclin destruction because of recycling of the thiophosphate group to produce GTPγS then the concentration of GTPγS required to inhibit cyclin destruction should be considerably less than 1 mM, the concentration at which ATPγS can inhibit cyclin destruction. To ascertain at what concentration GTPγS could inhibit cyclin destruction, assays were carried out in the presence of different concentrations of GTPγS. The mRNA encoding c-myc cyclin A was translated in CSF-arrested egg extract and either 0, 1, 10, 100 or 1000 μM of GTPγS was added to the translation reaction with the cycloheximide and incubated at 23°C for 10 minutes. Cyclin destruction was triggered by the addition of Ca²⁺ to the extract, and samples were collected for analysis by SDS PAGE and autoradiography. Figure 6.5 shows that although 100 pM GTPγS may have a slight effect on cyclin destruction (lanes 19 - 24) concentrations of GTPγS below this had no effect (lanes 7-18). 1 mM GTPγS had a much greater effect on cyclin destruction than 100 μM (lanes 25 - 30). Thus a concentration of GTPγS of somewhere between 100 μM and 1 mM was needed to markedly inhibit cyclin destruction. Since such a high concentration of GTPγS is required in order to inhibit cyclin destruction, it is unlikely that ATPγS is having its effect through the recycling of its thiophosphate group into GTPγS.

Okadaic acid can promote cyclin destruction in CSF-arrested egg extracts in the absence of Ca²⁺

Okadaic acid, a potent type 1 and 2A protein phosphatase inhibitor, was previously reported to release the cyclin degradation pathway from its inhibited state in CSF-arrested egg extracts and to switch on cyclin protease activity permanently in interphase egg extracts (Lorca et al., 1991a). To confirm these findings, the mRNA encoding c-myc cyclin A was translated in CSF-arrested egg extract, followed by the addition of different concentrations of okadaic acid to the translation reaction. Samples were frozen on dry ice until they were analysed for their histone H1 kinase activity using purified histone H1 and [γ-³²P]ATP, followed by SDS PAGE and autoradiography. Figure 6.6 shows that concentrations of up to 100 nM okadaic acid had no effect on cyclin destruction (lanes 7 - 24). There was a decline in histone H1 kinase activity 90 minutes after okadaic acid addition in all of these extracts, but this also occurred in the extract to which no okadaic acid had been added. The addition of 1 μM okadaic acid to CSF-arrested egg extract did, however, cause a decline in histone H1 kinase activity (lanes 25 - 30), so that by 45 minutes after okadaic acid addition the histone H1 kinase in the extract was completely inactive (lane 28). This is due to (or at least occurs concurrently with) destruction of the cyclin proteins (not shown). The addition of 1 μM okadaic acid to similar reactions at the same time as Ca²⁺ did not accelerate (or inhibit) the destruction of the cyclin proteins. Since
Figure 6.5 Concentration of GTPγS required to inhibit cyclin destruction
The mRNA encoding \textit{c-myc} cyclin A was translated in CSF-arrested egg extract in the presence of [35S]methionine. The standard destruction assay was used (see Chapter 2. The GTPγS was added with the cycloheximide and samples taken at the times indicated above the figure. Lanes 1 - 6, no GTPγS added; lanes 7 - 12, 1 µM GTPγS added; lanes 13 - 18, 10 µM GTPγS added; lanes 19 - 24, 100 µM GTPγS added; lanes 25 - 30 1 mM GTPγS added. Visualised by autoradiography. Asterisk on right indicates position of the endogenous B-type cyclins.
Figure 6.6  Okadaic acid can cause cyclin destruction in CSF-arrested egg extracts in the absence of Ca$^{2+}$

The mRNA encoding c-myc cyclin A was translated in CSF-arrested egg extract in the presence of [35S]methionine. Samples were taken at the times indicated above the figure and incubated with purified histone H1 and [$\gamma$-32P]ATP (see Chapter 2). Lanes 1 - 6, no okadaic acid added; lanes 7 - 12, 1 nM okadaic acid added; lanes 13 - 18, 10 nM okadaic acid added; lanes 19 - 24, 100 nM okadaic acid added; lanes 25 - 30, 1 µM okadaic acid added. Visualised by autoradiography.
the proteolysis of cyclins is triggered slightly faster by Ca^{2+} than it is by okadaic acid (data not shown), this suggests that okadaic acid and Ca^{2+} are not acting to trigger cyclin destruction in an identical manner.

**High levels of cyclin A kinase can inhibit cyclin destruction**

The effect of high levels of cyclin A kinase on cyclin destruction was studied using a bacterially expressed protein A-bovine cyclin A fusion protein (pAcycA, kindly provided by Dr Jörg Adamczewski) and a C-terminally deleted mutant of this construct, pAcycACΔ16, which is missing its last 16 amino acids, and cannot bind to or activate p34cdc2 (Kobayashi et al., 1992). Five translation reactions containing c-myc cyclin A mRNA were set up in CSF-arrested egg extract. To these reactions was added a 1/20th volume of 25 μM pAcycA or pAcycACΔ16 protein, either before translation started or at the end of the translation reaction, with the cycloheximide. To the reactions that did not receive protein was added a 1/20th volume of the buffer that the proteins were dissolved in (buffer A). The contents of each translation reaction were thus as follows:

<table>
<thead>
<tr>
<th>Reaction</th>
<th>At start of translation</th>
<th>At end of translation, with cycloheximide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reaction 1</td>
<td>Buffer A added</td>
<td>Buffer A added</td>
</tr>
<tr>
<td>Reaction 2</td>
<td>pAcycA added</td>
<td>Buffer A added</td>
</tr>
<tr>
<td>Reaction 3</td>
<td>Buffer A added</td>
<td>pAcycA added</td>
</tr>
<tr>
<td>Reaction 4</td>
<td>pAcycACΔ16 added</td>
<td>Buffer A added</td>
</tr>
<tr>
<td>Reaction 6</td>
<td>Buffer A added</td>
<td>pAcycACΔ16 added</td>
</tr>
</tbody>
</table>

Cyclin destruction was triggered in these reactions by the addition of Ca^{2+}, and samples were analysed by SDS PAGE and autoradiography. Figure 6.7 shows that while pAcycA protein inhibited cyclin destruction, whether added at the start of translation (lanes 7 - 12) or with the cycloheximide at the end of the translation reaction (lanes 13 - 18), pAcycACΔ16 did not inhibit cyclin destruction when added at either point (lanes 19 - 30). pAcycA that had been boiled also did not inhibit cyclin destruction (data not shown). This shows that cyclin A must be able to activate p34cdc2 in order to inhibit cyclin destruction.

The concentration of pAcycA that could inhibit cyclin destruction was investigated. The mRNA encoding c-myc cyclin A was translated in CSF-arrested egg extract and the translation reaction was split into five aliquots. To each aliquot was added cycloheximide and a different concentration of pAcycA protein. After a 10 minute incubation destruction was triggered by the addition of Ca^{2+} to the reaction. Samples were analysed by SDS PAGE and autoradiography. Figure 6.8 shows that pAcycA at a concentration of 0.1 μM had
Figure 6.7  Cyclin A kinase can inhibit cyclin destruction in CSF-arrested egg extract

The mRNA encoding c-myc cyclin A was translated in CSF-arrested egg extract in the presence of [35S]methionine. The standard destruction assay was used (see Chapter 2) and samples were taken at the times indicated above the figure. Lanes 1 - 6, buffer only added to reaction; lanes 7 - 12, 1.25 μM pAcycA protein added at the start of translation; lanes 13 - 18, 1.25 μM pAcycA protein added with the cycloheximide; lanes 19 - 24, 1.25 μM pAcycAΔ16 protein added at the start of translation; lanes 25 - 30, 1.25 μM pAcycAΔ16 protein added with the cycloheximide. Visualised by autoradiography. Asterisk on right indicates position of the endogenous B-type cyclins.
Figure 6.8 Concentration of pAcycA required to inhibit cyclin destruction
The mRNA encoding c-myc cyclin A was translated in CSF-arrested egg extract in the presence of \([^{35}S]\)methionine. The standard destruction assay was used (see Chapter 2). The pAcycA was added with the cycloheximide and samples were taken at the times indicated above the figure. Lanes 1 - 6, buffer added; lanes 7 - 12, 0.1 μM pAcycA protein added; lanes 13 - 18, 0.2 μM pAcycA protein added; lanes 19 - 24, 0.4 μM pAcycA protein added; lanes 25 - 30, 0.6 μM pAcycA protein added. Visualised by autoradiography. Asterisk on right indicates position of the endogenous B-type cyclins.
little effect on cyclin destruction (lanes 7 - 12), but that a concentration of 0.2 μM pAcycA markedly inhibited cyclin destruction (lanes 13 - 18). When pAcycA was added to a concentration of 0.4 μM or above, cyclin destruction was almost completely inhibited (lanes 19 - 30).

To see how long the extract had to be incubated with pAcycA before destruction was maximally inhibited, \textit{c-myc} cyclin A mRNA was translated in CSF-arrested egg extract and the translation reaction split into two aliquots; to one was added a 1/20th volume of 25 μM pAcycA protein and to the other a 1/20th volume of buffer A. Aliquots of these reactions were taken and Ca$^{2+}$ was added at various times after the protein. Samples were taken and analysed by SDS PAGE and autoradiography. The data was quantified by scanning densitometry of the autoradiograph. Each 90 minute time point was expressed as a proportion of its zero time point, and these values were plotted on a graph, see figure 6.9C. The histone H1 kinase activity in each zero time point was also measured to determine how long the pAcycA took to activate p34\textit{cdc2}. Figure 6.9B shows that the pAcycA activated p34\textit{cdc2} in the CSF-arrested egg extract within 5 minutes of addition to the extract (lane 8), and figures 6.9A and C show that pAcycA inhibited cyclin destruction to almost half the possible maximal value after only a five minutes. Thus, the inhibition of cyclin destruction by pAcycA is a process that can occur rapidly.

**High levels of cyclin A2 kinase can inhibit cyclin destruction**

The cyclin A construct pAcycA was made using bovine cyclin A and it was possible that it inhibited cyclin destruction because it was derived from a foreign cyclin A rather than \textit{Xenopus}. To test this hypothesis, the effect on cyclin destruction of a \textit{Xenopus} cyclin A2 protein in which glutathione-S-transferase was fused to the first residue of \textit{Xenopus} cyclin A2 (GST-cyclin A2, kindly provided by Dr Michael Howell) was studied. The mRNA encoding \textit{c-myc} cyclin A was translated in CSF-arrested egg extract and the translation reaction was split into five aliquots. Cycloheximide and a different concentration of GST-cyclin A2 protein were added to each aliquot, and after a 10 minute incubation, destruction was triggered by the addition of Ca$^{2+}$. Samples were removed and analysed by SDS PAGE and autoradiography. Figure 6.10 shows that, like bovine pAcycA protein, \textit{Xenopus} GST-cyclin A2 protein inhibited cyclin destruction in CSF-arrested egg extract. A concentration of 0.1 μM GST-cyclin A2 partially inhibited cyclin destruction (see lanes 7 - 12) and higher concentrations of this protein showed increasing inhibition (lanes 13 - 30).
**A**

with (+) or without (−) added pAcycA protein

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<td>−</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
</tbody>
</table>

Time since Ca\(^{2+}\) addition (minutes)

Length of time extract was incubated with pAcycA protein before Ca\(^{2+}\) was added (minutes)

**B**

Time since protein added (minutes)

<table>
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Histone H1

without pAcycA

with pAcycA
Figure 6.9 How rapidly can pAcycA inhibit cyclin destruction?
(A) The mRNA encoding c-myc cyclin A was translated in CSF-arrested egg extract in the presence of \(^{35}\text{S}\)methionine. The translation reaction was incubated in the presence (+) or absence (-) of pAcycA protein for the lengths of time indicated below the figure before Ca\(^{2+}\) was added and samples taken at the times indicated above the figure (Ca\(^{2+}\) addition to the extract being time zero). Analysis was by SDS PAGE and autoradiography. Asterisk on right indicates position of the endogenous B-type cyclins. (B) The samples from the odd numbered lanes in (A) were incubated with purified histone H1 and [\(\gamma^{32}\text{P}\)]ATP (see Chapter 2). Analysis was by SDS PAGE and autoradiography. (C) The intensity of the cyclin A bands on the autoradiography shown in (A) were quantified by scanning densitometry. Each 90 minute time point (odd numbered lanes on the autoradiograph shown in (A)) was expressed as a fraction of the intensity of its corresponding zero time point and plotted on a graph.

High levels of cyclin B1 kinase also inhibits cyclin destruction
To investigate whether cyclin A was unique in its ability to inhibit cyclin destruction, or whether it was a feature shared by other mitotic cyclins, the ability of cyclin B1 to inhibit cyclin destruction was tested. The cyclin B1 construct used was a *Xenopus* cyclin B1 to which glutathione-S-transferase was fused at the N-terminus (GST-cyclin B1, kindly provided by Dr Katsumi Yamashita). The mRNA encoding c-myc cyclin A was translated in CSF-arrested egg extract, and the translation reaction was split into five aliquots. To each aliquot was added cycloheximide and a different concentration of GST-cyclin B1 and after a 10
Figure 6.10 Concentration of GST-cyclin A2 protein required to inhibit cyclin destruction
The mRNA encoding c-myc cyclin A was translated in CSF-arrested egg extract in the presence of [35S]methionine. The standard destruction assay was used (see Chapter 2). The GST-cyclin A2 was added with the cycloheximide and samples were taken at the times indicated above the figure. Lanes 1 - 6, buffer added; lanes 7 - 12, 0.1 μM GST-cyclin A2 protein added; lanes 13 - 18, 0.2 μM GST-cyclin A2 protein added; lanes 19 - 24, 0.4 μM GST-cyclin A2 protein added; lanes 25 - 30, 0.6 μM GST-cyclin A2 protein added. Visualised by autoradiography. Asterisk on right indicates position of the endogenous B-type cyclins.
Figure 6.11  Concentration of GST-cyclin B1 protein required to inhibit cyclin destruction
The mRNA encoding c-myc cyclin A was translated in CSF-arrested egg extract in the presence of [35S]methionine. The standard destruction assay was used (see Chapter 2). The GST-cyclin B1 was added with the cycloheximide and samples were taken at the times indicated above the figure. Lanes 1 - 6, buffer added; lanes 7 - 12, 0.1 µM GST-cyclin B1 protein added; lanes 13 - 18, 0.2 µM GST-cyclin B1 protein added; lanes 19 - 24, 0.4 µM GST-cyclin B1 protein added; lanes 25 - 30, 0.6 µM GST-cyclin B1 protein added. Visualised by autoradiography. Asterisk on right indicates position of the endogenous B-type cyclins.
minute incubation destruction was triggered by the addition of Ca^{2+} to the reaction. Samples were removed and analysed by SDS PAGE and autoradiography. Figure 6.11 shows that GST-cyclin B1 partially inhibited cyclin destruction when added at a concentration of 0.4 µM (lanes 19 - 24) and showed greater inhibition at a concentration of 0.6 µM (lanes 25 - 30). Thus, the inhibition of cyclin destruction by very high level of added cyclin does not appear to be confined to cyclin A. It should be noted, however, that the levels of added cyclin in these experiments are 50-500 times higher than would be encountered in vivo (see Kobayashi et al. (1991) and Appendix 2).

**Discussion**

The studies described in this chapter represent preliminary findings and in order to draw any firm conclusions about any of them would require further work. I will, however, discuss what the data in this chapter could suggest to us about the mechanism of cyclin destruction.

**Inhibition of cyclin destruction by increased phosphorylation of proteins**

ATPγS, but not AMP-PNP, can inhibit cyclin destruction. This implies that the effect of ATPγS is probably not due to inhibition of an ATPase. Protein kinases can use this ATP analogue to thio-phosphorylate proteins, but the rate of dephosphorylation of thio-phosphorylated proteins is extremely slow (Eckstein, 1985). AMP-PNP cannot be used by kinases at all, as the kinase can bind the ATP-analogue, but are unable to hydrolyse the bond between the nitrogen and the final phosphate group. Since cyclin destruction can be inhibited by ATPγS but not by AMP-PNP, this suggests that the dephosphorylation of a protein or proteins is required to enable cyclin destruction either to be triggered or to occur in CSF-arrested egg extract.

Although the concentration of ATPγS required to inhibit cyclin destruction, 1 mM, is quite high, it should be noted that the concentration of free rATP in CSF-arrested egg extract has been measured to be between 12 and 18 mM (Dolores Harrison, pers. comm.); thus the added ATPγS would contribute less than 10% of the total ATP present.

High levels of cyclin A1, A2 or B1 kinase can also inhibit cyclin destruction. Cyclin A had previously been shown to delay cyclin destruction; Lorca et al. (1992) showed that cyclin A kinase (but not free cyclin A) at a concentration of about 100 nM could strongly delay the destruction of both A- and B-type cyclins. However, they stated that adding even a five fold higher concentration of cyclin B kinase to their assay system (I estimate this to be a final
concentration of 0.5 μM) had no effect at all on cyclin destruction. In contrast, in this chapter I showed that adding GST-cyclin B1 to a concentration of 0.4 μM in CSF-arrested egg extract inhibited cyclin destruction. This discrepancy could be due to a difference in the assay system used; Lorca et al. used Xenopus egg interphase extract in which cyclin degradation had been activated by the addition of cyclin B kinase, while I used Xenopus CSF-arrested egg extract in which cyclin destruction had been triggered by the addition of Ca\(^{2+}\).

Since the proportion of active cyclin protein in each bacterially produced protein solution is not accurately known, it is not possible to make a comparison of the concentration of each cyclin required to inhibit cyclin destruction, but the levels of cyclin A and B required appear to be quite similar.

It is not clear how the cyclin A1, A2 and B1 kinases are mediating their inhibition of cyclin destruction, but it is very unlikely that they are doing so because the high concentration of cyclin protein in the extract is overwhelming the cyclin destruction machinery, as the pAcycA construct does not contain a destruction box, which is required for the protein to be recognised by the cyclin destruction machinery (Glotzer et al., 1991). The more likely explanation is that the cyclin kinase is phosphorylating a protein that must be dephosphorylated in order to allow cyclin degradation to occur; certainly the cyclin A used must be able to bind to and activate p34\(^{cd2}\) in order to prevent cyclin proteolysis. As the concentration of cyclin required to inhibit cyclin destruction is at least 10 times greater than the physiological levels of this protein, the cyclin kinases are probably acting in a less specific manner than they would under physiological conditions. Thus the cyclin kinases may not be phosphorylating a physiological substrate. The idea that the cyclin kinases are inhibiting cyclin destruction by phosphorylation is consistent with the result that the addition of ATP\(_y\)S to a CSF-arrested egg extract can inhibit cyclin destruction, probably by causing stable thio-phosphorylation of a protein involved in triggering cyclin destruction.

The promotion of cyclin destruction by okadaic acid

The results discussed above show that high levels of cyclin kinase can inhibit cyclin destruction, which implies that for cyclin degradation to occur, a protein or proteins require dephosphorylation. The promotion of cyclin destruction and it constitutive activation by okadaic acid, however, implies that for cyclin destruction to occur a protein or proteins require phosphorylation! These results are not necessarily contradictory, however, and probably reveal that cyclin destruction is a complex process, involving several regulatory factors, all or some of which may be regulated by phosphorylation and dephosphorylation. It would be interesting to investigate the effects of adding both okadaic acid and ATP\(_y\)S, or okadaic acid and high concentrations of cyclin protein to CSF-arrested egg extract.

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at once. The inhibition or promotion of cyclin destruction in such extracts could give clues as to the relationship between the regulatory phosphorylation/dephosphorylation reactions.

At the concentration of okadaic acid used in these experiments, it has been shown that type 2A phosphatase in CSF-arrested egg extracts is almost completely inhibited, while type 1 phosphatase is inhibited by about 60% (Félix et al., 1990a). Thus, as concluded by Lorca et al. (1991a), it is likely that the effect of okadaic acid on CSF-arrested egg extracts (i.e. triggering cyclin destruction) is due to the inhibition of a type 2A phosphatase.

**The inhibition of cyclin destruction by GTPγS**

One of the original reasons for testing the effect of GTPγS on cyclin destruction was that the MAD2 gene, which is required to prevent budding yeast cells from leaving mitosis until spindle assembly is complete (Li and Murray, 1991), had been reported to encode a protein with significant homology to the α-subunit of prenyltransferase (Boguski et al., 1992) and to be required for the membrane association of two small GTP-binding proteins involved in protein trafficking (Li et al., 1993). Thus it appeared that small GTP-binding proteins might be involved in regulation of the cell cycle. It has recently been discovered, however, that there was a mistake in the cloning of the MAD2 gene and that it is not a prenyltransferase but is a protein of unknown function.

I do not have enough data to know why the addition of 1 mM GTPγS to CSF-arrested egg extract should inhibit cyclin destruction, but there are several possible explanations.

It is unlikely that GTPγS inhibits destruction due to its conversion into ATPγS, as the concentrations of both nucleotide analogues required to inhibit cyclin destruction are of the same order of magnitude. It is possible that the inhibition of cyclin destruction by GTPγS can be explained by the involvement of a GTPase, such as a heterotrimeric G protein or one of ras p21-related small GTP-binding proteins, even though we now know that there is not a precedent for the involvement of GTP-binding proteins in cell cycle control after all. The binding of both types of GTP-binding proteins to GTPγS causes their constitutive activation, because the GTP-bound forms of these enzymes are active and are usually inactivated by the hydrolysis of the GTP to GDP. As GTPγS is a non-hydrolysable form of GTP, the GTPγS bound proteins cannot be inactivated. Heterotrimeric G proteins are involved in transducing receptor-generated signals across the plasma membrane (for a review on heterotrimeric G proteins, see Hepler and Gilman (1992) and Kaziro et al. (1991)) while small GTP-binding proteins have been implicated in a diverse spectrum of intracellular processes, including cellular proliferation and differentiation, intracellular vesicle
trafficking, oxidase generation, and cytoskeletal control (see Bokoch and Der (1993) and Bourne et al. (1990)). As some protein kinases can use GTP as a source of phosphate as well as ATP, the most likely explanation for the ability of GTPγS to inhibit cyclin destruction is that stable thio-phosphorylation of the protein whose dephosphorylation is required for cyclin destruction (see above) can occur in the presence of either ATPγS or GTPγS.
Chapter 7

Cyclin A destruction (i)

The destruction box of *Xenopus* cyclin A is required for its destruction

It had previously been demonstrated that a short motif in the N-terminus of sea urchin cyclin B was required for the proteolysis of this protein at the end of M-phase (Glotzer *et al.*, 1991). This so-called ‘destruction box’ is conserved throughout B-type cyclins and a similar motif is present in the N-termini of A-type cyclins.

Figure 7.1 shows the destruction boxes of a selection of A- and B-type cyclins. The residues in bold fit the consensus sequence, which is shown at the bottom of the figure. The numbers under the consensus sequence indicate the number of A- and B-type cyclins that conserve each residue, out of a total of 23 cyclins studied. The arginine and leucine residues are conserved in all destruction boxes except that of CLB6, which has a lysine residue instead of an arginine. The main difference between A- and B-type cyclin destruction boxes seems to lie in the 6th residue of this region; A-type cyclins usually have a valine residue whilst B-type cyclins have a variety of residues including aspartic acid, glutamic acid, asparagine and lysine.

When the work described in this chapter was started, it had not been shown that the cyclin A ‘destruction box’ was indeed required for cyclin A destruction. In order to test this point, a *Xenopus* cyclin A mutant, ATVA, was constructed in which the conserved residues of the cyclin destruction box, arginine 41 and leucine 44 (see figure 7.1), were changed to alanine residues in c-myc cyclin A by PCR (see Chapter 2). The mRNAs encoding ATVA and wild-type (RTVL) c-myc cyclin A were translated in CSF-arrested egg extract and cyclin destruction was triggered by the addition of Ca$^{2+}$ to the translation reactions. Figure 7.2 shows that whilst wild-type cyclin A was almost completely destroyed by 20 minutes (lanes 1 - 5), ATVA cyclin A was still present in the extract 90 minutes after Ca$^{2+}$ addition (lanes 6 - 10). Note that in this experiment the endogenous B-type cyclins (indicated by an asterisk on the right of the figure) were destroyed at the same rate in both assays, indicating that the stability of
Figure 7.1 Consensus sequence of the mitotic cyclin destruction box
The sequences of the destruction boxes of a selection of A- and B-type cyclins are displayed, with the consensus sequence shown at the bottom. The destruction boxes of 23 A- and B-type cyclins were studied and the residues that corresponded to the consensus sequence were counted. The numbers of destruction boxes with each of the conserved residues are shown below the consensus sequence.

Consensus:
R-ALG-I-N

<table>
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<tr>
<th>Human B1</th>
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<th>Human B2</th>
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</table>

Figure 7.2 The destruction box of cyclin A is necessary for degradation of the cyclin A protein
The mRNAs encoding c-myc cyclin and the destruction box mutant of cyclin A, ATVA, were translated in CSF arrested egg extract in the presence of [35S]methionine. The standard destruction assay was used (see Chapter 2) and samples were taken at the times indicated above the figure. Lanes 1 - 5, c-myc cyclin A; lanes 6 - 10, ATVA cyclin A. Visualised by autoradiography. Asterisk on right indicates position of the endogenous B-type cyclins.
ATVA cyclin A must be due to its inability to be destroyed and not to a problem with the destruction reaction.

The degradation of *Xenopus* cyclin A thus requires an intact destruction box, like the proteolysis of B-type cyclins. This result was confirmed by the results of Lorca et al. (1992), who showed that mutating the conserved arginine residue in the destruction box of *patella* cyclin A to a cysteine residue made the protein resistant to proteolysis.

The destruction of non-p34<sup>cdc2</sup> binding mutants of cyclin A

In order to study what regions of cyclin A, other than the destruction box, were required for its destruction, the C-terminal deletions and point mutants of cyclin A, which were unable to bind to p34<sup>cdc2</sup>, were tested for their ability to undergo programmed proteolysis.

The C-terminal deletions of cyclin A are indestructible

The mRNAs encoding c-myc cyclin A and CA14 cyclin A were translated in CSF-arrested egg extract and their destruction studied using the standard destruction assay. Figure 7.3A shows that while c-myc cyclin A was destroyed by 60 minutes after Ca<sup>2+</sup> addition (lane 5), CA14 cyclin A was still present at the end of the assay, 90 minutes after Ca<sup>2+</sup> was added to the extract (lane 12).

To check whether this indestructibility was a property unique to CA14, or whether it was shared by the other C-terminal deletion mutants of cyclin A, the mRNAs encoding c-myc cyclin A and CA24, CA50, CA79, CA97 and CA137 cyclin As were translated in CSF-arrested egg extract in the presence of 10% reticulocyte lysate (to improve the translation of the added mRNAs, see Chapters 2 and 5). Cyclin destruction was triggered in these reactions by the addition of Ca<sup>2+</sup> in the standard way. The intensity of the cyclin A bands on the autoradiograph were quantified by scanning densitometry and expressed as a proportion of the intensity of the cyclin A band at time zero for each construct. Figure 7.3B shows the data plotted on a graph against time since the addition of Ca<sup>2+</sup> to the extract. This graph shows that while c-myc cyclin A was destroyed by 60 minutes after Ca<sup>2+</sup> addition to the extract, all the C-terminal deletion mutants of cyclin A were relatively stable.

It is worth noting, however, that the C-terminal cyclin A deletion proteins were gradually degraded during the cyclin destruction assay (see figure 7.3A, lanes 7 - 12 and figure 7.3B), whereas the ATVA mutant of cyclin A, was completely stable (see figure 7.2, lanes 6 - 10). The proteolysis of the C-terminally deleted cyclin A mutants, which was very much more gradual than that
Figure 7.3 The C-terminal deletions of cyclin A are indestructible

The mRNAs encoding c-myc cyclin A and the C-terminal deletions of cyclin A were translated in CSF-arrested egg extract in the presence of $[^{35}S]$methionine. The standard destruction assay was used (see Chapter 2). Samples were taken at the times indicated above the figure. (A) Visualised by autoradiography. Lanes 1-6, c-myc cyclin A; lanes 7-12, CA14 cyclin A. (B) The intensities of the cyclin A bands on autoradiographs of destruction assays of c-myc cyclin A, CA14, CA24, CA50, CA79, CA97 and CA136 were quantitated by scanning densitometry. The values were expressed as a proportion of the intensity of the cyclin band at time 0, and plotted against time. The key to the graph is shown on the right of the figure.
of wild-type cyclin A, is most likely to be due to the misfolding of these mutants (see Discussion).

Two plausible reasons could be advanced to explain the relative stability of the C-terminally deleted cyclin A mutants. Their indestructibility could have stemmed from the inability of the cyclin destruction machinery to recognise them as cyclin proteins, or being unable to proteolyse them, owing to their altered structure. The alternative explanation was that cyclin A must be able to bind to p34\(^{cdc2}\) in order to be destroyed when Ca\(^{2+}\) is added to CSF-arrested egg extract.

In order to distinguish between these two explanations for the abnormal proteolysis of the C-terminally deleted cyclin A mutants, it was important to test the destructibility of the cyclin A point mutations that were either unable to bind to p34\(^{cdc2}\), R197A and D226A, or able to bind only very weakly, R197K and D226E, as these proteins are less likely than the C-terminal deletion mutants of cyclin A to be badly misfolded (see Chapter 3).

The point mutations of cyclin A are indestructible

The mRNAs encoding R197A, R197K, D226A and D226E cyclin As were translated in CSF-arrested egg extract, and cyclin destruction was assayed as previously described. Figure 7.4A shows that while c-myc cyclin A was destroyed by 20 minutes after Ca\(^{2+}\) addition (lane 3), the point mutants of cyclin A, R197A and D226A, were completely stable throughout the whole destruction assay (lanes 7 - 18). A similar result is seen in the destruction assay of the point mutants R197K and D226E; these proteins were stable throughout the assay (figure 7.4B, lanes 7 - 18) while c-myc cyclin A was destroyed by 60 minutes after Ca\(^{2+}\) addition (lane 5). These two assays display a good example of the variability of the speed of cyclin destruction in different batches of CSF-arrested egg extract. While c-myc cyclin A was almost completely destroyed by 10 minutes after Ca\(^{2+}\) addition to the extract used for the experiment shown in figure 7.4A, in the extract used for the experiment shown in figure 7.4B, protein translated from the same construct was not destroyed until between 30 and 60 minutes after Ca\(^{2+}\) addition. Since these mutants, which are unlikely to be misfolded, were completely stable when Ca\(^{2+}\) was added to CSF-arrested egg extract, it is likely that cyclin A must be able to bind stably to p34\(^{cdc2}\) in order to be destroyed.

Other cyclin A mutants that cannot bind to p34\(^{cdc2}\) are also indestructible

That cyclin A must be able to bind to p34\(^{cdc2}\) in order to be destroyed was confirmed by the analysis of a number of other cyclin A deletion mutants that contain intact destruction boxes but cannot bind to p34\(^{cdc2}\). Dolores Harrison and
Figure 7.4  Point mutants of cyclin A that cannot bind to p34cdc2 are indestructible
The mRNAs encoding c-myc cyclin A and R197A, R197K, D226A and D226E cyclin As were translated in CSF-arrested egg extract in the presence of [35S]methionine. The standard destruction assay was used (see Chapter 2) and samples were taken at the times indicated above the figures. (A) Lanes 1 - 6, c-myc cyclin A; lanes 7 - 12, R197A cyclin A; lanes 13 - 18 D226A cyclin A. (B) Lanes 1 - 6 c-myc cyclin A; lanes 7 - 12, R197K cyclin A; lanes 13 - 18, D226E cyclin A. Visualised by autoradiography. Asterisks on right indicate positions of the endogenous B-type cyclins.
Hideki Kobayashi in the laboratory showed that three cyclin A proteins with larger deletions from the C-terminus, CΔ194, CΔ221 and CΔ295, and three cyclin A mutants containing internal deletions, Δ231-232, Δ101-169 and Δ80-201, all of which cannot bind to p34cdc2, are also indestructible (Stewart et al., 1994).

These proteins are, however, gradually proteolysed throughout the length of the cyclin destruction assay, like the C-terminal cyclin A deletions CΔ14, CΔ24 and CΔ50. In order to confirm that this gradual degradation was distinct from the specific cyclin proteolysis that is triggered by Ca$^{2+}$ in CSF-arrested egg extract, a derivative of the internal deletion mutant Δ101-169 was made in which the wild-type destruction box of the construct was replaced with the mutated destruction box from the cyclin A mutant ATVA. The mRNAs encoding this mutant construct, Δ101-169ATVA, and Δ101-169 with a wild-type destruction box were translated in CSF-arrested egg extract and Ca$^{2+}$ was added to trigger cyclin destruction. The samples were analysed by SDS-PAGE and autoradiography. Figure 7.5 shows that gradual proteolysis of Δ101-169 occurred in the presence (lanes 1 - 6) or absence (lanes 7 - 12) of an intact destruction box.

Figure 7.5  Δ101-169ATVA cyclin A is degraded to the same extent as Δ101-169 cyclin A
The mRNAs encoding Δ101-169 and Δ101-169ATVA were translated in CSF-arrested egg extract in the presence of [35S]methionine. The standard destruction assay was used (see Chapter 2) and samples were taken at the times indicated above the figure. Lanes 1 - 6, Δ101-169; lanes 7 -12, Δ101-169ATVA. Visualised by autoradiography. Asterisk on right indicates position of the endogenous B-type cyclins.
The slow degradation of this construct, and probably that of the other constructs with C-terminal deletions and those with large internal deletions, is therefore distinct from the rapid and specific cyclin proteolysis that is seen on the addition of Ca^{2+} to CSF-arrested egg extract.

**The destruction of cyclin A bound to p34^{cdc2} compared to that bound to p33^{cdk2}**

In the previous section it was shown that cyclin A must be able to bind to p34^{cdc2} in order to be destroyed at the end of M-phase. As cyclin A can bind to p33^{cdk2} in addition to p34^{cdc2}, I tested whether cyclin A bound to p33^{cdk2} was also destroyed.

The Assay

The destruction assay described in Chapter 5 and used above relied on the newly-translated cyclin A protein binding to endogenous p34^{cdc2} that is present in CSF-arrested egg extract. It was necessary to use a slightly different approach when testing the destructibility of cyclin A bound to p33^{cdk2}, as in egg extracts this protein is present at a much lower concentration than p34^{cdc2} (Gabrielli *et al*., 1992; Kobayashi *et al*., 1992) and is not bound to cyclin A (Minshull *et al*., 1990; Fang and Newport, 1991; this thesis Chapter 4).

The assay used to study the destruction of cyclin A bound to p33^{cdk2} was thus as follows. Bacterially produced GST-cdk2 was added to CSF-arrested extract at the beginning of the translation reaction, with the c-myc cyclin A mRNA. This allowed a fraction of the newly translated cyclin A to bind to GST-cdk2 rather than to the endogenous p34^{cdc2}. At the end of the translation reaction cyclin destruction was triggered in the usual manner and samples taken. Each of the samples was diluted into an excess of ice-cold bead buffer and stored on ice until the last sample had been taken. At this point the samples were incubated with glutathione Sepharose and the bead-bound proteins analysed by SDS-PAGE and autoradiography. All the cyclin A protein visible on the autoradiograph, therefore, was that which had been bound to GST-cdk2.

**Cyclin A protein is destroyed with the same kinetics whether bound to endogenous p34^{cdc2} or bacterially expressed GST-cdc2H6**

As the p33^{cdk2} protein to be used in the assay was a bacterially produced GST fusion protein, it was necessary to check that the destruction of cyclin A bound to such a protein was the same as that bound to the wild-type version. To investigate this point, the destruction of cyclin A bound to GST-cdc2H6 was
Figure 7.6 Destruction of cyclin A bound to GST-cdc2H6 and a mixture of GST-cdc2H6 and p34cdc2

The mRNA encoding c-myc cyclin was translated in CSF-arrested egg extract in the presence of [35S]methionine and 1.2 μM GST-cdc2H6 protein. The standard destruction assay was used (see Chapter 2). (A) Samples were taken at times indicated above the figure. Lanes 1 - 12, precipitation of samples with glutathione Sepharose; lanes 13 - 24, precipitation of samples with the monoclonal anti p34cdc2 antibody, A17; lanes 1 - 6 and 13 - 18, Ca2+ added to the destruction assay; lanes 7 - 12 and 19 - 24, H2O added to the assay in place of Ca2+ (i.e. no cyclin destruction was triggered). Visualised by autoradiography. Asterisk on right indicates position of the endogenous B-type cyclins. (B) The intensity of the cyclin A bands on the autoradiograph shown above were quantitated by scanning densitometry. The values were expressed as a proportion of the intensity of the cyclin band at time 0, and plotted against time on a graph. The key to the graph is shown on the right of the figure.
compared with that bound to both p34cdc2 and GST-cdc2H6, in the same destruction assay.

The mRNA encoding c-myc cyclin A was translated in CSF-arrested egg extract in the presence of 1.2 µM GST-cdc2H6 protein. At the end of the translation reaction the extract was split into two aliquots. To one Ca2+ was added to trigger cyclin destruction, while H2O was added to the other. Samples (5 µl each) were taken into ice-cold buffer and split into aliquots; one of which was incubated with glutathione Sepharose and the other with the anti-p34cdc2 monoclonal antibody, A17, and protein A Sepharose. The bead-bound proteins were analysed by SDS-PAGE and autoradiography (see Chapter 2) and the intensities of the cyclin A bands on the autoradiograph were quantitated by scanning densitometry. These values were expressed as proportions of the cyclin intensity at time zero for each assay, and plotted on a graph against time. Figure 7.6 shows that the destruction of cyclin A bound to GST-cdc2H6 was almost identical to that of the cyclin A bound to both GST-cdc2H6 and the endogenous p34cdc2. It is notable that there was a small fraction of cyclin A bound to GST-cdc2H6 and endogenous p34cdc2 that did not get destroyed in these assays.

This result does not absolutely prove that cyclin A bound to GST-cdc2H6 gets destroyed in a manner identical to that bound to wild-type p34cdc2, as it was not shown what proportion of the newly translated cyclin A bound to GST-cdc2H6 and what proportion bound to the endogenous p34cdc2. However, I think that the destruction of cyclin A bound to GST-cdc2H6 is very similar to the destruction of cyclin A bound to wild-type p34cdc2 for the reasons set out in the Discussion section at the end of this chapter.
Cyclin A bound to p33\textsuperscript{cdk2} is not destroyed to the same extent and with the same kinetics as that bound to p34\textsuperscript{cdc2}

To test whether cyclin A bound to GST-\textit{cdk2} is destroyed in a manner similar to cyclin A bound to GST-\textit{cdc2H}_{6}, translation reactions were set up containing the mRNA encoding \textit{c-myc} cyclin A and 1.2 \mu M of either GST-\textit{cdc2H}_{6} or GST-\textit{cdk2}. Each reaction was split into two aliquots, to one was added Ca\textsuperscript{2+} to trigger cyclin destruction and to the other was added H\textsubscript{2}O. Two samples were removed at each time point, one (1 \mu l each) was added to sample buffer and stored on dry ice, the other (5 \mu l each) was added to ice-cold buffer and stored on ice until the end of the destruction assay. The GST-bound proteins from these samples were affinity purified on glutathione Sepharose and the results analysed by SDS-PAGE and autoradiography. The intensities of the cyclin A bands on the autoradiograph were quantitated by scanning densitometry, the values expressed as proportions of the cyclin intensity at time zero for each assay, and plotted on a graph against time. As figures 7.7A and C show, cyclin A bound to GST-\textit{cdc2H}_{6} was almost totally destroyed by 20 minutes after the addition of Ca\textsuperscript{2+}. In contrast, the cyclin A that was bound to GST-\textit{cdk2} was destroyed to a lesser extent and with slower kinetics (figure 7.7B, lanes 13 - 18 and figure 7.7C). This result was confirmed in several different CSF-arrested egg extracts (not shown).

Thus in order to be destroyed rapidly and completely when Ca\textsuperscript{2+} is added to CSF-arrested egg extract, cyclin A must be bound to p34\textsuperscript{cdc2}, not p33\textsuperscript{cdk2}. Cyclin A bound to p33\textsuperscript{cdk2} is, however, partially proteolysed, unlike those mutants of cyclin A that cannot bind efficiently to either p34\textsuperscript{cdc2} or p33\textsuperscript{cdk2}.

\textit{Ubiquitination of cyclins during destruction assays}

Cyclin destruction is thought to occur via the ubiquitin pathway (Glotzer \textit{et al.}, 1991; Hershko \textit{et al.}, 1991). However, the ubiquitinated intermediates are very short-lived (Glotzer \textit{et al.}, 1991) and therefore difficult to detect. On long exposures of the polyacrylamide gels from experiments described above, however, faint bands were visible above those of the cyclins that had been affinity purified on the GST- fusion proteins.

Cyclin A bound to p34\textsuperscript{cdc2} or p33\textsuperscript{cdk2} is ubiquitinated

The autoradiograph shown in figure 7.6 was generated from an overnight (about 16 hours) exposure of the polyacrylamide gel. A seventy day exposure of the same gel is shown in figure 7.8A. Bands of higher molecular weight than that of cyclin A can be seen above lanes 2 - 4 and 14 - 16. These are the reactions to which Ca\textsuperscript{2+} was added to trigger cyclin degradation. In those reactions where cyclin destruction was not triggered, a similar pattern of bands can just be seen,
**A**

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without Ca\(^{2+}\) | 13 14 15 16 17 18 | 19 20 21 22 23 24 |

Translation

Precipitation with Glutathione Sepharose

- cyclin A
- +GST-cdc2H\(_6\)

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without Ca\(^{2+}\) | 13 14 15 16 17 18 | 19 20 21 22 23 24 |

Translation

Precipitation with Glutathione Sepharose

- cyclin A
- +GST-cdk2
Figure 7.7 Cyclin A bound to GST-cdc2H₆ is destroyed more rapidly and more completely than that bound to GST-cdk2

The mRNA encoding c-myc cyclin was translated in CSF-arrested egg extract in the presence of [³⁵S]methionine and 1.2 μM of either GST-cdc2H₆ or GST-cdk2 protein. The standard destruction assay was used (see Chapter 2). (A and B) Samples were taken at the times indicated above the figure. Lanes 1 - 12, the complete translation reaction; lanes 13 - 24 precipitation with glutathione Sepharose; lanes 1 - 6 and 13 - 18, Ca²⁺ added to the destruction assay; lanes 7 - 12 and 19 - 24, H₂O added to the assay in place of Ca²⁺ (i.e. no cyclin destruction was triggered). Visualised by autoradiography. (A) GST-cdc2H₆ added to the reaction. (B) GST-cdk2 added to the reaction. (C) The intensity of the cyclin A bands in lanes 13 - 24 on the autoradiographs shown above were quantitated by scanning densitometry. The values were expressed as a proportion of the intensity of the cyclin band at time zero, and plotted against time on a graph. The key to the graph is shown on the right of the figure.

but they are extremely faint in comparison to those in the lanes to which Ca²⁺ was added. These high molecular weight bands probably correspond to ubiquitinated forms of the A- and possibly B-type cyclins that are bound to GST-cdc2H₆ (lanes 1 - 12) or to GST-cdc2H₆ and endogenous p34cdc2 (lanes 13 - 24). This experiment shows that, if the high molecular weight bands really do correspond to ubiquitinated cyclins, then A- and possibly B-type cyclins can be ubiquitinated while still bound to p34cdc2.

Since cyclin A bound to p33cdc2 is destroyed to a lesser extent than that bound to p34cdc2, it was interesting to see whether cyclin A bound to p33cdc2 was also apparently ubiquitinated. The polyacrylamide gel of an experiment similar to that shown in figure 7.7B, lanes 13 - 18, where the destruction of GST-cdk2-bound cyclin A was studied, was exposed for 13 days. The autoradiograph of this
Precipitation with Glutathione Sepharose

Immunoprecipitation with α-p34cdc2 antibody

Ubiquitinated A- and B-type cyclins

Cyclin A

Minutes since Ca²⁺ or H₂O addition
Figure 7.8  Cyclin A bound to p34<sup>cdc2</sup>, GST-cdc2H<sub>6</sub> and to GST-cdk2 is ubiquitinated
The mRNA encoding c-myc cyclin was translated in CSF-arrested egg extract in the presence of [<sup>35</sup>S]methionine and 1.2 μM of either GST-cdc2H<sub>6</sub> or GST-cdk2 protein. The standard destruction assay was used (see Chapter 2) and samples were taken at times indicated above the figures. (A) GST-cdc2H<sub>6</sub> added to the reaction. Lanes 1 - 12, precipitation of samples with glutathione Sepharose; lanes 13 - 24, precipitation of samples with the monoclonal anti p34<sup>cdc2</sup> antibody, A17; lanes 1 - 6 and 13 - 18, Ca<sup>2+</sup> added to the destruction assay; lanes 7 - 12 and 19 - 24, H<sub>2</sub>O added to the assay in place of Ca<sup>2+</sup> (i.e. no cyclin destruction was triggered). Visualised by autoradiography (70 day exposure). (B) GST-cdk2 added to the reaction. The samples were precipitated with glutathione Sepharose. Visualised by autoradiography (13 day exposure). Asterisk on right indicates position of the endogenous B-type cyclins.
exposure is shown in figure 7.8B and it can be seen that higher molecular weight bands are present in lanes 2 - 4. These bands probably correspond to ubiquitinated cyclin A since GST-cdk2 cannot bind to B-type cyclins, at least under these conditions (compare the radio labelled bands bound to GST-cdc2H6 in figure 7.7A, lane 13, with those bound to GST-cdk2 in figure 7.7B, lane 13). Thus cyclin A can also be ubiquitinated while bound to p33cdk2. The proportion of ubiquitinated cyclin A compared to non-ubiquitinated cyclin A appears to be similar for cyclin A bound to GST-cdk2 or GST-cdc2H6 (compare figures 7.8A and 7.8B, but this is more easily seen in an experiment not shown).

The pattern of high molecular weight bands associated with GST-cdk2 appears to be the same as that associated with GST-cdc2H6. This implies that the high molecular weight bands associated with GST-cdc2H6 correspond to cyclin A only and not to any of the B-type cyclins that are translated from the endogenous cyclin B mRNA.

**Ubiquitinated cyclin A is also precipitated with anti-cyclin A antibodies**

It was possible that the high molecular weight bands seen associated with p34cyc2 and p33cdk2 in the experiments described above were not ubiquitinated cyclin A protein, but other radiolabelled proteins associated with p34cyc2 and p33cdk2. In order to check whether these bands were associated with cyclin A itself, the mRNA encoding c-myc cyclin A was translated in CSF-arrested egg extract and the reaction was split into two aliquots. To one was added Ca²⁺, to trigger cyclin destruction, to the other, H₂O. Samples were taken into ice-cold buffer and incubated with the anti-cyclin A monoclonal antibody, XLAl-3 and then protein A Sepharose. The products were analysed by SDS-PAGE and autoradiography. The autoradiograph shown was a 54 day exposure of the gel. As figure 7.9 shows, the high molecular weight bands seen in the previous experiments were associated with cyclin A (lanes 1 - 4 and 7 - 12). The pattern of these bands in this experiment is very similar to that seen in the previous experiments, implying that the majority of the labelled ubiquitinated cyclin bound to GST-cdc2H6 is cyclin A. In contrast to the previous experiments, however, ubiquitinated cyclin A was easily detectable in the reaction to which Ca²⁺ was not added (lanes 7 - 12) as well as in the reaction in which cyclin destruction was triggered (lanes 1 - 6). The reason for this variability is not clear.

**Cyclin B2 is also ubiquitinated under these conditions**

Since the ubiquitination of B-type cyclins was not noticeable in the experiments described above, I wanted to test whether *in vitro* translated cyclin B2 was ubiquitinated like *in vitro* translated cyclin A. As there were no good anti-cyclin
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Ubiquitinated cyclin A

Ubiquitinated cyclin A

cyclin A

Immunoprecipitation with \(\alpha\)-cyclin A antibody

Figure 7.9 Ubiquitinated cyclin A is precipitated with an anti cyclin A antibody

The mRNA encoding c-myc cyclin was translated in CSF-arrested egg extract in the presence of \(^{35}\)S\text{methionine}. The standard destruction assay was used (see Chapter 2) and samples were taken at times indicated above the figure. The samples were precipitated with the anti cyclin A monoclonal antibody, XLA1-3. Lanes 1 - 6, Ca\(^{2+}\) added to the destruction assay; lanes 7 - 12, H\(_2\)O added to the assay in place of Ca\(^{2+}\) (i.e. no cyclin destruction was triggered). Visualised by autoradiography (54 day exposure).
B2 antibodies available in the laboratory at this time, an N-terminally histidine tagged cyclin B2 construct was used. The clone of cyclin B2 in pET 16b, a gift from Katsumi Yamashita, was digested with NcoI and BamHI which cuts out the coding region of this construct along with about 200 base pairs of 3' untranslated region. This fragment was ligated into NcoI and BclI cut c-myc cyclin A to give a construct that would translate well in egg extract (see Chapter 2).

The mRNA encoding Hiocyclin B2 was translated in CSF-arrested egg extract with added 10% (v/v) reticulocyte lysate and the reaction was split into two aliquots. Ca\(^{2+}\) was added to one to trigger cyclin destruction, H\(_2\)O was added to the other. Samples (10 µl each) were taken into ice-cold buffer and the Hiocyclin B2 was affinity purified on Ni\(^{2+}\)-NTA agarose. The products were analysed by SDS-PAGE and autoradiography; the autoradiograph shown is a 40 day exposure of the gel. Figure 7.10 shows that ubiquitinated Hiocyclin B2 could be seen 10 and 20 minutes after Ca\(^{2+}\) addition (lanes 2 and 3). Unlike in the case of cyclin A, no ubiquitinated cyclin B2 could be seen in the lanes to which Ca\(^{2+}\) was not added. Thus cyclin B2 is also ubiquitinated when its mRNA is translated in vitro. The background on this autoradiograph is much higher than in the other similar experiments. This is partly because the Ni\(^{2+}\)-NTA agarose adsorbs more proteins than the other affinity resins since it has affinity for any proteins which contain repeated histidine residues. The washes used (see Chapter 2) should remove most of these proteins. The other reason is a purely practical one. Ni\(^{2+}\)-NTA agarose is much more difficult to handle than the other affinity resins as it is much lighter and therefore easier to suck up into the pipette. This means that it is difficult to wash effectively without losing the majority of the resin. This problem is obviously highlighted with a 40 day exposure of the polyacrylamide gel.

**Discussion**

**Cyclin A must be bound to p34\(^{cdc2}\) in order to be destroyed**

It was very surprising that those cyclin A mutants that could not bind to p34\(^{cdc2}\) were not proteolysed when cyclin destruction was triggered by the addition of Ca\(^{2+}\) to *Xenopus* CSF-arrested egg extract. It had previously been demonstrated that a construct containing residues 13-66 of sea urchin cyclin B (which includes the destruction box) fused to protein A, and a construct consisting simply of residues 13-91 of sea urchin cyclin B, were able to be destroyed in *Xenopus* egg extracts in a cell cycle-regulated manner (Glotzer *et al.*, 1991). When the destruction of these constructs was studied in our assay system, we obtained the same results as Glotzer *et al.*; that these constructs were degraded in a cell cycle-dependent and destruction box-dependent manner (Stewart *et al.*, 1994). Their
Figure 7.10  Cyclin B2 can be ubiquitinated
The mRNA encoding the histidine-tagged cyclin B2 construct, H<sub>10</sub>cyclin B2 was translated in CSF-arrested egg extract in the presence of [<sup>35</sup>S]methionine. The standard destruction assay was used (see Chapter 2) and samples were taken at times indicated above the figure. The samples were precipitated with Ni<sup>2+</sup>-NTA agarose. Lanes 1 - 6, Ca<sup>2+</sup> added to the destruction assay; lanes 7 - 12, H<sub>2</sub>O added to the assay in place of Ca<sup>2+</sup> (i.e. no cyclin destruction was triggered). Visualised by autoradiography (40 day exposure).
destruction was, however, somewhat slower than the destruction of wild-type cyclin B (Dolores Harrison, pers. comm.).

The indestructibility of the mutant cyclin A proteins could be explained if they all have conformational changes that simultaneously prevent them from binding to \( p^{34}\text{cdc}2 \) and from being recognised by the cyclin destruction machinery. This may be the case for the C-terminally deleted cyclin A proteins and for those mutants that have large internal deletions, such as \( \Delta 101-169 \). These mutant proteins were not completely stable when \( Ca^{2+} \) was added to CSF-arrested egg extract, although their proteolysis was unlike that triggered by \( Ca^{2+} \), as it was more gradual and occurred in the presence or absence of \( Ca^{2+} \) (data not shown).

Mutation of the destruction boxes of \( \Delta 101-169 \) or \( \Delta 194, \Delta 221 \) and \( \Delta 295 \) cyclin A had no effect on the destruction of the proteins (figure 7.5 and Stewart et al. (1994), confirming that this gradual proteolysis is unrelated to programmed cyclin destruction. There is evidence that C-terminally deleted cyclin A proteins are not folded correctly (see Discussion section in Chapter 3) and it is therefore likely that their gradual destruction is due to non-specific proteolysis of the misfolded protein.

Even if the C-terminally deleted cyclin A proteins and those mutants that have large internal deletions are indestructible due to misfolding of the protein, this does not explain the stability of the cyclin box point mutants of cyclin A, in particular the mutants containing the conservative substitutions, R197K and D226E. It is extremely unlikely that these changes would have such a drastic effect on the structure of the cyclin A protein so as to make it unable to bind to \( p^{34}\text{cdc}2 \) and unrecognisable to the destruction machinery. The cyclin A point mutants are not even slightly unstable in CSF-arrested egg extract, unlike the C-terminally deleted cyclin A mutants. This is another indicator that the point mutations do not affect the gross structure of the cyclin protein.

From these results it can be concluded that cyclin A must be able to bind to \( p^{34}\text{cdc}2 \) in order to undergo programmed proteolysis. I have not, however, shown that cyclin A must be bound to \( p^{34}\text{cdc}2 \) in order to be destroyed. To show this it would be necessary to translate wild-type cyclin A in CSF-arrested egg extract such that it was not bound to \( p^{34}\text{cdc}2 \), and then show that this protein was not destroyed when \( Ca^{2+} \) was added to the extract. The system that I used to test cyclin destruction presented difficulties in this respect. CSF-arrested egg extract contains between 450 and 600 nM of \( p^{34}\text{cdc}2 \), at least 80% of which is free (see Appendix 2) and so any cyclin that is translated in this system immediately becomes bound to \( p^{34}\text{cdc}2 \). The translation and cyclin destruction machinery in CSF-arrested egg extract appears to be delicate and can very easily be inactivated. Thus attempts to deplete CSF-arrested egg extract of its \( p^{34}\text{cdc}2 \) before the translation of cyclin mRNA and subsequent cyclin destruction were unsuccessful.
An alternative approach would be to occupy all of the p34\textsuperscript{cd2} before translating the cyclin A in the extract, so that there would be no free p34\textsuperscript{cd2} left for the newly-translated protein to bind to. I attempted to do this experiment using first a protein A-bovine cyclin A fusion protein (pAcycA) and then the glutathione-S-transferase-cyclin B1 fusion protein (GST-cyclin B1). CSF-arrested egg extracts were incubated with these proteins to allow the endogenous p34\textsuperscript{cd2} to bind to them before the mRNA encoding c-myc cyclin A was translated in the extract. Unfortunately for this experiment, however, I discovered that high levels of cyclin A or cyclin B1 kinase inhibit cyclin destruction (see Chapter 6). Thus although destruction of the \textsuperscript{35S}-labelled A- and B-type cyclins did not occur in these experiments, it was not due to their inability to bind to p34\textsuperscript{cd2}.

Although it was not directly demonstrated that free, wild-type cyclin A cannot be destroyed unless it is bound to p34\textsuperscript{cd2}, the data on the destruction of cyclin A bound to p33\textsuperscript{cdk2} indicates that this is indeed the case. In the assay system I used, cyclin A bound to p33\textsuperscript{cdk2} appears to be destroyed much less effectively than cyclin A bound to p34\textsuperscript{cd2} (see below for discussion on the validity and implications of this result). Thus cyclin A must not only be bound to a cdk subunit in order to be destroyed when Ca\textsuperscript{2+} is added to CSF-arrested egg extract, but it may have to be bound to the correct one.

These data suggests one of two models for the recognition of cyclin A by the destruction machinery. Either cyclin A changes its conformation on binding to p34\textsuperscript{cd2} such that the destruction box, which is 'masked' in non-p34\textsuperscript{cd2} bound cyclin A, becomes available for recognition by the destruction machinery. Alternatively the cyclin A destruction machinery recognises both cyclin A and p34\textsuperscript{cd2} simultaneously and cyclin A on its own is therefore not targeted for destruction. With the present results I am unable to distinguish between these two hypotheses.

**Cyclin A bound to p33\textsuperscript{cdk2} is not destroyed as efficiently as cyclin A bound to p34\textsuperscript{cd2}**

Cyclin A bound to GST-cdc2H\textsubscript{6} appears to be destroyed with near identical kinetics to that bound to a mixture of wild-type p34\textsuperscript{cd2} and GST-cdc2H\textsubscript{6} protein. It was not, however, shown what proportion of the newly translated cyclin A binds to GST-cdc2H\textsubscript{6} and what proportion binds to the endogenous p34\textsuperscript{cd2}. It is therefore possible that all of the labelled cyclin A was bound to GST-cdc2H\textsubscript{6}. This seems unlikely, though, given that the concentration of endogenous p34\textsuperscript{cd2} in CSF-arrested egg extract is between 450 and 600 nM, with over 80% of it unbound to any other protein (see Appendix 2), and that the concentration of added GST-cdc2H\textsubscript{6} was 1.2 \textmu{}M. Moreover, in destruction assays where GST-
cdk2, not GST-cdc2H6, protein was added at the beginning of the translation reaction, the difference between the destruction of cyclin A bound to both GST-cdk2 and p34cdc2 as compared to the destruction of that cyclin A bound specifically to the GST-cdk2 is marked (figure 7.7B, compare lanes 1 - 6 with lanes 13 - 18). Thus a reasonable proportion of cyclin A must be bound to the endogenous p34cdc2 in these assays. As such a difference is not seen in assays to which GST-cdc2H6 was added, it is likely that the destruction of cyclin A bound to p34cdc2 is the same as that bound to GST-cdc2H6. This data not withstanding, it is possible that cyclin A bound to GST-cdk2 behaves differently to cyclin A bound to wild-type p33cdk2. There is evidence that GST-cdk2 does not always mimic the behaviour of non-GST-tagged p33cdk2. Bacterially expressed GST-cdk2 could be efficiently phosphorylated on threonine 160 (a phosphorylation necessary, but not sufficient, for activation of the kinase) by p40MO15 kinase in the absence of bound cyclin A, whereas GST-cdk2 from which the GST 'tag' had been cleaved was not efficiently phosphorylated in the absence of cyclin A (R. Poon, pers. comm.). In all other respects so far tested, however, GST-cdk2 appears to behave the same as wild-type p33cdk2.

The less complete destruction of cyclin A bound to p33cdk2 compared to that bound to p34cdc2 could be explained in one of two ways. It is possible that there are two distinct subsets of cyclin A/p33cdk2 complexes; one in which the cyclin A can be destroyed, and one in which the cyclin A cannot be destroyed. These could differ, for example, by their location in the egg extract or by what other proteins they are associated with. Alternatively, it could be that the destruction of cyclin A bound to p33cdk2 is slower than that of cyclin A bound to p34cdc2. Since the cyclin destruction machinery is only active for a limited period of time in the assay system used here (see figure 5.4), that cyclin A which has not been destroyed by the time the machinery is turned off, becomes stable once again. This could be explained in terms of either of the cyclin A destruction hypotheses. If cyclin A changes its conformation when it binds to p34cdc2 such that its destruction box becomes available for recognition by the destruction machinery, when it binds to p33cdk2 it could change its conformation in a slightly different way such that its destruction box is less available, or not so easily recognised. If, on the other hand, the cyclin A destruction machinery requires simultaneous recognition of both cyclin A and p34cdc2 it could be that the destruction machinery recognises a complex between cyclin A and p33cdk2 less well than a complex between cyclin A and p34cdc2. It is also possible that the inefficient recognition or destruction of cyclin A bound to p33cdk2 in CSF-arrested egg extract is because the correct signal or destruction machinery that mediates the proteolysis of p33cdk2-bound cyclin A is not present in CSF-arrested egg extract, as under normal circumstances no cyclin A is bound to p33cdk2 in
The situation described above, where cyclin A is bound to p33cdk2 in CSF-arrested egg extract does not, therefore, usually exist. However, the result that cyclin A does not get destroyed as effectively at the end of M-phase when it is bound to p33cdk2 as when it is bound to p34cdc2 may be generally applicable. This raises several points. If cyclin A bound to p33cdk2 does not get destroyed properly at the end of M-phase it is possible that cyclin A dissociates from p33cdk2 and binds to p34cdc2 before cyclin A destruction is initiated. However, cyclin destruction in somatic cells may be somewhat different to that in embryonic systems. In *Xenopus* CSF-arrested egg extract cyclin destruction is only active for a short period of around 30-45 minutes (Chapter 5) and in clam embryos the cyclin destruction machinery is active for only 5 minutes (Hunt *et al*., 1992). In contrast, the destruction of B-type cyclins in *S. cerevisiae* continues until CDC28 is activated by the G1 CLN cyclins towards the end of the G1 phase of the cell cycle (Amon *et al*., 1994). Extrapolating the graph in figure 7.7C, the destruction of cyclin A bound to p33cdk2 would, in this case, take about 40-50 minutes to be complete. The length of the G1-phase of somatic vertebrate cells is quite variable, but if, like in *S. cerevisiae*, cyclin destruction continues on into G1 in these cells, it is likely to carry on for long enough to destroy all of the cyclin A bound to p33cdk2, despite its slow destruction.

**Cyclin ubiquitination**

The higher molecular weight bands seen on long exposures of polyacrylamide gels of experiments such as that shown in figure 7.6 are likely to be ubiquitinated forms of cyclin A and possibly the B-type cyclins. However, it was not possible to show this unequivocally, as the amount of protein contained in these bands was extremely low. At even the most generous estimate, the amount of protein contained in each these bands is less than 0.04 ng. It was therefore not possible to visualise the bands by Western Blotting, although I did attempt this once. Judging from Andrea Klotzbucher's results in the laboratory it is likely, nevertheless, that the bands do correspond to ubiquitinated cyclin. She added histidine-tagged ubiquitin, the mRNA encoding cyclin A, and [35S]methionine to an RNase A-treated CSF-arrested egg and allowed translation to occur. In this system, only the added mRNA can be translated, and the protein which it encodes is therefore the only protein to become [35S]-labelled. The histidine-tagged ubiquitin was then affinity purified on Ni²⁺-NTA agarose and the results visualised by SDS-PAGE and autoradiography. The autoradiographs showed ladders of [35S]-labelled bands of higher molecular weight than the cyclin construct being tested, similar to the ladder of bands that I see in my experiments.
These bands must contain the cyclin protein as they are $^{[35S]}$-labelled, and the bands must also contain histidine-tagged ubiquitin since the proteins were isolated by affinity purification on Ni$^{2+}$-NTA agarose. These bands therefore represent ubiquinated cyclin.

Ubiquitinated cyclin A is found associated with both GST-cdc2H6 and GST-cdk2 to roughly the same level (the data shown in figure 7.8 does not make this very clear, but from other data not shown it can be seen that this is the case). Yet cyclin A bound to p34cdc2 is destroyed more efficiently than cyclin A bound to p33cdk2. This suggests that the ubiquitination step is not likely to be the one that determines the rate of cyclin destruction. This is supported by results from Andrea Klotzbücher, who showed that mutants of cyclin A which cannot bind to p34cdc2 are ubiquitinated to the same extent as wild-type cyclin A, despite being indestructible.

It could be that the vast majority of ubiquitinated cyclin A is not bound to p34cdc2 or p33cdk2 and we are therefore seeing only a very small fraction of the total ubiquitinated cyclin A in figure 7.8. I do not think that this is the case, however, since the ratio of un-ubiquitinated cyclin A : ubiquitinated cyclin A bound to GST-cdc2H6 or total p34cdc2 is very similar (by eye) to that of un-ubiquitinated cyclin A : ubiquitinated cyclin A precipitated with the anti-cyclin A antibody.

Since the pattern of ubiquitinated cyclin bands is the same in those experiments using GST-cdc2H6 and GST-cdk2 and also when using an anti-p34cdc2 or anti-cyclin A antibody, it seems that the ubiquitinated cyclin bands that are visible in all of these experiments correspond only to cyclin A. This is probably because the ubiquitinated B-type cyclins were too faint to show up on these autoradiographs, or because the ubiquitinated B-type cyclins were hidden by the large band corresponding to non-ubiquitinated cyclin A on the autoradiographs. Alternatively, it is possible that the B-type cyclins were not still bound to p34cdc2 when they were ubiquitinated. My data does not distinguish between these three alternatives.

There is one result in this section that is extremely difficult to explain. The cyclin A precipitated by virtue of its association with p34cdc2 (figure 7.8) is hardly ubiquitinated at all in the reaction to which Ca$^{2+}$ was not added, or in the zero time lane of the '+Ca$^{2+}$' aliquot (if you study the figure hard, you can see very faint bands in these lanes, but much less intense than in the '+Ca$^{2+}$' lanes). The same is true of H10cyclin B2 when it is affinity purified on Ni$^{2+}$-agarose. In the experiment where cyclin A was precipitated using an anti-cyclin A antibody (figure 7.9), however, cyclin A is ubiquitinated equally well in '+' and '-' Ca$^{2+}$ reactions, and in the zero time point of both reactions. I can think of no explanation for this discrepancy. As noted above, I do not think that I am only
precipitating a sub-population of ubiquitinated cyclin A in figure 7.8, and although I have no definite proof, it is likely that all of the cyclin A precipitated with the anti-cyclin A antibody is bound to p34\textsuperscript{cdc2} (it can certainly nearly all be destroyed once Ca\textsuperscript{2+} is added to the extract, which requires p34\textsuperscript{cdc2} binding). This result shows that although ubiquitination of cyclin A protein may be necessary, it is not sufficient to trigger cyclin destruction. Andrea Klotzbücher in the laboratory has confirmed this result; in her experiments (similar to the one described above) cyclin is ubiquitinated equally well in the presence or absence of Ca\textsuperscript{2+}, but is only proteolysed in the presence of Ca\textsuperscript{2+}. 
Chapter 8

Cyclin A destruction (ii)

It was shown in the previous chapter that in order for cyclin A to get destroyed when Ca\(^2+\) is added to a CSF-arrested egg extract, the protein must contain an intact destruction box and be capable of binding stably to p34\(^{\text{cd}c2}\). This chapter describes studies of the destruction of internal deletion mutants of cyclin A that both retain the destruction box and can bind to p34\(^{\text{cd}c2}\). These mutants were constructed by Hideki Kobayashi (Kobayashi \textit{et al.}, 1992).

The internal deletion mutants of cyclin A are not destroyed properly

Figure 8.1 shows a schematic diagram of the internal cyclin A deletions, \(\Delta 88-144\), \(\Delta 90-147\), \(\Delta 102-158\) and \(\Delta 109-161\) (Kobayashi \textit{et al.}, 1992). All these constructs contain intact destruction boxes and can bind to p34\(^{\text{cd}c2}\). The sites of the deletions are shown by a Δ sign and their spatial relationships to the conserved sequence motifs, the destruction box (■), the cyclin box (□), a conserved motif known as FxxxVDE (□) and to a conserved phosphorylation site (□) (see below) are indicated.

The mRNAs encoding non-tagged cyclin A, \(\Delta 102-158\) and \(\Delta 88-144\) cyclin A were translated in CSF-arrested egg extract and Ca\(^2+\) was added to trigger cyclin destruction. Figure 8.2 shows that all three cyclin A constructs were stable in the absence of Ca\(^2+\) (lanes 7 - 12, 19 - 24 and 31 - 36). When Ca\(^2+\) was added, however, wild-type cyclin A was destroyed in less than 30 minutes (lanes 1 - 6), whereas \(\Delta 88-144\) and \(\Delta 102-158\) cyclin A were almost completely stable, with most of the \([35S]\)-labelled mutant proteins still being present 90 minutes after the addition of Ca\(^2+\) to the extract (lanes 13 - 18 and 25 - 30). The rate of destruction of these constructs (taken at the point of most rapid proteolysis) was estimated to be less than 10% of that of wild-type cyclin A. Another internal cyclin A deletion mutant, \(\Delta 90-147\), displayed similar characteristics, being virtually indestructible (data not shown).
Figure 8.1  Schematic diagram of internal deletions of cyclin A
The sites of the deletions from the constructs (Δ) are shown in relation to the position of the destruction box and the start of the cyclin box. The positions of the two conserved motifs in Xenopus cyclin A are also shown. The diagram is to scale.
Figure 8.2  The internally deleted mutants of cyclin A are virtually indestructible
The mRNAs encoding non-tagged cyclin A, Δ88-144 and Δ102-158 cyclin A were translated in CSF-arrested egg extract in the presence of [35S]methionine. The standard destruction assay was used (see Chapter 2) and samples were taken at the times indicated above the figure. Lanes 1 - 12, non-tagged cyclin A; lanes 13 - 24, Δ102-158 cyclin A; lanes 25 - 36, Δ88-144 cyclin A; lanes 1 - 6, 13 - 18 and 25 - 30, Ca2+ added to the destruction assay; lanes 7 - 12, 19 - 24 and 31 - 36, H2O added to the assay in place of Ca2+ (i.e. no cyclin destruction was triggered). Visualised by autoradiography. Asterisk on right indicates position of the endogenous B-type cyclins.
The ‘unreliable’ proteolysis of Δ109-161 cyclin A is mediated by its destruction box

The final internal deletion mutant of cyclin A that retained the destruction box and could bind to p34cdc2 is the construct Δ109-161. This protein showed variable destruction characteristics. In repeated destruction assays it was sometimes almost completely stable, like the Δ88-144, Δ90-147 and Δ102-158 cyclin A mutants, and in some assays it was completely destroyed, although later than wild-type cyclin A (data not shown).

Although many of the C-terminal deletions of cyclin A and Δ101-169 do not undergo specific cyclin proteolysis, they do get gradually proteolysed, probably due to their misfolding (see Chapter 3). The proteolysis of Δ109-161, when it did occur, did not show these characteristics, being quite rapid and the protein was stable if Ca\(^{2+}\) was not added to the CSF-arrested egg extract. Nevertheless, to check that the degradation of Δ109-161, when it did occur, was mediated by the destruction box, a construct was made in which the invariant residues in the destruction box of Δ109-161, R41 and L44, were mutated to alanine. The mRNAs encoding Δ109-161 and the destruction box mutant of this construct, Δ109-161ATVA, were translated in CSF-arrested egg extract and cyclin destruction was triggered with Ca\(^{2+}\). Figure 8.3 shows that in this experiment Δ109-161 was destroyed between 30 and 60 minutes after the addition of Ca\(^{2+}\) to the extract (lanes 1 - 6), but that Δ109-161ATVA cyclin A was completely stable through the length of the assay (lanes 7 - 12). Thus the destruction of Δ109-161, although variable in timing, requires the destruction box and therefore appears to be due to specific cyclin proteolysis as opposed to non-specific degradation caused by misfolding of the protein. It is not clear what accounts for the variability of the behaviour of Δ109-161.

A conserved sequence motif is missing from Δ88-144 and Δ90-147

In order to try to explain the unexpected stability of the internal deletion mutants of cyclin A, we carefully inspected the sequence that was missing from these constructs. The region that had been deleted from Δ88-144 and Δ90-147 contained a sequence motif that is conserved in all known cyclin A proteins except that from Drosophila. The sequence of this motif in cyclin A from several species is shown in figure 8.4, with the consensus sequence at the bottom. The F, V and D residues in this motif are absolutely conserved, and their spacing is the same in all species except in mouse cyclin A, which has an extra amino acid between the F and the V residues. The position of the ‘FxxxVD’ motif in cyclin A proteins is not absolutely conserved, but, like the destruction box, it always lies within a certain area; between the destruction box and the conserved SP motif.
Figure 8.3  The destruction box mutant of Δ109-161 is stable
The mRNAs encoding Δ109-161 cyclin A and the destruction box mutant version
of this construct, Δ109-161ATVA cyclin A, were translated in CSF-arrested egg
extract in the presence of [35S]methionine. The standard destruction assay was
used (see Chapter 2) and samples were taken at the times indicated above the
figure. Lanes 1 - 6, Δ109-161 cyclin A; lanes 7 - 12, Δ109-161ATVA cyclin A.
Visualised by autoradiography. Asterisk on right indicates position of the
endogenous B-type cyclins.

Figure 8.4  A conserved sequence motif found in the N-terminus of nearly all
cyclin As
The sequence of the FxxxVD sequence motif in cyclin A from eight different
animals is shown. The consensus sequence of this motif is shown at the bottom of
the figure. Those residues that conform to the consensus are shown in bold, the
three absolutely conserved residues are shown in bold and are boxed.
(see below), upstream of the start of the cyclin box. Its location in wild-type Xenopus cyclin A is shown in figure 8.1.

The conservation of this motif and its deletion from Δ88-144 and Δ90-147, which are unexpectedly stable, suggested that it might be important for cyclin A destruction. Although the 'FxxxVD' motif is present in Δ102-158 and Δ109-161 cyclin As, which also show abnormal destruction patterns, it is very close to the deletion sites of these mutants (see figure 8.1). It could be, therefore, that the sequence context surrounding the 'FxxxVD' motif in these deletion mutants was changed, and that this interfered with its function.

In order to test whether the 'FxxxVD' motif was required for the destruction of cyclin A, two mutants were constructed. In the first, the conserved F, V and D residues (residues 94, 98 and 99, see figure 8.4) were changed to alanines using PCR (see Chapter 2). In the second construct, made by Hideki Kobayashi, the conserved V and D residues were simply deleted (Δ98-100, see figure 8.1) (Stewart et al., 1994).

The destruction of these cyclin A mutants was tested. The mRNAs encoding c-myc cyclin A, the point mutant ('FVD mutant') and the deletion mutant (Δ98-100) were translated in CSF-arrested egg extract and cyclin destruction was triggered by the addition of Ca^{2+} to the translation reactions. Although the destruction of the FVD mutant of cyclin A appeared to be very slightly slower than that of wild-type cyclin A (figure 8.5A, compare lanes 1 - 5 with lanes 6 - 10), it was, nevertheless, rapidly destroyed. The destruction of Δ98-100 was identical to that of wild-type cyclin A (figure 8.5B, compare lanes 1 - 6 with lanes 7 - 12). Thus the absence of the FVD motif from Δ88-144 and Δ90-147, and the proximity of this motif to the deletion sites in Δ102-158 and Δ109-161, does not explain the abnormal proteolysis of these mutant cyclin A proteins.

**A major p34^cdc2 phosphorylation site is missing from Δ88-144, Δ90-147, Δ102-158 and Δ109-161**

When cyclin A mRNA is translated in CSF-arrested egg extract the product binds to the endogenous p34^cdc2 that is present in the extract. If the cyclin A/p34^cdc2 complexes are immunoprecipitated with anti-cyclin A antibodies and protein A Sepharose, and the immunoprecipitates incubated with [γ-^32P]ATP, the cyclin A protein gets phosphorylated. However, when the phosphorylation of the Δ88-144 and Δ109-161 cyclin A mutants was tested in this assay, they became phosphorylated to a much lesser extent than wild-type cyclin A (Stewart et al., 1994). This much lower level of phosphorylation of Δ88-144 and Δ109-161 is because these constructs, and Δ90-147 and Δ102-158, are missing the tripeptide sequence S_{136}PM, the S and P residues of which are conserved in all cyclin A proteins.
Figure 8.5  The FxxxVD motif of cyclin A is not required for destruction of the protein
The mRNAs encoding c-myc cyclin A, the 'FVD' mutant of cyclin A and ∆98-100 cyclin A were translated in CSF-arrested egg extract in the presence of [35S]methionine. The standard destruction assay was used (see Chapter 2) and samples were taken at the times indicated above the figure. (A) Lanes 1 - 5, c-myc cyclin A; lanes 6 - 10, the FVD mutant of cyclin A. (B) Lanes 1 - 6 c-myc cyclin A; lanes 7 - 12, ∆98-100 cyclin A. Visualised by autoradiography. Asterisks on right indicate positions of the endogenous B-type cyclins.
proteins studied. This motif is located about 30 amino acids upstream of the start of the region required for p34\textsuperscript{cdc2} binding (see figure 8.4) and is a major site of p34\textsuperscript{cdc2} phosphorylation \textit{in vitro}. The identification of the serine in this motif, S136, as a site of p34\textsuperscript{cdc2} phosphorylation is described in Appendix 3.

Another explanation for the unexpected stability patterns of the internal mutants of cyclin A, therefore, could be that phosphorylation at this site was required for cyclin destruction. Those mutants that could not be phosphorylated at this site by p34\textsuperscript{cdc2}, either because they were missing the site or because they could not bind to p34\textsuperscript{cdc2}, would therefore be stable.

**Phosphorylation on serine 136 is not required for cyclin A destruction**

To test whether phosphorylation on serine 136 was required for cyclin A destruction, a mutant was constructed in which serine 136 was replaced with an alanine residue in \textit{c-myc} cyclin A (S136A cyclin A) by PCR (see Chapter 2). The mRNAs encoding \textit{c-myc} cyclin A and S136A cyclin A were translated in CSF-arrested egg extract and cyclin destruction was triggered with Ca\textsuperscript{2+}. Figure 8.6 shows that the destruction of S136A cyclin A followed the same kinetics as that

![Phosphorylation at S136PM is not required for cyclin A destruction](image)

**Figure 8.6 Phosphorylation at S136PM is not required for cyclin A destruction**

The mRNAs encoding \textit{c-myc} cyclin A and S136A cyclin A were translated in CSF-arrested egg extract in the presence of [\textsuperscript{35S}]methionine. The standard destruction assay was used (see Chapter 2) and samples were taken at the times indicated above the figure. Lanes 1 - 6, \textit{c-myc} cyclin A; lanes 7 - 12, S136A cyclin A. Visualised by autoradiography. Asterisk on right indicates position of the endogenous B-type cyclins.
of wild-type cyclin A (compare lanes 1 - 6 with lanes 7 - 12). Thus phosphorylation of serine 136 is not required for cyclin A destruction.

**Discussion**

Cyclin A requires more than an intact destruction box and the ability to bind to p34\textsuperscript{cdc2} in order to be proteolysed normally when Ca\textsuperscript{2+} is added to CSF-arrested egg extract. The unexpected stability of Δ88-144, Δ90-147, Δ102-158 and Δ109-161 cyclin As could be for one of three reasons. Firstly, it could be that the spacing between the destruction box and the cyclin box, both of which are required for cyclin destruction (see Chapter 7), is important. Secondly, it could be that the correct conformation of the N-terminus is important for recognition of the destruction box, and that the N-termini of the internal cyclin A mutants are misfolded such that the destruction box is not recognised by the destruction machinery. Thirdly, it could be that the regions missing from the internal deletion mutants of cyclin A contain (an) additional elements important in cyclin destruction.

The final hypothesis was tested by the mutation of two conserved motifs that are present in the N-terminus of cyclin A, the FxxxVD motif and the conserved phosphorylation site at S\textsubscript{136}PM. These were deleted from some or all of the anomalously stable internal deletion mutants of cyclin A. However, since the ‘FVD mutant’ of cyclin A, Δ98-100, and S136A cyclin A were destroyed normally, it is unlikely that the presence of either of these conserved motifs is required for destruction of the protein. This advances the conclusion of Izumi and Maller (1991), who showed that the phosphorylation of cyclins B1 and B2 was not required for their destruction. As both of these motifs are conserved in the N-terminus of nearly all cyclin A proteins, a region that shows very little conservation other than the destruction box, I believe that both may be important in aspects of cyclin A function. These functions, however, have yet to be determined.

It is not possible to say that phosphorylation of cyclin A is not necessary for cyclin A destruction. The assay used to detect cyclin A phosphorylation will not detect stably phosphorylated sites or phosphorylation by any kinase that does not stably bind to the cyclin A/p34\textsuperscript{cdc2} complex (see Appendix 3). The destruction of both A- and B-type cyclins is unlikely to require phosphorylation of the cyclin subunit by the kinase to which they are bound, however, since A- and B-type cyclins bound to a ‘kinase dead’ mutant of p34\textsuperscript{cdc2} (one in which the ATP binding site is mutated such that it is no longer able to act as a kinase) are
destroyed as fully and with the same kinetics as cyclins bound to wild-type p34\textsuperscript{cdc2} (not shown).

Glotzer \textit{et al.} (1991) found evidence that, in addition to the destruction box, the region between residues 54 and 66 in sea urchin cyclin B was necessary for degradation of the protein. They suggested that this was because the lysine residues in this area were required as ubiquitin acceptor sites. Two lysine residues were deleted from the cyclin A constructs A88-144 and A90-147 and one from \(\Delta102-158\), but I believe that this is unlikely to be the reason for the stability of these mutants. The positioning of lysine residues in this region is not conserved between different species of cyclin A, and two lysine residues still remain between the destruction box and the deletion sites of these mutants. Chicken cyclin A does not have any lysine residues in the 50 amino acids following its destruction box, and only 2 in the next 77 residues, and its degradation presumably occurs in the usual fashion. For the reasons outlined above, it seems unlikely that there are sequence element(s) in the N-terminus of cyclin A that are required for cyclin A destruction.

From the data described in this chapter it is difficult to distinguish between the spacing and the conformation hypotheses explaining the stability of A88-144, A90-147, \(\Delta102-158\) and \(\Delta109-161\). It is possible that the precise spacing of the destruction box with respect to the start of the cyclin box is important, because \(\Delta109-161\) cyclin A, which can be degraded (although not in an entirely wild-type manner), has its destruction box slightly further away from the cyclin box than the other, more stable, mutants. However, the distance between the destruction box and the start of the conserved region in sea urchin cyclin A is 38 amino acids longer than the same region in \textit{Xenopus} cyclin A, so if spacing is important, the correct distance must vary between species. In retrospect, it would perhaps have been worthwhile to insert new sequences into A88-144, A90-147, \(\Delta102-158\) and \(\Delta109-161\) cyclin A mutants to move the destruction box further away from the cyclin box, to see if this restored correct destruction. Deciding what sequences to insert would have been difficult, however, as the structure of the cyclin A protein has not been determined, and one would not know whether the sequence inserted was causing distortion of the protein. It is also possible that the conformation of the destruction box and its surroundings are crucial, and are significantly altered by the exact location of the deletions in A88-144, A90-147, \(\Delta102-158\) and \(\Delta109-161\). In this case it could be argued that the conformation of the destruction box and its surroundings are more like wild-type in \(\Delta109-161\) than in the almost totally indestructible internal deletion mutants, which would explain the more normal, but not quite wild-type destruction of this mutant. These two hypotheses are by no means mutually exclusive and it is quite possible that the
spacing between the cyclin box and the destruction box and the local conformation of the destruction box are both important in cyclin A destruction.
Chapter 9

Sub-cellular localisation of mutant cyclin A proteins

In vertebrates, cyclin A protein accumulates in the nucleus as soon as it is synthesised (Girard et al., 1991; Pines and Hunter, 1991; Pagano et al., 1992; Zindy et al., 1992). At the time that the studies described in this section were started, very little was known about what was required of cyclin A for it to be targeted to the nucleus. In order to investigate these requirements, I looked at the sub-cellular localisation of a selection of the cyclin A mutants that Hideki Kobayashi and I had generated. In particular, I was interested in the ‘FVD mutant’ and the S136A mutants of cyclin A. The FxxxVD motif and the phosphorylation site at serine 136 are highly conserved amongst cyclin A proteins, but are not required for the binding of cyclin A to p34cdc2 or for the destruction of the protein. I thought that it was possible that one of both of these motifs would be required for the nuclear localisation of cyclin A.

Putting the cyclin A mutants into a mammalian expression vector

In order for the mutant cyclin A protein to be distinguished from the endogenous cyclin A present in the cells to be used for the sub-cellular localisation studies, it was necessary for the mutant cyclin A proteins to contain an epitope tag. All of the cyclin A mutants that I had constructed already contained c-myc tags, but the internal deletion mutant, Δ88-144, constructed by Hideki Kobayashi, did not.

The DNA encoding Δ88-144 was digested with BsmI and EcoRI, which cuts out all but the extreme N-terminus of the cyclin A coding region, and the whole of the 3' UTR. These fragment was ligated into BsmI and EcoRI cut c-myc cyclin A. The construct was now identical to c-myc cyclin A except for the internal deletion that it contained. It was called c-myc Δ88-144.

The plasmid vector to be used for expression of the Xenopus cyclin A mutants in mammalian cells was kindly provided by Dr Chris Norbury. It contained the rat CD2 gene under the SV40 early promoter and mouse cyclin B1 under the control of the human cytomegalovirus (hCMV) promoter (pCD2/CMV-mouse cyclin B1, see figure 9.1A). The mouse cyclin B1 gene was removed from
Figure 9.1  The mammalian expression vector pCD2/CMV-mouse cyclin B1 and the added cloning cassette
(A) The structure of the mammalian expression vector, pCD2/CMV-mouse cyclin B1, provided by Dr Chris Norbury.  (B) The sequence of the oligonucleotide cassette that was ligated into the above vector in place of the mouse cyclin B1 gene. The vector thus created was called pCD2/CMV.
this vector by digestion with SalI and NotI, and in its place was ligated a synthetic oligonucleotide containing sites for NheI, PacI, EcoRV and SpeI restriction endonucleases; the SalI and NotI sites were also preserved (see figure 9.1B). This was named pCD2/CMV. The DNA encoding the coding regions of c-myc cyclin A, CΔ14, R197K, D226E, ‘FVD mutant’, S136A, ATVA, c-myc NΔ133, and c-myc Δ88-144 cyclin A was cut out with NheI and SpeI and ligated into NheI and SpeI cut pCD2/CMV, to give pCD2/CMV-c-myc cyclin A, pCD2/CMV-CΔ14 cyclin A, pCD2/CMV-R197K cyclin A etc.

Expression of mutant Xenopus cyclin A constructs in mammalian tissue culture cells

The mutant cyclin A constructs in mammalian expression vectors were transiently transfected into Cos1 cells using the Calcium Phosphate method (see Chapter 2). 30 hours after the removal of the DNA-Ca\(^{2+}\) precipitate from the cells they were fixed with a 1:1 (v/v) mixture of methanol and acetone and incubated with TRITC-conjugated anti-c-myc antibody, 9E10, and FITC-conjugated anti-rat CD2 antibody, OX34, at 4°C for 16 hours. The cells were washed with phosphate buffered saline (PBS), the DNA was stained with Hoechst dye and the cells viewed by fluorescence microscopy. Figure 9.2 shows the subcellular localisation of the mutant cyclin A proteins. In all but figure 9.2C the left hand panel shows staining by FITC-conjugated anti-CD2 antibody, indicating which cells have been transfected with the pCD2/CMV-cyclin A vector, and the right hand panel displays staining by TRITC-conjugated anti-c-myc antibody, showing the subcellular localisation of that particular c-myc tagged cyclin A mutant. Figure 9.2C shows staining of the cells by the DNA stain, Hoechst, in the left hand panel, staining by FITC-conjugated anti-CD2 antibody in the middle panel and staining by TRITC-conjugated anti-c-myc antibody in the right hand panel. As can be seen from this figure, about 10% of the cells were transfected with the pCD2/CMV cyclin A plasmid in these experiments. Figure 9.2 shows that those cyclin A mutants that bind efficiently to p34\(^{cdc2}\); c-myc cyclin A, ‘FVD mutant’ S136A, ATVA, c-myc NΔ133, and c-myc Δ88-144 are localised to the nucleus of the Cos1 cells, but that those mutants that cannot bind to p34\(^{cdc2}\) or bind very weakly, CA14, R197K and D226E are excluded from the nucleus. Mutation of the ‘FVD’ motif or substitution of serine 136 with an alanine residue has no apparent effect on the subcellular localisation of these cyclin A constructs (compare figure 9.2B with figures 9.2F and G). Nor does the removal of the first 133 amino acids from cyclin A (figure 9.2I). Cells that have been transfected with pCD2/CMV (the construct that does not contain any gene under the control of the CMV promoter) show no staining with TRITC-conjugated anti-c-myc antibody (figure 9.2A).
FITC conjugated anti-CD2 (OX 34)

TRITC conjugated anti c-myc (9E10)

A

pCD2/CMV

B

pCD2/CMV-c-myc cyclin A

195
Hoechst (DNA staining)  
FITC conjugated anti-CD2 (OX 34)  
TRITC conjugated anti c-myc (9E10)  
pCD2/CMV-CA14 cyclin A
FITC conjugated anti-CD2 (OX 34)

TRITC conjugated anti c-myc (9E10)

pCD2/CMV-FVD cyclin A

pCD2/CMV-S136A cyclin A
FITC conjugated anti-CD2 (OX 34)

TRITC conjugated anti c-myc (9E10)

pCD2/CMV-ATVA cyclin A

pCD2/CMV-c-myc NΔ133 cyclin A
Figure 9.2 Sub-cellular localisation of the cyclin A mutants

The cyclin A mutants in the mammalian expression vector pCD2/CMV were transiently transfected into Cos1 cells using the Calcium Phosphate method (see Chapter 2). 30 hours later the cells were fixed with a 1:1 (v/v) mixture of methanol and acetone and stained with TRITC-conjugated anti-c-myc antibody, 9E10, and FITC-conjugated anti-rat CD2 antibody, OX34. The DNA was stained with Hoechst 33258 dye and the cells viewed by fluorescence microscopy. (A, B, D, E, F, G, H, I, J) The left hand panel shows staining by FITC-conjugated anti-CD2 antibody, OX34, and the right hand panel displays staining by TRITC-conjugated anti-c-myc antibody, 9E10. Cells transfected with constructs: (A) pCD2/CMV; (B) pCD2/CMV-c-myc cyclin A; (D) pCD2/CMV-R197K cyclin A; (E) pCD2/CMV-D226E cyclin A; (F) pCD2/CMV-FVD cyclin A; (G) pCD2/CMV-S136A cyclin A; (H) pCD2/CMV-ATVA cyclin A; (I) pCD2/CMV-c-myc NA133 cyclin A; (J) pCD2/CMV-c-myc Δ88-144 cyclin A. (C) The left hand panel shows staining of the cells by the DNA stain, Hoechst 33258, the middle panel shows staining by FITC-conjugated anti-CD2 antibody and the right hand panel shows staining by TRITC-conjugated anti-c-myc antibody. The cells were transfected with pCD2/CMV-CA14 cyclin A.
Discussion

In this chapter I have shown that for cyclin A to be localised to the nucleus, the cyclin A construct must be able to bind tightly to p34^cdc2. In addition, I have shown that the conserved sequence motif 'FxxxVD' and the conserved phosphorylation site S_{136}PM, are not required for the correct localisation of this protein. Indeed, removal of the first 133 amino acids of cyclin A gives a protein that is still able to be localised to the nucleus and the internal deletion mutants c-myc Δ88-144 and c-myc Δ109-161 (data not shown) are also nuclear. Thus it appears that nuclear localisation does not require any part of the N-terminus of cyclin A. While the studies described in this chapter were being carried out, Maridor et al. published a paper in which they showed that the nuclear localisation of chicken cyclin A correlated with its ability to form complexes with cdks, and that deletion of more than 100 residues from the N-terminus of this protein did not impair its nuclear localisation (Maridor et al., 1993). These results are in complete agreement with those described in this chapter.

Cyclins A and B show great similarity within their cyclin boxes and although their C-termini show much less conservation, there are still conserved residues throughout this region (see figure 3.9). Their N-termini, however, show virtually no conservation outside of the 9 amino acid destruction box, and even the positioning of this region is not absolutely conserved. The subcellular localisation of cyclins A and B are completely different, with cyclin A accumulating in the nucleus immediately after its synthesis (Girard et al., 1991; Pines and Hunter, 1991; Pagano et al., 1992; Zindy et al., 1992), and cyclins B1 and B2 remaining cytoplasmic until shortly before the onset of mitosis (Pines and Hunter, 1991; Gallant and Nigg, 1992). It seemed likely, therefore, that the region that showed the greatest difference between these two proteins was that which determined their different subcellular localisation. This is not the case, however, as the N-terminus of cyclin A is clearly not required for subcellular localisation, although a region in the N-terminus of cyclins B1 and B2 is required for the retention of these proteins in the cytoplasm (Pines and Hunter, 1994).

It is possible that those cyclin A mutants that are unable to bind to p34^cdc2 or p33^cdk2 are misfolded, and that this is the reason for both their incorrect localisation and their inability to bind to cdk subunits. This certainly could be the case for those C-terminally deleted mutants described by Maridor et al. (1993), as I believe that C-terminally deleted cyclin A proteins may be misfolded, as discussed in Chapter 3. I do not believe, however, that the conservative substitution mutants R197K and D226E, which can bind only very weakly to p34^cdc2 and p33^cdk2, are misfolded and these cyclin A mutants are also excluded from the nucleus. Thus I believe that cyclin A can only be localised to the nucleus if it is bound to a cdk subunit. I did not investigate the type of cdk
subunit that the *Xenopus* cyclin A associates with in Cos1 cells, but it is likely to
be p33cdk2.

That cyclin A must be able to bind efficiently to a cdk subunit if it is to be
localised to the nucleus could be explained by a number of mechanisms. It is
possible that the cdk subunit to which the cyclin A is bound when it is localised to
the nucleus contains a nuclear localisation signal (NLS), and the cyclin A protein
is thus indirectly targeted to the nucleus. This is unlikely as neither p34cdc2 or
p33cdk2 contain an obvious NLS (Maridor *et al.*, 1993). It is also possible that the
binding of cyclin A to a cdk subunit makes a ‘composite’ NLS, formed partly
from cyclin A sequences and partly from cdk sequences, or that a change of
conformation by the cyclin A protein when it binds to the cdk reveals a previously
hidden NLS. The final, and probably the most likely, explanation is that the
cyclin A/cdk complex associates with another protein that has a NLS.
General Discussion

Cyclin A structure

Determining the functional domains of cyclin A would be a great deal easier if the crystal structure of the protein were known, especially now that the structure of p33^cdk2 has been determined. It is not, however, and in the absence of knowledge about the three-dimensional structure of cyclin A, constructing deletion and point mutants is one way of probing the regions of the protein required for the various functions of cyclin A. Even if the structure of the protein were known, it would be necessary to construct cyclin A mutants to really determine what part of the protein is required for what function. In this situation, however, it would probably be easier to decide which residues to mutate and which regions to delete as at present we just have to guess as to which residues are likely to be on the surface of the protein and involved in p34^cdc2 and p33^cdk2 binding.

The cyclin box is the region of homology between all cyclins; although closely related cyclins may show similarity to each other outside of the cyclin box, it is the only region that all cyclins have in common. Since nearly all cyclins are known to bind to cdk subunits, it is likely that the cyclin box is the region that is involved in this binding. Within the cyclin box there are five residues which are conserved in nearly all cyclin proteins. I have shown that two of these residues, arginine 197 and aspartic acid 226 (numbering according to Xenopus cyclin A1), are required for Xenopus cyclin A to bind to p34^cdc2 and p33^cdk2. Even conservative substitutions of these residues (changing arginine 197 to lysine, and aspartic acid 226 to glutamic acid) gave proteins that were seriously impaired in their ability to bind to both p34^cdc2 and p33^cdk2 (Chapters 3 and 4). I do not think that the reason for the inability of these conservative point mutants to bind to p34^cdc2 or p33^cdk2 is due to misfolding of the protein for the reasons outlined in Chapter 3. In addition, these proteins were perfectly stable in cyclin destruction assays, unlike the C-terminally deleted cyclin A mutants, which were gradually degraded whether or not cyclin destruction had been triggered in the egg extract. I believe that the gradual proteolysis of the C-terminally deleted cyclin A mutants was due to misfolding of the protein, and thus since the conservative point mutants of cyclin A were stable, this suggests that these
proteins are not misfolded. It is perhaps interesting to note at this point that cyclin C, which does not show conservation of the arginine and aspartic acid residues, was identified at the same time as cyclins D and E, but a kinase subunit to which it binds has still not been found. Perhaps the non-conservation of otherwise almost absolutely conserved residues is an indication that, if cyclin C does bind to a kinase subunit, this kinase is less homologous to the cdk family than those cdks so far identified. This could be the reason that a partner for cyclin C has not yet been identified.

The cyclin box is not, however, the only region of cyclin A required for p34cdc2 or p33cdk2 binding. Small *Xenopus* cyclin A1 constructs consisting of just the cyclin box are unable to bind to p34cdc2 (data not shown), and deletion of as few as 14 amino acids from the C-terminus of *Xenopus* cyclin A gave a protein that was unable to bind to either p34cdc2 or p33cdk2 (Chapters 3 and 4). For the reasons outlined in Chapter 3, I would suggest that this result is a consequence of the misfolding of C-terminally deleted cyclin A proteins rather than because there are residues in the final 14 amino acids of the protein that are required for direct interaction with p34cdc2 or p33cdk2.

The C-terminus of cyclin A is important in cdk binding and shows some homology with the C-terminus of cyclin B, but comparison between the C-terminus of cyclin A and the C-terminus of cyclin B shows far fewer conserved residues than a comparison between the cyclin boxes of these proteins (see figure 3.9). For this reason I considered the possibility that the C-termini of cyclins A and B were the regions of the proteins that were required for distinguishing between p34cdc2 and p33cdk2. However, exchanging the C-termini of cyclins A and B1 at a run of alanine residues, which is conserved between cyclins A and B, gave proteins that were not only unable to bind to p33cdk2, but were also unable to bind to p34cdc2. Given that I believe that deletion of residues from the C-terminus of cyclin A gives a protein that is unable to bind to p34cdc2 or p33cdk2 because of misfolding, it is likely that the constructs cyclin A/B1 and cyclin B1/A are unable to bind to either p34cdc2 or p33cdk2 for the same reason. Perhaps the folding of the C-terminus of cyclin A is not the same as that of cyclin B1, or the ‘exchange sites’ were not equivalent. This result does not eliminate the possibility that the C-termini of these proteins are involved in distinguishing between p34cdc2 and p33cdk2.

If I were to start my PhD again, I would perhaps look for residues or groups of residues that were highly conserved amongst cyclin A proteins but not in cyclin Bs, and investigate their role in cdk binding, rather than mutating residues that are highly conserved amongst all cyclins, or deleting or ‘exchanging’ whole regions of the C-terminus of cyclin A. It would also be useful to develop some type of screening mechanism for cyclin A mutants. Since
overexpression of cyclin A in *S. cerevisiae* kills it, one could introduce a randomly mutagenised cyclin A clone on a high copy number plasmid and identify those cyclin A mutants that no longer kill the yeast. These would probably be cyclin A mutants that could not bind to p34^cdc28, but this method could also identify cyclin A mutants that could still bind to p34^cdc28, but that were unable to phosphorylate the substrate whose phosphorylation normally kills the yeast.

**Cyclin A destruction**

Cyclin destruction, although it is the property of cyclins that lead to their discovery, is still not well understood. In this thesis I have shown that for a cyclin A protein to be destroyed it must be able to bind to p34^cdc2 (Chapter 7). In addition, it appears that the conformation of the N-terminus of cyclin A is important for its destruction (Chapter 8). This data is an advance in understanding the requirements for cyclin A degradation, but does not bring us nearer to discovering its trigger, or the mechanism by which it occurs. It also does not tell us whether the trigger for cyclin A degradation differs from that of cyclin B. Although A- and B-type cyclins are both degraded at the end of mitosis, cyclin A is degraded before cyclin B. Furthermore, cyclin A and cyclin B2 need to be able to bind to p34^cdc2 in order to be destroyed, whereas at least some cyclin B1 mutants that cannot bind to p34^cdc2 appear to be destroyed with near normal kinetics (Stewart *et al.*, 1994). This indicates that either the signal that marks a cyclin molecule for destruction is different for different cyclins, or that the destruction machinery must be able to distinguish between different types of mitotic cyclins. I believe that, although the ubiquitin system may well be important for cyclin destruction, the ubiquitination of a cyclin protein is not the trigger for its proteolysis. In some instances cyclin proteins can be ubiquitinated before cyclin destruction is triggered, and those cyclin A mutants that cannot bind to p34^cdc2, and are therefore indestructible, are ubiquitinated to the same extent as wild-type cyclin A. We are therefore left with the question, what is the trigger for cyclin destruction? At the moment I fear that we are not in any position to answer this.

One major problem with the destruction assay described in this thesis is that it is reliant on 'good' CSF-arrested *Xenopus* egg extract. Egg extract that both translates exogenous cyclin mRNA efficiently and destroys cyclin proteins quickly and completely when Ca^{2+} is added is hard to make, mainly because we do not know what conditions give rise to these 'good' extracts. We suspect that the variability of extracts may mainly be due to the quality of eggs that we use, but so far we have been unable to find a way of distinguishing which eggs will make 'good' extract and which will make 'bad' extract by simple visual
inspection. If a way was found of consistently making 'good' extract it would
save a great deal of time and considerably reduce the levels of frustration felt in
the laboratory!

Cyclin A and p34cdc2 normally form a very stable complex (Kobayashi et
al., 1994), and one idea that integrates information about the regions of cyclin A
required for p34cdc2 binding and cyclin destruction, is that in order to dissociate
cyclin A from p34cdc2 during cyclin destruction, the protease first removes the C-
terminus of the cyclin. This would release the protein from its complex with
p34cdc2, and also cause it become misfolded, making it more accessible for
further proteolysis. This would be a very neat way of achieving two objectives at
once; dissociating two very tightly complexed proteins, and ensuring that the
proteolytic machinery did not first remove the N-terminus of cyclin A, thereby
giving rise to an indestructible and constitutively active protein.

The function of cyclin A
In this thesis the main assays used to determine the activity of the cyclin A
mutants that I have constructed have been whether the protein can bind to p34cdc2
and p33cdk2, whether the protein is proteolysed when Ca2+ is added to CSF-
arrested egg extract and whether the protein is localised to the nucleus. None of
these assays actually address the function of cyclin A. That is not to say that
these assays are irrelevant, as cyclin A must be able to bind to p34cdc2 or p33cdk2
in order to activate its kinase (which can be assayed by histone H1 kinase
activity) and therefore in order to perform its function(s) in the cell cycle;
versions of cyclin A that cannot be proteolysed at the end of mitosis arrest egg
extracts in mitosis; and it is likely that cyclin A must be localised to the nucleus in
order to perform its function. Nevertheless, they do not indicate what function(s)
cyclin A normally performs in the cell cycle. Indeed, it is still not altogether clear
even in which phase of the cell cycle cyclin A acts (see Chapter 1). The
confusion over this point may be solved if it is assumed that cyclin A acts in S
phase when complexed to p33cdk2 and in M phase when complexed to p34cdc2.
This assumption raises another problem, however; how does the cell determine
whether the newly translated cyclin A binds to p34cdc2 or p33cdk2? In the early
embryonic cycles of Xenopus it appears that the cyclin A binds almost exclusively
to p34cdc2, probably because the levels of p33cdk2 are very low and the p33cdk2
that is present in the embryos is bound to cyclin E (Michael Howell, pers.
comm.). However, in somatic cells the levels of p34cdc2 and p33cdk2 are very
similar (Kobayashi et al., 1992) and their abundance does not oscillate during the
cell cycle (Simanis and Nurse, 1986; Draetta and Beach, 1988; Rosenblatt et al.,
1992). It could be that the cdk to which cyclin A binds after it is synthesised is
not regulated at all, and therefore there are two pools of cyclin A in the cell; one
which acts in S phase and one which acts in M phase. Alternatively, it could be that there is some kind of mechanism that allows the cyclin A synthesised early in the cell cycle to bind to p33\(^{cdk2}\) and the cyclin A that is synthesised later in the cell cycle to bind to p34\(^{cdc2}\). It seems unlikely that cyclin A changes cdk partner during the cell cycle as cyclin A forms very tight complexes with p34\(^{cdc2}\) (Kobayashi et al., 1994) and there is evidence to suggest that cyclin A may also bind tightly to p33\(^{cdk2}\) (Hideki Kobayashi, pers. comm.). Either way, then, it would seem that later on in the cell cycle the cell may contain active complexes of cyclin A/p33\(^{cdk2}\) which have already performed their function in the cell cycle, unless there is a mechanism that specifically destroys the cyclin A bound to p33\(^{cdk2}\) at this time. These issues must be addressed if we are to begin to understand how cyclin A functions in the cell cycle.

Even once we understand at which point in the cell cycle cyclin A acts and how its function is regulated, if it does indeed perform two separate roles, we still need to know its direct function, that is, which proteins the cyclin A/cdk complex(es) phosphorylates. If we assume that cyclin A/p34\(^{cdc2}\) has a separate function from cyclin A/p33\(^{cdk2}\), then the cdk subunit within these complexes must play a role in determining their substrate specificities. However, the cyclin A subunit of these complexes must also play a part in determining substrate specificity if cyclin E/p33\(^{cdk2}\) has a different role in the cell cycle to cyclin A/p33\(^{cdk2}\), and if cyclin B/p34\(^{cdc2}\) has a different function from cyclin A/p34\(^{cdc2}\). Substrate specificity may partly be controlled by the availability of a certain complex at a certain time, for example, cyclin A protein levels only start to rise at the beginning of S phase, whereas cyclin E levels peak in G1, thus cyclin E/p33\(^{cdk2}\) kinase is present earlier in the cell cycle than cyclin A/p33\(^{cdk2}\) kinase. Another factor regulating the substrate specificity of the complex is its subcellular localisation. For example, cyclin A is nuclear through most of the cell cycle (we do not know whether this cyclin A is bound to p34\(^{cdc2}\), or p33\(^{cdk2}\), or both), whereas cyclins B1 and B2 are cytoplasmic throughout interphase and only get translocated to the nucleus at the onset of mitosis. Even within these compartments of the cell the complexes may be more specifically localised; for example, cyclin A/p33\(^{cdk2}\) complexes localise at subnuclear sites of DNA replication during S phase. Thus the actual substrate preference of a particular cyclin/cdk complex may play only a small role in determining which proteins the kinase phosphorylates \textit{in vivo}. The many ways in which the substrates of cyclin/cdk complexes can be controlled; by availability of the kinase, by subcellular localisation and specific localisation within that cellular compartment, and by the substrate preference of the complex, provide a means of controlling the substrate specificity of each different combination of cyclin and cdk.
I have suggested mechanisms by which the substrates of different cyclin/cdk complexes may be determined, but I have still not answered the question of what these substrates are. The simple answer is that the substrates of cyclin A/p34^cdc2 or cyclin A/p33^cdk2 are not known, and to identify them is by no means an easy matter. As discussed above, it is difficult to determine the substrates of cyclin A kinase in vitro, as all of the factors that control substrate specificity must be taken into account. In vivo it is even more difficult, however, as it is very difficult to distinguish between cyclin A/p34^cdc2 and cyclin A/p33^cdk2 complexes. If we are really to understand the function of cyclin A in the cell cycle, however, then this is probably the most fundamental question which must be answered.

Finally, a mention must go to cyclin A2. Cyclin A2 shows more similarity to those forms of cyclin A cloned from somatic cells than it does to Xenopus cyclin A1 (the ‘original’ cyclin A)(Michael Howell, pers. comm.). This would imply that cyclin A2 was a ‘somatic’ form of the protein, and it is indeed found in Xenopus somatic cell lines, where cyclin A1 is not. Cyclin A2 was, however, cloned from an oocyte library and is very much in evidence during embryogenesis, although, like cyclin A1, its concentration is very low in oocytes and eggs. In early embryos it, like cyclin A1, is found associated exclusively with p34^cdc2, but as embryogenesis proceeds cyclin A2 progressively associates with p33^cdk2, and in Xenopus somatic cells cyclin A2 is mainly found associated with p33^cdk2. This change in partner is likely to be due less to a change in ‘allegiance’ of cyclin A2 and more to the change in availability of its partners. In early embryos the level of p33^cdk2 is low in comparison to the level of p34^cdc2 and it appears that all of the p33^cdk2 is associated with cyclin E (Michael Howell, pers. comm.). As embryogenesis progresses, however, the concentration of p33^cdk2 rises and the protein becomes available for binding by cyclin A2. Thus the reason that cyclin A1 is never found in association with p33^cdk2 may be because it never gets the chance (cyclin A1 disappears from Xenopus embryos around the time of gastrulation and never reappears) rather than because of a preference for p34^cdc2. Nevertheless, the slight sequence differences between cyclins A1 and A2 could indicate that they could possibly have different binding preferences with regard to p34^cdc2 and p33^cdk2.

The characterisation of cyclin A2 and the experiments showing that cyclin A1 is only present during early embryogenesis, suggests that I have spent 4 years working on a minor form of cyclin A. This begs the question, is what I have discovered really relevant to cells other than those in early embryos? My answer is yes, I do believe that it is. Although cyclin A1 and cyclin A2 are different, they still show high homology to each other and I would be extremely surprised if the properties of cyclin A1 that I have discovered during the work for this thesis
differ significantly from those of cyclin A2. My confidence in this belief is supported by my work on the sub-cellular localisation of *Xenopus* cyclin A1 in Cos1 cells. Maridor *et al.* (1993) showed that the localisation of chicken cyclin A in HeLa cells was dependent on the ability of the protein to bind to cdk subunits. My work, described in Chapter 9, gives identical results, even though the cyclin A that I was using was the ‘embryonic’ form. It would be interesting to know whether the cyclin A1 was, in these circumstances, bound to p34^cdc2^ or p33^cdk2^ or to both. If I had had more time, this is one of many issues that I would have liked to address.
Appendix 1

Oligonucleotide sequences and binding sites on cyclin constructs

Table A1.1  Sequence of oligonucleotides mentioned in the text
The sequences of the oligonucleotides mentioned in the text are shown in a 5' to 3' direction. Mutagenic residues are shown in bold and restriction enzyme sites present in the sequence are underlined, with the name of the restriction enzyme that cuts at that site underneath. A brief description of the use of the oligonucleotide is also given.

A

Oligonucleotides for cyclin A

<table>
<thead>
<tr>
<th>Oligonucleotide number</th>
<th>Oligonucleotide sequence (mutagenic residues in bold) 5' → 3'</th>
<th>Direction of use of oligonucleotide in PCR and its use</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CACCATGGCTTCCAATGGGCA  Ncol</td>
<td>5' direction. To make an Ncol site at the N-terminus of full-length, wild-type cyclin A</td>
</tr>
<tr>
<td>2</td>
<td>ACACTGTTGATCAGAGGTAA    Beil</td>
<td>3' direction. Non-mutagenic, spans the Beil site just downstream of the end of the coding region of cyclin A</td>
</tr>
<tr>
<td>3</td>
<td>CATGGAGGAAAGCTCTATTCTGAAGAAGA TCTGAACAGTG</td>
<td>5' direction. Top strand of the double-stranded oligonucleotide encoding the c-myc epitope for cyclin A</td>
</tr>
<tr>
<td>4</td>
<td>CTTTCAGATTTTTCTGAGAAATGAGCTTT TGCTC</td>
<td>3' direction. Bottom strand of the double-stranded oligonucleotide encoding the c-myc epitope for cyclin A</td>
</tr>
<tr>
<td>5</td>
<td>AGAACGGATCCTTTTACATATACCTTGGTG   BamHI</td>
<td>3' direction. To make CΔ14 cyclin A</td>
</tr>
<tr>
<td>6</td>
<td>TGTACGGATCCTTTTATGCTTTGAAGCAAGC   BamHI</td>
<td>3' direction. To make CΔ24 cyclin A</td>
</tr>
</tbody>
</table>
7  CTATGGGATCTTATGGTAGCCAGTAAA  3' direction. To make CΔ50 cyclin A
     \[ \text{BamHI} \]
8  TGCAGATCTTTAAGTGAAAGGAAGCTTC  3' direction. To make CΔ79 cyclin A
     \[ \text{BamHI} \]
9  GAGTTGGATCTTATGGTTACATTGCAAG  3' direction. To make CΔ97 cyclin A
     \[ \text{BamHI} \]
10 GAACGGATCTTTAGCGAGAAAGGAGTTTT  3' direction. To make CΔ139 cyclin A
     \[ \text{BamHI} \]
11 CTCTAGATATCAGTGAAAGCTTCTCCGA  5' direction. Non-mutagenic. Spans the HindIII site in the N-terminus of cyclin A
12 TCAGCAATGGCCAGATACT  5' direction. To make R197A cyclin A
13 AGTATCGGTCATTTGGCTTA  3' direction. To make R197A cyclin A
14 TCAGCAATGAAAGCTTCTCCGA  5' direction. To make R197K cyclin A
15 AGTATCGTCCTTCTTTGGCTTA  3' direction. To make R197K cyclin A
16 ATTATTTGGGCCCCTTCCTTA  5' direction. To make D226A cyclin A
17 TAGGAAGGCCCAGAATAAT  3' direction. To make D226A cyclin A
18 ATTATTTGGGAAAGCTTCTCCGA  5' direction. To make D226E cyclin A
19 TAGGAAGGCCATCAAATAAT  3' direction. To make D226E cyclin A
20 CTCAAGCAACTGTGGGCGGTG  5' direction. To make RTVL to ATVA mutations in cyclin A
21 CACCCGCACACTGTGGCGGTG  3' direction. To make RTVL to ATVA mutations in cyclin A
22 AATACGACACATCTATAG 5' direction. Non-mutagenic. Spans the T7 RNA polymerase promoter
23 GTGCCTACAGCGCTGCTTT 3' direction. Used as a 3' primer in cyclin A; not used for mutagenesis
24 CCAAGTGTGCTGACCTAGCTGGACC  5' direction. To make the 'FVD mutant' of cyclin A
25 GGCTCAGGCGCATAGACTGTAGGACACTTTG  3' direction. To make the 'FVD mutant' of cyclin A
26  TGAAGCTGCTCCCATGGTGG  
    \textit{Ncol}  
5' direction. To make S136A cyclin A

27  CCACCATGGAGGAGCTTTCA  
    \textit{Ncol}  
3' direction. To make S136A cyclin A

28  ACATAATTGCATTGATGGAAGCT  
    \textit{BsmI}  
5' direction. To make c-myc NΔ133 cyclin A

29  GAACATTCATGCATTCCTAGATAT  
    \textit{BsmI}  
5' direction. To make c-myc NΔ129 cyclin A

30  GATTGTCCTGGTGATGA  
    \textit{BsmI}  
5' direction. To make c-myc NΔ114 cyclin A

31  GAAACCTATTCATGGAAATAGA  
    \textit{BsmI}  
5' direction. To make c-myc NΔ107 cyclin A

32  CTTACAGGCAGGCAGCCCAT  
    \textit{NotI}  
5' direction. To introduce a \textit{NotI} site into the C-terminus of cyclin A

33  ATAGGCTGCGGCCGCTGTAAG  
    \textit{NotI}  
3' direction. To introduce a \textit{NotI} site into the C-terminus of cyclin A

34  CCTATCATGGATCTGTACT  
    \textit{NsiI}  
5' direction. Non-mutagenic. Spans the \textit{NsiI} site in the cyclin box of cyclin A

B

Oligonucleotides for constructs other than cyclin A

<table>
<thead>
<tr>
<th>Oligonucleotide number</th>
<th>Oligonucleotide sequence (mutagenic residues in bold) 5' \rightarrow 3'</th>
<th>Direction of use of oligonucleotide in PCR and its use</th>
</tr>
</thead>
</table>
| 35                     | TCGACGCTAGTCCCATGGGTGG  
                          \textit{NheI} \hspace{1cm} \textit{PacI} \hspace{1cm} \textit{EcoRV}  
                          \textit{NsiI} \hspace{1cm} \textit{SpeI} \hspace{1cm} \textit{EcoRV} \hspace{1cm} \textit{PacI}  
                          Top strand of an oligonucleotide containing restriction enzyme sites, inserted into pCD2/CMV |
| 36                     | GGCCGCCTAGTCCCATGGGTGG  
                          \textit{SpeI} \hspace{1cm} \textit{EcoRV} \hspace{1cm} \textit{PacI}  
                          Bottom strand of an oligonucleotide containing restriction enzyme sites, inserted into pCD2/CMV |
| 37                     | CCAAATAGGCGGCTCTCCTCCTC  
                          \textit{NsiI}  
                          5' direction. To introduce a \textit{NotI} site into the C-terminus of cyclin B1 |
| 38                     | GAGGAAGGCGGCTATTTGG  
                          \textit{NotI}  
                          3' direction. To introduce a \textit{NotI} site into the C-terminus of cyclin B1 |
| 39 | TTGTAAGCTGATCACACATACAC | 5' direction. Non mutagenic. Spans the \textit{BclI} site in cyclin box of cyclin B1 |
| 40 | ATTGGAATGCCACGTAGTCC | 3' direction. Non mutagenic. Spans the \textit{BsmI} site just downstream of the end of the coding region of cyclin B1 |
| 41 | GATCTAGCCCTTGCCT | 5' direction. For making cyclin A/B2Δ2 |
| 42 | TAAGGCAAGGGCTA | 3' direction. For making cyclin A/B2Δ2 |
| 43 | CAAACAGACACCAGAGAT | 5' direction. Non mutagenic. Spans the \textit{NcoI} site at the start of the coding sequence of cdk2 |
| 44 | CACCGCTGGTACCACGATGATG | 3' direction. Adds an \textit{NcoI} site at the end of the coding sequence of cdk2 |
Figure A1.1 Position of annealing of oligonucleotides to c-myc cyclin A and cyclin B1 DNA
The open reading frames of cyclins A and B1 are represented as solid lines, while the 3' and 5' untranslated regions are shown as dotted lines. The region encoding the c-myc tag in c-myc cyclin A is shown as a darkly shaded box (■); the regions encoding the destruction boxes are shown as lightly shaded boxes (□) and the regions encoding the cyclin boxes of these constructs are shown as striped boxes (□). The annealing positions of the oligonucleotides are shown; the name of the oligonucleotide is shown beside the line representing the oligonucleotide. The arrow head indicates the direction of priming of the oligonucleotide. Non-mutagenic oligonucleotides are shown as ➔, while mutagenic oligonucleotides are shown as ➢. The sites of digestion of the restriction enzymes mentioned in the text are shown.
Figure A1.2  The cyclin A mutants whose construction is described in this thesis
All the cyclin A mutants whose construction are described in Chapter 2 are shown, in diagramatic form. The diagrams are to scale. c-myc cyclin A is shown at the top; the c-myc tag is shown as a pale grey box (■), the destruction box is shown as a black box (■) and the cyclin box is shown as a white box (□). Residues that have been mutated are also shown on the diagram of c-myc cyclin A and the amino acid that they were changed to is shown on the diagram of that mutant. Deletions are shown as spaces in the line that represents the protein.
Appendix 2

Concentration of p34cdc2 and cyclin A in egg extracts

For many of the experiments carried out in this thesis it was important to know the concentration of endogenous p34cdc2 and cyclin A in CSF-arrested egg extract, in addition to the concentration of cyclin A synthesised from added mRNA in translation reactions.

The concentration of endogenous p34cdc2 in CSF-arrested egg extract is between 450 and 600 nM

The concentration of endogenous p34cdc2 in CSF-arrested egg extracts was measured by immunoblotting. The concentration of bacterially expressed p34cdc2 was measured by comparison with bovine serum albumin (BSA) standards using scanning densitometry of a Coomassie Blue stained polyacrylamide gel (not shown). Volumes of 0.4, 0.3 and 0.2 µl of three different CSF-arrested egg extracts were run on a polyacrylamide gel, along with the bacterially expressed p34cdc2 concentration standards. The proteins on the gel were transferred to nitrocellulose and the membrane was incubated with the anti-p34cdc2 monoclonal antibody, A17, followed by a horseradish peroxidase (HRP)-conjugated rabbit anti-mouse second antibody. The immunoreactive bands were visualised by the Amersham Enhanced Chemiluminescence (ECL) system (see figure A2.1A). The absorbance of the p34cdc2 bands on the film was quantitated by scanning densitometry (see table A2.1). A standard curve was constructed using the data from the p34cdc2 concentration standards (figure A2.1B) and used to calculate the concentration of p34cdc2 in each of the CSF-arrested egg extracts (see the right hand lane of table A2.1B). This data shows that the concentration of endogenous p34cdc2 in CSF-arrested egg extract is in the range 450 - 600 nM.

Over 80% of the endogenous p34cdc2 in CSF-arrested egg extract is monomeric

In order to see what proportion of the endogenous p34cdc2 was monomeric and therefore (presumably) available to bind to newly translated cyclins, an aliquot of
Figure A2.1 Concentration of endogenous p34\textsuperscript{cdc2} in CSF-arrested egg extract

(A) Western blot using the anti p34\textsuperscript{cdc2} monoclonal antibody, A17. The immunoreactive bands were detected using the Amersham enhanced chemiluminescence (ECL) system. Lanes 1 - 7, bacterially expressed p34\textsuperscript{cdc2} concentration standards; lanes 8 - 10, extract A; lanes 11 - 13, extract B; lanes 14 - 16, extract C; lanes 8, 11 and 14, 0.2 µl of extract loaded; lanes 9, 12 and 15, 0.3 µl extract loaded; lanes 10, 13 and 16, 0.4 µl extract loaded. (B) The absorbance of the bands in lanes 1 - 7 on the film from (A) was determined by scanning densitometry (see table A2.1A) and plotted to give a calibration curve, which was used to convert the values of the absorbances of the bands in lanes 8 - 16 to the concentrations of p34\textsuperscript{cdc2} shown in table A1.1.
### Table A2.1  Concentration of endogenous p34cdc2 in CSF-arrested egg extract

(A) The absorbance of the p34cdc2 bands in lanes 1 - 7 on the film shown in figure A2.1A, determined by scanning densitometry. This is the data used to generate the calibration curve shown in figure A2.1B. (B) The absorbance of the p34cdc2 bands shown in lanes 8 - 16 on the film shown in figure A2.1A were determined by scanning densitometry (column 3). The calibration curve, shown in figure A2.1B, was used to calculate the amount of p34cdc2 loaded onto each of the lanes (column 4) and this was converted into concentration of p34cdc2 in each lane (column 5) taking into account the volume of extract loaded (column 2). This was then averaged to give the concentration of p34cdc2 in each extract (column 6).
Figure A2.2  The majority of p34<sup>cdc2</sup> is monomeric in CSF-arrested egg extract.
Translation reactions containing the mRNA encoding either c-myc cyclin A (top panel) or the non-p34<sup>cdc2</sup> binding mutant of cyclin A, D226E (bottom panel) were analysed on a Superdex 200 FPLC column, and fractions analysed by SDS PAGE and immunoblotting with the anti-p34<sup>cdc2</sup> monoclonal antibody, A17. Immunoreactive bands were detected using the ECL system from Amersham. The elution position of size markers run in parallel are indicated above the figure.

CSF-arrested egg extract was fractionated on a Superdex 200 gel filtration column. The column fractions were analysed by immunoblotting with the anti-p34<sup>cdc2</sup> monoclonal antibody, A17, followed by an HRP-conjugated rabbit anti-mouse second antibody. The immunoreactive bands were visualised by the Amersham ECL system. Two different extracts were analysed; one in which the mRNA encoding c-myc cyclin A had been translated, and one in which the mRNA encoding the D226E mutant of cyclin A, which can bind only very weakly to p34<sup>cdc2</sup> had been translated. As shown by figure A2.2, in both reactions over 80% of the p34<sup>cdc2</sup> showed the expected behaviour for a protein of 34 kDa, with only a small fraction migrating at higher apparent molecular weights. There is little visible difference between the extract in which c-myc cyclin A was translated and the extract in which D226E cyclin A was translated. There was also little difference in the behaviour of p34<sup>cdc2</sup> in these extracts and the behaviour of p34<sup>cdc2</sup> in an extract in which no exogenous mRNA had been translated (data not shown).
The concentration of cyclin A translated from synthetic mRNA in CSF-arrested egg extracts and mixtures of CSF-arrested egg extract and reticulocyte lysate

The concentration of cyclin A after translation of the mRNA encoding c-myc cyclin A in CSF-arrested egg extract, and in 9:1 and 1:1 (v/v) mixtures of CSF-arrested egg extract and reticulocyte lysate, was measured by immunoblotting. The concentration of a bacterially-synthesised cyclin A protein was measured by comparison with BSA standards using scanning densitometry of a Coomassie Blue stained polyacrylamide gel (not shown). This protein lacks the N-terminal 56 residues of cyclin A1, and has a molecular weight of 42.1 kDa (see figure A2.3A). Translation reactions were set up in pure CSF-arrested egg extract and in 9:1 and 1:1 mixtures of CSF-arrested egg extract and reticulocyte lysate, using two different batches of egg extract. To each translation reaction was added the mRNA encoding c-myc cyclin A. In addition, two reactions were set up in pure CSF-arrested egg extract to which no exogenous mRNA was added. Samples of the translation reactions were separated by SDS PAGE along with the bacterially expressed cyclin A concentration standards. The proteins on the polyacrylamide gel were transferred onto nitrocellulose and the membrane was incubated with the anti-cyclin A monoclonal antibody, XLA1-3, followed by an HRP-conjugated rabbit anti-mouse second antibody. The monoclonal antibody XLA1-3 reacts with an epitope lying between residues 88 and 106 of Xenopus cyclin A1 (D. Harrison and T. Hunt, unpublished data). The immunoreactive bands were visualised by the Amersham ECL system (see figure A2.3A). The absorbance of the cyclin A bands was quantitated by scanning densitometry (see table A2.2). A standard curve was constructed using the data from the cyclin A concentration standards (figure A2.3B) and used to calculate the concentration of cyclin A in each of the translation reactions (see table A2.2B). Figure A2.3A, lane 10, shows that the monoclonal antibody XLA1-3 could detect as little as 0.05 ng of cyclin A2. Cyclin A translated from endogenous mRNA (either before or during the translation reaction) was not detectable at the exposure length shown in figure A2.3A, when 0.4 μl of egg extract was loaded on the gel (figure A2.3A, lanes 11 and 18), although it could be recognised in very long exposures of similar ECL immunoblots (see figure A2.4). After translation of added c-myc cyclin A mRNA in the egg extract for 2 hours, however, cyclin A protein was easily detectable, at 1 - 2 nM (Figure A2.3, lanes 12, 13, 19 and 20). Addition of rabbit reticulocyte lysate to 10% (lanes 14, 15, 21 and 22) or 50% (lanes 16, 17, 23 and 24) by volume increased the translation of added c-myc cyclin A mRNA by about 5 and 25 fold respectively. There was considerable batch-to-batch variation between extracts prepared in parallel from the eggs of different frogs (compare extract W, 221
A

ng protein

kDa

wild-type cyclin A standards

cyclin A standards

extract W

extract X

66.0

57.5

2.00 1.75 1.50 1.25 1.00 0.75 0.50 0.25 0.1

0.05

2 3 4 5 6 7 8 9 10

11 12 13 14 15 16 17

18 19 20 21 22 23 24

CSF-arrested egg extract reticulocyte lysate

9.1 egg extract reticulocyte lysate

1.1 egg extract reticulocyte lysate

18.7 egg extract reticulocyte lysate
Figure A2.3 Concentration of cyclin A before and after translation of added c-myc cyclin A mRNA in CSF-arrested egg extracts

(A) Western blot using the anti-cyclin A monoclonal antibody, XLA1-3. The immunoreactive bands were detected using the ECL system from Amersham. Lanes 1 - 10, bacterially expressed cyclin A standards; lanes 11 - 17, extract W; lanes 18 - 24, extract X; lanes 11 and 18, pure egg extract, no added mRNA; lanes 12 - 17 and 19 - 24, 100 ng/μl added c-myc cyclin A mRNA; lanes 12, 13, 19 and 20, no added reticulocyte lysate; lanes 14, 15, 21 and 22, 10% by volume added reticulocyte lysate; lanes 16, 17, 23 and 24, 50% added reticulocyte lysate. In lanes 11 - 14, 16, 18 - 21 and 23, 0.4 μl of extract was loaded, lanes 15, 17, 21 and 24 were loaded with 0.2 μl of extract. Note that the bacterial cyclin A standard was missing 56 residues from the N-terminus and therefore migrates faster than wild-type cyclin A. (B) The absorbance of the bands in lanes 1 - 10 on the film from (A) were determined by scanning densitometry (see table A2.2A) and plotted to give a calibration curve, which was used to convert the values of the absorbances of the bands in lanes 11 - 24 to the concentrations of cyclin A shown in table A2.2.

Concentration of endogenous cyclin A in different types of Xenopus extract

Since the levels of endogenous cyclin A were so low in CSF-arrested egg extract, it was difficult to calculate its concentration with great accuracy. I did, however, attempt to gain an estimate of this value.
The concentration of cyclin A was studied in three different types of *Xenopus* extract: CSF-arrested egg extract; CSF-arrested egg extract which had been incubated with 0.4 mM Ca^{2+} to trigger cyclin destruction and cause the extract to enter interphase (see Chapter 5) and interphase extract, made from *Xenopus* eggs that had been induced to enter interphase before crushing by treatment with the Ca^{2+} ionophore A23187. Aliquots of CSF-arrested egg extract and interphase extract were thawed and the CSF-arrested egg extract was split.

<table>
<thead>
<tr>
<th>Cyclin A standards (ng)</th>
<th>Absorbance of band</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.00</td>
<td>85.86</td>
</tr>
<tr>
<td>1.75</td>
<td>91.78</td>
</tr>
<tr>
<td>1.50</td>
<td>89.65</td>
</tr>
<tr>
<td>1.25</td>
<td>90.42</td>
</tr>
<tr>
<td>1.00</td>
<td>82.80</td>
</tr>
<tr>
<td>0.75</td>
<td>71.53</td>
</tr>
<tr>
<td>0.50</td>
<td>49.58</td>
</tr>
<tr>
<td>0.25</td>
<td>34.69</td>
</tr>
<tr>
<td>0.10</td>
<td>14.82</td>
</tr>
<tr>
<td>0.05</td>
<td>3.66</td>
</tr>
</tbody>
</table>

Table A2.2 Concentration of cyclin A before and after translation of added c-myc cyclin A mRNA in CSF-arrested egg extracts

(A) The absorbance of the cyclin A bands in lanes 1 - 10 on the film shown in figure A2.3A, determined by scanning densitometry. This is the data used to generate the calibration curve shown in figure A2.3B. (B) The absorbance of the cyclin A bands shown in lanes 12 - 17 and 19 - 24 on the film shown in figure A2.3 was determined by scanning densitometry (column 4). The calibration curve, shown in figure A2.3B, was used to calculate the amount of cyclin A loaded onto each of the lanes (column 5) and this was converted into concentration of cyclin A in each lane (column 6), taking into account the volume of extract loaded (column 3). This was then averaged to give the concentration of cyclin A in each extract (column 7).
<table>
<thead>
<tr>
<th>Extract</th>
<th>Translation</th>
<th>Volume extract loaded (µL)</th>
<th>Absorbance of band</th>
<th>Calculated amount cyclin (ng)</th>
<th>Concentration in extract (nM)</th>
<th>Average concentration (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pure egg extract</td>
<td></td>
<td>0.4</td>
<td>1.73</td>
<td>0.021</td>
<td>1.14</td>
<td>1.16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.4</td>
<td>1.82</td>
<td>0.022</td>
<td>1.16</td>
<td></td>
</tr>
<tr>
<td>9:1 (v/v) egg extract :</td>
<td>reticulocyte lysate</td>
<td>0.4</td>
<td>13.87</td>
<td>0.095</td>
<td>5.11</td>
<td>4.54</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.2</td>
<td>4.70</td>
<td>0.037</td>
<td>3.97</td>
<td></td>
</tr>
<tr>
<td>1:1 (v/v) egg extract :</td>
<td>reticulocyte lysate</td>
<td>0.4</td>
<td>47.49</td>
<td>0.429</td>
<td>23.10</td>
<td>24.73</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.2</td>
<td>31.35</td>
<td>0.244</td>
<td>26.36</td>
<td></td>
</tr>
<tr>
<td>Pure egg extract</td>
<td></td>
<td>0.4</td>
<td>5.50</td>
<td>0.041</td>
<td>2.23</td>
<td>2.29</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.4</td>
<td>5.87</td>
<td>0.043</td>
<td>2.34</td>
<td></td>
</tr>
<tr>
<td>9:1 (v/v) egg extract :</td>
<td>reticulocyte lysate</td>
<td>0.4</td>
<td>28.13</td>
<td>0.213</td>
<td>11.49</td>
<td>12.91</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.2</td>
<td>18.95</td>
<td>0.133</td>
<td>14.34</td>
<td></td>
</tr>
<tr>
<td>1:1 (v/v) egg extract :</td>
<td>reticulocyte lysate</td>
<td>0.4</td>
<td>79.59</td>
<td>0.926</td>
<td>49.87</td>
<td>58.39</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.2</td>
<td>61.28</td>
<td>0.621</td>
<td>66.91</td>
<td></td>
</tr>
</tbody>
</table>
Table A2.3  Concentration of cyclin A after translation of added c-myc cyclin A mRNA in different egg extracts

The cyclin A concentration after the translation of c-myc cyclin A mRNA in four different egg extracts was determined by quantitative immunoblotting, as shown in figure A2.3 and table A2.2. Extracts W and X are those shown in figure A2.3 and table A2.2.

<table>
<thead>
<tr>
<th>Translation</th>
<th>Extract</th>
<th>Cyclin A concentration (nM)</th>
<th>Range (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pure egg extract</td>
<td>W</td>
<td>1.16</td>
<td>1.16 - 8.12</td>
</tr>
<tr>
<td></td>
<td>X</td>
<td>2.29</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Y</td>
<td>8.12</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Z</td>
<td>3.66</td>
<td></td>
</tr>
<tr>
<td>9:1 (v/v) egg extract :</td>
<td>W</td>
<td>4.54</td>
<td>4.54 - 26.72</td>
</tr>
<tr>
<td>reticulocyte lysate</td>
<td>X</td>
<td>12.91</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Y</td>
<td>22.52</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Z</td>
<td>26.72</td>
<td></td>
</tr>
<tr>
<td>1:1 (v/v) egg extract :</td>
<td>W</td>
<td>24.73</td>
<td>24.73 - 79.74</td>
</tr>
<tr>
<td>reticulocyte lysate</td>
<td>X</td>
<td>58.39</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Y</td>
<td>52.37</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Z</td>
<td>79.74</td>
<td></td>
</tr>
</tbody>
</table>

Into two aliquots. Ca\(^{2+}\) was added to one, which was incubated at 23°C for 20 minutes, while the other CSF-arrested egg extract aliquot and the interphase extract were incubated on ice. Each of these three different extracts was then split into three aliquots themselves. To one of each of these aliquots was added cycloheximide and a 1/10th volume of 0.2 mM EDTA and the extracts were incubated on ice; to the second set of aliquots a 1/10th volume of 0.2 mM EDTA was added and they were incubated at 23°C; to the third set of aliquots a 1/10th volume of the mRNA encoding c-myc cyclin A (in 0.2 mM EDTA) was added and these were also incubated at 23°C. All incubations were for 2 hours. At the end of the incubation period cycloheximide was added to those reactions to which it had not already been added. One μl of each reaction was removed, added to 24
μl of SDS sample buffer and stored on dry ice. The rest of each reaction (9 μl) was incubated with p13suc1 Sepharose 4°C for one hour. The concentration of cyclin A in the total reactions and that bound to half of the p13suc1 Sepharose was analysed by SDS PAGE, followed by immunoblotting with the anti-cyclin A monoclonal antibody, XLA1-3, and an HRP-conjugated rabbit anti-mouse second antibody. Cyclin A concentration standards were also run on the polyacrylamide gel and immunoblotted. The immunoreactive bands were visualised by the Amersham ECL system (figure A2.3) and their absorbance was quantitated by scanning densitometry. As figure A2.4 and table A2.4 show, the concentration of cyclin A was greatly increased in the extracts to which c-myc cyclin A mRNA had been added (compare lanes 14 - 16 with lanes 17 - 19). Translation of added mRNA appeared to be most efficient in the interphase extract (lane 19) and better in the CSF-arrested egg extract to which Ca^2+ had been added than in CSF-arrested egg extract to which Ca^2+ had not been added (compare lanes 17 and 18). After Ca^2+ had been added to the CSF-arrested egg extract and incubated for 20 minutes, the extract had a very low histone H1 kinase activity, in contrast to the same extract in the absence of Ca^2+ (data not shown), thus cyclin destruction and the consequent progression of the extract into interphase had been triggered. It is noticeable, however, that although the concentration of cyclin A decreased when Ca^2+ was added to the CSF-arrested egg extract, not all of the protein was destroyed (compare lane 11 with 12 and 20 with 21).

The concentration of endogenous cyclin A in the CSF-arrested egg extract was calculated in two ways. Firstly, a calibration curve was drawn using the cyclin A concentration standards, and the concentration of cyclin A in lane 11 was calculated using this graph (not shown). Using this method of calculation, the concentration of endogenous cyclin A was calculated to be 1.16 nM (see table A2.4). This is the same as the calculated value for the concentration of translated cyclin A in CSF-arrested egg extract W (figure A2.3, lanes 12 and 13); in this extract the concentration of endogenous cyclin A is lower than this as no band can be seen in the lane to which no mRNA had been added. This highlights the problem of obtaining accurate estimates of the concentration of cyclin A when the value is so low. In the second method, the concentration of cyclin A in the translation reaction to which c-myc cyclin A mRNA was added to CSF-arrested egg extract (figure A2.4, lane 17) was calculated using the standard curve, as was the concentration of cyclin A bound to p13suc1 Sepharose from this translation reaction (lane 26) and the concentration of cyclin A bound to p13suc1 Sepharose from the CSF-arrested egg extract which had been incubated at 4°C in the presence of cycloheximide (lane 20). The efficiency of precipitation of the cyclin A protein on p13suc1 Sepharose from the ‘+mRNA’ translation reaction occurring in CSF-arrested egg extract was calculated and this was used to deduce the
Figure A2.4  The concentration of cyclin A in different types of *Xenopus* extracts

Western blot using the anti cyclin A monoclonal antibody, XLAl-3. The immunoreactive bands were detected by using the ECL system from Amersham. Lanes 1 - 10, bacterially expressed cyclin A standards; lanes 11 - 19, translation reaction; lanes 20 - 28, precipitation of the translation reaction with p13suc1 Sepharose; lanes 11 - 13 and 20 - 22, incubation of the extract at 4°C in the presence of cycloheximide and in the absence of added mRNA; lanes 14 - 16 and 23 - 25, incubation of the extract at 23°C in the absence of cycloheximide and added mRNA; lanes 17 - 19 and 26 - 28, incubation of the extract at 23°C in the absence of cycloheximide and in the presence of the mRNA encoding for c-myc cyclin A; lanes 11, 14, 17, 20, 23 and 26, CSF-arrested egg extract; lanes 12, 15, 18, 21, 24 and 27, CSF-arrested egg extract that had been pre-incubated with Ca²⁺; lanes 13, 16, 19, 22, 25 and 28, interphase extract. Note that the bacterial standard was missing 56 residues from the N-terminus and therefore migrates faster than full-length cyclin A1.
Table A2.4  The concentration of cyclin A in different types of Xenopus extracts
The concentration of cyclin A in each of lanes 11 - 28 of figure A2.4 was determined by quantitative immunoblotting, using the cyclin A concentration standards shown in lanes 1 - 10.

<table>
<thead>
<tr>
<th>Incubation conditions</th>
<th>Extract</th>
<th>Cyclin A concentration in translation (nM)</th>
<th>Cyclin A concentration in p13suc1 Sepharose precipitation (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4°C</td>
<td>egg extract</td>
<td>1.16</td>
<td>2.75</td>
</tr>
<tr>
<td>no added mRNA</td>
<td>egg extract +Ca²⁺</td>
<td>0.89</td>
<td>1.23</td>
</tr>
<tr>
<td></td>
<td>interphase extract</td>
<td>2.19</td>
<td>8.89</td>
</tr>
<tr>
<td>23°C</td>
<td>egg extract</td>
<td>0.68</td>
<td>1.03</td>
</tr>
<tr>
<td>no added mRNA</td>
<td>egg extract +Ca²⁺</td>
<td>3.42</td>
<td>17.78</td>
</tr>
<tr>
<td></td>
<td>interphase extract</td>
<td>2.19</td>
<td>12.92</td>
</tr>
<tr>
<td>23°C</td>
<td>egg extract</td>
<td>5.20</td>
<td>18.67</td>
</tr>
<tr>
<td>added c-myc</td>
<td>egg extract +Ca²⁺</td>
<td>9.64</td>
<td>35.28</td>
</tr>
<tr>
<td>cyclin A mRNA</td>
<td>interphase extract</td>
<td>12.58</td>
<td>38.29</td>
</tr>
</tbody>
</table>

Theoretical concentration of cyclin A in CSF-arrested egg extract, based on the concentration of cyclin A from this reaction that was precipitated on p13suc1 Sepharose. This calculation gives the concentration of endogenous cyclin A in CSF-arrested egg extract to be 0.756 nM.

**Discussion**

The concentration of p34cdc2 in CSF-arrested egg extract is in the range 450 - 600 nM, with over 80% of it being monomeric. The exact p34cdc2 concentration appears to vary in different extracts, although this variation may, in part, be due to experimental error.

There is considerable variation in the ability of different CSF-arrested egg extracts to translate added mRNA, but even after translation of c-myc cyclin A mRNA in a CSF-arrested egg extract that is good at translating added mRNA, the concentration of cyclin A is well below 10 nM. The addition of only 10% (by
volume) rabbit reticulocyte lysate to CSF-arrested egg extract increases the concentration of cyclin A after translation by between 3.5 and 7 fold and the addition of 50% (by volume) of reticulocyte lysate increased this value by about 20 fold in most extracts. In the experiment shown in figure A2.3A, the concentration of cyclin A in the reactions to which no c-myc cyclin A mRNA had been added was well below 1.16 nM, as no band was apparent in these lanes (lanes 11 and 18) where it was in the lanes that contained cyclin A at 1.16 nM (lanes 12 and 13). This is in contrast to the figure of 1.16 nM calculated for the concentration of endogenous cyclin A in the CSF-arrested egg extract used for the experiment shown in figure A2.4. This could be due to variations in the concentration of cyclin A in different extracts, but is more likely to be due to inaccuracies in the measurement of these values. Another method of calculation gives the concentration of endogenous cyclin A in the CSF-arrested egg extract shown in figure A2.4 to be 0.77 nM. These results broadly agree with earlier results from the laboratory, which suggested that about 0.5 nM cyclin A was present in whole Xenopus eggs (Kobayashi et al., 1991). In this paper Kobayashi et al. also show that in unfertilised eggs the cyclin A protein cannot be affinity purified on p13 Suc1 Sepharose. The data shown in this Appendix do not entirely agree with this result; at least some of the endogenous cyclin A in extracts made from unfertilised eggs (CSF-arrested egg extract) clearly can bind to p13 Suc1 Sepharose, as shown in lane 20 of figure A2.4. This could, however, account for the discrepancy between the values for endogenous cyclin A concentration calculated by the two different methods.

Although it is difficult to gain accurate estimates of the concentration of cyclin A in CSF-arrested egg extract, both before and after the translation of c-myc cyclin A, the data described in this Appendix gives a some idea as to the range within which these values fall. The concentration of endogenous cyclin A in CSF-arrested egg extract is probably between 0.5 and 1 nM. After the translation of added c-myc cyclin A mRNA in CSF-arrested egg extract, this value is increased 2.5 - 10 fold to between 1.2 and 8.1 nM. The addition of 10% or 50% (by volume) of reticulocyte lysate to the CSF-arrested egg extract greatly increases the translation activity of the extract.
Appendix 3

Identification of a p34cdc2 phosphorylation site in cyclin A

As described in Chapter 8, when the internal deletion mutants of cyclin A, Δ88-144 and Δ109-161 are translated in CSF-arrested egg extract, immunoprecipitated with an anti-cyclin A antibody, and incubated with [γ-32P]ATP, the mutant cyclin A proteins become phosphorylated to a much lesser extent than wild-type cyclin A (Stewart et al., 1994). The N-terminal deletion mutant of cyclin A, NA133, which is missing its first 133 amino acids (Kobayashi et al., 1992), is not phosphorylated at all in such an assay (see figure A3.2, lane 20). Thus it was likely that these constructs were missing a major p34cdc2 phosphorylation site.

Construction of N-terminal deletion mutants of cyclin A

The region that had been deleted from both Δ88-144 and Δ109-161 (residues 109-144) is shown in figure A3.1A. In the deleted region there are 5 serine residues and one threonine, all of which were possible p34cdc2 kinase sites. Two of these serines and the threonine residue are present, however, in the N-terminally deleted construct NA133, which does not get phosphorylated. Nevertheless, these 3 residues were not ruled out as being the site of p34cdc2 phosphorylation as they are close to the N-terminus of NA133, and this proximity to the start of the protein could possibly interfere with their phosphorylation.

In order to identify the major in vitro p34cdc2 phosphorylation site, four N-terminally deleted cyclin A mutants were constructed using PCR. 5' oligonucleotides were used to create BsmI sites just upstream of the desired start sites of the constructs, and the 3' oligonucleotide was one which annealed downstream of the NsiI site in cyclin A. The PCR products were digested with BsmI and NsiI and ligated into BsmI and NsiI cut c-myc cyclin A. The N-terminally deleted constructs thus contained the first 18 amino acids of c-myc cyclin A (including the c-myc tag) followed by the desired start of the construct. The N-terminal 18 amino acids of these constructs is shown in figure A3.1B and the residues that follow for the four constructs, c-myc ΔA107, c-myc ΔA114, c-
**Figure A3.1** The starts of the N-terminally deleted cyclin A mutants

(A) The region deleted from both Δ88-144 and Δ109-161 cyclin As (residues 109 - 144) is shown, with one extra amino acid at the N-terminus. The start sites of the cyclin A portions of c-myc NA107, c-myc NA114, c-myc NA129 and c-myc NA133 are indicated by the arrows. The sequence upstream of the indicated start sites is the 18 amino acid sequence shown in (B). The residues that could be phosphorylated are shaded. (B) The N-terminus of the c-myc tagged cyclin A deletions. The epitope recognised by the 9E10 monoclonal antibody is shown in bold and underlined.

**Phosphorylation of the N-terminally deleted cyclin A constructs**

The mRNAs encoding c-myc cyclin A, c-myc NA133, c-myc NA129, c-myc NA114, c-myc NA107 and non-tagged NA133 were translated in a 1:1 (v/v) mixture of CSF-arrested egg extract and reticulocyte lysate in the presence or absence of [35S]methionine. The translation reactions were immunoprecipitated with the anti-c-myc antibody, 9E10 (all reactions except non-tagged NA133) or a polyclonal anti-cyclin A antibody (NA133) and protein A Sepharose. The non-[35S]-labelled immunoprecipitates were incubated with [γ-32P]ATP for 30
Figure A3.2  The c-myc tagged N-terminal deletions of cyclin A are phosphorylated
The mRNAs encoding the cyclin A constructs indicated above the figure were translated in a 1:1 (v/v) mixture of CSF-arrested egg extract and reticulocyte lysate in the presence or absence of [35S]methionine. Lanes 1 - 7, the complete translation reaction ([35S]-labelled); lanes 8 - 14, immunoprecipitation of the reactions shown in lanes 1 - 7 with the anti c-myc monoclonal antibody, 9E10; lanes 15 - 21, immunoprecipitation of unlabelled translation reactions with the anti c-myc monoclonal antibody, followed by incubation of the bead-bound proteins with [γ-32P]ATP. Visualised by autoradiography.
minutes at room temperature (see Chapter 2). As shown in figure A3.2, all of the c-myc tagged N-terminal deletions of cyclin A were phosphorylated to roughly the same (or greater) extent as wild-type cyclin A (lanes 15 - 19), while non-tagged NA133 was not phosphorylated at all (lane 20). c-myc NA133 was phosphorylated to a markedly greater extent than wild-type cyclin A. The reason for the higher incorporation of $^{32}$PO$_4$ in c-myc NA133 as compared to wild-type cyclin A is not entirely clear, but it is possible that the conformation of this construct was such that its phosphorylation site was more accessible to p34$^{cd}$ than any of the sites in the other cyclin A proteins. It is also feasible that one or both of the serines in the c-myc tag insert (see figure A3.1B) were phosphorylated in this construct. The phosphorylation of all four mutants, including c-myc NA133, suggested that the phosphorylation site was present in all four constructs, and was therefore likely to be either serine 136, serine 143 or threonine 142 (see figure A3.1). It was likely that non-tagged NA133 was not phosphorylated due to the proximity of the phosphorylation site to the N-terminus of the protein, as suggested.

The phosphorylated residue in c-myc NA133 is a serine

In order to investigate whether the residue(s) that were phosphorylated in c-myc cyclin A and c-myc NA133 were serines or threonines, [γ-$^{32}$P]ATP-labelled c-myc cyclin A and c-myc NA133 proteins were hydrolysed in 5.7 M HCl, as described in Chapter 2. The resulting amino acids were electrophoresed on a silica thin layer chromatography (TLC) plate at pH 3.5 with phosphoserine, phosphothreonine and phosphotyrosine as standards. A mixture of these standards was run with the two samples, and they were also run individually. The standard phosphoamino acids were stained with ninhydrin and their positions marked on the TLC plate. The plate was then exposed to film (see Chapter 2). Figure A3.3 shows that p34$^{cd}$-phosphorylated c-myc cyclin A contains mainly phosphoserine, although there is a small amount of phosphothreonine. c-myc NA133, on the other hand, contains only phosphoserine. Thus the residue phosphorylated by p34$^{cd}$, that is missing from Δ88-144 and Δ109-161, is almost certainly a serine residue.

V8 endopeptidase digestion of c-myc cyclin A

In order to investigate the phosphopeptides present in c-myc cyclin A, [γ-$^{32}$P]ATP-labelled c-myc cyclin A was digested with V8 endopeptidase in 50 mM ammonium bicarbonate (see Chapter 2). Under these conditions the protease cuts C-terminally to glutamic acid residues. Half of the resulting peptides were separated in two dimensions on a silica TLC plate by electrophoresis in pH 4.72
buffer, followed by chromatography in phospho-chromatography buffer (see Chapter 2). Autoradiography of the TLC plate showed that c-myc cyclin A contains two major phosphopeptides, designated spot A and spot B (see figure A3.4A). The presence of two phosphopeptides of about equal intensity could indicate that there are two distinct phosphorylation sites, or the presence of 2 spots could be due to incomplete digestion of the protein by the V8 endopeptidase.

In order to check the phosphoamino acid content of the two $[^{32}P]$-labelled spots, they were scraped off the TLC plate and hydrolysed in 5.7 M HCl. The resulting amino acids were analysed by one dimensional thin-layer electrophoresis at pH 3.5 (see Chapter 2). As shown in figure A3.4B, the phosphorylated residue in both spot A and spot B is exclusively phosphoserine. Thus the major in vitro p34cdc2 phosphorylation of c-myc cyclin A occurs on a serine residue, as suggested by the previous results.

**Edman degradation of the phosphorylated peptides**

All the information presented so far in this chapter suggested that the major in vitro p34cdc2 phosphorylation site in cyclin A was either serine 136 or serine 143. It was possible, however, that the phosphorylation that I had been investigating was occurring on in the c-myc tag of the constructs used in this analysis, although
Figure A3.4  Phosphopeptide mapping of V8 endopeptidase digested c-myc cyclin A and phosphoamino acid analysis of the two resulting major spots

(A) [γ-32P]ATP-labelled c-myc cyclin A was digested with V8 endopeptidase and the resulting peptides were separated by thin layer electrophoresis in pH 4.72 buffer in the first dimension and chromatography in the second dimension. See Chapter 2.

(B) The phosphorylated peptides seen in spots A and B were eluted from the silica coating the TLC plate (A) and hydrolysed in 5.7 M HCl. The resulting amino acids were analysed by one dimensional thin layer electrophoresis in pH 3.5 buffer (see Chapter 2 for further details). The positions of the standard phosphoamino acids are indicated on the left of the figure. Visualised by autoradiography.
I had also shown that non-tagged cyclin A and c-myc cyclin A gave similar patterns of phosphorylated peptides after V8 endopeptidase digestion (data not shown).

In order to check that the p34\(^{\text{cdc2}}\)-phosphorylation site in c-myc cyclin A was not in the c-myc tag, and to identify whether serine 136, serine 143, or both, were the site of p34\(^{\text{cdc2}}\) phosphorylation, the rest of the V8 endopeptidase digest that had been used in the previous section was analysed by thin layer electrophoresis at \(\text{pH} \ 4.72\) followed by chromatography in phosphochromatography buffer. The silica containing the two major phosphorylated peptides (spots A and B, see figure A3.4A) was scraped off the TLC plate and analysed by Edman degradation by the ICRF protein sequencing laboratory. In this process the amino acids in the phosphorylated peptide are sequentially removed and the amount of radioactivity associated with each determined, allowing the position of the phosphorylated residue in the peptide to be identified. The data from this analysis is shown in figure A3.5. Spot A released over 30% of its radioactive material on cycle 2 of the analysis (i.e. when the second residue in this peptide was removed), giving the clear result that the second amino acid in the peptide was phosphorylated. The results from spot B were less clear. There was not an obvious release of radioactivity on any of the cycles, although there does seem to be a small peak at cycle 9 and possibly an even smaller one at cycle 2. Despite spot B having an intensity similar to that of spot A on the TLC plate, there seemed to be less radioactivity associated with it when it was used for Edman degradation or phosphoamino acid analysis (see figure A3.4B).

Sequence analysis of c-myc cyclin A shows that digestion with V8 endopeptidase gives 32 different peptides, only two of which have a serine residue as their second residue (see figure A3.6). One of these serine residues is serine 136, the other is serine 119. There is only one peptide that contains a serine residue as its ninth residue, this serine is serine 143.

From all of the data shown in this appendix it appears that the major p34\(^{\text{cdc2}}\) phosphorylation site in cyclin A is serine 136, perhaps with minor phosphorylation on serine 143.

**Phosphorylation of the S136A mutant of c-myc cyclin A**

To confirm that serine 136 was indeed the major \(\text{in vitro}\) p34\(^{\text{cdc2}}\) phosphorylation site in \textit{Xenopus} cyclin A, it was replaced with an alanine residue in c-myc cyclin A by PCR (see Chapter 2). The mRNA encoding this mutant cyclin A construct (S136A cyclin A) was translated in a 1:1 (v/v) mixture of CSF-arrested egg extract and reticulocyte lysate, in the presence or absence of \([^{35}\text{S}]\)methionine, as was the mRNA encoding c-myc cyclin A. The translation reactions were immunoprecipitated with the anti-c-myc antibody, 9E10, and protein A

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Figure A3.5  Edman degradation of phosphorylated peptides contained in spots A and B (figure A3.4A)

V8 endopeptidase digested c-myc cyclin A that had been phosphorylated with [γ-32P]ATP was analysed by thin layer electrophoresis in pH 4.72 buffer, followed by chromatography in phospho-chromatography buffer. The silica containing the two major phosphorylated peptides, spots A and B (see figure A3.4A), was scraped off and the peptides extracted. Edman chemistry was performed for 20 cycles on the peptides and the radioactivity contained in each resulting phenyl thiohydantoin residue was determined (see Chapter 2). The radioactivity (in counts per minute) contained at each position in the peptides in spots A and B is shown as a bar graph. This identified the position of the [γ-32P]-labelled residue in each peptide.
Figure A3.6  The peptides resulting from V8 endopeptidase digestion of c-myc cyclin A in 50 mM ammonium bicarbonate

When proteins are digested with V8 endopeptidase in 50 mM ammonium bicarbonate, cleavage occurs C-terminally to all available glutamic acid residues. The peptides predicted to be produced when c-myc cyclin A is digested under these conditions are shown, the top of the figure is the N-terminus of the protein and the bottom is the C-terminus. The serine residues are shown in bold type and the second and ninth residues of each peptide is shaded.

Sepharose. The non-labelled immunoprecipitates were incubated with [γ-32P]ATP and the products analysed by SDS PAGE and autoradiography. As shown in figure A3.7, mutation of serine 136 to an alanine residue in c-myc cyclin A strongly reduced the phosphorylation of this construct, but did not entirely eliminate it (compare lanes 9 and 10). This is probably due to phosphorylation at other sites in c-myc cyclin A, like in the mutants Δ88-144 and Δ109-161. To check whether this additional phosphorylation was due to phosphorylation in the N-terminus of the protein, a serine 136 to alanine mutation was made in the N-terminally deleted c-myc NΔ107 construct. This mutant, NΔ107S136A, was not
The mRNAs encoding the cyclin A constructs indicated above the figure were translated in a 1:1 (v/v) mixture of CSF-arrested egg extract and reticulocyte lysate in the presence or absence of \([^{35}\text{S}]\)methionine. Lanes 1 - 4, the complete translation reaction (\([^{35}\text{S}]\)-labelled); lanes 5 - 8, immunoprecipitation of the reactions shown in lanes 1 - 4 with the anti-c-myc monoclonal antibody, 9E10; lanes 9 - 12, immunoprecipitation of unlabelled translation reactions with the anti c-myc monoclonal antibody, followed by incubation of the bead-bound proteins with \([\gamma-^{32}\text{P}]\)ATP. Visualised by autoradiography.

phosphorylated at all in the phosphorylation assay described above, in contrast to c-myc NA107 containing serine 136 (compare lanes 11 and 12 in figure A3.7). Thus the phosphorylation seen on S136A cyclin A must be due to phosphorylation in the N-terminus of the protein.

**Phosphopeptide analysis of S136A cyclin A**

To double check that S136 was the major *in vitro* p34<sup>cdc2</sup> phosphorylation site in cyclin A, \([\gamma-^{32}\text{P}]\)ATP-labelled c-myc cyclin A and S136A cyclin A were digested with V8 endopeptidase and analysed by thin layer chromatography at pH 4.72 and chromatography in phospho-chromatography buffer, as described in Chapter 2. The resulting peptide maps, shown in figure A3.8, are slightly different to that shown in figure A3.4. This is probably because thin layer electrophoresis of the peptide was carried out using a new thin layer electrophoresis apparatus that the laboratory had purchased. This apparatus gave much more consistent results, and allowed better separation of the phosphopeptides as the electrophoresis step could be continued for longer without the TLC plate drying out. Thus the rather
Figure A3.8 Phosphopeptide mapping of V8 endopeptidase digested c-myc cyclin A S136A cyclin A

\[\gamma^{32}\text{P}]\text{ATP}-labelled c-myc cyclin A and S136A cyclin A were digested with V8 endopeptidase and the resulting peptides were separated by thin layer electrophoresis in pH 4.72 buffer in the first dimension and by chromatography in phosphochromatography buffer in the second dimension.
strangely shaped spot A in figure A3.4A is probably several unresolved spots. In addition, the weaker spots seen on figure A3.8 may have been too faint to see in figure A3.4A. The result from Edman degradation of spot A from figure A3.4A was, however, clear. There was only one phosphorylated residue in the peptide(s) in this spot; the second amino acid. This result is confirmed by phosphopeptide analysis of S136A cyclin A; all but two very faint spots and one slightly darker one are absent from this TLC plate. The darkest spot is that which probably corresponds to spot B from figure A3.4A, but even this spot is greatly reduced in intensity in comparison to the corresponding one from c-myc cyclin A. The two faint spots probably correspond to phosphorylation at the other sites in cyclin A.

**Discussion**

In this appendix I have identified serine 136 of *Xenopus* cyclin A as the major p34\(^{cdc2}\) phosphorylation site *in vitro*. Serine 136 occurs as the first residue of an SPM motif, the SP of which is conserved in all cyclin As that have been studied (including *Drosophila*). The motif lies about 30 residues upstream of the start of the p34\(^{cdc2}\) binding region of cyclin A, and is a potential site for proline directed protein kinase such as p34\(^{cdc2}\) kinase.

It is strange that non-tagged N\(\Delta\)133 cyclin A does not get phosphorylated by p34\(^{cdc2}\) even though it contains the phosphorylation site. This is probably due to serine 136 being so close to the N-terminus of the protein (it is the 7th residue), since c-myc N\(\Delta\)133 can be phosphorylated. Perhaps the extreme N-terminus of N\(\Delta\)133 is too mobile to allow phosphorylation. There was the possibility, however, that phosphorylation of the c-myc tagged N-terminal deletions was not on the same site as in wild-type cyclin A, but due to phosphorylation on one or both of the serines in the c-myc tag, or other serine residues that were placed close to the kinase site of cyclin A/p34\(^{cdc2}\) complex as a result of the removal of the N-termini of these proteins. This possibility was eliminated by showing that while c-myc N\(\Delta\)107 was able to be phosphorylated, N\(\Delta\)107S136A was not phosphorylated at all. This result also indicates that the other p34\(^{cdc2}\) phosphorylation on cyclin A (*i.e.* that not on serine 136) occurs on residues within the first 107 amino acids of the protein. At least some of this phosphorylation is on threonine residue(s).

It should be noted that although I have identified serine 136 as the major p34\(^{cdc2}\) phosphorylation site in *Xenopus* cyclin A, this phosphorylation and the minor threonine (and possibly serine) phosphorylation in the N-terminus of the protein are not necessarily the only phosphorylations to take place on cyclin A *in vivo*. The assay used in this thesis, which was performed on immunoprecipitates, relied on *de novo* phosphorylation, or the turnover of pre-existing phosphate. If a
site was stably phosphorylated by endogenous (and therefore non-radioactive) ATP in the CSF-arrested egg extract during translation of cyclin A mRNA, it would not incorporate \( [\gamma^{32}\text{P}] \text{ATP} \) after immunoprecipitation. In addition, this assay only reliably detects the phosphorylation of cyclin A by the p34\(^{\text{cdc2}} \) to which it is bound. The possibility cannot be excluded that cyclin A is phosphorylated at other sites by other proteins kinases.
References


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Solomon, M. J., Harper, J. W. and Shuttleworth, J. (1993). CAK, the p34\textsuperscript{cdc2} activating kinase, contains a protein identical or closely related to \textsuperscript{p40}EMBO J. 72, 3133-3142.


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The specific and rapid destruction of cyclins A and B during mitosis is their most remarkable property. A short peptide motif of ~10 amino acids near the N-terminus, known as the destruction box, is absolutely required for programmed proteolysis. In this paper we show that although the destruction box is necessary for the degradation of cyclin A, it is not sufficient. Mutant versions of cyclin A that cannot form complexes with p34^{cd2} are stable, which we interpret to mean that this cyclin must bind to p34^{cd2} in order to undergo programmed proteolysis. Thus, N-terminal fragments of cyclin A containing little more than the destruction box and its surroundings are indestructible. p34^{cd2} binding also appears to be required for the destruction of cyclin B. In contrast, cyclin B1 does not require p34^{cd2} binding for specific proteolysis. The systems for the proteolysis of cyclins A, B1 and B2 thus appear to show important differences in the way they recognize their substrates.

Key words: cell cycle/phosphorylation/protease/protein kinase

Introduction

Cyclins were first identified in marine invertebrate eggs as newly synthesized proteins which accumulated rapidly during interphase and were abruptly destroyed in mitosis, just before the metaphase to anaphase transition (Evans et al., 1983; Swenson et al., 1986). These proteins have since been identified in eukaryotes from yeast to mammals (see Hunt, 1991). Cyclins appear to be activating subunits of protein kinases encoded by genes from the cdc family (see Nurse, 1990; Pines and Hunter, 1990). At least some of these kinases are important regulators of cell cycle transitions. The two best characterized groups of cyclins are those that regulate progression through mitosis (mitotic cyclins) and those that regulate progression through interphase to anaphase (G1 cyclins).

The transition from interphase to mitosis requires the activation of maturation-promoting factor (MPF) (Masui and Markert, 1971; Newport and Kirschner, 1984; Draetta and Beach, 1988; Langan et al., 1989) which is a heterodimer composed of a p34^{cd2} kinase subunit and a B-type cyclin (Dunphy et al., 1988; Gautier et al., 1988, 1990; Löhka et al., 1988; Draetta et al., 1989; Labbé et al., 1989). The transition from metaphase to anaphase requires specific proteolysis of the mitotic cyclins and consequent loss of the kinase activity of p34^{cd2} (Luca and Ruderman, 1989; Murray and Kirschner, 1989; Murray et al., 1989; Luca et al., 1991). Proteolysis can be activated by addition of MPF to interphase extracts (Félix et al., 1990) and the protease remains active for as long as MPF remains active. Thus, protease-resistant mutants of cyclin B arrest cells in mitosis and maintain the protease in its active state (Murray et al., 1989; Ghiara et al., 1991; Glotzer et al., 1991; Luca et al., 1991; Gallant and Nigg, 1992). In cleavage stage embryos, the protease seems to be inactive in interphase and rapidly activated at the end of metaphase, for example, in clam embryos, where accurate measurements are possible, proteolysis is active for ~5 min, by which time most of the cyclin has been degraded and MPF turned off (Hunt et al., 1992). The enzyme(s) responsible for the recognition of cyclin and initiation of its proteolysis have not been identified and the mechanism of their control is not known. Two lines of evidence, however, implicate the ubiquitin system in cyclin degradation. First, high molecular weight ubiquitinylated intermediates of sea urchin cyclin B (and constructs derived from it) were detected in crude extracts of frog embryos undergoing cyclin proteolysis, and the flux through these intermediates was adequate to account for the total loss of cyclin (Glotzer et al., 1991). Second, addition of carboxymethylated ubiquitin to clam extracts inhibited cyclin proteolysis (Hershko et al., 1991). It is not clear, however, that polyubiquitination is the sole signal for cyclin destruction and it is also not known whether the abrupt onset of proteolysis is due to the sudden activation of a cyclin- and cell cycle stage-specific ubiquitin ligase or of the substrate, or both. Nor has it yet been possible to chart the biochemical route of destruction of the ubiquitinated cyclin, although it is generally thought that the multifunctional proteasome is responsible. At present, the one point that is clear is that the N-terminus of mitotic cyclins contains a short conserved region, no more than 10 residues long, known as the destruction box. Mutation of the conserved arginine or leucine residues in this motif inhibits the destruction of both cyclins A and B (Murray et al., 1989; Glotzer et al., 1991; Lorca et al., 1991b, 1992a; Gallant and Nigg, 1992; Kobayashi et al., 1992). In the case of cyclin B, such modifications also inhibit its polyubiquitination, consistent with the idea that the destruction domain is recognized by some component of the ubiquitin-conjugating system.

Although the degradation of cyclins A and B both require an intact destruction box and occur at about the same time in the cell cycle, there are distinct differences between some aspects of their proteolysis. For example, cyclin A is degraded slightly earlier in the cell cycle than cyclin B (Luca and Ruderman, 1989; Minshull et al., 1990; Whitfield et al., 1990; Hunt et al., 1992), and whereas disruption of the mitotic spindle formation with colchicine strongly retards
cyclin B destruction, such treatment had no effect on cyclin A proteolysis (Whitfield et al., 1990; Hunt et al., 1992).

Another curious difference between the two types of cyclin, both of which appear to be associated with p34<sup>cdc2</sup> at this juncture of the cell cycle, is that cyclin B–p34<sup>cdc2</sup> triggers cyclin destruction, whereas cyclin A does not; indeed, cyclin A tends to delay the destruction of both A- and B-type cyclins (Luca et al., 1991; Lorca et al., 1992b).

Certain other properties of cyclin proteolysis have been discovered during searches for inhibitors of the process. For example, the adenosine analogue 6-dimethyl aminopurine stabilizes cyclins (Félix et al., 1989, 1990; Luca and Ruderman, 1989), possibly by virtue of its ability to inhibit the protein kinase activity of MPF. The well-known ability of ATP-γ-S to stabilize MPF (Lohka et al., 1988) is probably due to its ability to antagonize cyclin destruction. Other inhibitors include the trypsin inhibitor, tosyl-lysine chloromethyl ketone, certain sulfydryl-reactive agents, such as N-ethylmaleimide and ZnCl<sub>2</sub> and EDTA (Luca and Ruderman, 1989). Conversely, it has been reported that in Xenopus egg extracts cyclin destruction can be triggered by okadaic acid (Lorca et al., 1991a).

Unfertilized Xenopus eggs are arrested in metaphase of meiosis II with high MPF activity (Masui and Markert, 1971; Lohka and Masui, 1983). Cell cycle arrest in these eggs is mediated by an activity known as cytostatic factor (CSF) and is thought to involve the c-mos protein kinase (Masui and Markert, 1971; Sagata et al., 1989). Fertilization triggers a large increase in the intracellular Ca<sup>2+</sup> levels (Busa and Nuccielli, 1985), which leads to the specific proteolysis of the mitotic cyclins, MPF inactivation and resumption of the cell cycle (Newport and Kirschner, 1984; Murray et al., 1989; Lorca et al., 1991b). In this paper, we have used extract made from unactivated Xenopus eggs (CSF-arrested extract) in order to study cyclin destruction. For this analysis we used a number of deletion and point mutations of frog cyclin A, which we made recently to look at the requirements for binding to p34<sup>cdc2</sup> (Kobayashi et al., 1992). We find that for the proteolysis of cyclin A to proceed with normal kinetics, it must be capable of binding to p34<sup>cdc2</sup>. Surprisingly, cyclin B2 also needs to bind to p34<sup>cdc2</sup> for normal destruction, but cyclin B1 does not, confirming the results of Glotzer et al. (1991), who showed that constructs derived from sea urchin cyclin B containing a destruction box could be proteolysed even if they lacked the p34<sup>cdc2</sup> binding domain.

**Results**

**The assay for cyclin destruction**

In the experiments described below, we used concentrated extracts made from unactivated Xenopus eggs, called CSF-arrested extracts (Murray, 1991). Cyclin destruction can be triggered in these extracts by the addition of 0.4 mM CaCl<sub>2</sub> (Lohka and Maller, 1985; Murray et al., 1989; Lorca et al., 1992a; Vandervelden and Lohka, 1993), but if Ca<sup>2+</sup> is not added, the cyclin proteins remain stable. CSF-arrested extract can be frozen in liquid nitrogen and retains the ability to translate added mRNA and degrade cyclins in response to added Ca<sup>2+</sup> after thawing. In the experiments described in this paper, the standard assay for cyclin destruction was as follows. Synthetic mRNAs encoding wild type or mutant cyclins were translated in freshly thawed CSF-arrested extract. Some cyclin A constructs carried an N-terminal c-myc tag, and when the same CSF-arrested extract is used for a series of assays, the results are highly reproducible. However, batches of extract prepared from different females vary considerably both in their ability to translate added mRNA and in their ability to destroy cyclin proteins. The length of time required for the complete destruction of wild type cyclin, for example, varied between 15 and 40 min (compare Figure 1A, lanes 1–6 and Figure 6, top panel, first six lanes). For these reasons, wild type cyclins were included in every assay as positive controls and comparisons between the rate and extent of destruction of mutant constructs were always performed at the same time in the same batch of extract. Some cyclin A constructs carried an N-terminal c-myc tag, and non-c-myc tagged cyclin A (data not shown).

**Cyclin A destruction at the end of M phase requires p34<sup>cdc2</sup> binding**

Figure 1A shows that in the standard destruction assay wild type cyclin A was rapidly destroyed between 30 and 60 min after Ca<sup>2+</sup> addition (compare the intensity of lanes 4 and 5). In striking contrast to the wild type protein, however, two cyclin A mutants in which single highly conserved residues within the cyclin box had been conservatively

**Fig. 1.** Destruction of cyclin box mutants of cyclin A. (A) Destruction of R197K and D226E cyclin A. Lanes 1–6, c-myc tagged wild type cyclin A; lanes 7–12, R197K cyclin A; lanes 13–18, D226E cyclin A. (B) Destruction of Δ 231–232 cyclin A. Lanes 1–5, wild type cyclin A; lanes 6–10, Δ 231–232 cyclin A. Asterisk on left indicates position of the endogenous B-type cyclins. The standard destruction assay was used (see Materials and methods).

**Table**

<table>
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<th>Cyclin A</th>
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**Fig. 1A, top panel, first six lanes.**
mutated (R197 to K or D226 to E), were still present in the extract 90 min after Ca\(^{2+}\) addition (Figure 1A, lanes 12 and 18). These mutants are unable to bind to p34\(^{cdc2}\) (data not shown). Two other point mutations, R197A and D226A, also do not bind to p34\(^{cdc2}\) (Kobayashi et al., 1992) and are not destroyed (see Table I). Figure 1B shows that another mutant (∆231–232), which cannot bind to p34\(^{cdc2}\) (Kobayashi et al., 1992), is also indestructible in the CSF-arrested extract. This construct contains a deletion of two amino acids (C231 and M232) in the heart of the conserved cyclin box. It is unlikely that such conservative point mutations or such a small deletion seriously distort the structure of the protein. These results suggested that it may be necessary for cyclin A to bind to p34\(^{cdc2}\) in order to undergo programmed proteolysis. It has previously been shown that the destruction box of cyclin A is necessary for programmed proteolysis (Kobayashi et al., 1992; Lorca et al., 1992a).

In order to confirm this conclusion, we next studied the destruction of a number of C-terminally deleted cyclin A mutants (Figure 2A) which cannot bind to p34\(^{cdc2}\) (Kobayashi et al., 1992 and data not shown). As shown in Figure 2B, C and D, and summarized in Table I, constructs lacking 14, 79, 139, 194, 221 or 295 residues from the C-terminus of cyclin A are all much more stable in destruction assays than their wild type parent. CA194 cyclin A showed slight instability in the destruction assay (Figure 2D) and for this reason a parallel construct in which the essential residues within the destruction box of CA194 were mutated to alanine (see Materials and methods) was made (CA194-ATVA). CA194-ATVA showed similar instability in the destruction assay to CA194 (Figure 2D), thus the gradual proteolysis of CA194 appears to be non-specific, probably due to slight misfolding of the protein.

The result that cyclin A destruction requires p34\(^{cdc2}\) binding was confirmed by the analysis of two internal deletion mutants of cyclin A, ∆101–169 and ∆80–201 (formerly NA169 and NA201). These constructs are also unable to bind to p34\(^{cdc2}\), have intact destruction boxes (Kobayashi et al., 1992) and again are much more stable than wild type cyclin A (data not shown). ∆101–169 does display a slight instability in the destruction assay, like CA194, but this instability is not affected by mutation of the destruction box (data not shown). Therefore the instability of this mutant is probably due to non-specific proteolysis, as it is for CA194. These data and those from the degradation of cyclin B2-CA24 (see next section) suggest that some C-terminally truncated cyclins may be misfolded and are recognized by a constitutive cytoplasmic proteolysis pathway. This pathway is not dependent on the cyclin destruction box and occurs even when the specific cyclin proteolysis pathway is not activated (see next section).

From these results we conclude that the cyclin A destruction at the end of M phase requires p34\(^{cdc2}\) binding.

### Cyclin B2 destruction also requires p34\(^{cdc2}\) binding, but cyclin B1 destruction does not

At first sight, the results described in the previous section appear to disagree with those of Glotzer et al. (1991), who showed that short segments of the N-terminus of sea urchin cyclin B, which do not contain the p34\(^{cdc2}\) binding domain, could be destroyed in a cell cycle-regulated manner. To investigate further this apparent contradiction, we made mutants of cyclins B1 and B2 in which the final 24 residues of each protein were deleted. This removes part of the region shown to be required for the binding of p34\(^{cdc2}\) to cyclin A (Kobayashi et al., 1992). As expected, neither of these mutants could bind to p34\(^{cdc2}\) (data not shown). In agreement with the results of Glotzer et al. (1991), cyclin B1-CA24 was destroyed rapidly, albeit slightly later than wild type cyclin B1, when Ca\(^{2+}\) was added to the CSF-arrested extract, but remained stable in the absence of Ca\(^{2+}\) (Figure 3A). Remarkably, however, cyclin B2-CA24 was much more stable than wild type cyclin B2 when Ca\(^{2+}\) was added to the CSF-arrested extract (Figure 3B) and although this construct does show gradual proteolysis, this is unlike the rapid destruction of wild type cyclin B2. We believe this gradual proteolysis to be due to non-specific degradation, since cyclin B2-CA24 was destroyed at a similar rate in CSF-arrested extract in the absence of Ca\(^{2+}\). To check that the inability of cyclin B2-CA24 to undergo programmed proteolysis was due to its failure to bind to p34\(^{cdc2}\), and not simply because it folds incorrectly, we mutated R163 (the equivalent of R197 in cyclin A) to an alanine residue in full-length cyclin B2. This cyclin box mutant did not bind to p34\(^{cdc2}\) (data not shown) and was extremely stable in the standard destruction assay (Figure 4). Thus it appears that cyclins A and B2 require p34\(^{cdc2}\) binding in order to be destroyed at the end of M phase, whereas Xenopus cyclin B1 and sea urchin cyclin B do not.

The assay used by Glotzer et al. (1991) for studying cyclin

| Table I. Summary of the properties of the cyclin A constructs described in this paper |
|-------------------------------|-------------------------------|-------------------------------|-------------------------------|
| Mutant | p34\(^{cdc2}\) binding | Destruction box | Destruction |
| Wild type | + | + | + |
| c-myv tagged cyclin A | + | + | + |
| FVD mutant | + | + | + |
| ∆98−100 | ND | + | + |
| S136A | + | + | + |
| CA14 | + | + | + |
| CA24 | + | + | + |
| CA50 | + | + | + |
| CA79 | + | + | + |
| CA139 | + | + | + |
| CA194 | + | + | + |
| CA221 | + | + | + |
| CA295 | + | + | + |
| CA194-ATVA | + | + | + |
| CA221-ATVA | + | + | + |
| CA295-ATVA | + | + | + |
| NA133 | + | + | + |
| ∆88−144 | + | + | + |
| ∆90−147 | + | + | + |
| ∆102−158 | + | + | + |
| ∆109−161 | + | + | + |
| ∆101−169 | + | + | + |
| ∆80−201 | + | + | + |
| ∆36−245 | + | + | + |
| ∆109−161-ATVA | ND | - | - |
| ∆101−169-ATVA | ND | - | - |
| ∆231−232 | ND | - | - |
| R197A | + | + | + |
| D226A | + | + | + |
| R197K | + | + | + |
| D226E | + | + | + |
| ATVA | + | + | + |

ND, not determined.
Fig. 2. Destruction of C-terminally deleted mutants of cyclin A. (A) Schematic diagram of C-terminally deleted cyclin A mutants CA14, 79, 139, 194, 221 and 295 and the sea urchin cyclin B constructs 13–66 protein A (sea urchin cyclin B residues 13–66 fused to protein A) and 13–110Y (sea urchin cyclin B residues 13–110 followed by a single Y residue and no protein A) (Glotzer et al., 1991). (B) Destruction of CA14 cyclin A. Lanes 1–6, c-myc tagged wild type cyclin A; lanes 7–12 CA14 cyclin A. Asterisk on left indicates position of the endogenous B-type cyclins. The standard destruction assay was used (see Materials and methods). (C) Destruction of CA194 and CA139 cyclin A. Lanes 1–5, wild type cyclin A; lanes 6–9, CA139 cyclin A; lanes 10–13, CA194 cyclin A. Asterisk on left indicates position of the endogenous B-type cyclins. The standard destruction assay was used (see Materials and methods). (D) Destruction of CA194 cyclin A. The mRNAs encoding full-length c-myc tagged cyclin A (●); full-length c-myc tagged cyclin A with a mutated destruction box, ATVA (○); CA194 cyclin A (■) and CA194 cyclin A with a mutated destruction box, CA194-ATVA (□) were translated in nuclease treated CSF-arrested extract and the intensities of the cyclin bands on the autoradiographs were determined by scanning densitometry. A graph was plotted of the proportion of the cyclin A protein remaining against time (see Materials and methods).

Fig. 3. (A) Destruction of cyclin B1-CΔ24. Top panel, wild type cyclin B1. Middle panel, cyclin B1-CΔ24: left-hand seven lanes, Ca²⁺ added at time zero; right-hand seven lanes, no Ca²⁺ added to assay (i.e. no cyclin destruction triggered). Bottom panel, quantification by scanning densitometry: wild type cyclin B1 with Ca²⁺ added to assay (●), wild type cyclin B1 without Ca²⁺ added to assay (○), cyclin B1-CΔ24 with Ca²⁺ added to assay (■) and cyclin B1-CΔ24 without Ca²⁺ added to assay (□). (B) Destruction of cyclin B2-CΔ24. Top panel, wild type cyclin B2. Middle panel, cyclin B2-CΔ24: left-hand seven lanes, Ca²⁺ added at time zero; right-hand seven lanes, no Ca²⁺ added to assay (i.e. no cyclin destruction triggered). Bottom panel, quantification by scanning densitometry: wild type cyclin B2 with Ca²⁺ added to assay (●), wild type cyclin B2 without Ca²⁺ added to assay (○), cyclin B2-CΔ24 with Ca²⁺ added to assay (■) and cyclin B2-CΔ24 without Ca²⁺ added to assay (□). The standard destruction assay was used and the intensities of the cyclin bands on the autoradiographs were determined by scanning densitometry. Graphs were plotted of the proportion of the cyclin protein remaining against time (see Materials and methods).
destruction differed from the assay system used here in two important respects. First, Glotzer et al. used bacterially synthesized substrates labelled with 125I and secondly, destruction was assayed in interphase frog egg extracts in important respects. First, Glotzer et al. used bacterially synthesized indestructible sea urchin cyclin B constructs. It was therefore important to test these constructs in our assay system. One of the constructs contained sea urchin cyclin B residues 13–66 fused to protein A and the destruction boxes were mutated served as controls. We obtained these constructs from M.Glotzer and tested their destruction in an assay system in which their mRNAs were obtained these constructs from M.Glotzer and tested their destruction in an assay system in which their mRNAs were obtained these constructs from M.Glotzer and tested their destruction in an assay system in which their mRNAs were obtained these constructs from M.Glotzer and tested their destruction in an assay system in which their mRNAs were obtained these constructs from M.Glotzer and tested their destruction in an assay system in which their mRNAs were obtained these constructs from M.Glotzer and tested their destruction in an assay system in which their mRNAs were.

Some cyclin A mutants that have an intact destruction box and can bind to p34<sup>cdk2</sup> are nevertheless indestructible

The properties of some other mutant cyclin A constructs complicated the simple rule described above. Three clear examples were provided by the constructs Δ88–144, Δ90–147 and Δ102–158 cyclin A [previously named NΔ144, NΔ147 and NΔ158 (Kobayashi et al., 1992)] which have deletions of 57 or 58 residues between the destruction box and the start of the cyclin box (see Figure 5A). These three mutants were degraded extremely slowly compared with wild type cyclin A when Ca<sup>2+</sup> was added to CSF-arrested extract (Figure 6, first six lanes, and see Table I), although this destruction is specific since it did not occur in the assay in the absence of Ca<sup>2+</sup> (Figure 6, last six lanes). All three constructs have been shown to bind and activate p34<sup>cdk2</sup> with similar affinities and kinetics to wild type cyclin A (Kobayashi et al., 1992 and data not shown) and all contain intact destruction boxes (Figure 5A). A similar, but not identical result was obtained for the construct Δ109–161 cyclin A (previously NΔ161). This construct lacks 53 residues just upstream of the region of cyclin A required for binding to p34<sup>cdk2</sup> (see Figure 5A); it can activate p34<sup>cdk2</sup> (Kobayashi et al., 1992) and contains an intact destruction box. However, its proteolysis was 'unreliable' when compared with wild type cyclin A. In some assays, Δ109–161 was almost completely stable, like Δ88–144, Δ90–147 and Δ102–158 cyclin A, while in...
A major p34<sup>cdk2</sup> phosphorylation site is missing from ∆ 88–144 and ∆ 109–161 cyclin A

Another feature of the anomalously stable internal deletion constructs is that all four lack the tripeptide sequence SPM, a well-conserved motif ~30 residues upstream of the start of the p34<sup>cdk2</sup> binding region of almost all known cyclin A sequences, from molluscs to humans (see Figure 5A). Since this represented a potential phosphorylation site for proline-directed protein kinases, another hypothesis to explain the stability of some of the cyclin A mutant proteins was that phosphorylation at this S<sub>130</sub>M site was required for normal rapid destruction. Mutant cyclins would be stabilized in one of two ways: inability to bind to p34<sup>cdk2</sup>, or lack of the phosphorylation site. According to this model, phosphorylation of cyclin A would 'tag' it for destruction. If the protein were not tagged, it could not be destroyed. To investigate this hypothesis, we compared the phosphorylation of wild type cyclin A and mutants lacking the SPM motif. In vitro-transcribed mRNAs was translated in a mixture of CSF-arrested extract and rabbit reticulocyte lysate. The cyclin A was harvested with an anti-Xenopus cyclin A polyclonal antiseraum and incubated with [γ-<sup>32</sup>P]ATP. The products were analysed by SDS-PAGE followed by autoradiography, shown in Figure 8A; the <sup>32</sup>P-labelled cyclin A is shown in lanes 1–6 and the <sup>32</sup>P-labelled products in lanes 7–12.

The newly translated cyclin A binds to and activates p34<sup>cdk2</sup>, which is present at ~0.8 μM in egg extracts. This activated p34<sup>cdk2</sup> can then phosphorylate the cyclin subunit to which it is bound, so that the immunoprecipitates incorporate label from [γ-<sup>32</sup>P]ATP into cyclin A and into other tightly bound proteins. Since the egg extracts contained low levels of endogenous cyclin A protein and mRNA, these immunoprecipitates incorporated some <sup>32</sup>P even without added mRNA (Figure 8A, lane 12). When mRNA for cyclin A was added to these extracts, however, the total concentration of cyclin A protein was increased >15-fold after a 2 h incubation (determined by quantitative immunoblotting, data not shown). This accounts for the greatly increased <sup>32</sup>P labelling seen in lane 7 of Figure 8A compared with lane 12. By contrast, the cyclin A mutants, ∆ 101–169 and CA79, which cannot bind to p34<sup>cdk2</sup>, were not detectably phosphorylated in this assay (Figure 8A, lanes 10 and 11). Only the endogenous wild type cyclin A was labelled with <sup>32</sup>P in these lanes. Thus, the <sup>32</sup>P incorporation seen in the immunoprecipitates of kinase-binding constructs can probably be ascribed to p34<sup>cdk2</sup> or p33<sup>cdk2</sup>. Two of the internal deletion constructs, ∆ 88–144 and ∆ 109–161, which lack the SPM motif, can bind to p34<sup>cdk2</sup> and are phosphorylated in these reactions (Figure 8A, lanes 8 and 9), but they incorporated only ~10% as much <sup>32</sup>P as did wild type cyclin A (compare lanes 7, 8 and 9, 589
Fig. 8. Analysis of phosphorylation of cyclin A. (A) Phosphorylation of cyclin A mutants. Translations of cyclin A constructs were immunoprecipitated with anti-cyclin A antibodies. Left-hand panel, translation was carried out in the presence of [35S]methionine. The immunoprecipitates were eluted from the protein A-Sepharose with SDS sample buffer and analysed by SDS-PAGE and autoradiography (see Materials and methods). Right hand panel, translation was carried out in the absence of [35S]methionine. The immunoprecipitates were incubated with [γ-32P]ATP before elution with SDS sample buffer and analysis by SDS-PAGE and autoradiography. (B) Phosphoamino acid analysis of wild type and c-myc N133 cyclin A. The residue(s) that are phosphorylated by p34<sup>cdc2</sup> when p34<sup>cdc2</sup> bound wild type cyclin A or c-myc N133 cyclin A is immunoprecipitated and exposed to [γ-32P]ATP were analysed by acid hydrolysis of the phosphorylated proteins followed by thin layer electrophoresis in pH 3.5 buffer (see Materials and methods). The positions of the amino acid standards are indicated to the left of the figure. Lane 1, wild type cyclin A; lane 2, c-myc N133 cyclin A. (C) Phosphorylation of S136A mutant of cyclin A. Translations of c-myc tagged cyclin A constructs were immunoprecipitated with the anti c-myc monoclonal antibody, 9E10 (see Materials and methods). Left panel, [35S]methionine-labelled translations of cyclin A constructs; middle panel, immunoprecipitates of [35S]methionine-labelled translations; right panel, unlabelled immunoprecipitates of cyclin A translations were incubated with [γ-32P]ATP, eluted with SDS sample buffer and analysed by SDS-PAGE and autoradiography.

quantified by scanning densitometry). These results suggest that there are at least two phosphorylation sites in the N-terminus of Xenopus cyclin A, of which the major one is deleted in the mutant cyclin As, Δ88–144 and Δ109–161.

Although S136M is both a conserved motif and a potential site for proline directed phosphorylation, other serine and threonine residues are also deleted from Δ88–144 and Δ109–161. For this reason, it was necessary to check that the phosphorylation detected in the assay described above was indeed due to phosphorylation on S136, and not on some other nearby serine or threonine.

**Xenopus cyclin A is phosphorylated on S136**

To check that the phosphorylated residue in these assays was serine, we carried out phosphoamino acid analysis of phosphorylated wild type cyclin A. Figure 8B (lane 1) shows that the phosphoamino acid in cyclin A is indeed mainly serine, with only minor phosphorylation of threonine. The phosphorylated threonine residue was in the N-terminus of the cyclin A protein, since the N-terminally deleted construct c-myc NΔ133 (see Materials and methods) contained only phosphoserine (Figure 8B, lane 2).

To check that S136 in the sequence SPM was the major in vitro phosphorylation site in Xenopus cyclin A, it was replaced with an alanine in a c-myc tagged wild type cyclin A (S136A cyclin A). Replacement of S136 with alanine strongly reduced the phosphorylation of this construct but did not eliminate it (Figure 8C, compare lanes 9 and 10). This is probably due to phosphorylation at the other site(s) in the N-terminus of cyclin A. To check this, the same serine (136) to alanine mutation was made in the construct c-myc NΔ107, which lacked the 107 N-terminal residues of cyclin A. This construct was not phosphorylated in the immunoprecipitation assay (Figure 8C, compare lanes 11 and 12).

To confirm that S136 was the major phosphorylation site in cyclin A, wild type cyclin A protein was doubly labelled with [γ-32P]ATP and [35S]methionine, isolated and digested with V8 endopeptidase as described in Materials and methods. The resulting peptides were resolved by thin layer electrophoresis followed by chromatography. Wild type cyclin A gave two major [γ-32P]ATP-labelled spots on the autoradiograph, which were eluted from the TLC plate and used for progressive Edman chemistry to identify the positions of both the phosphorylated residue and any 35S-labelled residues within the peptide (see Materials and methods). The data from this analysis (data not shown, but see Materials and methods for a more detailed description).
confirmed that the site in cyclin A that is phosphorylated by the assay described above, is indeed S136.

Phosphorylation on S136 is not required for cyclin destruction

To test whether phosphorylation on S136 was required for the degradation of cyclin A, mRNA encoding full-length S136A mutant cyclin A protein was translated in CSF-arrested extract, followed by addition of Ca^2+ to trigger cyclin destruction. Proteolysis of the mutant followed the same kinetics as wild type cyclin A (Figure 9). Thus, S136 is not required for the destruction of cyclin A. This eliminates the hypothesis that phosphorylation at this site could account for the requirement for binding to p34^cd2.

Discussion

The major finding of these studies is that mutant cyclin A proteins that cannot bind to p34^cd2 are stable when cyclin proteolysis is triggered by the addition of Ca^2+ to Xenopus egg extracts. It is possible that these mutations cause severe conformational changes in the cyclin A protein which simultaneously prevent binding to p34^cd2 and impair their recognition by the cyclin destruction machinery. However, it is highly unlikely that all the mutations introduced into cyclin A which prevent binding to p34^cd2 (Kobayashi et al., 1992) cause serious conformational changes. Two constructs in particular, one in which R197 is mutated to a lysine residue (R197K) and the other in which D226 is mutated to glutamic acid (D226E), neither bind to p34^cd2 nor get destroyed. It seems improbable that these extremely conservative changes would have such a drastic effect on cyclin A conformation as to render the mutant proteins unrecognizable by both p34^cd2 and the cyclin destruction machinery. This suggests either that the recognition of cyclin A by the destruction machinery requires the simultaneous recognition of p34^cd2, or alternatively that when cyclin A binds to p34^cd2 it alters its conformation so as to make the destruction box accessible for recognition by the proteolytic system. Our data do not allow us to distinguish between these hypotheses.

We were somewhat surprised that cyclin A must bind to p34^cd2 in order to be destroyed, as previous studies on the stability of sea urchin cyclin B in Xenopus extracts did not suggest any such requirement (Glotzer et al., 1991). To investigate this point further, we made C-terminal deletions of both Xenopus cyclins B1 and B2, which removed their ability to bind to p34^cd2. As might have been expected from the results of Glotzer et al. (1991), cyclin B1-CA24 underwent quite normal programmed proteolysis upon addition of Ca^2+ and was stable in the absence of added Ca^2+. To our surprise, however, cyclin B2-CA24 behaved more like non-p34^cd2 binding mutants of cyclin A, and did not show the rapid destruction of wild type cyclin B2. Cyclin B2-CA24 appeared to undergo slow, non-specific proteolysis which occurred at a similar rate in the presence or absence of Ca^2+. This suggests that cyclin B2-CA24 may not be folded correctly, which could explain its indestructibility. However, a point mutant of cyclin B2 (R163A) that did not bind to p34^cd2 was completely stable, both in the presence and absence of Ca^2+. This mutation is unlikely to cause great conformational changes to the protein and it therefore appears that like cyclin A, cyclin B2 must be bound to p34^cd2 in order to be destroyed at the end of M phase. This result is in agreement with the results of Vanderwelden and Lohka (1993) who showed that the N-terminus of cyclin B2 was not destroyed when Ca^2+ was added to a CSF-arrested extract, in contrast to wild type Xenopus cyclin B2. We thus conclude that the recognition of cyclins A and B2 for destruction differs from that of cyclin B1. Differences in the proteolysis of cyclins A and B have been noted previously; the destruction of cyclin A normally occurs before that of cyclin B in vivo (Luca and Ruderman, 1989; Minshull et al., 1990; Whitfield et al., 1990; Hunt et al., 1992) and colchicine arrests cell entry into mitosis at a point where cyclin A is being continually destroyed but the destruction of cyclin B is delayed (Whitfield et al., 1990; Hunt et al., 1992). It is quite surprising that the proteolysis of cyclins B1 and B2 should show such a marked difference, considering how similar they are. It should, however, be noted that the N-termini of Xenopus cyclins B1 and B2 show only 35% identity up to the beginning of the cyclin box (and only 23% in the first 80 residues), compared with 62% identity throughout the rest of the protein, which may explain the differences. Further mutational studies will be required to understand this.

Cyclin A, however, needs more than an intact destruction box and the ability to bind to p34^cd2 for normal proteolysis. Three mutant cyclin A proteins, Δ 88—144, Δ 90—147 and Δ 102—158, possess intact destruction boxes and can bind to (and activate) p34^cd2; however, all three constructs get destroyed extremely slowly compared with wild type cyclin A. We estimate that the rates of destruction (taken at the point of most rapid destruction, see Materials and methods) of these mutants are <10% of that of wild type cyclin A. Δ 109—161 cyclin A can also bind to p34^cd2 and contains an intact destruction box, but it shows slower and less reliable proteolysis compared with wild type cyclin A protein. Two hypotheses could account for these anomalous examples. (i) Given that dual recognition of the destruction box and p34^cd2 is required for the correct degradation of cyclin A, the spatial relationship between the two recognition elements might be important. (ii) The deleted region in the constructs may contain additional element(s) whose presence is important for programmed proteolysis. We tested the second hypothesis quite extensively, because the region deleted in the anomalously stable cyclins contained at least two conserved motifs which might have been important for destruction. The first motif has the sequence PTVYVDE (the residues in bold are conserved in all examples of cyclin A except for Drosophila), and is situated 40–50 amino acids downstream of the destruction box. It is deleted in Δ 88—144 and Δ 90—147. When the conserved residues F, V and D
were mutated to alanine, or residues V, D and E were deleted, however, the mutant proteins were destroyed with near normal kinetics.

The tripeptide sequence, SPM, is the second conserved motif deleted from the cyclin A mutants ∆ 99−144, ∆ 90−147, ∆ 102−158 and ∆ 109−161. This SPM motif is conserved in all known examples of cyclin A at similar (although not identical) positions in the molecule, ~50 residues upstream of the start of the p34<sub>cdk</sub> binding domain. We showed here that S136 is a major in vitro site for phosphorylation by p34<sub>cdk</sub>. When we mutated S136 to alanine, however, the mutant cyclin A protein was destroyed at exactly the same time and with exactly the same kinetics as wild type cyclin A. Thus phosphorylation on this residue is clearly not required for the destruction of cyclin A. It has previously been shown that phosphorylation of Xenopus cyclins B1 and B2 is not required for destruction (Izumi and Mallifer, 1991), but the relationship between phosphorylation and destruction has not been studied for cyclin A. As the requirements for cyclin A and B2 destruction differ from those of cyclin B1, we considered it important to investigate this point. It should be noted that phosphorylation on S136 and the minor threonine phosphorylation in the N-terminus of cyclin A are not necessarily the only phosphorylations to take place on cyclin A in vivo. Our assay, which was performed on immunoprecipitates, relied on de novo phosphorylation or the turnover of a pre-existing phosphate. If a site was stably phosphorylated by endogenous (and therefore non-radioactive) ATP in the CSF-arrested extract during translation of cyclin A mRNA, it would not incorporate [γ-<sup>32</sup>P]ATP after immunoprecipitation. In addition, our assay only reliably detects the phosphorylation of cyclin A by the p34<sub>cdk</sub> to which it is bound. We cannot exclude the possibility that cyclin A is phosphorylated by other protein kinases, some of which may target it for destruction.

What other features necessary for destruction might be deleted in ∆ 88−144, ∆ 90−147 and ∆ 102−158? Glotzer et al. (1991) found evidence that the region between residues 54 and 66 in sea urchin cyclin B was necessary for its degradation, in addition to an intact destruction box, possibly because the lysine residues in this region were required as ubiquitin acceptor sites. Two lysine residues were deleted from cyclin A mutants ∆ 88−144 and ∆ 90−147 and one from ∆ 102−158, but we do not consider this very significant, for the following reasons. First, the positioning of lysine residues in this region is not conserved between different species of cyclin A and moreover, two lysine residues remain between the destruction box and the deleted region in these constructs. Finally, chicken cyclin A does not have any lysine residues in the 50 amino acids following its destruction box, and only two in the next 77 residues, although presumably it is degraded in the usual fashion.

Thus, we did not identify any conserved motifs or residues between the destruction box and the start of the cyclin box whose presence was essential for normal rapid proteolysis. It is possible that the precise spacing of the destruction box with respect to the cyclin box is important, because ∆ 109−161 cyclin A, which can be degraded (although not in an entirely wild type manner), has the destruction box slightly further away from the cyclin box than those of the other anomalously stable mutants. It would, perhaps, be worthwhile to insert new sequences into these mutants to place the destruction box further away from the cyclin box. However, deciding what sequences to insert would be difficult, as we do not know the structure of the cyclin protein and one would not know whether the sequence inserted was causing distortion of the protein. It is also possible that the conformation of the destruction box and its surroundings are crucial and are significantly altered by the exact location of the deletions in ∆ 88−144, ∆ 90−147, ∆ 102−158 and ∆ 109−161 cyclin A mutants.

We had not expected to find that cyclin A needed to be bound to p34<sub>cdk</sub> in order to be destroyed, and are still confused by the anomalous stability of some of the internal deletion mutants, even after many experiments to test what we considered to be reasonable explanations. It is difficult to distinguish between a model in which the destruction apparatus recognizes both cyclin A and p34<sub>cdk</sub>, and one in which binding to p34<sub>cdk</sub> causes some sort of conformational change in the presentation of the destruction box. If the latter is true, small changes in sequence could have large effects on stability. The problem in this case is to explain why ∆ 109−161 is, at times, destructible and why the cyclin B1 proteolysis system shows so much less stringent requirements for specific recognition than those of cyclins A and B2. Elucidation of these points will probably require both structural information about the conformation of cyclins bound to p34<sub>cdk</sub> and a more detailed knowledge of the recognition and effector components of the mitotic protease.

Perhaps the most intriguing question of all is this: what is the normal signal for cyclin A destruction? Does the disappearance of cyclin A mark the successful passage through a checkpoint, the completion of some essential cellular process? Failure to destroy cyclin A certainly delays the onset of anaphase (Luca et al., 1991), but would its premature loss accelerate passage through mitosis?

**Materials and methods**

**Cyclin A constructs**

The construction and structure of N- and C-terminal deletion mutants of cyclin A were described by Kobayashi et al. (1992).

- **c-myc NA107** cyclin A was constructed using PCR. A 5' primer was constructed with a BamHI site upstream of the desired start of the protein and this was used in conjunction with a 3' primer which recognized a sequence downstream of the naturally occurring Nsil site in cyclin A. The fragment of cyclin A thus generated was digested with BamHI and Nsil and inserted between the BamHI and Nsil sites of c-myc tagged cyclin A (Kobayashi et al., 1992). The N-terminus of this construct therefore had the following sequence: MDPEKIKISEELNLSF, followed by residue 107 of cyclin A and the rest of the cyclin A protein. The bold portion is the epitope recognized by the 9E10 monoclonal antibody (Evan et al., 1986). c-myc NA133 was constructed in a similar way using non-c-myc tagged NA133, and its N-terminus had the following sequence: MDPEKIKISEELNLSAFMDPM, followed by residue 133 of cyclin A.

Mutagenesis of individual residues within cyclin A was carried out using PCR essentially as described by Horton and Pease (1991). The altered segments were checked by sequencing the double-stranded template DNA with the United States Biochemicals Sequenase kit (Cleveland, OH).

The long C-terminal deletions of cyclin A, CA149, CA221 and CA295, were constructed using PCR. 3' primers were constructed to produce a stop codon at the desired place, followed by a BamHI restriction enzyme site and were used in conjunction with 5' primers which annealed to the T7 RNA polymerase promoter upstream of the parent cyclin A construct. The PCR product was digested with Nsil and BamHI and inserted between the Nsil and BglII sites of c-myc tagged cyclin A (Kobayashi et al., 1992). The amino acid sequences of the C-termini of the constructs are as follows: CA221: ...,DSNV*; CA221: ...,ITSAM*; CA295: ...,YLAN*.

The destruction box mutants of these constructs were made using the same primers, but instead of using wild type c-myc tagged cyclin A, the template used for the PCR was a c-myc tagged cyclin A construct in which the invariant residues within the destruction box, 841 and 144, had been mutated to alanine residues as previously described (Kobayashi et al., 1992).
The vinyl-100 mutant of cyclin A was constructed using the Amersham oligonucleotide-directed in vitro mutagenesis kit (Amersham, UK). The oligonucleotide:

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GGTTCCTGTTGGATAGACTGTAAAAC
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was used to delete nucleotides N GGTA GGAG (position of the deletion is indicated by the arrow) from c-myc tagged cyclin A. This deletes the tripeptide V98 D99 E100 from the protein.

**Cyclin B constructs**

CA24 cyclins D1 and B2 were constructed using PCR. 3' primers were constructed to produce a stop codon at the desired position, followed by a BamHI restriction enzyme site and were used in conjunction with 5' primers that produced a NcoI site at the start of the coding sequence of the cyclin protein. The PCR product was digested with NcoI and BamHI and inserted between the NcoI and BstXI sites of c-myc tagged cyclin A, thus giving the cyclin B mutants the 5' and 3' untranslated regions of this construct, which gives good translation of the proteins (Kobayashi et al., 1992). The amino acid sequences of the C-termini of the constructs are as follows: cyclin B1-CA24: ...KVASS*; cyclin B2-CA24: ...KVASS*.

**Mature cyclin B mRNAs**

The mRNAs encoding the cyclin A constructs to be tested were translated in 1:1 mixtures of CSF-arrested extract and nuclease-treated rabbit reticulocyte lysate for 2 h at 23°C. If the products were to be 35S-labelled, then 1 mM Cef (final concentration) of [35S]methionine was included in the incubation. Either 2 μl of polyonal rabbit anti-cyclin A serum (Kobayashi et al., 1991) or 1 μl of protein A-purified monoclonal anti-c-myc antibody, 9E10 (17 mg/ml) (Evan et al., 1986), was then added to 5 μl of the extract, which was diluted 4- to 6-fold with bead buffer (10 mM Tris–CI pH 7.4, 5 mM NaF, 250 mM NaCl, 5 mM EDTA, 5 mM EGTA, 0.1% Nonidet P-40, 1 μg/ml leupeptin, 1 μg/ml soybean trypsin inhibitor and 1 mM benzamidine). This was incubated on ice for 1 h. The immunocomplexes were diluted with 100–200 μl of bead buffer and recovered on 15–20 μl of protein A-Sepharose (Pharmacia, Uppsala, Sweden) for 30 min at room temperature. The immunocomplex loaded beads were washed four times with bead buffer and transferred into a fresh tube. [35S]Methionine-labelled immunocomplexes were eluted with SDS sample buffer and analysed by SDS–PAGE and autoradiography. Un labelled immunocomplexes were used for phosphorylation assays as described below.

**Phosphorylation assays**

Unlabelled immunocomplexes bound to protein A–Sepharose (see above) were washed twice with Cicerelli buffer (50 mM β-glycerophosphate, 7 mM NaF, 0.3 mM EDTA, 15 mM MgCl2 and 2 mM DTT, pH 7.3) before incubation in 10 μl Cicerelli buffer with 0.5 μg of the cyclin antibody (Kobayashi et al., Amersham, UK) for 30 min at 20°C. The bound proteins were eluted with SDS sample buffer and analysed by SDS–PAGE and autoradiography.

**Phosphoamino acid analysis**

The mRNA encoding c-myc tagged wild type cyclin A and c-myc NA133 was translated, without [35S]methionine, in 1:1 mixtures of frog egg extract and nuclease-treated reticulocyte lysate for 2 h at 23°C. The translation products were immunoprecipitated with the anti-c-myc antibody 9E10 (Evan et al., 1986) and collected on protein A–Sepharose beads (Pharmacia, Uppsala, Sweden). The bead-bound immunoprecipitates were washed twice with Cicerelli buffer and incubated with [32P]ATP in 10 μl Cicerelli buffer for 30 min at 20°C. The proteins were eluted with SDS sample buffer and analysed by SDS–PAGE, followed by electrophoretic transfer to Immobilon membrane (Millipore, Bedford, MA). Phosphoamino acid analysis was carried out according to Kamps (1991), using electrophoresis in pH 3.5 on silica thin layer plates to resolve the phosphoamino acids.

**Phosphopeptide mapping**

Phosphopeptide mapping was carried out essentially to the protocol of Luo et al. (1991) for tryptic peptide mapping of immobilized proteins, except that the protein was digested with 6 μg of staphylococcal V8 protease in 50 mM ammonium bicarbonate–5% acetonitrile overnight. The resulting peptides were analysed by electrophoresis on thin layer silica plates in pH 4.72 buffer and chromatography in phospho chromatography buffer, according to Boyle et al. (1991). The plate was then autoradiographed. To ensure that only [γ-32P]ATP-labelled peptides and not [35S]methionine-labelled spots were detected on the autoradiograph, a sheet of paper was placed between the silica plate and the film.

**Edman degradation of phosphopeptides**

The silica containing the phosphorylated peptides was scraped off the plate and the peptides were extracted by incubation in 100 μl of 7% ammonium–50% methanol for 2 h. This was filtered through a 0.45 μm Ultrafree unit and freeze dried. The freeze-dried material was dissolved in 30 μl of 1:1 (v/v) acetonitrile–water and covalently attached to an anly amine coated PVDF membrane (Coall et al., 1991). Edman chemistry was performed for 20 cycles with the resulting phenyl thiohydantoin residue being diverted for collection in a fraction collector. The radioactivity in these fractions was determined in a scintillation counter.

When this procedure was carried out on the spots resulting from V8 endoproteinase digestion of phosphorylated cyclin A, one of these spots released ~30% of its radioactive material at position number 2 in the peptide (20 cycles in total). The procedure was repeated with protein labelled with both [γ-32P]ATP and [35S]methionine. Edman degradation of the same phosphorylated peptide showed that there was a methionine residue at position number 4. There are only two predicted peptides in V8-digested c-myc tagged cyclin A with a serine residue at position number 2, only one of which has a methionine residue at position number 2. This peptide contains S126 as its second residue. Thus we concluded that S126 is the major site of phosphorylation against time. To analyse the differing rates of cyclin destruction, the slopes of the graphs at the points of most rapid destruction were determined.
phosphorylation in cyclin A in the assay described above and in the results section.

Acknowledgements

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References


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Note added in proof

Since the experiments described in this paper were performed, we have made a Xenopus cyclin B1 mutant in which arginine 168 (equivalent to R197) in cyclin A and R163 in cyclin B2 was changed to an alanine residue (cyclin B1 R168A). This mutant does not bind to p34cdc2. Much to our surprise, and in contrast to the CLB4 cyclin B1 mutant described in this paper, cyclin B1 R168A is not destroyed in our standard destruction assay.

Note added in proof
Identification of the Domains in Cyclin A Required for Binding to, and Activation of, p34^{cdc2} and p32^{cdk2} Protein Kinase Subunits

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The binding of cyclin A to p34^{cdc2} and p32^{cdk2} and the protein kinase activity of the complexes has been measured by cell-free translation of the corresponding mRNA in extracts of frog eggs, followed by immunoprecipitation. A variety of mutant cyclin A molecules have been constructed and tested in this assay. Small deletions and point mutations of highly conserved residues in the 100-residue "cyclin box" abolish binding and activation of both p34^{cdc2} and p32^{cdk2}. By contrast, large deletions at the N-terminus have no effect on kinase binding and activation, until they remove residues beyond 161, where the first conserved amino acids are found in all known examples of cyclin A. At the C-terminus, removal of 14 or more amino acids abolishes activity. We also demonstrate that deletion of, or point mutations, in the cyclin A homologue of the 10-residue "destruction box," previously described in cyclin B (Glotzer et al., 1991) abolish cyclin proteolysis at the transition from M-phase to interphase.

INTRODUCTION

Cell cycle transitions require the activity of protein kinases that contain catalytic subunits encoded by members of the cdc2 gene family together with a regulatory and activating subunit corresponding to a member of the cyclin family (reviewed by Nurse (1990) and Pines and Hunter (1990). The association of a mitotic cyclin with p34^{cdc2} appears to be necessary to turn on its protein kinase activity (Solomon et al., 1990; Desai et al., 1992), and the destruction of cyclin turns off the protein kinase activity of p34^{cdc2} just before the onset of anaphase (Luca and Ruderman, 1989; Murray et al., 1989; Luca et al., 1991). It is now known that there is more than one cdc2 gene in higher eukaryotes (Lehner and O'Farrell, 1990; Elledge and Spottswood, 1991; Paris et al., 1991; Tsai et al., 1991) and at least eight different kinds of cyclin (mitotic cyclins A, B1, and B2 in vertebrates; G1 cyclins CLN1/2 and CLN3 in budding yeast, and three cyclin homologues known as cyclins C, D (CYL1 or PRAD1) and E have recently been discovered in humans (Hadwiger et al., 1989; Léopold and O'Farrell, 1991; Lew et al., 1991; Matsushima et al., 1991; Motokura et al., 1991; Xiong et al., 1991). It is not yet clear what combinations of cyclin plus p34^{cdc2}-like subunits can associate to form active protein kinases or what special properties different cyclin subunits confer on their partners. Indeed, for some of the most recently discovered cyclins, particularly C and D, the evidence for association with protein kinase subunits is at present somewhat indirect. It is thought likely that cyclins are in some sense targeting subunits for p34^{cdc2} as well as controlling the timing of turning on and off its kinase activity (Minshull et al., 1990; Solomon et al., 1990). So far, most of the known substrates for cyclin/cdc2 protein kinases are able to be phosphorylated by any combination of cyclin and cdc2 subunit (Minshull et al., 1990) provided their kinase activity has been turned on by the appropriate phosphorylation state of the cdc2 subunit (Ducommun et al., 1991; Featherstone and Russell, 1991; Gautier et al., 1991; Gould et al., 1991; Parker et al., 1991, 1992; Strausfeld et al., 1991; Kumagai and Dunphy, 1992; Millar and Russell, 1992) (for reviews see Fleig and Gould, 1991 and Maller, 1991). The cellular targets of

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these kinases are not well characterized, however, and at the relatively low concentrations of kinases and their target substrates found in cells, the specificity may be much higher.

The sequence homology between different members of the cyclin family is not extensive and is largely confined to a stretch of 100 residues in the middle of the linear sequence that is commonly known as the "cyclin box" (Nugent et al., 1991). Even in this core, however, only five well-spaced residues (RDLKF) show complete conservation in all cyclins from yeast to man, and in Xenopus cyclin A and p32 but larger deletions, which encroach on the first conserved residues, cause complete loss of activity. By contrast, removal of as little as 14 amino acids at the N-terminus of the mitotic cyclins A and B, which is required for their regulated sudden destruction at the metaphase-anaphase transition (Murray et al., 1989; Glotzer et al., 1991; Lorca et al., 1992). As a first step to investigating structure-function relationships in cyclin A, we have made a number of N- and C-terminal deletions of Xenopus and bovine cyclin A1 and various small deletions and point mutations in the cyclin box. We have tested these constructs for their ability to promote Xenopus oocyte maturation, for their ability to bind and activate p34\(^c2\) or p34\(^d2\)-like proteins, it is likely that these conserved residues and the region spanned by them are important in the cyclin-p34\(^c2\) interaction, and if this is so, questions are raised about the function of the remaining 300 or so amino acids. One other clearly marked domain has been defined in the N-terminus of the mitotic cyclins A and B, which is required for their regulated sudden destruction at the metaphase-anaphase transition (Murray et al., 1989; Glotzer et al., 1991; Lorca et al., 1992). As a first step to investigating structure-function relationships in cyclin A, we have made a number of N- and C-terminal deletions of Xenopus and bovine cyclin A1 and various small deletions and point mutations in the cyclin box. We have tested these constructs for their ability to promote Xenopus oocyte maturation, for their ability to bind and activate p34\(^c2\) and p32\(^d2\), and for their ability to be destroyed. We find that the first 161 residues of cyclin A are dispensable for binding to and activation of p34\(^c2\), but larger deletions, which encroach on the first conserved residues, cause complete loss of activity. By contrast, removal of as little as 14 amino acids at the C-terminus of cyclin A renders it completely inactive. Point mutations of the completely conserved R197 and D226 and deletions of two or more residues from the cyclin box cause serious or complete loss of activity. There is a perfect correlation between the ability of cyclin A to bind with p34\(^c2\) and its activity, both in vivo and in vitro. We also confirm that the N-terminal destruction box in cyclin A is necessary for programmed destruction triggered by addition of Ca\(^{2+}\) to an egg extract (Glotzer et al., 1991; Luca et al., 1991; Lorca et al., 1992).

Finally, although we and others were previously unable to detect newly synthesized cyclin A associated with immunoprecipitates of p32\(^d2\) (Minshull et al., 1990; Gabrielli et al., 1992), we now report that when Xenopus cyclin A1 and p32\(^d2\) are capable of binding together to give an active histone kinase whose concentrations are increased by cell-free translation of added mRNA. By contrast, B-type cyclins do not appear to form complexes with p32\(^d2\) under the same conditions. The cyclin A mutations that abolish p34\(^c2\)-binding also abolish p32\(^d2\)-binding.

MATERIALS AND METHODS

Construction of Cyclin A Deletions

A full-length clone of Xenopus cyclin A1 in pGEM1 (clone XL4) (Minshull et al., 1990) was digested at the unique EcoRI site. The linearized DNA (10 \(\mu\)g) was incubated with 5 \(\mu\)l of Bal 31 nuclease (Boehringer, Mannheim, Germany) in a final volume of 100 \(\mu\)l in 20 mM tris-(hydroxymethyl)aminomethane (Tris)\(^{-}\)Cl, 100 mM NaCl, 5 mM CaCl\(_2\), 5 mM MgCl\(_2\), 1 mM EDTA, pH 8.0, at 30\(^\circ\)C. Samples of 15 \(\mu\)l were taken at 2-3 min intervals into an equal volume of 20 mM ethylene glycol-bis(\(\beta\)-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA); pH 7.3, to stop further digestion. The DNA was repaired with T4 DNA polymerase in the presence of 100 \(\mu\)M dNTPs, ligated with T4 DNA ligase, and introduced into Escherichia coli strain TG1 by standard Ca\(^{2+}\)-mediated transformation. DNA was prepared from individual colonies and the size of deletion assessed by restriction enzyme analysis followed by agarose gel electrophoresis. Selected clones were transcribed (see below) and translated in the nuclelease-treated rabbit reticulocyte lysate, and the \(^{15}\)S methionine-labeled products analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The clones that gave translation products of the expected size (as opposed to prematurely terminated products resulting from out-of-frame deletions) were selected for further analysis and were sequenced with an oligonucleotide with the sequence 5' ACAAGCT-TCAGTITTCGCT, corresponding to the amino acid sequence GKLQ LV of the cyclin A1 construct in which the first 21 residues of cyclin A1 were replaced with 15 amino acids of the c-myc epitope described by Evan et al. (1986), and the translational leader was derived from influenza virus NS protein as described by Dasso and Jackson (1989). The plasmid backbone was derived from pGEM2 (Promega, Madison, WI), modified by removal of the 252 bp EcoRI-NcoI fragment, in whose place was added an EcoRI-EcoRV fragment from a pP8 construct that included the T7 transcription terminator (the idea was to avoid the linearization step in the transcription protocol, but we found that there was too much readthrough, so we did not exploit this feature in practice). The sequence from the start of the T7 promoter to the start of the cyclin sequence was as follows:

The c-myc tag sequence is underlined. In this construct there is a unique EcoRI site at the 3' end of the cyclin 3' UTR (untranslated region), and the Nhe I, BamHI, BamHI, Nco I, and Bgl II in the sequence above are all unique in the plasmid (addition of the T7 terminator

We refer to cyclin A1 to describe the form of Xenopus cyclin A used throughout this paper. Xenopus has at least one other cyclin A gene, which may have different properties (M. Howell and T.H., unpublished data).

Abbreviations used: CSF, cytostatic factor; EGTA, ethylene glycol-bis(\(\beta\)-aminoethyl ether)-N,N,N',N'-tetraacetic acid; GVBD, germlinal vesicle breakdown; HEPES, N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid; IgG, immunoglobulin G; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; UTR, untranslated region of mRNA.
Table 1. Details of cyclin deletion constructs

<table>
<thead>
<tr>
<th>Name</th>
<th>Length of deletion</th>
<th>Location of deletion</th>
<th>Junction sequence</th>
</tr>
</thead>
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<tr>
<td>Mutants of Xenopus cyclin A1</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Δ21</td>
<td>21</td>
<td>1–21</td>
<td>MEKLIUSEDLN*SAF</td>
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<td>Δ62</td>
<td>39</td>
<td>24–62</td>
<td>ASSA/PKS</td>
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<td>57</td>
<td>88–144</td>
<td>NPAF/QTSP</td>
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<td>58</td>
<td>90–147</td>
<td>AVPA/PEDD</td>
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<td>57</td>
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<td>VDEP/VAYS</td>
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<td>229–235</td>
<td>LDRF/RGKL</td>
</tr>
<tr>
<td>Δ257</td>
<td>10</td>
<td>228–237</td>
<td>YLDR/KLQL</td>
</tr>
<tr>
<td>Δ261</td>
<td>16</td>
<td>226–261</td>
<td>MNYL/DEFY</td>
</tr>
<tr>
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<td>14</td>
<td>405–419</td>
<td>KTTKYM*</td>
</tr>
<tr>
<td>CA24</td>
<td>24</td>
<td>395–419</td>
<td>QAQQAA*</td>
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<tr>
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<td>50</td>
<td>369–419</td>
<td>AFYGIA*</td>
</tr>
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<td>79</td>
<td>340–419</td>
<td>KXVPSL*</td>
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<tr>
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<td>219–419</td>
<td>HLTETL*</td>
</tr>
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<td>Mutants of Bovine cyclin A*</td>
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</tr>
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<td>AFEO/VDDT</td>
</tr>
<tr>
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<td>255–275</td>
<td>EEEY/QVLR</td>
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<td>24</td>
<td>255–278</td>
<td>SKEO/OMEH</td>
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<td>CA16</td>
<td>16</td>
<td>403–419</td>
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</tr>
</tbody>
</table>

* Underlined sequence denote the c-myc tag. This leader was present in all the Xenopus C-terminal deletion series of constructs except CA200.

* Italic indicates altered residues from vector sequences; the wild-type sequence is LDS.

* M replaces V.

* An asterisk denotes a termination codon.

* The numbering system is that of the equivalent Xenopus sequences.

* The S is a T in the wild-type sequence.

* The G is an R in the wild-type sequence.

removed an Nhe I site in the vector), as are the internal EcoRV, HindIII, Nsi I, and Bcl I sites present in Xenopus cyclin A1.

The C-terminal deletion CA200 was derived from the Nsi I series of Bal 31 deletions. Closure of the deleted molecule formed a termini­nation codon (Table 1).

Construction of Point Mutations

Mutagenesis of individual residues in cyclin A1 was either performed with the Amersham (Arlington Heights, IL) Kit, or with a polynucleotide chain-based reaction strategy as described by Horton and Pease (1991). The altered segments were checked by sequencing the double-stranded template DNA with the United States Biochemicals Sequenase kit (Cleveland, OH).

Construction and Expression of Bovine Cyclin A

A partial clone encoding all but the first 24 residues (compared with the sequence of human cyclin A) of bovine cyclin A was isolated from a bovine lymphocyte cDNA library. The large Sau3AI fragment from this clone was inserted into the in-frame BamHI site of a vector derived from pET3a (Studier et al., 1990) encoding the IgG (immunoglobulin G) binding domain of staphylococcal protein A (derived by PCR from pRIT2T [Pharmacia, Uppsala, Sweden]). This construct encoded protein A at the N-terminus and bovine cyclin A at the C-terminus minus the first 76 residues of the cyclin A (the junction sequence is FQSLKDPPGNSRLPDINEYYPVPP, cyclin residues underlined). The fusion protein comprised 620 amino acids and was soluble when induced with 0.1 mM isopropyl-β-D galactoside (IPTG) at an A280 of 0.4 in E.coli / BL21 (DE3) at 20° overnight. After a freeze-thaw cycle, the bacteria were digested with lysozyme, and the protein A-cyclin A constructs were purified from the 100 000 g supernatant by chromatography on IgG Sepharose as described by Solomon et al. (1990). Mutations were introduced into the construct by Bal 31 deletions at a unique Acc I site, and deletion of 16 residues at the C-terminus was performed by means of the polymerase chain reaction. The structures of these constructs are summarized in Table 1 and Figure 5.

Preparation of mRNA for Translation

Plasmid DNA was linearized at the end of the 3' UTR with the appropriate restriction enzyme (BamHI for the untagged cyclin A constructs, EcoRI for the tagged ones) and used as templates for transcription in vitro using T7 RNA polymerase essentially as described by Nielsen and Shapiro (1986). Capped mRNAs were made in 50-μl reactions that contained 4–5 μg of DNA, 40 mM Tris-Cl, pH 8.0, 15 mM MgCl2, 5 mM dithiothreitol (DTT), 1 μM each of ATP, cytidine triphosphate (CTP), and uridine triphosphate (UTP), 0.1 mM guanosine triphosphate (GTP), 0.5 mM mGTP/pGpp/50G (New England Biolabs, Beverly, MA), 50 units of RNasin (Boehringer), and 25 units of T7 RNA polymerase (purified from E.coli BL21 harboring PAR1219 according to a protocol supplied by Dr. J.J. Dunn, Brookhaven National Laboratory, Upton, New York; see Studier et al., 1990) or purchased from New England Biolabs). After 30 min at 37°, the GTP concentration was increased to 1 mM and incubation continued for a further 60 min. The reactions were extracted twice with phenol/chloroform, and the RNA was recovered by ethanol precipitation. The RNA pellets were resuspended in 0.2 mM EDTA, pH7.0, at a concentration of 1-2 μg/μl.

Microinjection of mRNA into Xenopus Oocytes

Stage VI oocytes were obtained from female Xenopus laevis, with the use of mild digestion with collagenase as described by Colman (1984). We injected ~50 nl of 1 ng/nl mRNA into each oocyte, which were kept at 22° in modified Barth's medium. Maturation was assessed by the appearance of the characteristic white spot on the animal pole and checked in doubtful cases by dissection.

Translation in Cell-Free Extracts of Xenopus Eggs

For cell-free translation of mRNA, we prepared "cytostatic factor (CSF)-arrested" extracts from unactivated Xenopus eggs according to the protocol of Murray (1991). Sucrose was added to the extracts to a final concentration of 200 mM, and small aliquots were frozen in liquid nitrogen. For translation, mRNA was added at 100 μg/ml and [35S]methionine to 0.5 mCi/ml in a reaction of 10 μl, which was incubated at 23° for 1 h. Part of the reaction mix was used for SDSPAGE and autoradiography (typically, the equivalent of 0.2–0.5 μl was analyzed on one gel lane) and another part for immunoprecipitation or p134* affinity chromatography as described below. In some cases, the frog cell extract was mixed with an equal volume of nuclease-treated rabbit reticulocyte lysate, which stimulated translation of certain added mRNAs, some of which (for example the mRNAs for p134*.

The sequence of this bovine cyclin A clone has been deposited in the GENBANK/EMBL database with accession number X68321.

Functional Domains of Cyclin A

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and p32\textsuperscript{cyclin A} were translated extremely poorly in CSF extracts. We found that placing the coding regions of such mRNAs within the 5' and 3' untranslated regions of cyclin A mRNA strongly reduced their translation in the Xenopus cell-free system, although it did not significantly reduce its translation in the reticulocyte system.

We estimated the concentration of proteins produced by cell-free translation of added mRNA in pure reticulocyte lysate in two independent ways: by measuring the radioactivity in the protein, knowing the specific activity of the methionine pool, and also by immunoblotting and comparison with bacterial cyclin A standards. The two methods gave estimates of 0.4-1 \mu g/ml for wild-type cyclin A after 60 min translation. We did not perform these measurements in frog extracts.

**Affinity Chromatography on p13\textsuperscript{cyclin A}-Sepharose**

The affinity matrix for p34\textsuperscript{cyclin A}, p32\textsuperscript{cyclin A}-Sepharose, was prepared by modifications of the procedure described by Briuela et al. (1987). The p13\textsuperscript{cyclin A} was purified from \textit{E. coli} BL21(DE3) carrying the intronless p13\textsuperscript{cyclin A} gene in the modified pET3 vector pRK172, grown in 2xTY medium and induced with 0.5 mM IPTG. The bacteria were lysed on ice in 20 mM HEPES (N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid), 2 mM EDTA, 1 mM DTT, and 1 mM PMSF, pH 7.2, by addition of 2 mg/ml lysozyme followed by sonication. The extract was clarified by centrifugation for 20 min at 10,000 rpm in the Sorvall SS34 rotor (Sorvall Instruments, Newton, CT), dialyzed against the NaCl/carbonate coupling buffer. This p34\textsuperscript{cyclin A} appeared extremely pure by SDS-PAGE, as described by Dunphy et al. (Villeneuve-la-Carenne, France) equilibrated with the NaCl/carbonate coupling buffer: 500 mM NaCl, 100 mM Na\textsubscript{2}CO\textsubscript{3}. This solution was diluted with 400 \mu l bead buffer. In both cases, the bound proteins were eluted with SDS gel sample buffer. In both cases, the bound proteins were eluted with SDS gel sample buffer and analyzed by SDS-PAGE. Anti-cyclin A antibodies were the same as described by Kobayashi et al. (1991a). The anti-cdk2 antibody was raised in rabbits against full-length cdk2 expressed in pET3. The anti-c-myc epitope monoclonal (Evan et al., 1986) was obtained from the ICRF monoclonal production unit.

Proteins were transferred electrophoretically onto Immobilon filters (Millipore, Bedford, MA) and PSTAIRE-positive polypeptides were detected with the monoclonal antibody of Yamashita et al. (1992), with the Amersham (Aylesbury, England) ECL detection system.

**Histone H1 Kinase Assay**

To assay the histone H1 kinase activity associated with cyclin A translated in vitro, the CSF extracts were made mRNA-dependent essentially as described by Murray (1991). In brief, the CSF extracts were treated with 0.5 \mu g/ml RNase A (Boehringer) for 10 min at 10\textdegree C followed by one-fourth volume of 50% (vol/vol) RNSatin (Boehringer) for 10 min at 10\textdegree C. Next, 0.4 mM CaCl\textsubscript{2}, 200 mM sucrose, and 50 \mu g/ml calf liver RNA (Boehringer) were added and the reaction incubated for 15 min at 23\textdegree C. Aliquots were frozen in liquid nitrogen and stored at ~80\textdegree until use.

All of these nuclease-treated extracts were incubated with 100 \mu g/ml added cyclin mRNA and [\textsuperscript{35}S]methionine, and at 30-min intervals, samples were taken for affinity absorption on p13\textsuperscript{cyclin A} beads as described above, except that the buffer in this case was 80 mM Na \textbeta-glucopyrophosphatase, pH 7.3, 20 mM EDTA, 15 mM MgCl\textsubscript{2}, and 1 mM DTT. The beads were assayed in 10 \mu l of H1 kinase buffer (50 mM Na \textbeta-glucopyrophosphatase, pH 7.3, 0.3 mM EDTA, 15 mM MgCl\textsubscript{2}, 2 mM DTT (Cicirelli et al., 1988) with 25 \mu M ATP, 0.25 \mu Ci/\mu l \gamma\textsuperscript{32}P[ATP] (Amersham PB 10218), and 275 \mu g/ml histone H1 (Boehringer). After 30 min at 23\textdegree C, the reaction was terminated by addition of 25 \mu l of SDS sample buffer. The samples were analyzed for cyclin synthesis and histone phosphorylation by SDS-PAGE and autoradiography.

Activation of H1 kinase by protein A-bovine cyclin A was achieved by addition of 0.4 \mu g of wild-type or mutant protein to 25 \mu l of interphase Xenopus egg extract. After incubation for 10 min at 21\textdegree C, 250 \mu l of H1 kinase buffer was added, followed by 20 \mu l of IgG-Sepharose slurry (Pharmacia). The samples were rotated at 4\textdegree C for 1 h. The beads recovered, washed, and assayed for kinase activity as described above.

**Xenopus Cultured Cells**

A line of Xenopus fibroblasts (WAK) were obtained from R.A. Laskey (Cambridge) and propagated in 75% (vol/vol) Eagle medium supplemented with 10% (vol/vol) fetal calf serum at 27\textdegree C. At a density of ~5 × 10\textsuperscript{5} cells/14-cm plate, the cells were washed with serum-free medium and lysed on ice with buffer containing 50 mM Tris-Cl, 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 0.1% NP40, 10 \mu g/ml cytochalasin B, 1 mM PMSF, 1 \mu g/ml leupeptin, 2 \mu g/ml aprotinin, 10 \mu g/ml soybean trypsin inhibitor, 15 \mu g/ml benzamidine, and 10 \mu g/ml pepstatin, pH 7.5. The cells were scraped off and debris removed by centrifugation at 17,000 g for 30 min. The supernatant was used for immunoblotting.

**Cyclin Destruction Assays**

To measure the ability of cyclin A mutants to undergo programmed proteolysis, the test mRNA was translated in "CSF extracts" as described above. Translation was allowed to proceed for 1 h in the presence of [\textsuperscript{35}S]methionine at which point 0.1 mM cycloheximide was added to block further protein synthesis. The sample was divided in two, and one sample was made 0.4 mM in CaCl\textsubscript{2}, which triggers the destruction of cyclin A after a few minutes' lag (Lohka and Mallar, 1985; Murray et al., 1989; Shamu and Murray, 1992). Samples were taken for analysis on SDS-PAGE at intervals after adding the CaCl\textsubscript{2}.
The Conservation Plot for Comparing Cyclin A Sequences

Figure 1 contains a plot that compares the degree of polypeptide sequence conservation in examples of cyclin A from a variety of species: chicken, clam, cow, frog, fruit fly, hamster, human, limpet, mouse, and sea urchin (Swenson et al., 1986; Lehner and O'Farrell, 1989; Minshull et al., 1990; Wang et al., 1990; van Loon et al., 1991), and see ACKNOWLEDGMENTS). The sequences were aligned by the Intelligenetics Geneworks® (Mountain View, CA) protein alignment module and adjusted by eye. At each position in the sequence, the number of different amino acids was counted. Thus at position 197, all 10 examples of cyclin A have arginine, so the score is 1. At position 204, where the other nine sequences have an L, limpet cyclin A has an M. This position therefore scored 2. These scores were then converted (arbitrarily) as follows: 1 remained 1, 2 became 0.8, 3 became 0.6, 4 became 0.4, and lower scores were transformed into their reciprocal. These numbers were plotted against residue number, and the conservation axis was relabeled as 1, 2 etc. In this way, peaks of conservation and troughs of nonconservation in the sequence comparisons are easily visualized. One additional adjustment was made. The peaks produced by the "destruction box" and the conserved FXXVDE sequence do not appear when cyclin A sequences are strictly aligned because of slight differences in the spacing of these elements with respect to the cyclin box and each other. When locally aligned, they give the values shown in Figure 1. Their positions in the plot are those found in frog cyclin A1.

RESULTS

The cDNA for cyclin A1 from Xenopus contains unique Sty I, EcoRV, HindIII, and Nsi I sites as shown in Figure 1, which, with Table 1, summarizes most of the deletions and mutations we have made. Figure 1 shows how these mutations map in relation to the sequence conservation shown by a variety of examples of cyclin A. For example, mutant Δ133 removed all of the N-terminal 133 residues of cyclin A1 by fusing the efficient translational start site of influenza virus NS protein to the HindIII site (Dasso and Jackson, 1989). Other mutants contained internal deletions of varying size and retained some

![Figure 1](image-url)
portion of the correct N-terminus. They are named for their most C-terminal missing residue. The C-terminal deletion series was constructed with the 9E10 epitope from human c-myc replacing the N-terminal 21 amino acids of cyclin A1. All the constructs contained a T7 promoter upstream to allow cell-free synthesis of capped mRNA, and all ended with a termination codon, as indicated in Table 1.

The activity of cyclin A mutant mRNAs were tested by microinjection into stage VI Xenopus oocytes, which were incubated and scored for their ability to undergo meiotic maturation. Controls of untreated, progesterone-treated and wild-type cyclin A mRNA-injected oocytes were included in every experiment. The results of this test are given in Table 2. Wild-type cyclin mRNA typically caused maturation in all oocytes in a sample of ≥20, starting at 2–3 h and completing by 5–6 h. Deletions up to and including Δ161 were as active as full-length cyclin A1, whereas all deletions from Δ169 onwards were completely inactive. As shown in Figure 1, the first conserved residue in all known examples of cyclin A is Y164, closely followed by I168. These residues are conserved in the majority of known mitotic cyclin sequences, including cdclS of Schizosaccharomyces pombe and the CLB genes of budding yeast. Δ161 preserves both these residues, whereas they are deleted from Δ169. We conclude that these residues are necessary for the activity of cyclin A, although we have not tested this point by making point mutations.

**Deletion Mutants of Cyclin A That Fail to Promote Oocyte Maturation Cannot Bind to p34^CDC2**

We next translated several of the cyclin constructs described in Table 1 in the Xenopus cell-free system (Figure 2A), and tested which of them could bind to p13NUC. Sepharose, an affinity resin for p34^CDC2 and associated cyclins (Brizuela et al., 1987; Draetta et al., 1989; Labbé et al., 1989; Pondaven et al., 1990). Figure 2B shows that the cyclin A deletion mutants that were active in the oocyte maturation assay bound to p13NUC beads, whereas the inactive mutants failed to bind. It is still not completely clear how p34^CDC2 binds to p13NUC1, however, and it was possible that the mutant cyclins might form stable complexes with p34^CDC2 that were no longer capable of associating with p13NUC. Figure 2D shows that this was not the case, because when we used a monoclonal anti-p34^CDC2 antibody to immunoprecipitate the various cyclin A constructs, the results exactly followed those of the activity and p13NUC-binding assays. Constructs Δ133, Δ144, and Δ161 bound to p34^CDC2, whereas Δ169 and deletions extending beyond the start of the cyclin box did not bind.

**The C-Terminus of Cyclin A is Required for Binding to p34^CDC2**

To define the C-terminal end of cyclin A that is necessary for binding to p34^CDC2, we constructed a series of C-terminal deletion mutants (see MATERIALS AND METHODS). The ability of these constructs to bind with companion kinase subunits was tested in the same way as the experiment shown in Figure 2. Immunoprecipitation was performed with the A17 monoclonal anti-p34^CDC2 antibody. Figure 3 shows that the shortest C-terminal deletion, lacking only 14 amino acids from its C-terminus, failed to bind to p34^CDC2, and removal of 24 and 50 residues did not restore binding. Larger deletions of 79, 139, and 200 residues were similarly inactive. We also made a construct that deleted the 16 C-terminal residues of bovine cyclin A (see Figure 5), which showed no detectable cdcl2 binding activity in experiments like the one shown in Figure 3, or histone HI kinase activity, as Figure 6 shows. When these C-terminal deletions were tested by the oocyte maturation assay (Table 2), we found that ΔA14 and CA24 had slight residual activity; ~10% of the oocytes matured with normal kinetics. This may indicate that the in vivo assay is capable of detecting weaker interactions between p34^CDC2 and cyclin A than the in vitro binding assay. Clearly, however, these C-terminally deleted constructs are seriously impaired in activity.

**Activation of Histone H1 Kinase by Mutants of Cyclin A**

The ability of full-length cyclin A1 and the two mutants Δ161 and Δ169 were further tested for their ability to activate histone kinase activity in interphase extracts of
Xenopus eggs. Figure 4 shows that there was a gradual increase in histone H1 kinase activity associated with p13^sep^ Sepharose beads prepared from extracts that were incubated with mRNA encoding wild-type cyclin A1 or Δ161, but no increase in kinase activity was observed when Δ169 or no mRNA was added to the reactions. Similar results were obtained when immunoprecipitates of myc-tagged cyclin A1 produced by cell-free translation were assayed for their associated histone kinase activity. Wild-type cyclin A1 displayed activity (for example, see Figure 9), whereas C-terminal truncations completely lacked it.
Small Deletions in the Cyclin Box Reduce or Abolish Activity of Cyclin A

We made another set of internal deletions in *Xenopus* cyclin A1 with its unique *Nsi* I site or in bovine cyclin A with its unique *Acc* I site, as indicated in Figure 5. Removal of two or more amino acids at the *Nsi* I site completely inactivated frog cyclin A, as measured by the oocyte maturation assay (Table 3) or by the in vitro binding assay. The deletion of two amino acids further down the sequence in bovine cyclin A changed the sequence VYITD in the italicized sequence below to VSD, i.e., loss of Y1 and changing the next T to S: ASKFEEL-YIPPEAEVFLYTEDDYTKQVRME (underlined letters indicate the residues conserved in cyclin A from clam, fly, frog, and man). This construct (Δ267) gave ~10% residual kinase activity when the construct was added to an interphase extract derived from *Xenopus* eggs (Figure 6), which we interpret as showing that weak binding to p34^{cdk2} gives weak activation. Longer deletions in this location completely abolished the activity of bovine cyclin A. It is noteworthy that so far we have not found a mutant cyclin A that can bind to p34^{cdk2} but not activate it as a protein kinase. It thus appears that, if cyclin A can bind to p34^{cdk2}, it turns on the kinase activity of the complex.

Single Point Mutations of Conserved Residues in the Cyclin Box Abolish the Ability of Cyclin A1 to Bind and Activate p34^{cdk2}

Five residues in the cyclin box are conserved in essentially all known cyclins, including the yeast CLN genes (the major exception is cyclin C). They are (in *Xenopus* numbering) R197, D226, L241, K252, and E281. So far we have constructed, confirmed by sequencing, and tested R197 → A and D226 → A (Figure 5). Both of these point mutations caused complete loss of ability of binding p34^{cdk2} and the ability to activate the histone H1 kinase of p34^{cdk2}.
Figure 6. Histone H1 kinase activity associated with protein A-cyclin A mutants. Wild-type protein A-cyclin A or the mutant constructs shown in Table 1 and Figure 5 were added to CSF extract that had been activated by addition of CaCl₂. After 10 min, the cyclin-p34^cdc2 complexes were recovered on IgG beads and assayed for histone H1 kinase as described in MATERIALS AND METHODS.

the mutant cyclin A1 to form complexes with p34^cdc2 in the CSF-extract translation and immunoprecipitation assay.

Xenopus Cyclin A1 Can Bind Both p34^cdc2 and p32^cdk2

Xenopus eggs contain two well-characterized members of the cdc2 family, p34^cdc2 and p32^cdk2, the latter originally known as Egl, because its mRNA was polyadenylated and translated only during the time of progesterone-induced maturation until the time of fertilization (Paris et al., 1991). The sequences of these two polypeptides are very similar in the regions where all examples of 'cdc2' proteins are conserved and differ in the regions in between. Although it was originally found that p32^cdk2 did not substitute for cdc2 in S. pombe (Paris et al., 1991), more recent evidence indicates that the human homologue of Eg1, now known as cdks2 (cyclin dependent kinase) is capable of such substitution, particularly when certain other mutations were present in the tester strains (Elledge and Spottswood, 1991; Meyerson et al., 1992).

To test whether Xenopus cyclin A1 could associate with p32^cdk2 as well as p34^cdc2, we co-translated mRNA encoding myc-tagged cyclin A1 with cdc2 or cdk2 mRNA in the CSF extract. At the end of the incubation with [35S]methionine; antibodies against cyclin A were added and the immunoprecipitates collected on protein A-Sepharose beads. Figure 7 shows that both p34^cdc2 and p32^cdk2 were immunoprecipitated with anti-myc or anti-cyclin A antiserum when full length myc-tagged cyclin A1 (Δ21) mRNA or the mRNA for the active Δ161 construct was present. But when mRNAs encoding the C-terminally truncated cyclin A construct CA14 or the inactive Δ169 mRNA were used, the binding of the kinase subunits was the same as background reactions in which no cyclin A mRNA was added. Thus mutants that fail to associate with p34^cdc2 also cannot bind p32^cdk2.

We conclude that under the conditions of these assays, when the levels of both cyclin A and p32^cdk2 have been increased by translation of added mRNA, cyclin A can bind to either p34^cdc2 or to p32^cdk2. We should stress that we stand by our previous data (Minshull et al., 1990), which has been confirmed by Fang and Newport (1991) and by Maller and his colleagues (Gabrielli et al., 1992), that in frog egg extracts, no cyclin A1 can be detected in association with p32^cdk2. Presumably this is partly a matter of relative concentrations

<table>
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Figure 7. Cyclin A mutants that fail to bind to p34^cdc2 cannot bind to p32^cdk2. Cyclin A1 mRNA was co-translated with mRNA for either p34^cdc2 or p32^cdk2 in a cell-free translation system composed of equal volumes of Xenopus CSF extract and nuclease-treated rabbit reticulocyte lysate. Reactions were immunoprecipitated with rabbit polyclonal anti-cyclin A antiserum. (A) Complete translation reactions. (B) Immunoprecipitates.
because as Figure 8A shows, the levels of p34<sup>cdc2</sup> are 10–20 times higher than those of the p32<sup>cdc2</sup> in normal frog eggs, in agreement with Gabrielli <i>et al.</i> (1992). By contrast, extracts of somatic <i>Xenopus</i> cells (WAK fibroblasts) contain roughly equal concentrations of p34<sup>cdc2</sup> and p32<sup>cdc2</sup>. To get an idea of the relative affinities of p34<sup>cdc2</sup> and p32<sup>cdc2</sup> subunits for cyclin A, soluble protein A-bovine cyclin A protein produced by expression in bacteria was added to a frog egg extract in which the mRNA for p32<sup>cdc2</sup> or p34<sup>cdc2</sup> had been translated with added [35S]methionine, and the cyclin (together with any bound proteins) recovered by incubation with IgG-Sepharose beads. Figure 8B shows that both p34<sup>cdc2</sup> and p32<sup>cdc2</sup> bound to the beads, but considerably more of the labeled p34<sup>cdc2</sup> was retained. Apart from the background of labeled polypeptides produced by translation of endogenous mRNAs, we included negative controls of a truncated cyclin B2 mRNA and a <i>Xenopus</i> PCI AIRE clone (Figure 8, lanes 3 and 4). Compared with p32<sup>cdc2</sup> and p34<sup>cdc2</sup>, these did not bind significantly to the protein A-cyclin A. Because the amount of label in each kinase subunit was adjusted to be the same, this means that the specific activity of the p34<sup>cdc2</sup> was roughly one-tenth of that of the p32<sup>cdc2</sup>. Thus even though the cyclin A was added in excess, there was a strong tendency for p34<sup>cdc2</sup> to bind in preference to p32<sup>cdc2</sup> in the crude egg extract, which may explain why it is normally impossible to find cyclin A associated with p32<sup>cdc2</sup> in these extracts.

**Cyclin A-p32<sup>cdc2</sup> Complexes Possess Histone H1 Kinase Activity**

To measure the protein kinase activity of p32<sup>cdc2</sup> with histone H1 as the substrate, we used a C-terminally c-myc-tagged version (Kobayashi <i>et al.</i>, 1991a). The mRNA for this tagged p32<sup>cdc2</sup> was translated in the <i>Xenopus</i> egg/reticulocyte lysate mixture either in the presence, or the absence, of wild-type cyclin A1 mRNA (Figure 9A, lanes 2 and 3). The C-terminally c-myc-tagged p32<sup>cdc2</sup> construct used for these experiments showed a small increase in mobility when co-translated with cyclin A1 mRNA (lane 3), probably associated with its activation (Gu <i>et al.</i>, 1992). This shift was not observed when wild-type cdk2 mRNA was used (Figure 7A).

When no mRNA was added (the negative control), no kinase activity was associated with the 9E10 anti-c-myc antibody (Figure 9C, lane 1) and only very low activity with polyclonal anti-cdk2 antibody (Figure 9C, lane 3). But as Figure 9C, lane 4 shows, the anti-c-myc antibody carried strong histone H1 kinase activity when p32<sup>cdc2</sup>-c-myc was translated together with wild-type cyclin A1. When c-myc-cyclin A was translated with no other added mRNA (in which case, the newly translated cyclin A can associate with the endogenous, unlabeled p34<sup>cdc2</sup>), strong histone H1 kinase activity was present in the 9E10 immunoprecipitate (Figure 9C, lane 5). The low kinase activity detected when cdk2 mRNA was translated without added cyclin A mRNA suggests that there cannot be a large concentration of a free, unlabeled (and unidentified) companion subunit of cdk2.
Functional Domains of Cyclin A

Figure 9. Cyclin A can activate the H1 kinase activity of both p34<sup>cdc2</sup> and p32<sup>cdk2</sup>. Four translation reactions were set up in a 1:1 mixture of reticulocyte lysate and frog egg extract. (A) Lane 1, no mRNA; lane 2, c-myc tagged cdk2; lane 3, mixture of wild-type cyclin A and c-myc tagged cdk2; lane 4, c-myc tagged cyclin A (A21). (B) Immunoprecipitates with anti-c-myc antibody 9E10 in lanes 1, 2, 4, and 5, and rabbit polyclonal anti-p32<sup>cdk2</sup> antiserum in lane 3. (C) Histone H1 kinase activity of these immunoprecipitates.

The Sequence RTVLGVIGD is Required for Programmed Cyclin Proteolysis

The most peculiar property of mitotic cyclins is their destruction at the onset of anaphase. It was previously shown by Glotzer et al. (1991) that the short conserved N-terminal sequence in sea urchin cyclin B, RAALGNISN, was required for this proteolysis. More recently, Luca et al. (1991) showed that removal of the N-terminal 60 amino acids from clam cyclin A had a similarly stabilizing effect on the protein. We tested three constructs to check that the destruction of <i>Xenopus</i> cyclin A1 required the closely related peptide motif RTVLGVIGD at positions 41–50. The first construct was Δ23 in which the N-terminal 133 amino acids are completely deleted; in the second construct, a unique Sty I site at the N-terminus of <i>Xenopus</i> cyclin A1 was used to make Bal 31 deletions. One of the in-frame mutants, Δ62 (Figure 10A), deleted the whole of the putative destruction box. Finally, we altered the conserved R and L residues of the destruction box to alanine by site-directed mutagenesis. Destruction was tested in unactivated frog egg extracts in which cyclin proteolysis and concomitant loss of histone H1 kinase activity can be triggered by addition of 0.4 mM Ca<sup>2+</sup>. Figure 10 shows that all these mutants were stable, so we conclude that the N-terminal RTVLGVIGD motif is necessary for the destruction of cyclin A1. A similar conclusion has recently been reported for limpet cyclin A by Lorca et al. (1992), who mutated the R of the related RSALGTITNQ sequence to C. We will report elsewhere on the stability of the rest of the mutants described in this paper, all of which have an intact destruction box. Their story is somewhat more complicated, for several of them are unexpectedly stable.

DISCUSSION

As discussed in the INTRODUCTION, the cyclin family of proteins share a common region of about 100 residues known as the cyclin box, which contains five very highly conserved residues. The first important data in this paper show that point mutations of the first two of these residues completely abolishes the ability of cyclin A to bind to p34<sup>cdk2</sup>. Similarly, deletions of only two residues in
two different parts of the cyclin box in one case completely inactivated and in the other 90% inactivated the ability of the cyclin to bind to p34\textsuperscript{cdc2} or to activate its kinase activity. Thus, both the composition and exact spacing of residues in the cyclin box are implicated in interactions with the companion kinase subunit. Together with sequence comparison data, these results strongly suggest that the cyclin box represents the heart of the p34\textsuperscript{cdc2} binding domain in the cyclin family.

Comparison of the sequences of members of the cyclin A family (Figure 1) suggests that with the exception of two small conserved islands, the destruction domain at residues 41-50 and the motif FXXXVDE at positions 91-100, there is practically no sequence conservation in the N-terminal 163 residues. We show here that complete removal of the first 133 residues, or an internal deletion of 53 residues from 109 to 161, has no detectable effect on the ability of the cyclin to bind to and activate p34\textsuperscript{cdc2}. The larger deletion, removing 69 residues from positions 101 to 169, is completely inactive and deletes the first two residues that are found in all known examples of cyclin A. The cyclin box proper starts with the conserved M 196, and it is not clear what role residues 164-195 play in the function of cyclin A.

It is noteworthy that for the most part, the equivalent residues in the B-type cyclin family (which bind p34\textsuperscript{cdc2}) are different from those in the cyclin A family, but the equivalents of EY 163-4 and I 168 are present in Xenopus cyclins B1 and B2 and also in Saccharomyces cerevisiae CLN1 and 2. CLN3 has EY, but not the I. Our data suggest that these residues are crucial for binding to members of the cdc2 family of kinases. This line of reasoning leads to a problem, however: if this is so, how is it possible for cyclin E, which lack these residues and shows essentially no similarity with other cyclins until the MRAIL sequence, to bind to p32\textsuperscript{cdc7}? Only structural information can clarify this puzzle.

Whereas large stretches of the N-terminus of both cyclins A and B can be lost without losing p34\textsuperscript{cdc2} binding, the C-terminus is indispensable for reasons that are hard to understand, given the lack of sequence similarity between cyclins in this region. Even within the cyclin A family, the homology plot in Figure 1 suggests that the C-terminal 120 residues represents a separate structural domain, with a region of great diversity between positions 300-315 (the position where B-type cyclins have their characteristic FLRRXSK). Considering how much various cyclins differ from one another in the sequences that flank the cyclin box, the intolerance of cyclin A to changes well outside the central conserved cyclin box is strikingly odd. It may be that these outlying motifs represent built-in safety checks, so to speak, which serve to prevent fragments or other altered forms of cyclins from binding to cdc2, and thereby make it more difficult to generate "plain vanilla" forms of the kinase, whose activity would probably be dangerous if they were able to be formed. It would be most helpful to know the structure of the molecule, to see how cyclin binds to cdc2.

We have confirmed that the homologue of the cyclin B destruction box in cyclin A is indeed necessary for
programmed proteolysis at the end of mitosis, both by removing relatively large domains including this sequence and also by point mutations that change QRTLGVIG to QATVAGVID.

It seems improbable that the only function of the first 161 residues is to provide a correctly spaced and presented destruction signal. For one thing, there is another small region of conservation at positions 91–100, which has some echoes in cyclin E, but not in cyclins B or D. The assays that we have used in these studies do not necessarily measure all the cellular functions of cyclin A; the ability to bind to p34\textsuperscript{cdc2} or p32\textsuperscript{cdk2} represents the minimum possible activity of cyclin A and does not measure the more subtle properties of subcellular targeting of the complex to specific cellular compartments and particular substrates. It will be important to test these properties in the future.

In this regard, it is important to note that the oocytes that were induced to mature by injected cyclin A1 mRNA underwent abnormal maturation. Unlike what happens in response to progesterone, the white spot stage was transient even when indestructible forms of cyclin were injected. The stimulated oocytes generally took on a marbled appearance, as though they were unable to arrest stably at meiosis II, and after several hours, there were often signs of necrosis. This deserves further study.

**How Does Cyclin A mRNA Lead to Oocyte Maturation?**

Ever since Swenson et al. (1986) showed that clam cyclin A mRNA could cause GVBD (germinal vesicle breakdown) and white spot formation in *Xenopus* oocytes, there has been a question of how it acts. This property of cyclin A mRNA (and more recently, protein [Roy et al., 1991]) has become even more puzzling as we learn more about the state of *Xenopus* oocytes before maturation and as claims emerge to suggest that cyclin A is normally involved in the control of S-phase (Fang and Newport, 1991; Girard et al., 1991; Pagano et al., 1992). Stage VI *Xenopus* oocytes do not contain detectable amounts of cyclin A protein, and the translation of the stockpile of maternal mRNA for cyclin A does not begin until about the time of GVBD (Kobayashi et al., 1991b). Moreover, oocyte maturation can occur completely normally after >95% of the endogenous cyclin A mRNA has been ablated with antisense oligonucleotides (Minshull et al., 1991). Assuming that maturation requires active p34\textsuperscript{cdk2} (an unexamined point, although it would be exceedingly heretical to question it), we argued that the preformed store of cyclin B2 and p34\textsuperscript{cdk2} must be adequate to perform the whole process. So, how does cyclin A promote maturation?

We show here that mutant cyclin A molecules that cannot bind to p34\textsuperscript{cdk2} fail to promote maturation. This makes it highly probable that cyclin A acts by combining with free p34\textsuperscript{cdk2} and activating its protein kinase activity. How is it that newly synthesized cyclin A can activate p34\textsuperscript{cdk2}, whereas the maternal stockpile of cyclin B/cdc2 is devoid of activity? We suppose that cyclin A directly activates free p34\textsuperscript{cdk2} in the oocytes, which then tips the balance of a positive feedback loop, leading to activation of the endogenous cyclin B2-p34\textsuperscript{cdk2}. Precisely how this occurs is not yet clear. As recently discussed in some detail by Devault et al., (1992), it is presumably necessary either to activate the tyrosine 15 phosphatase encoded by cdc25, or to inhibit the tyrosine 15 kinase encoded by a wee1-like enzyme, or both, to activate preformed cyclin B-p34\textsuperscript{cdk2} protein kinase (Dunphy and Newport, 1989; Dunphy and Kumagai, 1991; Gautier et al., 1991). Further work is necessary to discover what happens in frog oocytes.

**Why Did Previous Studies Fail to Detect Cyclin A-p32\textsuperscript{cdk2} Association in Xenopus Extracts?**

We were at first surprised to find that *Xenopus* p32\textsuperscript{cdk2} could form complexes with cyclin A, considering that studies in three other laboratories besides our own failed to detect newly synthesized cyclin A bound to p32\textsuperscript{cdk2} (Minshull et al., 1990; Fang and Newport, 1991; Paris et al., 1991; Gabrielli et al., 1992). More recently, data from mammalian cells has shown that cyclin A is often found predominantly associated with cdk2 (Tsai et al., 1991; Desai et al., 1992). The data in this paper clearly show that *Xenopus* cyclin A and p32\textsuperscript{cdk2} can, like their mammalian counterparts, bind tightly together and produce active histone H1 kinase. However, there are potentially important differences in the way our more recent experiments were performed and the situation in intact *Xenopus* eggs and embryos. First, to obtain high level translation of cdk2 mRNA, we found it necessary to add reticulocyte lysate to the *Xenopus* extract; it is thus possible that the presence of reticulocyte lysate may promote the interaction in some way, although it does not do so in the case of inactive mutants, like CA14. More relevant, perhaps, is that the concentration of p32\textsuperscript{cdk2} is normally much less (perhaps ~5–10%) of p34\textsuperscript{cdk2} in frog extract (see Figure 8), and the concentration of cyclin A is probably lower still. In the cell-free translation experiments, the concentrations of both components are much increased, which tends to promote interaction. There is very little cyclin A in normal eggs, and they contain a large excess of p34\textsuperscript{cdk2}, so it is very likely that most of the cyclin is "mopped up" by the p34\textsuperscript{cdk2}. The experiment shown in Figure 8 suggests that in the frog egg extract, the relative affinities of p34\textsuperscript{cdk2} for cyclin A is higher than that of p32\textsuperscript{cdk2} for cyclin A. These results by no means exclude the possibility that p32\textsuperscript{cdk2} has other, as yet unidentified, cyclin or cyclin-like subunits. What is perhaps more puzzling is that in human fibroblasts, cyclin A is mainly found associated with p32\textsuperscript{cdk2} and not with p34\textsuperscript{cdk2} (Tsai et al.,
One possible explanation is that the cyclin A found in eggs is different from that found in somatic cells, and we have preliminary evidence that this is true. It will be necessary to design experiments to compare the relative affinities of cyclins A1 (the oocyte cyclin A) and A2 (the presumptive somatic cyclin A) for various kinase subunits.

Do Some Mutations Cause Misfolding?
Both the reviewers of the submitted version of this paper raised the following question: how do we know that proteins with greatly altered sequences fold up correctly? Could nonbinding of cyclin A to p34<sup>cdc2</sup> be caused by gross misfolding, rather than deletion of, or alterations in key interacting residues in the subunit interface domain? We did not discuss this question because we thought it was very difficult, if not impossible, to answer in the absence of structural assays that are independent of functional ones. In the case of point mutations in the cyclin box, we would argue that single alanine replacements are generally well tolerated (see Gibbs and Zoller, 1991), but deletions of two amino acids might, we imagine, cause large changes in conformation. At the same time, large deletions of up to 133 amino acids in the N-terminal 161 residues of cyclin A are perfectly tolerated and presumably do not induce misfolding, because they are able to bind and to activate p34<sup>cdc2</sup> and p32<sup>cdc2</sup>. We are more puzzled by the failure of the short C-terminal truncations of cyclin A to bind to p34<sup>cdc2</sup>, given the variations in sequence between different members of the cyclin family in this region of the molecule. We are exploring the use of mild protease digestion, which may provide a way to detect gross structural alterations. In truth, however, solving the crystal structure of cyclin A-p34<sup>cdc2</sup> kinase is the only sure way to obtain precise, accurate and detailed information.

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