THE MECHANISMS OF c-MYC FUNCTION

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CORRIGENDA
The work presented here was carried out in the Growth Control and Development laboratory at the Imperial Cancer Research Fund between October 1991 and October 1995 under the supervision of Dr. Hartmut Land. Professor Martin Raff acted as internal supervisor.
ABSTRACT

The c-Myc protein (Myc) is involved in the control of cell proliferation, differentiation and programmed cell death. It has considerable structural similarity to transcription factors, including a helix-loop-helix and a leucine zipper (both known dimerisation motifs), a basic region able to bind sequence-specifically to DNA and a region in the N-terminus that can activate transcription when fused to a heterologous DNA-binding domain. A related protein (Max) was identified as a dimerisation partner for Myc; both Myc/Max and Max/Max can bind to DNA of sequence CACGTG.

I show herein that Myc can activate transcription in mammalian cells in a sequence-specific manner and show that the basic region, N-terminal regions and dimerisation domains are essential for transactivation, as is dimerisation with Max.

I have investigated the DNA-binding properties of Myc and Max and have determined the preferred binding sites for the Myc/Max and Max/Max dimers. Although both bind to CACGTG, the Myc/Max complex is more discriminatory than the Max homodimer at positions flanking this core sequence. In particular a 5' flanking T residue (or a 3'A) is disfavoured. A model for gene regulation by the Myc family of proteins is discussed.

In an effort to understand the means by which Myc acts, I have also investigated possible target genes for Myc that mediate proliferation and show that cyclin E, but not cyclin D1, is induced by Myc independently of new protein synthesis. Moreover, Myc induces cyclin E associated kinase activity. This regulation occurs at two levels: increased transcription of the cyclin E gene and activation of the kinase activity of cyclin E complexes, at least partly due to loss of the inhibitor p27^kip1. Induction of cyclin E protein alone is sufficient to induce apoptosis but not proliferation. The role of cyclin E in Myc function is discussed.
ACKNOWLEDGEMENTS

There are many people who have contributed immensely to this work both at a scientific and a personal level.

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ABBREVIATIONS

°C  degrees Celsius
Ab  antibody
ABTS  2-2’-azino-bis-(3-ethylbenzthiazoline-6-sulphonic acid)
bp  base pair(s)
BHI  brain heart infusion (broth)
BrdU  5-bromo-2’-deoxyuridine
BSA  bovine serum albumin
cDNA  complementary deoxyribonucleic acid
CIP  calf intestinal phosphatase
(d)NTP  (2’-deoxy)ribonucleoside 5’-triphosphate
(d)ATP  (2’-deoxy)adenosine 5’-triphosphate
(d)CTP  (2’-deoxy)cytidine 5’-triphosphate
(d)GTP  (2’-deoxy)guanosine 5’-triphosphate
(d)TTP  (2’-deoxy)thymidine 5’-triphosphate
DEPC  diethyl pyrocarbonate
DMSO  dimethyl sulfoxide
DNA  deoxyribonucleic acid
DNase  deoxyribonuclease
DTT  dithiothreitol
E4  Dulbecco’s modified Eagle’s medium
E. coli  Escherichia coli
ECACC  European collection of animal cell cultures
ECL  enhanced chemi-luminescence
EDTA  ethylene-diamine-tetraacetic acid (disodium salt)
EGF  epidermal growth factor
ELISA  enzyme-linked immunosorbent assay
EtOH  ethanol
EtBr  ethidium bromide
FACS  fluorescence activated cell sorter
FCS  foetal calf serum
FGF  fibroblast growth factor
FITC  fluorescein isothiocyanate
g  gramme
GAPDH  glyceraldehyde-3-phosphate dehydrogenase
HBS  hepes buffered saline (for transfection)
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<td>N-2-hydroxy-ethyl-piperazine-N’-2-ethane sulphonic acid</td>
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<td>HRP</td>
<td>horseradish peroxidase</td>
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<tr>
<td>Ig</td>
<td>immunoglobulin</td>
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<tr>
<td>kb</td>
<td>kilobase</td>
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<tr>
<td>kD</td>
<td>kiloDalton</td>
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<tr>
<td>l</td>
<td>litre</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani medium</td>
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<tr>
<td>LTR</td>
<td>long terminal repeat</td>
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<tr>
<td>M</td>
<td>molar</td>
</tr>
<tr>
<td>m</td>
<td>milli</td>
</tr>
<tr>
<td>µ</td>
<td>micro</td>
</tr>
<tr>
<td>mA</td>
<td>milliAmpere</td>
</tr>
<tr>
<td>MMTV</td>
<td>Mouse mammary tumour virus</td>
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<tr>
<td>Mo-MuLV</td>
<td>Molony murine leukaemia virus</td>
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<tr>
<td>MOPS</td>
<td>3-{N-morpholino}propane sulphonic acid</td>
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<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<td>n</td>
<td>nano</td>
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<td>nonidet P-40</td>
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<tr>
<td>OAc</td>
<td>Acetate</td>
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<tr>
<td>OE</td>
<td>17β-oestradiol</td>
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<td>OHT</td>
<td>4-hydroxy-tamoxifen</td>
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<td>P</td>
<td>pico</td>
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<td>poly-acrylamide gel electrophoresis</td>
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<td>PBS</td>
<td>phosphate-buffered saline</td>
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<td>PCR</td>
<td>polymerase chain reaction</td>
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<td>PDGF</td>
<td>platelet-derived growth factor</td>
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<tr>
<td>PEG</td>
<td>poly-ethylene-glycol</td>
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<tr>
<td>PMA</td>
<td>phorbol 12-myristate 13-acetate (also known as TPA)</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenyl-methyl-sulphonyl fluoride</td>
</tr>
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<td>PNK</td>
<td>poly-nucleotide kinase</td>
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<tr>
<td>PVP</td>
<td>polyvinyl-pyrrolidone</td>
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<td>RNA</td>
<td>ribonucleic acid</td>
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<td>ribonuclease</td>
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<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>RSB</td>
<td>resuspension buffer (nuclei)</td>
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<td>RSV</td>
<td>Rous sarcoma virus</td>
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<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>RT</td>
<td>room temperature</td>
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<td>Sarkosyl</td>
<td>sodium dodecyl sarkosinate</td>
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<tr>
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<td>sodium dodecyl sulphate</td>
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<tr>
<td>SET</td>
<td>SDS EDTA Tris buffer</td>
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<tr>
<td>SSC</td>
<td>saline sodium citrate</td>
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<td>TAE</td>
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<td>Tris-buffered saline</td>
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<tr>
<td>TE</td>
<td>Tris-EDTA buffer</td>
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<tr>
<td>TEMED</td>
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<tr>
<td>Tfb</td>
<td>transformation buffer</td>
</tr>
<tr>
<td>Tris (Trizma base)</td>
<td>Tris[hydroxymethyl]aminomethane</td>
</tr>
<tr>
<td>Tween 20</td>
<td>polyoxyethylenesorbitan monolaurate</td>
</tr>
<tr>
<td>U</td>
<td>unit (of enzyme activity)</td>
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<tr>
<td>V</td>
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<tr>
<td>v/v</td>
<td>volume for volume</td>
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<td>w/v</td>
<td>weight for volume</td>
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Chapter One

1 INTRODUCTION

1.1 Cancer, cells and genes

The theory that the cell is the basic unit of life was first proposed in 1838 by Theodor Schwann and Matthias Schleiden, and was later developed by Rudolf Virchow in 1858. We now know that the behaviour of a cell is a function of the genes it expresses; and cancer, a disease of cells in which they grow autonomously irrespective of outside signals, is a genetic disease.

1.2 Oncogenes and proto-oncogenes

A tremendous amount of information on the individual genes involved in cancer and growth control has been provided through the study of viruses. Two classes of viruses can transform cells in culture, and are associated with tumorigenesis in animals: the DNA tumour viruses and the retroviruses. In particular it is the retroviruses that have revealed the cellular genes involved in the control of proliferation.

The simplest retroviruses consist of 5 kb of RNA inside a capsule and the retroviral genome can consist of as few as three genes: Group antigen (gag), encoding the major structural proteins; env, encoding the envelope glycoproteins; and pol, encoding reverse transcriptase (for review see Bishop, 1978\(^1\)). As such these viruses are not particularly tumorigenic and do not upset the normal growth of their hosts, although they are slightly mutagenic by virtue of their integration into the host genome. However, there are also acutely transforming versions of these viruses, initially identified as filterable agents causing leukaemias and sarcomas in chickens. Mutational analysis\(^2\) revealed that the part of the genome responsible for transformation was not required for the normal viral life-cycle. Such genes, dominant inducers of transformation, are termed oncogenes. Interestingly, the oncogene from the Rous Sarcoma
Virus (RSV, which causes sarcoma in chickens) called \textit{v-src}, is homologous to normal avian DNA\textsuperscript{6} as well as to that from other species\textsuperscript{7,8}. In fact, when the oncogenes from acutely transforming retroviruses are used to probe cellular DNA libraries they are found to correspond to genes from normal cells\textsuperscript{9}. These corresponding genes are termed proto-oncogenes. Analysis of these transforming genes has identified over twenty oncogenes as well as their original normal cellular counterparts (for review see Bishop, 1983\textsuperscript{10}). Other methods, such as insertional mutagenesis and the introduction of DNA from cancer cells into untransformed cells, identified further oncogenes\textsuperscript{11-18}, often mutants of the proto-oncogenes identified by homology to the viral oncogenes.

Over sixty proto-oncogenes have now been discovered, which play a role in the growth of cells and which, when mutated or aberrantly expressed, are important in the development of cancer.

1.3 Myc: a key oncogene

The \textit{myc} gene was first discovered as the oncogene of MC29, a retrovirus causing myelocytomatosis (hence myc), and was subsequently found in other avian leukaemia viruses (for review see Erisman and Astrin\textsuperscript{19}) as well as feline leukaemia viruses\textsuperscript{20-22}. From the viral gene the cellular homologue (\textit{c-myc}) was identified, and the two are essentially identical. Although the viral genes usually contain mutations and are more potent than the cellular gene in transformation assays, deregulated expression of the normal protein is most usually responsible for carcinogenesis. The \textit{c-myc} gene is a common integration site for other viruses which do not carry their own oncogene such as the avian leukosis virus (ALV)\textsuperscript{23,24}, reticuloendotheliosis virus, myeloblastosis-associated virus-1 and murine MCF247 virus. Following integration into the gene, \textit{myc} expression is driven by read-through from the viral long
terminal repeat (LTR) or is cis-activated by the viral enhancer elements, resulting in Myc levels 20-100 fold higher than usual\textsuperscript{25}. Another member of the Myc family N-Myc (see below) is also oncogenic, and is frequently activated by viral insertion (by Moloney murine Leukaemia virus\textsuperscript{26,27} and Woodchuck hepatitis virus\textsuperscript{28,29}).

In mouse plasmocytoma and Burkitt's lymphoma in humans, two diseases of B-cells, Myc is activated by chromosome rearrangement\textsuperscript{30-33}. In plasmocytoma a translocation often brings together the \textit{c-myc} gene and either the \(\kappa\) light- or heavy-chain immunoglobulin genes. Since these genes are very highly expressed in B-cells, such a translocation results in greatly augmented Myc expression. The rearrangements in Burkitt's lymphoma are similar, involving the immunoglobulin heavy (on 14q32), \(\kappa\) light (on 2p12) or \(\lambda\) light (on 22q11) chain genes and the \textit{c-myc} locus (8q24).

In addition to the highly oncogenic activation of \textit{myc} by viral insertion or gross physical changes, the progression of some tumours to more aggressive states correlates with increased Myc levels. In a number of human tumour cell lines, \textit{myc} genes are amplified; for example a ten to fifty fold DNA amplification of \textit{c-myc} and a correspondingly increased expression level have been found in the promyelocytic leukaemia(HL60)\textsuperscript{34} and colon carcinoma (COLO 320)\textsuperscript{35} cell lines in humans as well as in mouse osteosarcoma lines\textsuperscript{36}. This is often typified by visible cytogenetic abnormalities. High \(N\text{-}myc\) or \textit{c-myc} amplification is associated with the rapid progression of neuroblastomas\textsuperscript{37} and poor prognosis in head and neck squamous cell carcinoma\textsuperscript{38} respectively and is also implicated in small cell lung carcinoma, retinoblastoma, promyelocytic leukaemia and breast carcinoma. In other cases, for example haematopoietic and colon cancers\textsuperscript{39,40}, Myc levels are elevated although there is no obvious change in the \textit{myc} gene; presumably the
mutation lies in other regulatory genes and is sufficient to elevate Myc expression.

1.4 The Myc family

Following its discovery in transforming viruses and the subsequent identification of a cellular homologue in chickens, Myc has been found throughout the vertebrates\textsuperscript{41-48} and recently in fruit flies\textsuperscript{49} but not so far in yeasts. As well as being highly conserved in evolution, myc genes are part of a large family with seven members so far discovered in higher vertebrates: c-, N-, L-, P-, R- and S-myc. c-myc, N-myc and L-myc are all capable of transforming rat embryo fibroblasts (REFs) in cooperation with activated ras\textsuperscript{50-54}, although neither N-myc nor L-myc have been found in transforming viruses. N-myc is not merely oncogenic \textit{in vitro}, however, since it is amplified or otherwise mutated in neuroblastomas\textsuperscript{55-57}, small cell lung carcinomas\textsuperscript{58}, retinoblastomas\textsuperscript{59} and Wilm's tumours\textsuperscript{60}. L-myc is found amplified or rearranged in small cell lung cancer\textsuperscript{61,62}, albeit at low frequency. These three myc genes (c-, N- and L-) have a conserved three-exon structure, and code for proteins of a similar size with homologous domains. They probably function in a similar manner although at different times and/or in different cell types. The N-Myc protein is not expressed in the adult, but instead is seen at high levels during embryogenesis, especially in the brain, retina, kidney and lung tissues. The same is true of L-Myc, again particularly highly expressed in the foetal brain yet in the adult, only expressed in the lung.

The remaining members of the family are not associated with cancers and yield considerably smaller proteins. B-myc is conserved in mice, rats and humans, and is expressed in a number of tissues but particularly in the brain\textsuperscript{63}. Corresponding to the N-terminal putative trans-activation domains of Myc, but without the essential C-terminal
dimerisation and DNA binding motifs (see below), this protein can interfere with *c-myc* induced transformation. The *S-myc* gene is highly homologous to exons two and three of *c-myc*, and although expression in cells has not been established, it suppressed the tumorigenicity (assayed in nude mice) of RT4-AC cells into which it had been transfected. Little is known about the last two members of the family: *R-myc* is homologous to exons two and three of *c-myc* and can cooperate with *ras* to transform REFs. *P-myc* is homologous to parts of exon three of *L-myc* and the 3' untranslated region. Most work has been carried out on *c-myc*, and unless otherwise stated it is to this gene that I will now refer.

1.5 Myc expression

The control of the levels of Myc protein is complex yet reasonably well understood. It takes place at almost every imaginable level: initiation of transcription, transcriptional elongation, RNA processing, RNA stability, translation initiation and protein turnover. The gene contains two major promoters (P1 and P2) each with TATA boxes, and two (one in non-humans) minor TATA-less ones. A number of elements are known to control transcription, including an E2F binding site, which may confer part of the response of the promoter to serum. PDGF stimulates transcription through a response element in P1; TGF-β interacts with an element near P1 and may also act via Rb, which in turn may repress through the E2F site. Many other signals are also known to affect transcription initiation, though through as yet undetermined mechanisms. Additionally, a number of oncoproteins, both cellular and viral, are implicated in Myc regulation, including Abi, c-Myb, AP-1, Polyoma middle T-antigen and E1A. The production of Myc RNA is further controlled at the level of
transcriptional elongation. In HL60 or MEL cells that have been induced to differentiate, the RNA polymerase stops prematurely at a defined site near the end of exon I (see Marcu, 1992 for review), leaving an RNA molecule that is not polyadenylated and becomes rapidly degraded. In some cases, the RNA polymerase is held back right at the initiation point. Myc RNA stability is also regulated by sequences in the first exon and the 3′ untranslated region and increased stability may contribute to elevated Myc levels in some tumours.

Another level of control of Myc expression, was first described in cells from Burkitt's Lymphomas: Myc expression is predominantly from the translocated gene, with the normal allele silent. We now know that there is an autoregulatory loop by which Myc protein represses expression from the *myc* gene, found in many cell types. Auto regulation is a dose-dependent phenomenon, that is evident in normal primary cells and some, but not all, established cell lines. It is however found in few tumour cell lines (exceptions include those from transgenic mice with a constitutive *myc* gene and normal Myc expression is suppressed in a number of tumours in which one allele is activated and not subject to autoregulation. The autoregulation system is therefore commonly disrupted in carcinogenesis.

Members of the Myc family can suppress each other, but expression of the Myc family members is not mutually exclusive.

1.6 Myc structure

The c-, L- and N-Myc proteins exist as a number of forms, varying according to their phosphorylation state and length. The c-Myc protein is found as a 64 or a 67 kDa form depending on which of two translational start sites is used. These two forms are conserved in evolution from...
Xenopus to man, and there is some evidence for a limited difference in function\textsuperscript{116}. The actual size of the proteins is 439 and 453 amino acids in man (calculated molecular weight of about 48 kDa – the higher apparent value being due to phosphorylation). Phosphorylation occurs on serine and threonine residues but not on tyrosines\textsuperscript{117,118}, and is probably mediated by casein kinase II (CK-II) on the C-terminal sites, and possibly by glycogen synthase kinase 3\alpha on Thr-58, Ser-62 and Ser-71. These N-terminal phosphorylations are implicated in cell cycle control and transformation\textsuperscript{119}.

Like its RNA, Myc protein is particularly unstable, with a half-life of 20-30 minutes\textsuperscript{25,120} due to a ubiquitin-dependent process\textsuperscript{121,122}. Myc is a nuclear protein with a nuclear localisation signal (PAAKRVKLD)\textsuperscript{123} in the third exon; there is also a secondary signal\textsuperscript{123} in the C-terminus.

Sequence alignment of different Myc proteins within and between species revealed a number of conserved regions (see Fig. 1.1): two in the N-terminus and one in the C-terminus. The latter consists of three structural elements: a basic region (br), a helix-loop helix (hlh) and a leucine zipper (lz). The most C-terminal of these, the leucine zipper is a motif first described in the transcription factor C/EBP, and now known to be common in nuclear oncoproteins and transcription factors\textsuperscript{124}, including Fos, Jun, Fra-1 and GCN4. The leucine zipper has an amphipathic helical structure with a leucine residue at every seventh position. These lie on one side of the helix creating a hydrophobic surface which mediates protein dimerisation with another leucine zipper in a sequence-specific fashion\textsuperscript{125-127}.

The helix-loop-helix is immediately N-terminal to the leucine zipper and consists of two amphipathic helices connected by a loop. Like the leucine zipper, helix-loop-helix domains are a common dimerisation
Figure 1.1 Myc structure and function
motif found in a number of proteins such as MyoD, E12, E47 and the *Drosophila* achaete-scute, daughterless and twist proteins.

The basic region – a short stretch of basic amino acids – is found adjacent to the helix-loop-helix and has been shown to be a DNA-binding motif. The br-hlh and br-lz families of proteins had been well characterised, but Myc was the first protein described with both an hlh and an lz. In each family, the hlh or lz mediates specific dimerisation bringing two br's together in such a way as to present them for DNA binding. Br-hlh-lz regions have now been found in other proteins, TFE3 and TFEB, which bind the μE3 sequence motif of immunoglobulin gene enhancers and USF, which binds to the adenovirus major late gene promoter. Both are sequence-specific transcription factors. In the br-hlh, br-lz and br-hlh-lz families, both the dimerisation motif(s) and the basic region are essential to transcriptional activation and are in fact essential for Myc function (see below and chapter three).

Apart from DNA binding, the other requirement for a transcription factor is a domain that activates transcription when targetted to DNA. In the Myc N-terminus lie two further regions that are well conserved in all Myc proteins, known as Myc boxes I and II. All or part of these regions are essential in functional assays of Myc including co-transformation with activated ras, autoregulation, apoptosis and inhibition of differentiation *in vitro*. The first Myc box contains the phosphorylated Thr-58 and Ser-62 residues, while the second is a hydrophobic region frequently mutated in tumours. A region spanning the two boxes has been shown to activate transcription when fused to the DNA binding section of the yeast Gal-4 protein. The Myc protein therefore has all the hallmarks of a transcription factor: regions that should be able to mediate dimerisation, DNA binding and transcriptional activation.
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1.7 Myc dimerisation and DNA binding

Since Myc had strong structural similarity to transcription factors, it was important to identify whether it bound DNA in a sequence-specific fashion. A Myc binding site had been obtained, the sequence CACGTG\(^{139-142}\), discussed in more detail in chapters 4 and 7. The binding activity of c-Myc is weak however, requiring a high concentration, possibly due to poor homodimerisation efficiency\(^{143,144}\).

Two groups identified a dimerisation partner for Myc, termed Max\(^{145}\) or Myn\(^{146}\), which associates in vitro specifically via the hlh-lz domains either with itself or with the three Myc proteins (c-, L-, N-) but not with other br-hlh-lz proteins\(^{145}\). Furthermore, the Myc/Max dimer binds to the DNA sequence CACGTG under conditions in which neither protein alone binds significantly, and this is dependent on both the dimerisation and DNA binding domains\(^{139,145,146}\). Essentially all of the Myc molecules in vivo are found complexed to Max\(^{147}\), which is expressed constitutively in cells\(^{148}\).

Max exists in a variety of alternatively spliced forms either with (Max-2), or without (Max-1), a 9 amino-acid insert in the N-terminus\(^{145,146}\); each of these also exists in a truncated form (ΔMax-1 and ΔMax-2) which lacks the last 62 amino acids\(^{149}\). All forms are able to homodimerise or to heterodimerise with Myc and bind DNA, but the ΔMax forms lack a nuclear localisation signal and, as such, are only transported to the nucleus when bound to Myc.

1.8 Myc function

The first clue as to the functions of Myc (see Kelly and Siebenlist, 1986 for review\(^{150}\)) was the finding that it is an immediate early gene\(^{151,152}\), induced by growth factors in the absence of protein synthesis; although its induction is later\(^{151-153}\) (peaking some two hours after...
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stimulation) than most other immediate early genes\(^{151}\). In contrast to other immediate early genes whose expression falls away rapidly however, Myc RNA expression declines after the initial peak to a moderate level at which it remains, provided the mitogenic signal is maintained\(^{151,154}\) and there is little correlation in cycling cells between expression level and cell cycle phase\(^{155,156}\), one exception being T-lymphocytes\(^{157}\). So while most other immediate early genes are involved in the transition from quiescence, Myc is involved in maintaining the non-quiescent state. Further, while a number of immediate early genes can be induced by a variety of non-mitogenic stimuli, Myc is almost always associated with proliferation.

This relationship between mitogens and Myc expression has been accurately measured in fibroblasts and is detailed below. In these cells, there are fewer than 300 Myc molecules per quiescent cell. The addition of growth factors increases this to about 10,000 over two hours after which the level then falls to about 4,000 in late G1, a level that is then maintained all the time the cells are proliferating. If growth factors are removed, the cell will stop growing and become quiescent once more. Provided the restriction point (the stage after which the cell is committed to the next cycle – see section 1.10) has not been reached, arrest is rapid; once the cell has crossed this point however, arrest will take place at the next G1, after completing DNA synthesis and mitosis. Expression of Myc RNA and protein falls rapidly as soon as mitogens are removed\(^{154,158}\). If Myc levels are artificially maintained when mitogens are transiently removed, the cell behaves as if it had been continuously exposed to the mitogens, suggesting that Myc acts as an indicator of mitogen exposure. Myc is also rapidly down-regulated following anti-proliferative cues such as \(\gamma\)-interferon or TGF-\(\beta\)\(^{159-161}\), independent of the position in the cell cycle.
Enforced Myc expression prevents growth arrest in response to either withdrawal of mitogens or application of inhibitory cytokines. Myc therefore seems to be required continuously to override an intrinsic tendency to arrest. Expression of Myc is sufficient not only to maintain cell proliferation after mitogen withdrawal, but also to drive quiescent cells into the cycle\(^{162}\). Myc is also necessary for both of these processes. Inhibition of Myc expression with antisense nucleotides prevents quiescent lymphocytes from being stimulated to enter the cell cycle\(^{163}\). In proliferating keratinocytes\(^{161}\), haematopoietic cells\(^{164,165}\) and embryonal cells\(^{166}\), antisense Myc causes growth arrest in G1 and often triggers subsequent differentiation. In tumour cells, elevated Myc expression is common, and is typically uncoupled from mitogenic signals, such that expression continues even when signals do not. This correlates well with the observation that tumour cells fail to quiesce when deprived of growth factors or when contacted by their neighbours.

Although levels of Myc increase following mitogen exposure, Myc expression is decreased by a wide variety of differentiation inducing agents (including retinoic acid, hydroxyurea, phorbol ester and DMSO) in many different cell types. Since antisense c-myc triggers the differentiation of HL-60 cells\(^{167-171}\), the decrease in Myc is probably responsible for differentiation rather than merely being a by-product of the terminally differentiated, non-proliferative state. A general model in which cells differentiate in response to a decline in Myc levels seems oversimplistic however as many cells can arrest in response to mitogen withdrawal without differentiating. In other systems such as MEL cells (also L6E9\(^{172}\) and P19 cells\(^{173}\), the situation is complicated by the biphasic response of Myc to differentiating agents: Myc levels fall rapidly then rise again before falling away permanently. The initial rise and fall seems to be involved in priming differentiation, and marks the point of
commitment to differentiation. Myc expression can therefore accelerate commitment to differentiate, and antisense myc can impede differentiation, possibly by slowing commitment, although another group sees an induction of differentiation by antisense\textsuperscript{165,174}. Similar findings come from groups working on F9 teratocarcinoma cells and it is clear that in most systems (including quail embryo myoblasts\textsuperscript{175}, embryonic heart cells\textsuperscript{176} and most MEL cell lines\textsuperscript{105,177-179}, enforced Myc expression prevents withdrawal into the terminally differentiated state.

The above observations \textit{in vitro} correlate well with \textit{in vivo} studies. Myc is found in almost all proliferating tissues but hardly any differentiated ones (an exception being avian post-mitotic neurons\textsuperscript{180}) and as shown above, tumour cell lines with de-regulated Myc often display only a partially differentiated appearance.

Thus, \textit{in vitro}, Myc expression is sufficient to inhibit differentiation and maintain the proliferative state. Observations on fibroblasts constitutively expressing Myc and grown in the absence of growth factors have revealed yet another function of Myc. These cells do indeed continue to cycle but their numbers do not necessarily increase: large numbers of cells die. The process by which this occurs is programmed cell death (apoptosis), characterised by its rapidity (20-40 minutes), cell-surface blebbing, cell shrinkage and fragmentation, and the cleavage of DNA into pieces of nucleosome length\textsuperscript{136}. The association of Myc with apoptosis is not restricted to fibroblasts in culture, but rather is a more general phenomenon. Following oestrogen ablation of MCF-7 breast cancer cells, elevated Myc expression accompanies regression\textsuperscript{181} as it does in normal rat prostate following androgen removal\textsuperscript{182,183}. Myc is also induced during receptor-mediated death of embryonic thymocytes\textsuperscript{184}. The observation that Myc-expressing fibroblasts die only when factors are removed underlies the fact that two
processes are at work: Myc expression activates the cell death pathway, but another pathway responding to extracellular signals protects from death. In fact protection from apoptosis is separable from mitogenesis, and although some factors such as PDGF and EGF are both mitogens and survival factors, others such as IGF-1 for example, protect from apoptosis but are not mitogenic\textsuperscript{185}. That Myc contributes to both proliferation and death is perhaps not surprising given the absolute requirement for preventing inappropriate growth in multi-cellular organisms. A cell with unwarranted Myc will die before it can divide – note that apoptosis can take place at any stage of the cell cycle\textsuperscript{136}.

1.9 **Possible targets for Myc**

The timing of Myc expression in cells in response to signals is clear, as are the biological effects of Myc. What is missing however, is an understanding of the mechanism(s) by which Myc expression impinges upon the cell cycle machinery. Some evidence is available. The same domains of Myc are required for autoregulation\textsuperscript{135} as for cell cycle progression and transformation, suggesting that Myc autoregulates via the same mechanism it uses to perform its other functions and so the *myc* gene is a target, but not necessarily a direct one, of the Myc protein. Unfortunately, studies on autoregulation have not so far yielded any direct Myc target genes and Myc does not bind to sequences in its own gene.

Autoregulation apart, the expression of a number of genes is found to be down-regulated in association with Myc expression. Of potential importance is the effect of Myc on major histocompatibility complex (MHC) class I gene expression\textsuperscript{186-189}, which may allow Myc-expressing tumour cells to escape recognition by the immune system: down-regulation of MHC class I by Myc alters the appearance of the cell
surface, rendering the cells sensitive to natural killer cells. The mechanism of down-regulation seems to be different for N-Myc, which may act through enhancer A\textsuperscript{186,190} than for Myc, which does not \textsuperscript{191}. Neither effect is likely to be direct however. The LFA-1 gene is also down-regulated by Myc\textsuperscript{192}, as are the mouse pro-alpha 2 collagen\textsuperscript{193} and human c-neu\textsuperscript{194} genes. In MEL cells expressing exogenous Myc, two histone genes are down-regulated\textsuperscript{195}. There is no evidence that these genes are directly regulated by Myc however or that they are relevant to the proliferative, apoptotic or differentiating effects of Myc. The best candidate transcriptional target in 1991 was the \textit{α-prothymosin} gene, which is activated by Myc in the absence of protein synthesis\textsuperscript{162}, suggesting a direct transcriptional effect. This particular case, which has been further characterised over the past five years, is discussed further in the introduction to chapter five and in chapter seven.

1.10 The cell cycle

A major effect of Myc is the induction and maintenance of the proliferative state and Myc must interface in some way with the cell cycle. Dissecting the mechanisms by which Myc acts will therefore require an understanding of the cell cycle.

The cell cycle varies in time \textit{in vitro} from about ten hours to two days depending on cell type, but can be considered as four distinct phases: a period of growth and expansion following the previous division (G1), a period during which the DNA is replicated (S phase), a second growth phase (G2) and finally M phase when the cell divides (Fig. 1.2). The cycle is mainly regulated during G1 and G2, which vary greatly in length. In early G1 especially, a number of signals are
**Figure 1.2** The cell cycle (after Graña and Reddy)
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integrated in a cell-type specific fashion (for review see Feramisco et al., 1985 and Alberts et al., 1983): for example in fibroblasts, cell density and specific growth factors such as EGF, PDGF and IGF-1 are important, while IL-2 and antigen binding are important for T cells. Once cells have progressed through a certain point in G1 known as the restriction point (R), they are committed to S-phase entry even in the absence of continued growth signals, although they may arrest in the next G1.

1.11 Cyclins and cdk’s

At the core of the cell cycle, controlling the checkpoints are protein kinases known as cyclin dependent kinases or cdk’s. Their activity is regulated by cyclins expressed at precise periods in the cycle, by site-specific phosphorylation and dephosphorylation and by inhibitory proteins. The cyclin/cdk complexes are protein kinases, able to phosphorylate other proteins in a sequence-specific fashion, and their functional targets are now being characterised.

The cyclins were initially identified in invertebrate eggs, as proteins specifically degraded at mitosis. There are now over twelve distinct cyclins in mammalian cells, with varying levels of homology. All contain a 'cyclin box' through which they contact a cdk. The cyclins confer specificity to their partner kinases, acting to activate them (when associated) and de-activate them (when degraded). They also target the kinase within the cell, namely to the nucleus (the A-type cyclins) or to the mitotic apparatus (the B-type cyclins) which may be crucial given the similar in vitro consensus target sequence for phosphorylation by most cyclin/cdk complexes: (K/R)-S/T-P-X-(K/R) (see Nigg, 1993 for review).
Different cdk/cyclin complexes are active at different phases of the cycle. When mammalian cells are arrested in G1 by the removal of growth factors and then stimulated to progress into the cycle, then the first cyclin/cdk complex to be activated is generally a D-type cyclin bound to cdk4 or cdk6\textsuperscript{215,216}. D-cyclins are typically expressed throughout the cycle, and down-regulated only when the supply of growth factors is stopped (see Sherr, 1993\textsuperscript{217} for review). The function of cyclin D1 is most likely to be required in early to middle G1 since cyclin D1 antibodies prevent S-phase entry\textsuperscript{218,219} when applied prior to the restriction point but have no effect subsequently. It is likely therefore that cyclin D1 complexes phosphorylate a substrate required for G1-S transition, most probably the retinoblastoma (Rb) protein\textsuperscript{220} since in cells lacking Rb, D-type cyclins are not required for progression through the restriction point and p16 (a cyclin D1/cdk inhibitor protein, see below) does not cause cell cycle arrest\textsuperscript{221-227}.

Cyclin E, which like the D-type cyclins can complement for G1 cyclin mutations in yeast, is also expressed early after mitogen application in quiescent cells. In proliferating cells, cyclin E expression is periodic and regulates the G1-S transition at which point its levels are maximal\textsuperscript{228,229}. It is associated predominantly with cdk-2, a kinase which is in a genetically different class to cdk4 or cdk6 (since these cannot complement cdc2 in yeast while cdk2 can)\textsuperscript{230-232}.

As well as being a target for the cyclin D complexes, Rb is also a likely target for cyclin E complexes, and is phosphorylated on a number of cdk consensus sites during G1. When phosphorylated, Rb releases proteins bound to it such as E2F-1 (for review see Lam and La Thangue, 1994\textsuperscript{233} and Nevins, 1994\textsuperscript{234}), a transcription factor that regulates genes required for S-phase.
The cyclin E gene is up-regulated by E2F and so a positive feedback system operates whereby cyclin E-dependent phosphorylation of Rb releases E2F and drives cyclin E expression. In this way, initial phosphorylation of Rb by cyclin D complexes, which is mitogen-dependent, becomes mitogen-independent as cyclin E expression becomes established. Inactivation or absence of Rb reduces a cell’s growth factor requirements but does not eliminate them and so there must be additional levels of restriction point control\textsuperscript{219,235-238}. Proteins related to Rb and E2F-1 have since been identified, and are classified into the Rb and E2F families respectively. The other Rb-family members (p107 and p130) which are also putative cdk/cyclin targets seem to bind to and inactivate other E2F family members such as E2F-4. It is unlikely that the activities of the E2F proteins are controlled solely by the phosphorylation state of the Rb-family members: the phosphorylation timing of p130 is subtly different to that of Rb\textsuperscript{239} and p107 doesn't seem to be regulated by phosphorylation. It has been suggested that p107 expression levels may control E2F-4 availability\textsuperscript{240-242}.

After, or just prior to, cells entering S-phase, cyclin E is degraded and cyclin A is produced, with which cdk2 now complexes. Cyclin A/cdk2 can phosphorylate DP-1 (another E2F family member), abolishing DP-1/E2F-1 DNA binding which may be the mechanism for turning off the cyclin E autoregulatory loop. Later in the cycle, cyclin A binds to cdk-1 (originally known as cdc-2) and is then degraded prior to division.

Cyclin B/cdk-1 controls division, and the destruction of cyclin B at the end of M-phase is required before exit to the next G1. Degradation at M-phase is conferred by a destruction box\textsuperscript{243} though only cdk-1 associated cyclins are destroyed\textsuperscript{244,245}. The mitotic cyclin destruction system is inactivated by G1 cyclin/cdk activity which ensures that in
each round of the cell cycle, G1 cyclins must accumulate before the mitotic cyclins.

Other cyclin/cdk pairs also have important roles related to cell cycle control, especially cdk7(MO15 or CAK)/cyclin H which regulates the phosphorylation and hence activity of other cdk's. A number of other cyclins and cdk's have also been described. The properties of the mammalian cdk/cyclins are summarised in the table below (Fig. 1.3 after Pines).

1.12 Cell cycle arrest and cyclin/cdk regulation

Cells may arrest the cycle at either G1 (typically) or G2 in response, for example, to a lack of proliferative signals, cell density or to DNA damage. One mediator of arrest, the tumour suppressor gene p53, is a key regulator of a G1 checkpoint. In response to irradiation, p53 protein levels increase and the cells arrest. Cells lacking p53 fail to arrest but if the gene is replaced, the arrest phenotype is partially rescued. Two genes implicated in cell cycle arrest are trans-activated by p53: GADD45 and p21. GADD45 stimulates DNA repair in vitro and inhibits DNA synthesis until repair is complete. p21 is a member of an exciting family of recently discovered proteins (the cyclin dependent kinase inhibitors or cdi's) that inhibit the cyclin/cdk complexes. There are a large number of cdi proteins each of which inhibit the kinase activity of particular cdk's (see figures 1.2 and 1.3). Cdi's are implicated in many forms of growth arrest including senescence, terminal differentiation, cyclic-AMP induced arrest, the quiescence of T cells in the absence of IL-2, TGF-β mediated arrest, and contact inhibition as well as p53 mediated arrest.

In addition to the control of cyclin and cdi levels, the activity of the cdk/cyclin complexes is dependent on regulatory phosphorylation.
<table>
<thead>
<tr>
<th>Cyclin</th>
<th>Subfamily</th>
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<th>CDI</th>
<th>Phase</th>
<th>Substrates</th>
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<td>p21?</td>
<td>S, G2, M</td>
<td>RF-A?</td>
<td>Interacts with p107, p130, E2F, E2F-1</td>
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<td></td>
<td></td>
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<td>cdk-1</td>
<td>p21?, p24?</td>
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<td>Rb?</td>
<td>PRAD1 and Bcl1 proto-oncogene</td>
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<td>cdk-2</td>
<td>p21, p27</td>
<td>G1/S</td>
<td>Rb?</td>
<td>Interacts with p107, p130, E2F, RF-A?</td>
</tr>
<tr>
<td>F</td>
<td></td>
<td></td>
<td></td>
<td>?</td>
<td>?</td>
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<td></td>
<td>T-loop Threonine</td>
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**Figure 1.3** Mammalian Cyclins after Pines, 1995
and dephosphorylation. Cyclin B/cdk1 complexes are present but inactive prior to mitosis, when the cdc-25 phosphatase specifically dephosphorylates certain sites on cdk1, activating the cyclin B/cdk1 complex and thereby allowing mitosis to proceed (see Morgan, 1995 for review). This provides another checkpoint for control that may be used to prevent division in the case of improper completion of S-phase (for example unreplicated DNA or incorrect assembly of the mitotic apparatus).

The cell cycle is thereby regulated at a number of levels. The interactions between the Myc protein and the cell-cycle machinery are examined in chapter 5 and more particularly in chapter 6.

1.13 Aims: the mechanisms of Myc action

For years following its discovery as an oncoprotein, the mechanisms of c-Myc function remained obscure. Initially oncoproteins were classified as being either nuclear, as Myc is, or cytoplasmic. Other nuclear oncoproteins such as Jun, Fos, Myb, Ets and Erb-A were shown to be transcriptional regulators, and at the start of this project, the structure/function analysis of Myc had revealed it to be a putative transcription factor with a partially determined DNA binding site (reviewed in Collum, 1990). My initial aim was to determine whether Myc could indeed activate transcription in mammalian cells. The second aim was to determine the exact DNA binding sites for the Myc/Max and Max/Max complexes as a prelude to identifying the target genes controlling the cell cycle. The final aim was to find the targets of Myc action and to understand the mechanisms by which Myc acts.
## Chapter Two

### 2 MATERIALS AND METHODS

#### 2.1 MATERIALS

##### 2.1.1 Chemicals and solvents

Chemicals used were all of analytical grade and obtained from either Sigma Chemicals, UK or the suppliers listed below.

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<tr>
<td>β-Mercaptoethanol</td>
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</tr>
<tr>
<td>Bis acrylamide (EASIgel)</td>
<td>Scotlab, UK</td>
</tr>
<tr>
<td>Bis acrylamide (Protogel)</td>
<td>National Diagnostics, USA</td>
</tr>
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<td>Bromophenol blue</td>
<td>Biorad, USA</td>
</tr>
<tr>
<td>Caesium chloride</td>
<td>Fisons, UK</td>
</tr>
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<td>Coomassie brilliant blue</td>
<td>Biorad, USA</td>
</tr>
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<td>ECL kits</td>
<td>Amersham International, UK</td>
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<td>Ethanol (Absolute alcohol)</td>
<td>Hayman, UK</td>
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<td>Polaroid (supplied by Sigma, UK)</td>
</tr>
<tr>
<td>Film (X-omat)</td>
<td>Kodak (supplied by Sigma, UK)</td>
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<tr>
<td>Film (RX)</td>
<td>Fuji (supplied by Sigma, UK)</td>
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<td>Filtration units (0.2 &amp; 0.45µm)</td>
<td>Nalge Company, USA</td>
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<td>G418, Geneticin</td>
<td>Life Technologies, Sweden</td>
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<td>GeneClean kits</td>
<td>BIO 101 Inc., USA</td>
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<tr>
<td>Glycogen</td>
<td>Boehringer Mannheim, UK</td>
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<tr>
<td>4-Hydroxy-tamoxifen</td>
<td>Laboratoires Isovesco, France</td>
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<td>Membrane (Hybond-ECL/N)</td>
<td>Amersham International, UK</td>
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<tr>
<td>Membrane (Nitrocellulose)</td>
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</tr>
<tr>
<td>Membrane (Immobilon P)</td>
<td>Millipore, UK</td>
</tr>
<tr>
<td>Microcapillary loading tips</td>
<td>Bioscience Inc., USA</td>
</tr>
<tr>
<td>MicroSpin columns</td>
<td>Pharmacia, Sweden</td>
</tr>
</tbody>
</table>
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| Protein A-sepharose          | Pharmacia, Sweden |
| Protein G-sepharose          | Pharmacia, Sweden |
| Qiagen kits                  | Qiagen, Germany  |
| RNAsin                       | Promega, UK      |
| Sequenase sequencing kit     | United States Biochemical corp., USA |
| Temed                        | Biorad, USA      |
| TNT kits                     | Promega, UK      |
| Tween 20                     | Biorad, USA      |
| Ultima Gold liquid scintillant| Packard Instruments, Netherlands |
| Urea                         | Fisons, UK       |
| Xylene cyanol FF             | Biorad, USA      |

2.1.2 Enzymes

Enzymes were purchased from among the following suppliers interchangeably: New England Biolabs, USA; Promega, UK; Stratagene, UK; Boehringer Mannheim, UK; Sigma, UK.

2.1.3 Radiochemicals

All radiochemicals were purchased from Amersham International, UK.

2.1.4 Antibodies

Myc (rabbit polyclonal pan-myc, XM-1) Trevor Littlewood (PM\textsuperscript{147})

Max (rabbit polyclonal, Max 2.1) Trevor Littlewood (MX\textsuperscript{147})

HRP-labelled secondary antibodies Dakopatts, Denmark, or Amersham International, UK
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Fluorochrome labelled Jackson Immunoresearch secondary antibodies

Rat cyclin E (rabbit polyclonal, sc-481) Santa Cruz, USA

Human p21 (rabbit polyclonal, sc-397) Santa Cruz, USA

Human p27 (rabbit polyclonal, sc-528) Santa Cruz, USA

Human cyclin E (western only) Pharmingen, USA (mouse monoclonal HE12)

Human cyclin E Pharmingen, USA (immuno-precipitation only) (mouse monoclonal, HE67)

2.1.5 Escherichia coli strains

The following strains were used routinely for propagating and amplifying plasmid DNA.

DH5α^{280}: F-psi80d, lacZM15, endA1, recA1, hsdR17, (rK^-mK^-), supE44, thi-1 k-, gyrA96, relA1 (lacZYA-argF)U69

JM101 (Stratagene)^{281}: supE, thi-1, Δ(lac-proAB), [F^- traD36, proAB, lacI^QZΔM15]
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XL-1 blue (Stratagene)\(^282\):

\(\text{recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac, }\{\text{F proAB, lacI^qZ\Delta}{\text{M15, Tn10 (tet^r)}}\}\)

### 2.1.6 Oligonucleotides

Oligonucleotides were provided as a service, synthesised using an Applied Biosystems Model 380B automated synthesiser.

The following pairs were used for gel retardation assays:

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence, 5' to 3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>CM1-1</td>
<td>CCCCCCACCACGTTGGTCCTGTA</td>
</tr>
<tr>
<td>CM1-2</td>
<td>TCAGGCACCACGTGGTGCCCCG</td>
</tr>
<tr>
<td>wt-1</td>
<td>TGGACGCGCCACGGTTGGGTTCCTC</td>
</tr>
<tr>
<td>wt-2</td>
<td>TCGAGGCGACACGGTGCCG</td>
</tr>
<tr>
<td>GTA-1</td>
<td>TGGACGCGACACGGTGCCG</td>
</tr>
<tr>
<td>GTA-2</td>
<td>TCGAGGGGATCAGGTCCCG</td>
</tr>
<tr>
<td>Twt-1</td>
<td>TGGAGGCGATCGGTCCCTC</td>
</tr>
<tr>
<td>Twt-2</td>
<td>TCGAGGGGACACGGTGCG</td>
</tr>
<tr>
<td>TwtA-1</td>
<td>TGGAGGCGATCGGTCCCTC</td>
</tr>
<tr>
<td>TwtA-2</td>
<td>TCGAGGGGACACGGTGCG</td>
</tr>
</tbody>
</table>

### 2.1.7 Plasmid constructs

Myc and Max plasmids were obtained from Bruno Amati except where noted in the text. For in vitro transcription and translation, pKSM-Myc* and Max* were used\(^283\). MLV-Myc and BJ3-Max expression vectors are also as described in Amati, 1993\(^283\). Other expression vectors using BJ9 and BJ3 were made by exchanging the cDNA insert from the DOR, BJ3 or MLV vectors as appropriate.

Other plasmids are listed overleaf:
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pBLCAT2  
pGL2 Basic  
GalER system  
pBabe vectors  
J-series vectors  
murine cyclin D1*  
pVZ-1 Mad*  
pMxi-1*  
pUHG cyclin E*  
PRLCGAP*  
pBJ4-cyclin A*  

Plasmids marked * were used as a source of cDNA's for probing northern blots.

2.1.8 Cell lines

The following cell lines were obtained from the source listed below:

Rat-1  
Rat-1 MycER  
Rat-1 ΔMycER  
Secondary rat embryo fibroblasts  
Swiss 3T3  
Swiss 3T3 MycER  
Murine erythroleukaemia cells, strain 585  

GCD lab\textsuperscript{284}  
Promega, UK  
Braselmann\textsuperscript{285}  
GCD lab\textsuperscript{286}  
GCD lab\textsuperscript{287}  
Anton Andrews\textsuperscript{288}  
Bob Eisenmann\textsuperscript{289}  
A. Zervos\textsuperscript{290,291}  
GCD lab\textsuperscript{292}  
GCD lab\textsuperscript{293}  
GCD lab (containing human cyclin A fragment from Pines\textsuperscript{294})  
GCD lab.\textsuperscript{103}  
GCD lab.\textsuperscript{136}  
GCD lab.\textsuperscript{136}  
GCD lab.\textsuperscript{50}  
GCD lab.\textsuperscript{295}  
GCD lab. derived from Swiss 3T3 by infection with pMV7-MycER  
Dr. Bill Wood, Oxford
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MRC5 fibroblasts

ICRF established cell stocks

GP+E cells

GCD lab.

2.1.9 Stock solutions

All buffer solutions were prepared using deionised, distilled water and stored at room temperature unless indicated otherwise.

<table>
<thead>
<tr>
<th>Buffer Name</th>
<th>Description and Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin (500x)</td>
<td>50 mg/ml ampicillin (Na salt)</td>
</tr>
<tr>
<td>β-galactosidase buffer (2x)</td>
<td>120 mM Na₂HPO₄·7H₂O, 80 mM NaH₂PO₄·H₂O, 1.33 mg/ml O-nitrophenyl-β-D-galactopyranoside, 2 mM MgCl₂, 2.7 ml/litre β-mercaptoethanol</td>
</tr>
<tr>
<td>BHI</td>
<td>3.7% (w/v) Brain heart infusion powder</td>
</tr>
<tr>
<td>BHI agar</td>
<td>400 ml BHI, 6 g agar</td>
</tr>
<tr>
<td>Blocking buffer</td>
<td>PBSA, 2% milk, 0.1% Tween</td>
</tr>
<tr>
<td>CAT/LacZ lysis Buffer</td>
<td>0.1% Triton X-100, 250 mM Tris-HCl, pH 8.0</td>
</tr>
<tr>
<td>CIP buffer</td>
<td>50 mM Tris-HCl, pH 9.0, 1 mM MgCl₂, 0.1 mM ZnCl₂, 1 mM spermidine</td>
</tr>
<tr>
<td>Church buffer 45% (RNA)</td>
<td>200 mM Na₂HPO₄·NaH₂PO₄ pH 7.2, 1 mM EDTA, 1% (w/v) BSA, 7% (w/v) SDS, 45% (v/v) formamide</td>
</tr>
<tr>
<td>Denaturation buffer</td>
<td>1.5 M NaCl, 0.5 M NaOH</td>
</tr>
<tr>
<td>Denhardt’s solution (10x)</td>
<td>0.2% (w/v) Ficoll, 0.2% (w/v) poly-vinyl-pyrrolidone, 0.2% (w/v) BSA (Fraction V)</td>
</tr>
<tr>
<td>Dextran hybridisation buffer</td>
<td>1% (w/v) SDS, 10% (w/v) dextran sulphate, 1M NaCl</td>
</tr>
<tr>
<td>DNA gel loading buffer (10x)</td>
<td>0.25% (w/v) bromphenol blue, 0.25% (w/v) xylene cyanol, 25% Ficoll type 400</td>
</tr>
</tbody>
</table>

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**E4 medium**

All ingredients in mg/l:
- CaCl$_2$.2H$_2$O, 265; Fe(NO$_3$)$_3$, 0.1;
- KCl, 400; MgSO$_4$.7H$_2$O, 200;
- NaCl, 6,400; NaH$_2$PO$_4$.2H$_2$O, 140;
- L-Arginine mono. HCl 84;
- L-Cystine, 48; L-Histidine mono. HCl, 42;
- L-Glutamine, 584;
- Glycine, 30; L-Isoleucine, 104.8;
- L-Leucine, 104.8; L-Lysine mono. HCl, 146.2;
- L-Methionine, 30; L-Phenylalanine, 66;
- L-Serine, 42;
- L-Threonine, 95.2;
- L-Tryptophan, 16; L-Tyrosine, 72.4;
- L-Valine, 93.6; Inositol, 7;
- Choline Chloride, 4; Folic acid, 4;
- Nicotinamide, 4; DL-pantothenic acid, Ca salt, 4;
- Riboflavin, 0.4; Thiamine HCl (aneurine), 4;
- D-Glucose, 4,500;
- Phenol red, 15; NaHCO$_3$, 3,700;
- Penicillin, 100,000 units;
- Streptomycin Sulphate, 100;
- Sodium pyruvate, 110;
- Antimyocotic (Butyl-p-hydroxybenzoate), 0.2;

**Formaldehyde running buffer (10x)**

200 mM MOPS pH 7.0, 50 mM NaOAc, 10 mM EDTA pH 8.0

**Gel Loading Buffer (6x)**

TBE plus: 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, 30% (v/v) glycerol

**Glutamate buffer**

125 mM potassium glutamate, 10 mM HEPES pH 8.0, 0.5 mM spermidine, 0.15 mM spermine, 2 mM DTT, 1 mM EDTA, 0.5 mM EGTA, 40% (v/v) glycerol. Store at -70°C.
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Guanidium solution
6 M guanidium isothionate, 5 mM sodium citrate pH 7.0, 100 mM β-mercaptoethanol, 0.5% (w/v) sarkosyl

HBS
0.8% (w/v) NaCl, 0.037% (w/v) KCl, 0.0135% (w/v) Na₂HPO₄, 0.1% (w/v) dextrose, 0.5% (w/v) HEPES; adjusted to pH 7.05 with 0.5N NaOH

Histone H1 solution
10 mg/ml histone H1 in 50 mM Tris-HCl, pH 7.5

Histone H1 kinase buffer
50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 1 mM DTT

Hybridisation solution (run-on)
10 mM Tris pH 7.5, 250 g/ml Torula RNA, 0.02% BSA, 0.02% Ficoll 400, 0.02% PVP 360, 0.5% non-fat dry milk, 300 mM NaCl, 1% SDS, 10 mM EDTA

Ligase buffer (10x)
500 mM Tris-HCl pH 7.8, 100 mM MgCl₂, 200 mM DTT, 10 mM ATP, 5% (w/v) BSA, store at -20°C in aliquots

Luciferase lysis buffer
0.65% NP40, 10 mM Tris-HCl, pH 8.0, 1mM EDTA, pH 8.0, 150 mM NaCl

Luciferase reaction buffer
25 mM Glycylglycine, pH 7.8, 5 mM ATP (pH corrected to 8.0), 15 mM MgSO₄

Luria Broth
1% (w/v) bacto-tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl

Lysis buffer NP40
500 mM NaCl, 1% (v/v) NP40, 50 mM Tris-HCl, pH 8.0

Neutralisation buffer
1.5 M NaCl, 500 mM Tris-HCl, pH 7.2, 1 mM EDTA

P1 buffer (resuspension buffer)
100 µg/ml RNase A, 50 mM Tris-HCl, 10 mM EDTA, pH 8.0, store at 4°C
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<table>
<thead>
<tr>
<th>Buffer Description</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>P2 buffer (lysis buffer)</td>
<td>200 mM NaOH, 1% (w/v) SDS</td>
</tr>
<tr>
<td>P3 buffer (neutralisation buffer)</td>
<td>3.0 M KAc, pH 5.5, store at 4°C</td>
</tr>
<tr>
<td>PAGE-Running Buffer</td>
<td>20 mM Trizma base, 190 mM glycine, 0.1% (w/v) SDS, pH 8.6</td>
</tr>
<tr>
<td>PBSA</td>
<td>140 mM NaCl, 2.5 mM KCl, 10 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.2</td>
</tr>
<tr>
<td>PCR buffer</td>
<td>500 mM KCl, 100 mM Tris-HCl, pH 8.3, 15 mM MgCl₂, 0.1% (w/v) gelatin</td>
</tr>
<tr>
<td>Phenol/chloroform/isoamyl alcohol</td>
<td>25 parts redistilled phenol, equilibrated with TE pH 8.0 and mixed with 24 parts chloroform and 1 part isoamyl alcohol</td>
</tr>
<tr>
<td>Ponceau-S red stain</td>
<td>0.1% (v/v) Ponceau S in 5% (v/v) acetic acid</td>
</tr>
<tr>
<td>PNK buffer</td>
<td>70 mM Tris-HCl (pH 7.6), 10 mM MgCl₂, 5 mM DTT</td>
</tr>
<tr>
<td>QBT buffer (equilibration buffer)</td>
<td>750 mM NaCl, 50 mM MOPS, 15% (v/v) ethanol, pH 7.0, 0.15% (v/v) Triton X-100</td>
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<tr>
<td>QC buffer (wash buffer)</td>
<td>1 M NaCl, 50 mM MOPS, 15% (v/v) ethanol, pH 7.0</td>
</tr>
<tr>
<td>QF buffer (elution buffer)</td>
<td>1.25 M NaCl, 50 mM Tris-HCl, 15% (v/v) ethanol, pH 8.5</td>
</tr>
<tr>
<td>Restriction buffer 1 (NEB)</td>
<td>10 mM Bis Tris Propane-HCl, 10 mM MgCl₂, 1mM DTT, pH 7.0 at 25°C</td>
</tr>
<tr>
<td>Restriction buffer 2 (NEB)</td>
<td>10 mM Tris-HCl, 10 mM MgCl₂, 1 mM DTT, 50 mM NaCl, pH 7.9 at 25°C</td>
</tr>
<tr>
<td>Restriction buffer 3 (NEB)</td>
<td>50mM Tris-HCl, 10mM MgCl₂, 1 mM DTT, 100 mM NaCl, pH 7.9 at 25°C</td>
</tr>
<tr>
<td>Restriction buffer 4 (NEB)</td>
<td>20 mM Tris-acetate, 10 mM magnesium acetate, 50 mM potassium acetate, 1mM DTT, pH 7.9 at 25°C</td>
</tr>
<tr>
<td>Material/Buffer</td>
<td>Description</td>
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<tr>
<td>----------------</td>
<td>-------------</td>
</tr>
<tr>
<td><strong>RIPA buffer</strong></td>
<td>150 mM NaCl, 1% (v/v) Nonidet P-40, 0.5% DOC, 0.1% (w/v) SDS, 50 mM Tris, pH 8.0</td>
</tr>
<tr>
<td><strong>RNA gel loading buffer</strong></td>
<td>50% (v/v) glycerol, 1 mM EDTA, 0.4% (w/v) bromophenol blue, 0.4% (w/v) xylene cyanol</td>
</tr>
<tr>
<td><strong>RSB</strong></td>
<td>10 mM Tris-Cl, pH 7.5, 10 mM KCl, 0.5 mM spermidine and 0.1% NP40 store at -20°C; PMSF 50 μg/ml and DTT 1 mM added just prior to use.</td>
</tr>
<tr>
<td><strong>SDS loading/lysis buffer (1x)</strong></td>
<td>60 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 0.1% (w/v) bromophenol blue, 10% (v/v) glycerol, 5% (v/v) β-mercaptoethanol</td>
</tr>
<tr>
<td><strong>SDS PAGE buffer</strong></td>
<td>25 mM Tris base, 192 mM glycine, 0.1% (w/v) SDS</td>
</tr>
<tr>
<td><strong>Sequenase buffer (5x)</strong></td>
<td>200 mM Tris-HCl (pH 7.5), 100 mM MgCl₂, 250 mM NaCl</td>
</tr>
<tr>
<td><strong>Sequenase enz. dil. buffer</strong></td>
<td>10 mM Tris-HCl (pH 7.5), 5 mM DTT, 0.5 mg/ml BSA</td>
</tr>
<tr>
<td><strong>Sequenase labelling mix (5x)</strong></td>
<td>7.5 μM of each: dGTP, dCTP, dTTP</td>
</tr>
<tr>
<td><strong>Sequenase Mn buffer</strong></td>
<td>0.15 M Sodium isocitrate, 0.1 M MnCl₂</td>
</tr>
<tr>
<td><strong>Sequenase stop solution</strong></td>
<td>95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol</td>
</tr>
<tr>
<td><strong>Sequenase termination mix</strong></td>
<td>XTP termination mix (for each of G, A, T and C): 80 μM of each dNTP, 8 μM ddXTP, 50 mM NaCl</td>
</tr>
<tr>
<td><strong>SET (10x)</strong></td>
<td>10% (w/v) SDS, 100 mM Tris-HCl, pH 7.5, 50 mM EDTA</td>
</tr>
<tr>
<td><strong>SOC medium</strong></td>
<td>0.5% (w/v) yeast extract, 2% (w/v) tryptone, 10 mM NaCl, 2.5</td>
</tr>
<tr>
<td><strong>Chapter Two</strong></td>
<td><strong>Materials and Methods</strong></td>
</tr>
<tr>
<td>----------------</td>
<td>--------------------------</td>
</tr>
<tr>
<td><strong>Solution 1</strong></td>
<td>mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, 20 mM glucose</td>
</tr>
<tr>
<td><strong>Solution 2</strong></td>
<td>50 mM glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA</td>
</tr>
<tr>
<td><strong>Solution 3</strong></td>
<td>200 mM NaOH, 1% (w/v) SDS</td>
</tr>
<tr>
<td><strong>SSC (20x)</strong></td>
<td>5 M KOAc, pH 4.8</td>
</tr>
<tr>
<td><strong>T4 DNA polymerase buffer (10x)</strong></td>
<td>330 mM Tris-acetate, pH 8.0, 660 mM KOAc, 100 mM Mg(OAc)₂, 5 mM DTT, 1 mg/ml (w/v) BSA, stored in aliquots at -20°C</td>
</tr>
<tr>
<td><strong>TAE buffer (1x)</strong></td>
<td>40 mM Tris-acetate, 2 mM EDTA</td>
</tr>
<tr>
<td><strong>TBE buffer (10x)</strong></td>
<td>1.3 M Tris base, 440 mM boric acid, 25 mM EDTA, pH 8.8</td>
</tr>
<tr>
<td><strong>TE (10x)</strong></td>
<td>100 mM Tris-HCl, pH 8.0, 10 mM EDTA pH 8.0</td>
</tr>
<tr>
<td><strong>Tfb 1</strong></td>
<td>30 mM KOAc, 100 mM RbCl, 10 mM CaCl₂, 50 mM MnCl₂·4H₂O, 15% (v/v) glycerol, pH adjusted to 5.8 with 200 mM acetic acid, filter sterilised, stored at 4°C</td>
</tr>
<tr>
<td><strong>Tfb 2</strong></td>
<td>20 mM MOPS, 75 mM MgCl₂, 10 mM RbCl, 15% (v/v) glycerol, pH adjusted to 6.5 with KOH, filter sterilised, stored at 4°C</td>
</tr>
<tr>
<td><strong>TM Buffer</strong></td>
<td>20 mM Tris-HCl, pH 8.0, 2 mM MgCl₂</td>
</tr>
<tr>
<td><strong>Tris saline</strong></td>
<td>24 mM Trizma base, 0.8% (w/v) NaCl, 0.38% (w/v) KCl, 0.01% (w/v) Na₂HPO₄, 0.1% (w/v) dextrose, 0.001% (w/v) phenol red, pH 7.7 (adjusted with HCl)</td>
</tr>
<tr>
<td><strong>Trypsin</strong></td>
<td>Tris saline plus: 0.25% (w/v) trypsin, 100 U/ml penicillin (Na salt), 0.01% (w/v) streptomycin</td>
</tr>
</tbody>
</table>
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TYM broth 2% (w/v) Bacto-Tryptone, 0.5% (w/v) Bacto Yeast Extract, 0.1 M NaCl, 0.01 M MgCl₂ or MgSO₄

Versene PBSA plus: 0.54 mM EDTA, 0.001% (w/v) phenol red, pH 7.2

Western transfer buffer 25 mM Tris-HCl pH 8.3, 192 mM glycine, 20% (v/v) Methanol, 0.1% (w/v) SDS

2.2 METHODS

The following protocols were used except as described in the text. They are presented with technical notes to enable them to be followed in practice. For more information, see the relevant manufacturer’s notes or ‘Molecular Cloning: a laboratory manual’

2.2.1 Preparation of competent bacteria

Grow a single fresh colony of the desired strain of *E. coli* for 2 hours in 5 ml of TYM broth shaking vigorously at 37°C.

Transfer to 100 ml fresh TYM broth. Shake at 37°C for a further 2-3 hours until OD₅₅₀=0.5. Spin the cells in two 50 ml Falcon tubes at 3,500 rpm, 0°C for 10 minutes. Pour off the supernatant and aspirate remaining liquid from the tube walls. Resuspend the pellet vigorously in 20-40 ml of ice cold Tfb I.

Leave the resuspended bacteria on ice for 10 minutes. This step is the most critical one for maximising transformation efficiency. Spin the cells as gently as possible (10 minutes at 3,000 rpm, 0°C). Remove the supernatant, dry the tubes and resuspend in 4 ml of ice cold Tfb II. It is important to be gentle and patient during resuspension. Aliquot the competent cells into pre-cooled (on ice) sterile tubes and place in the -70°C freezer (i.e. do not flash freeze and store in liquid nitrogen).
2.2.2 Transformation of competent bacteria

Place a tube of competent bacteria on ice and allow it to thaw (this takes about 10-15 mins). Add the DNA (10 µl of a ligation or 0.1 µl of a plasmid solution) to the bacteria, mix gently and incubate for 30 minutes on ice. Note when efficiency is not a concern, a 5 minute incubation is sufficient. If transforming a ligation performed in low-melt agarose, heat the ligation to 68°C for a few minutes to melt, then place it at 37°C to cool, finally adding it to the competent bacteria. Heat shock the bacteria at 42°C for 1 minute then cool 1 minute on ice. Add 1 ml of BHI broth and incubate at 37°C for 45 minutes with occasional agitation. Spin at 6,500 rpm in a microfuge for 1 minute. Remove all but 100 µl of the supernatant, gently resuspend the pellet in the remaining supernatant and spread the bacteria onto appropriate selective plates.

2.2.3 Plasmid preparation I - miniprep

Pick a single colony, transfer to 3 ml BHI and shake at 37°C overnight. Spin 1.5 ml of the culture in a microfuge tube (for a greater yield add a further 1.5 ml and spin again) and resuspend the pellet in 100 µl solution I. Add 200 µl solution II and mix by inversion. Add 150 µl ice-cold solution III and incubate 5 minutes on ice. Spin 5 minutes to pellet the cell debris and chromosomal DNA and remove the supernatant to a fresh tube containing 1 ml EtOH. Spin 5 minutes, wash pellet with 500 µl 70% EtOH and resuspend in 50 µl TE + RNAses A and T1 (20 µg/ml). 3 µl is usually sufficient for a diagnostic digest.

2.2.4 Plasmid preparation II - CsCl

A sound if extremely lengthy and laborious method, providing excellent quality DNA.
Inoculate 100 ml of BHI (with appropriate antibiotics) with the bacteria and shake vigorously overnight at 37°C.

Transfer 50 ml to a 50 ml Falcon tube (polypropylene only), spin 5-10 minutes, 5,000 rpm, 4°C. Discard supernatant. Top up with remainder of the culture. Spin as before and again discard the supernatant.

Resuspend in 7.5 ml of Solution I, making sure the bacteria are well in suspension. Add 15 ml Solution II. Invert (do not vortex) several times. The mix should become a homogeneous, translucent slime after a few minutes. Add 11.25 ml Solution III. Invert several times. Leave on ice 10 minutes. Spin 10 minutes 5,000 rpm 4°C.

Transfer the supernatant * (this supernatant may be used instead in the PEG purification protocol below) to a fresh 50 ml tube. Use a 25 ml plastic pipette, taking care to push the layer of white material on the top to the side. It will stick to the pipette, do not allow it to be transferred to the new tube. Top up to 50 ml with isopropanol (usually 25 ml). Mix well by inversion. Spin 6,000 rpm, 10 minutes. Remove the supernatant.

Resuspend the pellet in 5.2 ml of CsCl/EtBr mix (120 g CsCl + 100 ml water + 10 ml (5 mg/ml) EtBr + 200 μl 0.5M EDTA). This will require vigorous vortexing. Continue to mix until all the white lumps are gone. Spin 6,000 rpm, 5 minutes to pellet insoluble protein and RNA. Transfer the supernatant to a polypropylene (ultraclear tubes are not recommended) Vti65.2 tube using a syringe and top up with CsCl/EtBr mix. Heat seal.

Spin at either: 65,000 rpm 4 hours 25°C or 55,000 rpm overnight 20°C.

Use a trans-illuminator on its side to illuminate the tube with long-wave UV and visualise the DNA. Puncture the bottom of the tube, then the top. Allow the liquid to flow out, controlling the rate with a
finger over the top hole. Collect the plasmid (lower) band in a microfuge tube. The volume should be 0.5-1.5 ml.

Add to a new Vti65.2 tube, topping up with aqueous CsCl solution (120 g CsCl + 110 ml water, no EtBr). Respin as before. Collect the DNA as before. The band should come out in up to 1.5 ml, usually about 0.5 ml.

Extract with aqueous CsCl-saturated isobutanol until the pink colour has completely gone, usually 4-5 times but up to 8 times; this step is essential to efficiently extract the EtBr from the DNA. Transfer to a 15 ml Falcon tube and top up to 2 ml with TE to prevent CsCl precipitation. Add 5 ml EtOH and mix by inversion. Spin 6,000 rpm, 10 minutes. Wash with 70% EtOH. Resuspend the pellet in 400 µl TE.

Phenol extract twice, then chloroform/isoamyl alcohol extract twice (this removes EtBr and residual bacterial proteins which can adversely affect transfection efficiencies). Precipitate with 40 µl 3 M NaOAc and 1 ml EtOH. Resuspend in 200 µl TE, take OD\textsubscript{260} reading to ascertain concentration. (The OD of a 1/200 dilution x 10 = concentration in mg/ml).

2.2.5 Plasmid preparation III - PEG

A method which gives high yields but frequently results in DNA contaminated with PEG and not recommended for transfection.

Following the preparation of a plasmid solution by alkaline lysis (the supernatant marked * as prepared above), add RNase A to 20 µg/ml and incubate 30 minutes, 37°C. Precipitate the DNA with an equal volume of isopropanol with a 20 minute spin at 5,000 rpm. Resuspend the pellet in 800-1,000 µl TE, add an equal volume of 1.6 M NaCl, 13% PEG M.W. 800. Mix well and keep on ice for 10 minutes. Spin 10 minutes, discard the supernatant. Resuspend the pellet in 400-500 µl
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TE and transfer to a microfuge tube. Spin 10 minutes. Now the pellet will contain the remains of chromatin and denatured proteins that will not dissolve. Transfer the supernatant to a fresh tube; again add an equal volume of 1.6 M NaCl, 13% PEG M.W. 800, mix well and keep on ice 10 minutes. Spin 10 minutes. Wash the pellet with 80% EtOH. Dissolve in 200 μl TE and chloroform extract to remove contaminating PEG, repeating as necessary. Reprecipitate and measure the OD260.

2.2.6  Plasmid preparation IV - Magic resin

In principle, similar to the Qiagen method below yet unreliable in my hands.

Perform alkaline lysis, isopropanol precipitation as above with the Promega versions of solutions 1, 2 & 3 (it is necessary to have 100 μg/ml RNAse A in the resuspension solution).

Resuspend the DNA pellet in 2 ml TE and add 10 ml of Magic resin. Swirl to mix. Transfer to a Magic Maxicolumn and apply a vacuum (to pull the mix down). Wash with 25 ml Wash solution. Add 5 ml 80% EtOH and continue to apply the vacuum for 10 minutes after this has passed through. Place the column in a 50 ml tube. Apply 1.5 ml of TE or water preheated to 67°C and wait 1 minute. Elute by centrifugation at 2,500 rpm 5 minutes in a swinging bucket centrifuge. Measure the OD260 to ascertain the DNA concentration.

2.2.7  Plasmid preparation V - Qiagen column

The method of choice: much more reliable than Magic resin, faster than CsCl and cleaner than PEG although somewhat expensive.

Inoculate 100 ml of LB (with appropriate antibiotics) with the bacteria and shake vigorously overnight at 37°C.
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Transfer 50 ml to a 50 ml Falcon tube (polypropylene only), spin 5-10 minutes 5,000 rpm, 4°C. Note: the use of BHI rather than LB to grow the culture, or of volumes greater than 50 ml, will not increase the yield and may compromise the preparation. Discard the supernatant. Resuspend in 10 ml of Solution P1, making sure the bacteria are well in suspension. Add 10 ml Solution P2. Invert (do not vortex) several times. The mix should become a homogeneous, translucent slime after a few mins. Add 10 ml Solution P3. Invert several times. Leave on ice 10 minutes. Spin 10 minutes 5,000 rpm 4°C. Equilibrate a Qiagen column with 10 ml QBT during the spin. Transfer the supernatant to the column. Use a 25 ml pipette, taking care to push the layer of white material on the top to the side. It will stick to the pipette-don't let it be transferred to the new tube. Allow the liquid to flow through unaided and wash with 60 ml QC. Elute into a 50 ml tube with 15 ml QF and add 10.5 ml isopropanol. Spin 6,000 rpm 30 minutes 4°C, wash with 70% EtOH and resuspend the DNA in 200 μl TE. Take OD$_{260}$ reading to ascertain concentration. (The OD of a 1/200 dilution x 10 = concentration in mg/ml)

2.2.8 DNA digestion with restriction endonucleases

Dilute the DNA (typically 1-3 μg) in the appropriate buffer and add 5-10 units of enzyme as required but no more than 10% (v/v) of a 50% glycerol enzyme stock. Incubate for 30-60 minutes at the relevant temperature. Multiple digests may be performed simultaneously provided the maximum glycerol concentration is not exceeded and the buffer is compatible with all the enzymes. For double digests requiring different buffers, do the lower salt reaction first, heat inactivate (65°C, 20 minutes), adjust the salt concentration for the second enzyme and perform the second digest. When this is not possible, extract the DNA
from the first reaction with phenol/chloroform, add 0.1 M NaCl and precipitate with 2.5 volumes of EtOH before resuspending in the desired buffer. Blunt-ending with Klenow fragment *E.coli* DNA polymerase or T4 DNA polymerase can be carried out in any of the buffers used for restriction digests as can the removal of terminal phosphates with Calf intestinal phosphatase (CIP). Restriction fragments can be analysed and purified by agarose gel electrophoresis as described below.

### 2.2.9 Preparation of blunt-ended fragments

To remove 5' overhangs, add 33 μM dNTP's and 1 unit of Klenow fragment *E.coli* DNA polymerase per μg of DNA. Incubate 15 minutes (no longer) room temp. and purify on a gel or heat inactivate and phenol/chloroform extract to stop the reaction.

To remove 3' overhangs, add 100 μM dNTP's and 1-5 units of T4 DNA polymerase per μg of DNA. Incubate 20 minutes 12°C and purify on a gel or heat inactivate and phenol/chloroform extract to stop the reaction.

### 2.2.10 Removal of terminal phosphates

When cloning into a vector that can self-ligate (either with compatible ends such as *BamHI-BamHI* or *BamHI-BgII* or with blunt ends) it is necessary to dephosphorylate the ends to prevent recircularisation.

CIP works well in most restriction buffers so it can simply be added directly after digestion. Add 1 unit CIP per pmol DNA ends (1 pmol ends = 1 μg for a 3 kb plasmid fragment) and incubate 1 hour 37°C for 5' overhangs or 50°C for 3' overhangs or blunt ends.
**2.2.11 Agarose gel electrophoresis of DNA**

Melt agarose in 1x TAE to give a 0.8% solution (low melting point agarose should be used if the fragments are to be used later). This is good for general use, for the analysis of small fragments a higher concentration may be required. Allow the solution to cool slightly, add 1 µg/ml EtBr and pour into a casting tray. When the gel has set transfer to the tank and submerge in 1x TAE + 1 µg/ml EtBr.

Add 1/4 volume 5x loading buffer to the DNA samples, mix and load into the wells of the gel. Include DNA fragments of known size such as 1 kb ladder as a marker. Perform electrophoresis at about 80-120 V (7.5 V/cm).

Use a UV transilluminator to see the DNA; note use long-wave radiation if the bands are to be excised and used for cloning.

To isolate the fragments, cut them from the gel on the transilluminator using a clean scalpel blade in as small a volume of agarose as possible. They can now be used directly in ligations and random priming reactions. If pure DNA is required, dissolve the gel slice in 3 volumes 6 M NaI at 50°C. Add 5 µl vortexed glassmilk solution (‘Geneclean’ silica matrix in water) and incubate on ice 5 minutes. Pellet glassmilk and wash 3 times with 500 µl chilled ‘New Wash’ buffer. Resuspend the pellet in 5 µl TE at 50°C, incubate 5 minutes, spin, remove supernatant to a new tube and repeat. Combine the supernatants to give 10 µl purified DNA.

**2.2.12 Ligation of DNA fragments into cloning vectors**

Prepare DNA fragments as described above. For blunt-ended ligations or cohesive-ended ligations with the same site at both ends use phosphatased vector DNA to prevent self ligation. In any case a control ligation with vector alone should always be performed in parallel.
Melt the agarose slices containing the DNA fragments at 65°C and store at 37°C while assembling the reactions. Set up each reaction as follows in a microfuge tube at 37°C:

- 2 μl 10x ligation buffer
- 14 μl water
- 1 μl vector DNA
- 2 μl insert DNA
- 1 μl T4 DNA ligase

Incubate at 37°C 2 hours and transform bacteria with 10 μl. If it is a cohesive-ended ligation then add 0.5 μl ligase and place at 16°C o/n to obtain maximum efficiency. In the morning, if the plates from the previous day’s transformation give unsatisfactory results then transform bacteria with the overnight ligation.

For simple ligations, the above usually works well; for cloning into retroviral vectors or if results are poor, set up a range of reactions with different amounts of insert DNA and/or use purified DNA.

2.2.13 Oligonucleotide purification and annealing

Oligonucleotides were supplied by the ICRF Oligonucleotide Synthesis Service fully deprotected and dried down.

Resuspend in 200 μl 0.3 M NaOAc, 10 mM MgCl₂ and add 600 μl cold ethanol. After 15 minutes at -70°C or overnight at -20°C, spin down and wash with 80% cold ethanol. Resuspend at 1 μg / ml in TE or water and store at -20°C.

To prepare double stranded oligonucleotides, mix an equimolar amount of each strand in a small volume of TE + 10 mM MgCl₂ and heat to 95°C 3 minutes. Allow to cool slowly to room temperature over about 2 hours.
2.2.14 Radio-labelling of DNA probes

Oligonucleotides for use in band-shifts were 5’ end-labelled with $^{32}$P using Polynucleotide Kinase (PNK).

To 1 µl (100 ng) double-stranded oligonucleotide add 1 µl 10x PNK buffer, 7 µl $^{32}$P gamma ATP and 1 µl T4 PNK. Incubate 30 minutes 37°C and separate the unincorporated nucleotides by adding 190 µl TE and passing the mix down a Sephadex G50 spin column. Count 1 µl of the flow-through in a scintillation counter to determine the yield.

2.2.15 Spin column preparation and use

Remove the plunger from a 1 ml syringe and plug the barrel with a small quantity of siliconised glass wool. Stand the barrel in a 15 ml Falcon tube and fill with a 50% slurry of TE-equilibrated Sephadex G-50 beads. Spin 1,000 rpm 1 minute. Fill and spin again until a 1 ml column is formed. Place a screw-cap microfuge tube in the bottom of a fresh 15 ml tube, stand the column inside, add the sample to the top of the column and spin 1 minute, 1,000 rpm. Remove the microfuge tube carefully and replace the lid. Alternatively use a Pharmacia ‘S-300 HR’ ready-made spin column.

2.2.16 DNA sequencing

Double stranded supercoiled plasmid DNA was sequenced manually using the dideoxy chain termination method with Sequenase kits according to the Manufacturer’s instructions.

DNA from a maxiprep is sufficiently pure for sequencing directly. To sequence miniprep DNA, treat with RNAse A (10 µg/ml) and then phenol/chloroform extract repeatedly until no interface material is evident, chloroform extract twice then add NaCl to 0.1 M and EtOH precipitate. Wash in 70% EtOH and resuspend in 40 µl TE. Use 5 µl for
sequencing (20 µl if a pUC origin plasmid). For maxiprep DNA use 5 µg in 20 µl.

Primers: use 1.5-2 pmol (this is 10-13 ng for a 20mer).

Add 5µl 1M NaOH/1mM EDTA to plasmids, 30 minutes 37°C. Neutralise with 2.5 ml of 2M NH₄OAc (pH 4.6). Mix quickly. Add 60 µl 100% EtOH. Put on ice 5 minutes (can be kept overnight). Microfuge 5 minutes. Wash 70% ethanol. Dry pellet.

To anneal, resuspend in 10 µl, including:

- 2 µl 5x Sequenase buffer
- 1 µl 5% NP40
- 6 µl water (adjust for correct primer concentration)
- 1 µl primer

Warm to 68°C, leave to cool down to 30°C in about 30 minutes. Add the Mn²⁺ buffer (0.5-1 µl) now, to shorten reaction products if a short read is desired.

While annealing primer-template, make up 9 µl of enzyme-label mix per sequence reaction:

- 0.1M DTT 1 µl
- Enz. Dil. Buffer 1.7 µl
- Water 5.1 µl
- Labelling Mix 0.4 µl
- ³⁵S dATP 0.5 µl
- Sequenase 0.3 µl (add last)

Add to template/primer and leave 5-10 minutes room temp. Meanwhile quickly prepare the termination reaction: add 2 µl of each termination mixture (ddG,A,T,C) to the wells of a Microtiter III plate (Falcon).

Aliquot the reaction (4x) onto the termination mixes and incubate 37°C at least 15 minutes. Add 2 µl stop solution to each well. Heat at 85°C for 4 minutes (without the lid to the plate; use Saran Wrap).
Load on a 6% w/v acrylamide:bisacrylamide (19:1), 48% w/v urea gel in 1x TBE.

2.2.17 *In vitro* transcription

This protocol is suitable for the production of 5-10 μg of RNA per μg of plasmid DNA. Use DNA from a plasmid maxiprep. Digest to completion with an enzyme cutting at the 3' end of the sense strand of the insert, phenol/chloroform extract and EtOH precipitate. Assemble the following reaction at room temp. in the order given using nuclease-free solutions throughout:

- RNase free water to final volume 100.0 μl
- 5x transcription buffer 20.0 μl
- 100 mM DTT 10.0 μl
- RNasin 100 Units, approx. 2.5 μl
- 2.5 mM each rNTP 20.0 μl
- linearised plasmid 2-5 μg 2.0 μl
- T7 RNA Polymerase @ 15-20 U/μl 2.0 μl

Inclusion of cap analogue (0.5 mM m7G(5')ppp(5')G) improves the subsequent translation efficiency.

Incubate 1-2 hours 37-40°C. Extract with 1 volume of TE saturated phenol/chloroform then with 1 volume chloroform. Add 1/2 volume 7.5 M NH₄OAc and 2.5 volumes of EtOH. Place on dry ice 10 minutes, spin 5 minutes 4°C. Wash pellet with 70% EtOH, dry and resuspend in 10-20 μl TE or water.

Run 1 μl on a 1% agarose gel to determine yield and verify size and quality; use formamide loading buffer (eg from Sequenase kit) and heat to 68°C 3 minutes prior to loading.

Store at -70°C.
2.2.18 \textit{In vitro} translation

Allow the reagents to thaw on ice. Heat 2 \( \mu l \) template RNA to 67°C 10 minutes, cool on ice. Add the following:

- Rabbit reticulocyte lysate (nuclease treated) \( 35 \mu l \)
- Water \( 7 \mu l \)
- RNasin \( 1 \mu l \)
- Amino acids (minus methionine) @ 1 mM \( 1 \mu l \)
- Methionine @ 100 mM \( 4 \mu l \)

Incubate at 30°C 60 minutes. Aliquot and snap freeze in a dry ice/MeOH slurry. Store at -70°C. Labelled protein can be translated by replacing the methionine with 10 mCl/ml \(^{35}\)S-methionine (>1,000 Ci/mmol).

2.2.19 \textit{In vitro} combined transcription and translation

The Promega TNT system is the method of choice for preparing protein, being faster and more robust than separate transcription and translation reactions. It also typically produces more protein.

Allow the reagents (except the TNT reticulocyte lysate which should be thawed rapidly by hand warming and then placed on ice) to thaw on ice.
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Set up the following reaction:

- TNT Rabbit reticulocyte lysate: 12.5 µl
- TNT Reaction buffer: 1.0 µl
- TNT Polymerase: 0.5 µl
- DNA Substrate: 0.5 µg
- RNasin: 0.5 µl
- Amino acids (minus methionine) @ 1 mM: 0.5 µl
- Methionine @ 100 mM: 2.0 µl

Add water to the final volume of 25.0 µl.

Incubate at 30°C for 60-120 minutes. Aliquot and snap freeze in a dry ice/MeOH slurry. Store at -70°C. Labelled protein can be created by replacing the methionine with 10 mCi/ml ^35^S-methionine (>1,000 Ci/mmol).

2.2.20 Gel retardation assays

Mix in vitro translated proteins as required (use 2 µl) and incubate at 30°C for 60 minutes. Add to the following binding reaction in 25 µl: 10 mM Tris-HCl pH 7.4, 50 mM NaCl, 1 mM DTT, 1 mM EDTA, 5% glycerol, 25 ng sheared salmon sperm DNA and 0.5 µg poly(dl). Finally add 0.25-0.5 ng labelled probe pre-mixed with any competitor DNA. Allow the binding reaction to proceed at room temp. for 1 hour.

Resolve the protein/DNA complexes on 4% acrylamide:bis-acrylamide (19:1) gels in 1x TBE. Fix and dry prior to autoradiography or exposure to a phosphor-imager screen.

2.2.21 Immuno-precipitation of protein/DNA complexes

Protein-DNA interactions were analysed after McKay\(^{299}\). Unlabelled Max1, Max2 and Myc were synthesised as above using...
unlabelled methionine. Unprogrammed reticulocyte lysate was used as a negative control. The binding reaction was assembled as above for gel retardation in the presence of antibody or antibody blocked by pre-incubation with its cognate immunogenic peptide.

Add 15 μl of a 50% slurry of protein A-Sepharose CL4B equilibrated in DNA binding buffer supplemented with 1% BSA and 0.25% NP40. Incubate 60 minutes with occasional mixing. Spin 13,000 rpm; wash three times with 250 μl DNA binding buffer supplemented with 1% NP40. Elute labelled probe with 20 μl TE + 0.2% SDS 30 minutes room temp. and count in 4 ml of Aquasol in a scintillation counter (Beckman 1215 Rackbeta). To recover the precipitated DNA during binding site selection, elute in 5mM EDTA, 0.5% SDS, 100 mM NaOAc, 50 mM Tris-Cl pH 8.0 for 60 minutes at 45°C. Phenol/chloroform extract, add 1 μl glycogen as carrier and NaOAc to 250 mM. Ethanol precipitate and Cerenkov count to quantify the amount of probe brought down. Take up in an appropriate volume of water.

2.2.22 Polymerase chain reaction

The polymerase chain reaction (PCR) was used to amplify the small amounts of DNA precipitated during the site selection procedure (chapter 4) and for generating novel sequences for cloning purposes. Both reactions are described overleaf.
To amplify DNA selected during binding site selection, take 1 pg and add:

- 2.0 μl 10x PCR buffer
- 3.2 μl dA,dG,dT (all at 0.5 mM)
- 2.0 μl 0.04 mM dC
- 4.6 μl primer F (35 ng/ml)
- 4.6 μl primer R (35 ng/ml)
- 1.0 μl 32P α-dCTP (10 mCi)
- 0.5 μl Taq polymerase
- 2.0 μl water
- 20.0 μl total volume.

Overlay with a drop of mineral oil to prevent evaporation and use the following PCR program: 94°C, 1 minute; 62°C, 1 minute; 72°C, 1 minute for 15 cycles.

Add 120 μl TE and 10 μl 0.1 mM EDTA. Chloroform extract. Phenol extract. Add 1 μl glycogen, 40 μl 5M NH₄OAc and EtOH ppte. Take up in 10 μl TE and add 10 μl loading dye. Run on an 8% acrylamide gel. Place Saran wrap over the gel. Autoradiograph and draw around the gel so as to be able to orient it with the film later. Cut out the bands from the film and overlay on top of the gel using the marks made previously. Excise the portion of gel under the holes in the film very carefully and transfer the slices to microfuge tubes.

Elute overnight at 37°C in 0.5 to 1.5 ml elution solution: 0.5M NH₄OAc, 5 mM EDTA, 0.1% SDS. Add glycogen carrier and EtOH precipitate. Take up pellet in 10 μl TE and Cerenkov count 1 μl. Quantify synthesis and dilute to 0.1 ng/μl.

PCR was also used to generate new DNA constructs. Use between 50 ng to 1 μg of template DNA and 0.5 μM primer. Use the lowest annealing temperature of the two primers as the annealing temperature
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for the PCR reaction and 15-35 cycles. Analyse the results on an agarose gel and sequence ALL plasmids generated this way.

2.2.23 Procedures to prevent RNAse contamination

Clean electrophoresis tanks, casting trays and combs with detergent, rinse in ddH2O, dry with EtOH and fill with 3% H2O2. After 10 minutes wash extensively with DEPC treated water (DtH2O).

Wear gloves and change them regularly.

Prepare all solutions with ddH2O, autoclave them if possible and reserve them for RNA work. Wherever possible, treat solutions with 0.1% DEPC overnight at 37°C and autoclave. Purchase solutions that cannot be DEPC treated such as Tris from a reliable source who test them for RNAse activity.

2.2.24 RNA preparation I - CsCl centrifugation

Original method, rather slow and easy to lose RNA.

Prepare LiCl/GTC solution, add 0.5 g GTC (Fluka) to 0.58 ml of 25% LiCl for each ml required; filter 0.45 μm. Add 20 μl β-mercaptoethanol/ml. Store at room temperature in the dark. (Supposedly only stable for a week but I've used it satisfactorily after a month). Do not autoclave. Prepare CsCl solution: 239.91 g CsCl, 25 ml 1M EDTA, pH 7.0 (100 mM final - can be as low as 10 mM), water to 250 ml. It is very important to have a neutral pH. Filter through 0.45 μm filter. Autoclave.

Aspirate medium from plate(s) of adherent cells or pellet suspension cells in RNAse-free tubes. Wash with PBS and add 1.5 ml LiCl/GTC to each plate/pellet (max. 5x10⁷ cells/ml). Scrape GTC-treated adherent cells to edge of plate with autoclaved rubber/plastic scraper. Shear DNA by passage 7-10x through 19-23 g needle. The LiCl/GTC
lysate can theoretically be left at room temperature for days without harming the RNA, however I prefer to process as soon as possible or freeze at -70°C (stable for months)

Add 1.5 ml CsCl to autoclaved SW55 tube. Gently overlay the CsCl with the LiCl/GTC cell lysate using a syringe. Centrifuge for 2 hours, 50,000 rpm, 15°C in an SW55 rotor. Do not spin longer as this sometimes results in excess salt/debris collecting near the pellet. Aspirate the supernatant to CsCl level. Use a clean pipette to remove the remainder. Invert the tube on tissue. Cut off the top 3/4 of the tube with a sterile scalpel. Wash the pellet once with 70% EtOH. Be VERY gentle here. A clear/glassy RNA pellet should be visible if you expect >100 µg RNA but even if it is not, proceed with the rest of the protocol.

Resuspend in 300 µl 10 mM Tris-HCl, pH 7.0, 1 mM EDTA/0.1% SDS. Transfer immediately to a 1.5 ml tube containing 800 µl EtOH + 30 µl 3 M NaOAc and place at -20°C or -70°C for 15 minutes (can store at -20°C until needed.) Pellet the RNA precipitate at 13,000 rpm 5-10 minutes. Wash the pellet with 800 µl 70% EtOH. Resuspend in TE + 0.1% SDS and store at -20°C.

Measure the concentration and purity by measuring the absorbance ratio at OD_{260}/OD_{280}.

### 2.2.25 RNA preparation II - Acid phenol

A very rapid method with a high yield and little chance of contamination or loss of RNA^{300}.

Wash the plate of cells with PBS. Add 1.5 ml of the denaturation buffer (5x10^7 cells/ml). Scrape the cells off the plate with a rubber policeman.

Add in the following, mixing each time:
1.5 ml 2 M sodium acetate, pH 4.0
1.5 ml water saturated, acidic phenol
1.5 ml chloroform:isoamyl alcohol (49:1)

Shake for 10 seconds and put on ice for 15 minutes. Spin for 20 minutes at 4,000 rpm, 4°C. Take the upper aqueous phase and mix it with an equal volume of cold isopropanol. Keep at -20°C for 60 minutes. Spin RNA down at 6,000 rpm for 20 minutes, 4°C.

Resuspend the pellet in 20-200 μl of TE, transfer to a microfuge tube, add 4 μl 5M NaCl and 500 μl cold EtOH. Spin 15 minutes, 4°C, wash with 80% EtOH and resuspend pellet in 200 μl 0.1% SDS. Measure concentration and store at -20°C.

2.2.26 mRNA preparation

Pellet 10^7-3×10^8 suspension cells and wash with PBS or alternatively, wash a 15 cm plate of adherent cells with PBS. Add 10 ml of lysis buffer per 10^8 cells and transfer to a 50 ml tube (use a scraper for adherent cells). The lysate can be frozen and stored at this point for over a month.

Shear the lysate through 21 g needle (about 7 times or until it becomes very fluid). Incubate 2 hours, 45°C rotating in a hybridisation oven.

In the meantime, weigh out 20 mg oligo dT cellulose (dT) per 10^8 cells and add to 10 ml binding buffer. Incubate 1 hour, room temperature on shaker. Pellet dT and take up in fresh binding buffer; split into 20 mg aliquots and pellet.

Add 600 μl 5 M NaCl / 10 ml lysate, mix and add to dT. Incubate 1 hour, room temp. on shaker. Pellet dT; wash 3x 10 ml binding buffer then aspirate all but 500 μl buffer, resuspend and transfer to a spin column. Rinse with 400 μl binding buffer, spin 5 seconds and repeat twice more. Place the column in a fresh tube, add 200 μl elution buffer
and vortex. Spin 5 seconds, add a further 200 µl of elution buffer and mix again. Spin 20 seconds.

Add 40 µl 3 M NaOAc, 1 µl glycogen (1 mg/ml) and 880 µl EtOH. Freeze on dry ice 10 minutes, thaw and spin 30 minutes, 0°C. Wash the pellet with ice cold 70% EtOH and resuspend in 20 µl 0.1% SDS. Measure the concentration by taking the OD$_{260}$.

### 2.2.27 Northern blotting

For 200 ml of 1% gel mix, add 2 g agarose, 10 ml 20x MOPS and 110 ml water to a conical flask and melt. Separately combine 36 ml 38% formaldehyde with 44 ml water and put at 37°C. Mix the two solutions and pour into a clean casting tray.

Use 10-30 µg total RNA or 1-10 µg of mRNA per lane in formaldehyde/ formamide loading buffer. Heat at 65°C 5 minutes to denature. Either prepare an extra sample of total RNA or 5 µl of RNA ladder as a marker lane. After transfer, this lane can be cut off and stained (15 minutes in 5% acetic acid followed by 5-10 minutes in 500 mM NaOAc, pH 5.2, 0.04% methylene blue). Rinse in water to destain.

Run the gel in a fume hood at 120 V 2-4 hours (changing the buffer every 45 minutes) until the bromophenol blue approaches the bottom. The xylene cyanol will run close to the 18s ribosomal band (about 2 kb).

Transfer to a Hybond N+ membrane in 10x SSC. After transfer, either bake 2 hours, 85°C and UV treat 5 minutes in a tissue culture hood (old method) or treat with 50 mM NaOH 5 minutes followed by a wash in 2x SSC (new, more efficient method).

Hybridisation can be performed in dextran hybe or Church buffer ($3\times10^6$ cpm/ml, 1 hour prehybridisation and overnight hybridisation) or Stratagene ‘Quick hybe’ which is the method of choice, requiring only
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$10^6$ cpm/ml, a 20 minute pre-hybridisation and a 1 hour hybridisation at 65°C.

Wash the filter in 2x SSC, 0.1% SDS, 5 minutes room temp. followed by 2 washes for 15 minutes at 65°C. For same species probes or if the signal is still strong continue to wash in 0.2x SSC, 0.1% SDS at 65°C.

Seal the blot in a plastic bag and expose to film or a phosphor-imager screen.

To strip the blot prior to re-probing, wash 5 minutes in boiling 0.1% SSC, 1% SDS.

2.2.28 Random priming

Digest appropriate plasmid DNA (use an amount calculated to release approximately 1 μg of fragment) and purify the probe fragment on a low-melt agarose gel.

Combine 2 μl probe DNA fragment (about 20 μg), 1 μl random primers (1 mg/ml) and 7 μl water. Boil for 5 minutes. Cool to 37°C.

Add 2.5 μl 10x Klenow buffer, 1 μl (33 mM) cold dG/TTP's, 4.75 μl (3,000 Ci/mmol, 10 μCi/μl) $^{32}$P α-dATP, 4.75 μl (3,000 Ci/mmol, 10 μCi/μl) $^{32}$P α-dCTP and 2 μl (10-15 U) Klenow fragment (DNA polymerase). Incubate 37°C, 1-3 hours. Purify via Sephadex G50 fine spin column or a Pharmacia MicroSpin column (S-300 HR).

Place 1 μl in 10 ml of scintillant and measure the radioactivity.

2.2.29 Preparing cell lysates

Wash plate with PBS.

Add 1 ml ice-cold RIPA buffer (with inhibitors) to a 10 cm dish. Scrape & transfer to a microfuge tube (pipette up and down to break up clumps). Vortex briefly, keep on ice. Spin 2 minutes 13,000 rpm 4°C.
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Take supernatant = total cell lysate. Aliquot as required and snap-freeze by placing in a slurry of dry ice and MeOH. Store at -70°C. Measure the protein concentration using either the Bradford protein assay system (for lysates containing low levels of detergent such as NP40 lysis buffer) or the DC Protein assay system (for lysates containing high levels of detergent such as RIPA buffer).

Solutions:
RIPA buffer: PBS + 1% NP40 + 0.5% Sodium deoxycholate + 0.1% SDS
NP40 lysis: PBS + 1% NP40 (should be compatible with Bradford assay)

Add inhibitors freshly each time:

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Compound</th>
<th>Stock Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 µg/ml</td>
<td>PMSF</td>
<td>(10 µl/ml of 10 mg/ml stock)</td>
</tr>
<tr>
<td>3% (w/v)</td>
<td>Aprotinin</td>
<td>(30 µl/ml of Sigma cat# A6279)</td>
</tr>
<tr>
<td>1 mM</td>
<td>Sodium orthovanadate</td>
<td>(5 µl/ml of 200 mM stock)</td>
</tr>
<tr>
<td>20 mM</td>
<td>Sodium Fluoride</td>
<td>(40 µl/ml of 0.5 M stock)</td>
</tr>
</tbody>
</table>

2.2.30 Measuring protein concentration (DC assay - Biorad)

Add 20 µl of reagent S to each ml of reagent A that will be needed. Prepare 5 dilutions of BSA protein standard: 0, 0.25, 0.50, 0.75, 1.00, 1.25 and 1.50 mg/ml.

Pipet 10 µl of standards and samples into cuvettes. Add 50 µl of reagent A/S mix. Add 400 µl of reagent B, cover with parafilm and invert several times.

Wait 15 minutes and read the absorbances at 750 nm. Calculate the sample concentrations from the standard results.

2.2.31 SDS-PAGE

One dimensional SDS polyacrylamide gel electrophoresis (PAGE) was conducted as described by Laemmli\(^{302}\).
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Prepare a resolving gel of 10%, 12.5%, 15% or 17.5% acrylamide:bis-acrylamide (37.5:1) as required in 0.375 M Tris-HCl, pH 8.8, 0.1% SDS, 0.125% TEMED and 0.02% APS. Pour between the glass plates of the gel apparatus leaving a 1 cm gap between the top of the mix and the lowest point the comb will reach. Overlay with TE saturated butan-2-ol. After polymerisation, remove the butan-2-ol layer and wash with water. Pour a stacking gel on top: 4% acrylamide:bis-acrylamide (37.5:1), 125 mM Tris-HCl, pH 6.8, 0.1% SDS, 0.1% TEMED and 0.2% APS.

Heat samples for 3 minutes prior to loading and run the gel in 1x PAGE-running buffer at 80 V until the samples enter the separating gel and then at up to 250 V (maximum). Include Rainbow marker molecular weight standards to measure the extent of the run.

2.2.32 Immuno-precipitation

Prior to starting, block enough antibody for the controls 1:5 with cognate peptide 60 minutes room temperature. Also wash and equilibrate enough protein A-sepharose beads with RIPA buffer.

Transfer 1/4 of lysate from a confluent dish to a fresh tube and add cold RIPA buffer (+ inhibitors) to 1 ml (use same amount of protein for each sample).

Add antibody (for rat cyclin E, use Santa Cruz # sc-481 at 1%). Incubate 60 minutes 4°C on the wheel. Add 50 µl protein A-Sepharose beads. Rotate 60 minutes 4°C. Spin high speed 8 seconds.

Wash three times 1 ml RIPA (+ inhibitors) 4°C. Wash twice 1 ml (kinase buffer + 1 mg/ml BSA) 4°C. Spin high speed and resuspend beads in 50 µl kinase buffer + 30 µM ATP + 5 µCi [γ-32P]ATP + 10 µg histone H1.
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Incubate 37°C, 30 minutes. Agitate occasionally to mix. Add 17 µl 4xSDS sample buffer. Boil 3 minutes.

Run on 12.5% SDS-PAGE gel. Fix in 15% MeOH, 15% acetic acid 15 minutes. Dry. Expose to film or Phosphor-imager.

2.2.33 Western blotting

Use an appropriate amount of cell lysate depending on the quality of the antibody and abundance of the protein and add SDS sample buffer to 1x. It is important not to overload as the system can easily become saturated with differences becoming undetectable. For cyclin E, cdk-2 or p27, use approximately 15 µg total cell lysate. Heat samples at 95°C 3 minutes and load on to an appropriate SDS-PAGE gel.

After electrophoresis take a PVDF membrane, rinse in MeOH, wash with water until it loses its 'greasy' appearance and equilibrate 5 minutes in Transfer Buffer. Place the gel onto two sheets of 3MM paper wetted with Transfer Buffer and overlay with the membrane. Cover with two further sheets of wetted 3MM and squeeze out all bubbles. Invert the 'sandwich' onto a Biorad semi dry blotter and transfer using 15 V 30 minutes for a 12.5% 0.75 mm gel (lower voltage if the current goes over 75 mA, use a longer time for thicker or higher percentage acrylamide gels or if using a lower voltage).

Block non-specific binding by soaking the membrane in blocking buffer for at least 15 minutes (can leave for up to 48 hours at 4°C in 0.02% NaN3).

Incubate in blocking buffer with primary antibody for 45 minutes and wash twice, 7 minutes in PBS + 0.05% Tween 20.

Incubate 30 minutes with horseradish peroxidase-conjugated secondary antibody in blocking buffer.
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Wash three times 5 minutes with PBS + 0.05% Tween 20 then once with PBS alone.

Incubate 1 minute exactly in ECL reagent, drain and seal in clear film.

Expose to film.

2.2.34 Nuclear run-on

Method after Justin Blau, modified from Roberts and Bentley, 1992\textsuperscript{303}.

Take care to ensure that all solutions are RNAse free.

Prepare sufficient filters in advance: first denature the DNA to be applied by adding one fifth volume of 1 M NaOH / 1 mM EDTA and leave for 5 minutes at ambient temperature. Neutralise with one tenth volume of 2 M ammonium acetate, pH 4.6. Dilute in 1x SSC to about 400 µl per µg of DNA. Use Genescreen filters cut to fit on a slot blotter (Schleicher and Schuell) and lay over 2 layers of 3MM paper. Wash the slots with 1x SSC three times, apply 1 µg of denatured DNA fragment in 400 µl, wash three times with 400 µl 1x SSC and allow to dry thoroughly. Crosslink the DNA to the filter by exposing to UV (tissue culture hood is fine) for 2 minutes. Store filters at -20°C until needed.

Use about 20 million cells per assay. Harvest adherent cells by scraping not trypsinisation. Spin down gently, wash with ice-cold PBS and resuspend in 20 ml ice-cold RSB in a 50 ml centrifuge tube.

Leave to swell 5 minutes on ice then lyse by vigorous shaking for 30 seconds. Douncing is not necessary for Rat-1 cells.

Spin 2,000 rpm, 5 minutes, 4°C to pellet the nuclei. Loosen the cap to allow the bubbles to burst. Carefully aspirate the supernatant, spin again and remove the remaining supernatant.
Resuspend the white pellet in 500 μl of cold glutamate buffer and 1 μl of 1 M DTT by pipetting with a 1 ml Gilson and transfer to a 1.5 ml screw cap tube. Spin at 3,000 rpm in a pre-cooled centrifuge for 30-60 seconds to pellet the nuclei and aspirate the supernatant. Briefly vortex to resuspend. The nuclei can be frozen at -70°C for several months without loss of activity (Justin Blau, personal communication).

Briefly thaw the nuclei (30 seconds, 30°C) and add 1 μl 50 μg/ml DNAse free RNAse A, tap gently, add 1 μl 80 mM DTT, tap gently and incubate at room temperature for 5 minutes. Add 100 units of Pharmacia RNAsin and tap gently to mix.

Add 200 μCi 32P UTP, 0.5 μl 1 M creatine phosphate (in 10 mM HEPES pH 8.0 stored in aliquots at -70°C), 1.2 μl 2 mg/ml creatine kinase (in glutamate buffer), 1 μl nucleotide triphosphate solution (50 mM ATP, 25 mM CTP, 25 mM GTP) and 0.4 μl 1 M MgCl₂. Incubate at 30°C for 5 minutes, tap gently to mix and incubate for a further 5 minutes.

Note the limiting factor in the procedure is the concentration of the labelled nucleotide.

Add 0.5 μl of 1 M calcium chloride, 5 μl 50 mg/ml Torula RNA and 5 μl of 10 U/ml RNAse free DNAse (from Worthington, dissolved in 20 mM sodium acetate pH 6.5, 5 mM CaCl₂, 50% glycerol). Incubate a further 20-30 minutes at 37°C.

Add 18.5 μl 10x SET, 121.5 μl water and 10 μl 10 mg/ml proteinase K. Heat briefly at 65°C until the mix clears then incubate 45 minutes at 37°C.

Extract with 200 μl phenol/chloroform and re-extract the organic phase with 100 μl 1x SET to maximise recovery. Precipitate the RNA by adding 100 μl of 10 M ammonium acetate and 400 μl isopropanol to the 300 μl pooled aqueous phase. Leave 15 minutes on ice and pellet 5 minutes 12,500 rpm 4°C. Wash pellet with cold 70% ethanol. Redissolve
in 150 µl 0.5% SDS, 1 mM EDTA and repeat the 2.5 volumes ammonium acetate, 1 volume isopropanol precipitation.

Dissolve the RNA pellet in 100 µl 0.5% SDS, 1 mM EDTA. Meanwhile prehybridise the filter for 30 minutes.

(Note if using cells transfected with Bluescript vectors (or similar) and using whole plasmid rather than fragments to hybridise to, remember to compete out sequences that hybridise to M13 by hybridising to 25 µg single stranded M13 vector for 1 hour at 65°C.)

The probe is now ready to be heated at 95°C for 2 minutes and added to the hybridisation bag. Use at least 10 million cpm per ml.

Hybridise overnight at 45°C. Wash the filter in 2x SSC, 0.1% SDS, 5 minutes room temperature followed by 2 washes for 15 minutes at 65°C. If the signal is still strong or the background is high, wash at a lower SSC concentration.

After hybridisation and washing, it is helpful to digest the filters with RNase. Rinse in 2x SSC to remove the SDS and seal in a bag with 10 µg/ml RNase A in 300 mM NaCl, 10 mM Tris-HCl, pH 7.5, 5 mM EDTA at 37°C for 30 minutes. Wash 15 minutes at 65°C in 2x SSC. Expose to film.

2.2.35 Cell culture

Cells were grown in humidified incubators with 10% CO₂ at 37°C. Unless specified, cells were maintained in Dulbecco's modified Eagle's medium (E4) containing 2 µg/ml gentamycin and 100 µg/ml kanamycin and 10% foetal calf serum (FCS). Cells containing oestrogen receptor (ER) fusion proteins were grown in medium without phenol red, and charcoal-stripped serum was used to prevent activation of the ER domain. The medium was changed every 3-4 days.
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The following drug concentrations were routinely used.

- **Geneticin (G418):** 1 mg/ml (for neomycin resistant lines)
- **Hygromycin:** 150 μg/ml
- **Puromycin:** 1 to 5 μg/ml (depending on the cell type)
- **17 β-oestradiol:** 2 μM
- **OHT:** 200 nM
- **Insulin:** 5 μg/ml

All manipulations with living cells were performed in class II laminar flow hoods. Cells were passaged by first washing them in PBS and then incubating them with 1:1 (v/v) trypsin/versene. Detached cells were diluted with fresh medium and replated. For storage and subsequent recovery, near-confluent 90 mm dishes were trypsinised and resuspended in 3 ml of ice-cold 45% FCS/45% E4/10% DMSO (v/v). Cells were then wrapped in paper tissue or placed into polystyrene boxes and kept at -70°C for a minimum of 24 hours, before being transferred to liquid nitrogen for permanent storage. Cells were recovered by thawing at 37°C and dilution with 20 ml E4+10% FCS. Following attachment, the medium was changed to remove the DMSO.

2.2.36 Ca^{2+} transfection

The transfection procedure is based on the protocol by Graham, 1973 modified by Wigler, 1977.304,305

Prior to transfection, medium change the plates with fresh medium and leave to equilibrate in the incubator for at least 30 minutes. Suspend the DNA (maximum 20 μg) in 500 μl HBS in a Falcon 2058 tube and add 23-29 μl 2.5 M CaCl₂ (optimised for each batch of HBS).

Incubate for 10-20 minutes until the mix takes on a cloudy appearance and add dropwise to the cells. Spread evenly over the
monolayer by gently rocking the plate, not by swirling which will accumulate the precipitate in the centre. Do no more than 8 plates at a time to avoid an increase in pH.

Leave the precipitate overnight before medium changing as normal.

To generate stable transfectants, medium change with 0.5% FCS instead of 10% after incubation overnight with the precipitate and change to 10% FCS after 2 hours. The next day, split the cells at a range of dilutions (1-5 to 1-20) to ensure that there will be well spaced colonies on some plates; the following day (48 hours post transfection) add the appropriate drug selection. Follow the selection with an untransfected plate, if these cells survive increase the drug concentration.

2.2.37 Combined CAT and β-galactosidase assay

Lyse the cells as follows.

Wash the transfected plates with PBS. Drop 10 ml TM buffer on to each plate. Leave for about 5 minutes. Remove the buffer. Leave the plates inclined for a few seconds and suck off the rest of the buffer.

Drop 150 µl lysis buffer on to each plate. Shake the plates gently. Leave them horizontal for about 3-5 minutes (until only nuclei are visible under the microscope).

Scrape the plates with rubber policemen and transfer the lysates to microfuge tubes, on ice. Freeze the collected lysates on dry ice and thaw them at 37°C, then vortex and spin for 3 minutes at high speed. Remove the supernatants to fresh tubes. Store frozen at -70°C (both enzymes are stable enough for several repeat assays).

Never heat the extract before performing the β-galactosidase assay as it could inactivate the enzyme.
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To perform the β-galactosidase assay, mix the following, note the time and incubate at 37°C:

150 µl 2x β-galactosidase buffer
150 µl extract (if less, make up the difference with lysis buffer)

Add 500 µl of 1 M Na₂CO₃ to stop the reaction when it becomes obviously yellow and note the time.

Spin hard (13,000 rpm for 15 minutes) as there is a fine precipitate which will otherwise interfere with the spectrophotometer measurement. Transfer 400 µl to a cuvette without disturbing the pellet. Measure the absorbance at 420 nm against a control incubated with a mock extract. The assay is linear up to an absorbance of 0.7.

To perform the CAT assay, heat 30 µl of extract to 68°C for 15 minutes. Cool down and add to the following, freshly prepared:

1 µl 50 mg/ml chloramphenicol from the stock made up in EtOH.
2 µl 14C acetyl CoA (54 mCi/mmole)
1.6 µl 5 mg/ml unlabelled acetyl CoA
7.5 µl 1 M Tris-HCl pH 8.0
38.5 µl water

Incubate at 37°C for 1-4 hours (judge the period of incubation required from the strength of the β-galactosidase assay.)

The following part should be carried out on ice.

Cool the reaction on ice for 3-5 minutes. Add 200 µl of ice-cold ethyl acetate to each sample (do no more than 8 samples at a time as the ethyl acetate is extremely volatile). Immediately cap the tubes tightly. Vortex vigorously for 30 seconds, spin for 5 minutes then remove 150 µl to a scintillation vial containing 5 ml of liquid scintillant. Make sure that no aqueous layer is transferred; it is preferable to take the same smaller volume from all samples than to risk touching the interface. Count the level of radioactivity in a scintillation counter.
For controls a blank with 30 μl lysis buffer usually gives less than 300 cpm. For a positive control 0.025 units of purified CAT enzyme gives about 15,000 cpm and the assay is linear to about 100,000 cpm. Complete conversion of acetyl CoA in this assay occurs at about 150,000 cpm; samples which give a high degree of conversion should be diluted appropriately.

2.2.38 Combined CAT and luciferase assay

Lyse the cells as follows.

Wash the transfected plates twice with PBS (at room temperature). Add 150 μl lysis buffer to each plate. Leave plate horizontal for about 2 minutes until only nuclei are visible under the microscope.

Transfer lysate to a microfuge tube, cool, then spin for 1 minute. Remove supernatant to a fresh tube. Add BSA to a final concentration of 6%. Keep on ice. Note that while luciferase activity is stable for months at 4°C, this seems to apply only to purified enzyme; in cellular extracts it seems to have a half life of about a day at 4°C. The addition of BSA seems to stabilise the activity somewhat, but it is best to analyse activity as soon as possible. In contrast, CAT activity remains stable in these extracts, but is not inhibited by BSA. Protease inhibitors are less effective at stabilising luciferase than BSA, and PMSF actually inhibits luciferase activity.

To perform the luciferase assay, add 20 μl of extract to 350 μl of luciferase reaction buffer in a luminometer cuvette. Load samples into the luminometer (an LKB 1251) and inject 33 μl of 3 mM luciferin (sodium salt from Sigma). Controls with extracts from mock transfected cells give zero rate and have peak activities of less than 0.6 units. The assay is linear up to at least 5,000 units.
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To perform the CAT assay, heat 20 µl of extract to 68°C for 5 minutes. Add to this:

- 20 µl 8 mM chloramphenicol.
- 20 µl 0.5 mM acetyl CoA (made by diluting ¹⁴C acetyl CoA (54 mCi/m mole) from Amersham with cold 0.5 mM stock at 1 in 10)
- 10 µl lysis buffer.
- 30 µl 250 mM Tris-HCl, pH 7.8

Incubate at 37°C for 1 hour. Add 100 µl of cold ethyl acetate to each sample. Do no more than 8 samples at a time as the ethyl acetate is extremely volatile. Immediately cap tubes tightly. Vortex vigorously, spin for 1 minute then remove 80 µl to a scintillation vial containing 5 ml of liquid scintillant. Make sure that no aqueous layer is transferred. Count the level of radioactivity in a scintillation counter.

2.2.39 Viral infection I - co-cultivation

Proviral vectors are analogues of defective transforming viruses with exogenous genes inserted in place of excised viral coding sequences. To propagate such a provirus, the proviral DNA is transfected into a producer cell line providing the full complement of viral polypeptides needed for packaging. The GP+E cell line was used to produce infectious virus. Transfection with proviral DNA and subsequent drug selection was performed as above.

2.2.40 Viral infection II - incubation with virus solution

Grow the producer cell line to full confluence in a 10 cm dish. Remove spent media from the cells. Add 4 to 5 ml fresh media to the producer cells. Incubate the producer cells overnight to 3 days (the cells may look awful but the titre improves).
Split the recipient cells one wishes to infect the night before at either $10^5$ or $7.5 \times 10^5$ cells per 10 cm dish. Remove the media containing the virus from the producer cells with a 5 ml syringe and filter through a Millex (or equivalent) 0.22 μm-0.45 μm filter to remove any cells.

Remove the medium from the recipient cells and replace with 2 ml of viral supernatant containing 8 μg/ml polybrene (20 μl of 800 μg/ml). Allow the infection to proceed for 2 hours in the incubator. Remove the virus and add 10 ml fresh media or simply add 8 ml of media to dilute the polybrene. Incubate overnight.

If the cells were seeded at $1 \times 10^5$, initiate drug selection by removing the medium and adding fresh selective medium. Cells seeded at $7.5 \times 10^5$, are split 1:20 into selective media. If one is not interested in the absolute titre of virus and simply wants colonies quickly, don't add selective medium until two or three days post-infection. G418 resistant colonies appear about three days earlier if one waits several days before adding the antibiotic.

### 2.2.41 Ring cloning

Draw around the colonies to be picked with a marker pen. Place sterile cloning rings on a tray of sterile grease using flamed forceps.

Wash the plate twice with PBS, removing all the liquid and place a ring over each colony (up to 10 can easily be done per 10 cm dish). Press gently to obtain a good seal. Add 1 drop of trypsin/versene to each ring and wait 30 seconds for the cells to begin rounding up; follow this under the microscope as REFs for example are relatively sensitive while Rat-1’s are much more resilient. Add 3 drops of E4+10% FCS with a plugged Pasteur and pipette up and down to dislodge the colony. Transfer to 1ml E4+10% FCS in a 24 well plate for sensitive cells such as REFs or directly to 5 ml in a 6 well dish for hardy cells such as Rat-1’s.
2.2.42 FACS analysis

Treat the cells (one 9 cm dish of cells is ample) with 10 µM BrdU (from a 200x frozen stock) for at least two hours or the period of the experiment. Trypsinise, resuspend the cells in media with serum (to inactivate the trypsin) and spin 1,500 rpm for 3 minutes. Remove the supernatant and wash with PBS. Remove all but 200 µl of the saline and resuspend well. Gently add 2 ml of ice-cold 80% EtOH, while mixing. Leave on ice for at least 30 minutes; they are now ready for analysis. Analysis was performed by the FACS laboratory on a Beckton Dickinson FACStar Plus machine.

2.2.43 Time-lapse cinemicroscopy

Filming was performed (by Chris Gilbert) on cells in 5 cm dishes using an Olympus time-lapse unit, a 16 mm Bolex H16 camera and an Olympus IMT inverted microscope fitted with x10 phase objectives. The stage was wholly enclosed in a transparent environment chamber, maintained humidified at 37°C and in 10% CO₂.
Chapter Three

3 TRANS-ACTIVATION

3.1 Introduction

The Myc protein has strong structural similarities to known transcription factors. It possesses a classical basic region that can bind DNA (discussed in detail in chapter 4) and two dimerisation domains: a helix-loop-helix and a leucine zipper\textsuperscript{124,306-308}. In addition, Myc is located in the nucleus and has a dimerisation partner in Max\textsuperscript{145,146}. Finally Myc possesses a putative trans-activation domain in the N-terminus. This domain is conserved to some extent in c-, N- and L-myc as well as across species and is required for the biological effects of Myc: the transformation of mammalian cells in cooperation with activated Ras\textsuperscript{134}, apoptosis\textsuperscript{136}, inhibition of differentiation\textsuperscript{309} and autoregulation\textsuperscript{103,310}. This N-terminal region can activate transcription from a Gal4-responsive promoter when fused to the DNA binding domain of Gal4\textsuperscript{138} and a LexA-Myc fusion protein drives transcription of a gene with a LexA binding site in the promoter in yeast\textsuperscript{311}.

Max lacks an obvious trans-activation domain, suggesting that since Max complexes can recognise the same E-box sequence as Myc/Max\textsuperscript{145}, they may act repressively by blocking Myc/Max access.

Myc was expected to act as a transcription factor and at the commencement of this project, work in the laboratory using budding yeast as a model system (subsequently published\textsuperscript{312}) demonstrated sequence-specific transcriptional activation by Myc with Max. It remained to be demonstrated however that Myc could activate transcription in mammalian cells and it was therefore crucial to ascertain this as the next step in determining the mechanisms of Myc action.
3.2 Choice of a model system - cells and constructs

The simplest means to determine whether Myc can trans-activate in mammalian cells is to measure the expression of a gene to which Myc binds in response to various levels of Myc. Secondary rat embryo fibroblasts (REFs) can be transformed in culture by the co-transfection of Myc and Ras expression vectors\(^50\), demonstrating that they respond biologically to Myc. Since these cells are derived directly from normal tissue and are neither immortalised nor derived from a tumour, they do not have deregulated Myc expression. In addition, from a technical viewpoint, they can be transfected readily and the Myc/Ras cooperation assay had proved that the expression vectors available worked efficiently\(^283,312,313\). These properties made them ideally suited for the following experiments.

In the absence of a natural Myc-responsive promoter, detecting Myc trans-activation required a reporter construct to which I anticipated Myc would bind specifically. I therefore engineered a plasmid containing a Myc binding site (CACGTG) upstream from a minimal promoter, the thymidine kinase (tk) promoter from Herpes Simplex Virus (HSV), driving expression of chloramphenicol acetyl transferase (CAT). Plasmid pBLCAT\(^284\) was used as a source of HSV-tk driven CAT and I replaced the \(PstI\) fragment with a sequence derived by PCR from pBLCAT2 using the primers shown in Fig. 3.1. The resulting plasmid, pPml-tkCAT contains the best available Myc binding site at that time (a palindrome containing the sequence CACGTG – a \(Pml\) site)\(^141\), 20 base pairs upstream of the TATA box.
Figure 3.1 Construction of a Myc reporter

The plasmid pPml-tkCAT was derived from pBLCAT2 by replacement of the Psfl tk promoter fragment with the sequence shown, created by PCR using the following primers and pBLCAT2 as template. The changes from pBLCAT2 are shown.

5'->3':
AATGCCCTGCAGTCGACCACGTGGTCGACCACTTCGCATATTAAGGTGA
and
ATTACCATTGCTGCAGGGTCGCTCGGTGT
Changes from pBLCAT2

TATA Box

Figure 3.1 Construction of a Myc reporter
A variety of expression vectors were used to express proteins including Myc and Max in REFs. These were kindly provided by Bruno Amati (see chapter 2). The promoters driving expression in these constructs are summarised in Fig. 3.2 below:

<table>
<thead>
<tr>
<th>Name*</th>
<th>Promoter</th>
</tr>
</thead>
<tbody>
<tr>
<td>J3/BJ3</td>
<td>Simian Virus 40 Origin/Early promoter</td>
</tr>
<tr>
<td>J4/BJ4</td>
<td>Moloney Murine Leukaemia Virus LTR</td>
</tr>
<tr>
<td>J6/BJ6</td>
<td>Rat β-Actin promoter</td>
</tr>
<tr>
<td>J9/BJ9</td>
<td>Rous Sarcoma Virus LTR</td>
</tr>
<tr>
<td>Babe series</td>
<td>Moloney Murine Leukaemia Virus LTR</td>
</tr>
</tbody>
</table>

* Plasmids titled J are made from the pBR322 backbone whereas the BJ vectors contain the Bluescript backbone and therefore give greater plasmid yields in bacteria due to their increased copy number.

**Figure 3.2** Summary of the expression vectors used.

### 3.3 Effect of Myc on expression from pPml-tkCAT.

To determine whether Myc could activate transcription, I co-transfected REFs with either the BJ9-Myc expression vector or the empty BJ9 vector together with pPml-tkCAT (the reporter containing the CACGTG site), and measured CAT activity.

An increased level of transcription in the cells transfected with Myc compared with the cells transfected with the empty vector would not alone indicate sequence-specific transcription. It could instead be an effect of a general increase in the level of transcription. As a control therefore, to investigate the sequence specificity of any observation, cells were transfected in parallel with pBLCAT2 from which pPml-tkCAT had been made. Additionally, to provide an internal control of transfection
efficiency, cells were simultaneously transfected with J4-LacZ. Cells were harvested and assayed for both CAT and β-galactosidase activity. Shown in the figures is the normalised CAT activity, that is CAT activity divided by the β-galactosidase activity.

REFs were plated at 70% confluence and transfected with reporter (either pPml-tkCAT or pBLCAT2), BJ9-Myc or BJ9 and J4-LacZ. The system was optimised by performing a number of experiments with varying amounts of the different plasmids. Optimal results (high signal compared with background and a detectable, reproducible effect of Myc) were obtained using 3 μg of reporter and 5 μg of the expression vectors.

Myc increases expression from pPml-tkCAT about 4-fold while it has significantly less effect on the control reporter lacking a CACGTG site (Fig. 3.3). This is evidence that Myc can act as a sequence-specific transcription factor.
Subconfluent REFs in E4 + 10% serum were co-transfected with 5 \( \mu \text{g} \) each of BJ9 or BJ9-Myc along with 1 \( \mu \text{g} \) of J4-LacZ and 5 \( \mu \text{g} \) of the indicated reporter. Cells were harvested after 48 hours and assayed for CAT and \( \beta \)-galactosidase activity. Shown is the CAT activity over background normalised for \( \beta \)-galactosidase activity.
**Figure 3.3** Sequence-specific trans-activation by Myc
3.4 MycER – another means of examining Myc's effects

A second means of demonstrating trans-activation by Myc is afforded by the MycER chimaera. This consists of the human c-Myc protein fused to the hormone-binding domain of the human oestrogen receptor (Fig. 3.4A).

In cells expressing MycER, its activity is strictly dependent upon the application of exogenous 17β-oestradiol (OE). MycER binds to Max in a hormone-dependent manner, allowing tight regulation of transcriptional activation by Myc in vivo. Induction of transformation, cell-cycle entry and apoptosis, both in serum-starved cells and in response to TNF-α are all hormone dependent in MycER-expressing cells.

I made use of Rat-1 cells infected with either a MycER-expressing virus or a Δ106-143 MycER-expressing virus. When density arrested and serum starved, Rat-1 MycER cells re-enter the cell cycle in response to OE and die by apoptosis when serum starved. The Rat-1 ΔMycER cells in which residues 106-143 of Myc (a putative trans-activation domain) have been deleted exhibit neither of these behaviours. By using the MycER system, it is therefore possible to examine the response of a reporter in cells that are known to be behaving in a biologically relevant manner. Further, measurement of the MycER expression level in Rat-1 cells shows that it is similar to the level of Myc expression seen in serum-stimulated cells of about 10,000 molecules per cell. As such the responses of MycER cells treated with OE are to a physiological level of Myc. Lastly, with the MycER system, one can examine the effects of Myc activity rapidly following activation. With transient transfections, the effects can only be observed some 2 days later.

I stably co-transfected either pPml-tkCAT or pBLCAT2 into each of these Rat-1 cell lines with pJ6-puro (an expression vector in which
Figure 3.4

A Schematic representation of MycER and ΔMycER proteins

The numbers refer to the amino acids of the full-length proteins from which the fusion proteins are derived.

B Trans-activation by MycER

Shown is the CAT activity in MycER and ΔMycER cells stably transfected with pPml-tkCAT or pBLCAT2 and treated with either ethanol (EtOH) or 17β-oestradiol (OE). Cells were arrested 48 hours in 0% serum + 5 μg/ml insulin and treated for 17 hours as shown. They were then harvested for CAT activity. Each point represents the mean of 4 samples.
**Figure 3.4 MycER**

A Schematic representation of MycER and ΔMycER proteins

B Trans-activation by MycER
the puromycin-resistance gene is downstream of the β-actin promoter) and pooled the resultant puromycin-resistant clones to give four cell populations: Rat-1 MycER pPml-tkCAT, Rat-1 ΔMycER pPml-tkCAT, Rat-1 MycER pBLCAT2 and Rat-1 ΔMycER pBLCAT2. I grew each cell line to confluence and withdrew serum for 48 hours to arrest the cells and down-regulate endogenous Myc. I then treated the cells with 2 μM OE for increasing times prior to harvesting for CAT assays. The result (Fig. 3.4B) shows that only the full-length MycER protein trans-activates and that it does so in a sequence-specific manner. Note that activity of the pBLCAT2 plasmid increases slightly upon MycER activation, possibly because general transcription increases as Myc drives the cells into cycle.

3.5 Effect of Max expression on Myc trans-activation.

The Max protein forms homodimers more efficiently than does Myc (it is unlikely that Myc homodimers can form at physiological concentrations) but the preferred form is the Myc/Max heterodimer. In cells, essentially all Myc molecules are found complexed to Max which is constitutively expressed. Both Myc/Max and Max/Max dimers can bind to the CACGTG sequence, but as Max lacks an obvious trans-activation domain, its dimers were not expected to trans-activate. However, since Myc is most probably active only as a heterodimer with Max, it was possible that Max expression would enhance Myc trans-activation.

Expression of Max in REFs in addition to Myc does not in fact increase Myc trans-activation, but instead lowers it slightly (Fig. 3.5). This apparent repressive function of Max is concentration dependent (Fig. 3.6). Max exists in a number of forms (see chapter 1), but in the experiments shown above, I have used the longer Max2 form as it has a
Figure 3.5  Effect of Max co-transfection

REFs were co-transfected with 5 µg each of BJ9 or BJ9-Myc and BJ3 or BJ3-Max2 along with 1 µg of J4-LacZ and 5 µg of the indicated reporter. Cells were harvested after 48 hours and assayed for CAT and β-galactosidase activity. Shown is the CAT activity over background normalised for β-galactosidase activity.
Figure 3.5 Effect of Max co-transfection.
Figure 3.6  Dose-dependent effect of Max on Myc trans-activation

Subconfluent REFs in E4 + 10% serum were transfected with 5 μg of pPml-tkCAT, 1 μg of J4-LacZ and 5 μg of either BJ9 or BJ9-Myc as well as a total of 20 μg of BJ3/BJ3-Max2 made up as indicated. Cells were harvested after 48 hours and assayed for CAT and β-galactosidase activity. Shown is the CAT activity over background normalised for β-galactosidase activity.
**Figure 3.6** Dose-dependent effect of Max on Myc trans-activation
slightly higher affinity for DNA. I chose not to use the ΔMax forms as their homodimers were unlikely to enter the nucleus because they lack a nuclear localisation signal.

These findings are consistent with a model in which Max is constitutively expressed at a higher level than the amount of exogenously produced Myc, and therefore is available to interact with all of these Myc molecules; and in which Max can also homodimerise and bind the Myc/Max binding site, thereby either actively repressing transcription or passively blocking the action of Myc/Max complexes (and possibly other transcription factors). This is also consistent with studies in yeast performed in the laboratory, showing that Max homodimers do form in yeast but fail to trans-activate although they can block Myc-dependent trans-activation; and also with the fact that Gal4-Max fusion proteins fail to trans-activate in mammalian cells.

3.6 Domains of Myc required for trans-activation

Using a number of Myc mutants we attempted to identify the domains of Myc required for trans-activation in mammalian cells. Data from several experiments are compiled in Fig. 3.7. Note that for some of the data shown, different reporters (MinCAT and M4MinCAT analogous to pBLCAT2 and pPml-tkCAT respectively) obtained from Bob Eisenmann were used. These reporters give more consistent results with less DNA per transfection (1 μg instead of 3 μg) and a higher level of trans-activation by wild-type Myc. The main difference is that M4MinCAT contains four CACGTG binding sites compared to only one in pPml-tkCAT, Fig. 3.8. There is however no qualitative difference in the results.
Figure 3.7  Functional requirements for Myc trans-activation

Subconfluent REFs in E4 + 10% serum were co-transfected with 5μg of the appropriate expression construct, 1 μg of LacZ expression construct and the indicated reporter. Cells were harvested after 48 hours. Shown is the CAT activity over background normalised to β-galactosidase in arbitrary units.
Figure 3.7 Functional requirements for Myc trans-activation
Figure 3.8 Comparison of the M4MinCAT and pPml-tkCAT reporters.
Chapter Three

Trans-activation

The following mutant Myc proteins were tested for their trans-activation potential:

i) Myc Δ7-91\textsuperscript{136} which removes Myc Box I, a conserved region between all Myc proteins important in REF transformation and in trans-activation when fused to Gal4 and which contains two phosphorylation sites\textsuperscript{119}.

ii) Myc Δ106-145 which removes Myc Box II, a second conserved domain also important in transformation as well as auto-suppression\textsuperscript{135} and apoptosis\textsuperscript{136}.

iii) Myc Δhlh which partly removes the helix-loop-helix region (amino acids 371-412\textsuperscript{135}) and can no longer dimerise with Max\textsuperscript{324}.

iv) Myc Δlz which partly removes the leucine zipper region (amino acids 414-433\textsuperscript{135}) and can no longer dimerise with Max\textsuperscript{324}.

v) Myc 364/6/7 R-A in which residues crucial for DNA binding have been changed from a basic arginine to the pH-neutral alanine, resulting in a protein that can still dimerise with Max but can not bind to DNA\textsuperscript{312,313}.

vi) MycEG and MaxEC\textsuperscript{283,313}, which interact with each other but not with their wild-type counterparts. By analogy with the solved crystal structure of a GCN4 leucine zipper dimer\textsuperscript{325}, the leucine zippers of these proteins have had the positions of the electrostatic charges at positions e and g (using O'Shea's nomenclature\textsuperscript{325}) swapped. As a result, these charges will no longer line up with, and attract those of, the natural proteins but will only do so with another mutant in which the charged positions have also been swapped.

Mutation of the DNA binding domain or either of the two dimerisation domains abolishes trans-activation. Mutation of either N-terminal domain (Myc box I or II) significantly but not completely reduces trans-activation. Trans-activation by MycEG requires MaxEG, demonstrating that Myc/Max dimerisation is essential and that the
Chapter Three

Trans-activation

Myc/Max dimer that is the active form of Myc. This is consistent with
data from similar experiments in yeast, in which trans-activation by Myc
requires Max expression\textsuperscript{312}.

3.7 Discussion

The data described above show that Myc activates transcription in
a sequence-specific manner and that Myc Boxes I and II contribute to the
activation, while DNA binding and dimerisation with Max, mediated by
the C-terminus, are essential. Two papers in particular\textsuperscript{323,326} have
demonstrated that Myc can trans-activate in mammalian cells, with
results essentially the same as those presented here. Myc trans-activates
from CACGTG-driven promoters but not from controls lacking this site.

Mutation of either the DNA binding or dimerisation domains
abolishes transcription. Additionally, using reciprocal mutants of Myc
and Max which dimerise with each other but not with their wild-type
counterparts, it has been shown that in transcriptional activation\textsuperscript{312}, Ras
co-transformation\textsuperscript{283,327,328} and apoptosis assays\textsuperscript{313} the Myc/Max dimer
is the active form. Max is essential for Myc action but only in providing a
means to bind DNA: only the br-hlh-lz domain of Max is required to
complement Myc\textsuperscript{283,312}.

Despite a large body of deletion data\textsuperscript{134,135,138}, the role of the Myc
N-terminus is still unclear and demarcation of the trans-activation
domain is still incomplete. Kato's data\textsuperscript{138} using Myc-Gal4 fusion proteins
defined three distinct regions in trans-activation assays: amino acids 1-43
(region 1) were moderately active, 43-103 (region 2) were inactive but
regions 1 and 2 together were more active than region 1 alone. A third
region, amino acids 103-143, had weak activity but again was powerful
in combination with region 2. In my hands Myc Δ106-143 had
significantly reduced trans-activation potential in agreement with Amin
et al.\textsuperscript{326} as did Myc Δ7-91; these findings are also in agreement with earlier work looking at Myc activity in a REF co-transformation assay\textsuperscript{134}. Thus there are at least two active trans-activating regions in the N-terminus although the mechanisms by which they act remain unclear. Further data on the proteins that can interact with these regions may explain the molecular mechanisms by which they activate transcription. It has been shown for example that TBP can interact with Myc \textit{in vivo}\textsuperscript{329-331} and this may be the basis for Myc-dependent transactivation. It is also possible that N-terminal phosphorylation of Myc is relevant, the phosphorylation sites at Thr-58 and Ser-62 seem important for transformation\textsuperscript{332} and are frequently mutated in Burkitt's Lymphoma\textsuperscript{333}, but mutant Myc proteins in which these positions are mutated to non-phosphorylated alanines trans-activate normally\textsuperscript{119}.

Kretzner et al. find that expression of Max alone represses transcription from M4MinCAT (and that the effect is CACGTG-dependent and requires the basic region of Max) while I didn't see this with my reporter. This is probably because the background level of expression I see is too low compared with the error in the system to make such an observation whereas M4MinCAT has a higher basal expression level; it might also be because Max homodimers bind more efficiently or cooperatively to the four sites in M4MinCAT. If there is a contribution from endogenous Myc to the basal level then repression by Max could be simply due to Max/Max complexes blocking Myc/Max from the CACGTG site. It is also possible, however, that Max is acting to block the binding of some other E-box specific factor such as USF\textsuperscript{133,334} from the site; this possibility is intriguing given that little is known about the binding preferences of Max complexes (see chapter 4), and they may regulate the targets of a range of transcription factors in addition to Myc. Lastly, it is also possible that Max acts as a repressor of transcription
per se by interacting with other transcription factors or the general transcription machinery.
Chapter Four

4 DNA BINDING SPECIFICITIES

4.1 Introduction

In the related br-hlh and br-hlh-lz families of transcription factors, the basic regions of protein dimers recognise sequences containing CANNTG (the E-box), see Littlewood and Evan, 1995 for review. In view of the large size of these families, it is likely that there may be several factors present in a single cell that potentially recognise the same target sequence; yet these proteins must exercise a high degree of specificity in recognising their own targets, given the diversity (such as growth or differentiation) and fundamental importance of their effects. The identification of a transcription factor’s binding site is crucial in understanding its function, not least as it can indicate potential target genes.

Previous work using purified GST-Myc(br-hlh-lz) proteins which bind to DNA as homodimers, had identified a 12 base pair DNA binding site containing the sequence CACGTG. In vitro translated Myc br-hlh-lz peptides also recognise a palindromic CACGTG motif. A similar peptide corresponding to the br-hlh-lz region of v-Myc (61 amino acids long) also binds CACGTG. Another approach in which the basic region of Myc was substituted for the same region of E12 generated a fusion protein that also bound CACGTG sequences, and this was inhibited by CpG methylation.

Myc dimers are however undetectable in vivo and are only formed at extreme concentrations with in vitro translated or bacterially produced Myc. Studies of Myc trans-activation in both yeast and mammalian cells (see also chapter 3), as well as biochemical analysis of the Myc protein, had demonstrated that Myc acts as a complex with Max. Although it was likely that the sites identified by Halazonetis and Kandil were actually bound by a heterodimer
between the Myc peptide and endogenous Max present in the reticulocyte lysate\textsuperscript{147}, no DNA binding site had been determined for the Myc/Max heterodimer using full-length proteins, and I decided to identify such a site.

There are several possible mechanisms to achieve specificity of DNA binding: differential interactions with other DNA-binding proteins on a promoter may restrict the set of sites at which a given factor may act; specific E-boxes (such as CACGTG but not CAGCTG) may be recognised by different factors or combination of factors in a heterodimer\textsuperscript{131,132,338-344}; or preferences for sequences outside the E-box may be factor specific. Blackwell and Weintraub\textsuperscript{344} demonstrated that br-hlh-containing proteins recognise 3 bases on either side of the hexanucleotide core, and it therefore seemed important to determine such extended DNA-binding sequences for the full-length Myc/Max protein complex to provide the best information as to potential target genes.

The Max dimer had been shown to also recognise the CACGTG sequence\textsuperscript{145,146} and to affect trans-activation mediated through such sites\textsuperscript{312,323,326,337} (see also chapter 3). Since Max RNA and protein are very stable compared with Myc and there is an excess of Max over Myc in vivo\textsuperscript{345-348} it seemed likely that Max/DNA complexes would form in cells. However, a Max binding site had never been determined from scratch, rather Max had only been shown to be able to bind to the Myc binding site. The possibility remained that Max would bind preferentially to other sequences which may be important functionally, and I therefore decided to analyse Max binding as well.

To determine complete consensus sequences requires either a binding-site selection from an initially random pool\textsuperscript{349} or comprehensive testing of all possible binding site variants. I decided on the former
approach to define the Myc/Max and Max/Max DNA binding sites. Further, since Myc is a phospho-protein, I concluded that in vitro translation was superior to purification from bacteria as the protein produced would be modified to an extent (for example phosphorylation by casein kinase II (CKII)\textsuperscript{350,351}. Work with in vitro translated, full-length Myc and Max in the laboratory (subsequently published\textsuperscript{147}) had established a gel retardation and immuno-precipitation assay for DNA binding. This confirmed that Myc/Max can bind to CACGTG and I decided to use this system to analyse the binding specificities of the full-length Myc and Max proteins.

4.2 Selection of binding sites

In an attempt to investigate the DNA binding preferences of the Myc/Max and Max/Max complexes, I performed repeated rounds of selection and amplification from an initially random oligonucleotide pool (R76), as previously described\textsuperscript{349}. Accordingly, in vitro translated full-length Myc and Max or Max alone were allowed to bind to a random oligonucleotide pool consisting of 26 random bases flanked by PCR primers (see Fig. 4.1). This was then co-immuno-precipitated with the appropriate antibody (anti-Myc for the Myc/Max complex and anti-Max for the Max/Max complex). Any bound DNA is therefore precipitated with the protein complex bound. I then amplified the selected DNA by PCR and performed further rounds of selection in the same manner by using the selected DNA in place of R76. The Max2 form of Max was used since it binds DNA more efficiently than Max1, both as a homodimer and as a heterodimer with Myc\textsuperscript{147}.
Figure 4.1 Binding-site selection protocol
At each round parallel reactions were run with a labelled control CACGTG probe (wt) known to bind efficiently to both Myc/Max and Max/Max and also able to mediate trans-activation (see chapter 3). A high level of immuno-precipitated labelled wt, and a low level precipitated by antibody pre-incubated with its cognate peptide, indicated that the binding and precipitation were both efficient and specific. Additionally I measured the extent of selection by performing another pair of immuno-precipitations (with and without peptide) using labelled probe amplified from the previous round. The fraction of radioactive counts blocked by peptide with this probe, compared with the fraction with the control probe, is a simple measure of the extent of selection - if this ratio is 1:1 then the test probe is as efficiently bound as the control. I continued selection for one round after recording a significant level of peptide blockable precipitated probe (five rounds with Max, eight with Myc/Max, Fig. 4.2). The final oligo. pools in each case were about half as efficiently precipitated as the control probe.

To preserve low-affinity sites, I cloned and sequenced selected oligonucleotides from the penultimate as well as the final rounds (Figs 4.3 and 4.4). In addition a parallel selection was performed with unprogrammed reticulocyte lysate to control for any endogenous DNA binding activity, and no enrichment was measured, nor were any recurring motifs observed, other than an increased proportion of T and A residues (not shown).
Figure 4.2  Extent of selection

Shown is the amount of probe from each successive round of selection that is brought down by the antibody or by the antibody when blocked with its cognate peptide. A similar value in each case suggests that the probe is not specifically recognised whereas a significantly higher value with the antibody alone suggests a probe that is specifically brought down. Note the first round (R76) was performed with double the amount of probe.
Figure 4.2 Extent of selection.
Figure 4.3  Sequences selected by Myc

PCR products from the appropriate round of selection were digested with *BamHI* and *EcoRI*. They were then gel purified and cloned into Bluescript for sequencing. Only the central 26 bases are shown.
Figure 4.3 Sequences selected by Myc.
Figure 4.4  Sequences selected by Max

PCR products from the appropriate round of selection were digested with BamHI and EcoRI. They were then gel purified and cloned into Bluescript for sequencing. Only the central 26 bases are shown.
After 4 rounds:

```
AAAGCTTCGGAAATTAC CAGTG ACA
TTATGTGAAC CACGTG ACTTCCGTGTT
CTACGTGACGTGAA CAGTGGCCT
ATCGCTGGA CAGTG ATTCGAGCGCA
ACGAAGCGGCC CAGTG GAACATTG
GAGGAATACAGGC CAGNG TCAGACAGATA
ATGATACACTAA CAGTG TT
GACCTCTGGAACGCTGA CAGTG ACT
AAAA CACGTG TTTATACTTACNCAA
CTATGAAA CACGTG TTNCATGTTGCG
CGCTGAA CACGTG ATTCTGAGGCA
ACGAACCGCGCC CACGTG GTACAATTG
GAGGAATAGAGGC CAGNG TCAGACAGATA
ATGATATCACTAA CACGTG TT
GAACCTGGAACGGTGAA CAGTG ACT
.......................AAAA CACGTG TTTATACTTACNCAA
CTATGAAA CACGTG TTNCATGTTGCG
CGCTGAA CACGTG ATTCTGAGGCA
ACGAACCGCGCC CACGTG GTACAATTG
GAGGAATAGAGGC CAGNG TCAGACAGATA
ATGATATCACTAA CACGTG TT
GAACCTGGAACGGTGAA CAGTG ACT
```

After 5 rounds:

```
GAAATCCATTGAGTGGGT CAGTG CT
TTTCGACTTGGT CAGTG TCGTGTGCGCTGCT
TCAAAACACCACCTGAA CAGTG GT
.............AAAC CACGTG GAGTAATCGAGATAAA
GATCGCATTGTACTCA CAGTG CTCTACGTGGCTAA
.........TTTTAATATTGATAC CACGTG ACTC
GTACTTAGTAGAC CACGTG ACATGAT
........................TTTACATAA CACGTG GTGTTAAGA
.............CAGTCGGTAGAA CACGTG GTAATAG
AGCAAAGAC CACGTG ACCTGACGTGA
ACCTCAAGC CACGTG ACTG CACGTG CAAT
.GTACAATAGTTCACGC CACGTG ACA
.......................ACCTCAAGC CACGTG ACTG CACGTG CAAT
.............GTACAAATGTTTCAAGC CACGTG A
.............CCAAATATGCTAT CACGTG TTTCT
.............GGAGCCGAC CACGTG ACTAA
.............GAGGTGAATNCNGAA CACGTG GAC
.....................ATACAGAAGT CAGTG CTCTCCTATT
.....................TNCCCTATACAAA AAG CAGTG TTTGAGCC
.............GTACACTAGTAGAG CACGTG TTAAACAC
.............AACACCAAGA CACGTG GCCTGATNGAC
.............CAGT CACATG CCATTATCACCCGC
.............GCCGAAGCTAAATTTAAAATTACCA
.....................CGATAGGATATGAAGTTCAGATTTCGA
.............TAATTACATAGTACCAATTATNCGTA
.............TCATGGCAATATAAGCGGTATTTGT
.............GTGGCTCCCTCTCCCTTTGATAAT
...............CAAAACTCTTTTCTCCCTTTGATAAT
AGCTAAATCAGGTAAGTGAATAC
.....................ACATAAATAGTTATATACCT
.............CCACAATTTACCACGATTATATCACA
.....................GAGATGAACTCGTGATTTGCGCC
```

Figure 4.4 Sequences selected by Max.
4.3 Results of site selection

Both the Myc/Max and the Max/Max complexes selected sequences containing the previously described hexanucleotide CACGTG (73% and 90% for Myc/Max in the penultimate and final rounds respectively; 55% and 91% for Max/Max). To investigate the extended binding sites, I studied the base composition flanking this core. Since the sequence CACGTG is a palindrome, the orientation of the selected molecules is indeterminate. To account for this I considered each half-site independently. For example the sequence AAAG CACGTG TTTG was considered as one AAAG CAC half-site and one CAAA CAC half-site. All the half-sites were aligned and the number of occurrences for each base at each position was determined and is shown in Fig. 4.5. This was used to derive a half-site consensus binding sequence. The full consensus sequence is composed of two mirror image half-sites as indicated (Fig. 4.5).

Surprisingly the two complexes exhibited very different preferences at the -1 position. Of the sequences containing the CACGTG motif, only one of the 76 selected by Myc/Max had a T residue at this position, demonstrating that the TCAC half-site is strongly disfavoured. The Max dimers show no significant preference at this position.

Both complexes have a clear preference for purines at positions -2 and -3, with the -2 position being especially well defined: A is the preferred residue but a G is allowed, while either pyrimidine is strongly disfavoured. At -3, the preference appears less strong and only a C is significantly disfavoured. The consensus binding site of the Myc/Max dimer is GACCACGTGGTC, while the consensus for Max homodimers is RANCACGTGNTY. Neither complex shows any preference for particular bases at the -4 position nor at positions further out.
Figure 4.5  Half-site tables

A  Myc/Max
B  Max/Max

The number of occurrences of each base is shown for each position relative to the CACGTG core. Also shown are the consensus sequence and those flanking sequences that are particularly disfavoured.
Figure 4.5 Half-site tables.

**A Myc/Max**

<table>
<thead>
<tr>
<th>Position relative to the CACGTG core</th>
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</tbody>
</table>

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<td>19</td>
<td>49</td>
<td>7</td>
</tr>
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<td>T</td>
<td>21</td>
<td>12</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
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**B Max/Max**

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</thead>
<tbody>
<tr>
<td>-4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
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<th>19</th>
<th>24</th>
<th>22</th>
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</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>28</td>
<td>31</td>
<td>45</td>
<td>22</td>
</tr>
<tr>
<td>T</td>
<td>15</td>
<td>11</td>
<td>4</td>
<td>17</td>
</tr>
<tr>
<td>C</td>
<td>12</td>
<td>7</td>
<td>2</td>
<td>24</td>
</tr>
</tbody>
</table>

Full consensus sequence

NGAC CACGTG GTCN NRAN CACGTG NTYN

Disfavoured flanking sequences

-CYT ....... ARG- -CY- ....... -RG-
Of those sequences without a CACGTG, the only obvious feature was the CACATG motif in three of 52 sequences selected by Max, none of them in the final round of selection. I tested whether the Myc/Max and Max/Max dimers could bind to this sequence by using increasing amounts of either CACATG or CACGTG as competitor in a gel retardation assay. Both Myc/Max and Max/Max complexes were competed by CACATG although less efficiently than by the wild-type sequence (Fig. 4.6). As a control, two mutant sequences (CAATTG and CAAGTG) failed to compete at all, even at 160 times the concentration of the wild-type DNA.

Computer analysis of those sequences lacking either CACGTG or CACATG motifs failed to reveal any recurrent feature other than AT-rich sequences similar to those brought down by the unprogrammed lysate. Specifically the sequence TCTCTTA reported as being associated with Myc was not present.

4.4 Effects of flanking sequences on DNA binding

To confirm the difference in binding preference of the Myc/Max and Max/Max complexes, I analysed the DNA binding activities of these complexes in gel-retardation assays using a labelled CACGTGG probe in the presence of increasing unlabelled competitor DNA (either CACGTGG or TCACGTGA). A 100-fold excess of TCACGTGA was only able to compete weakly for the Myc/Max complex in comparison with competition by the CACGTGG sequence itself, while both sequences competed efficiently for the Max/Max complex (Fig. 4.7).
Figure 4.6  Affinities of the Myc/Max and Max/Max complexes for CACGTG and CACATG

*In vitro* translated Myc and Max2 or Max2 proteins were incubated together with both radio-labelled probe (CGCCGAC CACGTG GTCCCTC) and increasing amounts of the indicated competitor which differs only at the underlined positions. Complexes were resolved by gel retardation and quantified on a phosphor-imager. Shown is the fraction of counts bound compared to the reference sample with no competitor. Values indistinguishable from background are recorded as zero.
Figure 4.6 Affinities of the Myc/Max and Max/Max complexes for CACGTG and CACATG
Figure 4.7  Affinities of the Myc/Max and Max/Max complexes for CCACGTGG and TCACGTGA

*In vitro* translated Myc and Max2 or Max2 proteins were incubated together with both radio-labelled probe (CGCCGA CCACGTGG TCCCTC) and increasing amounts of the indicated competitor which differs only at the underlined positions. Complexes were resolved by gel retardation and quantified on a phosphor-imager. Shown is the fraction of counts bound compared to the reference sample with no competitor. Values indistinguishable from background are recorded as zero.
Figure 4.7 Affinities of the Myc/Max and Max/Max complexes for CCACGTGG and TCACGTGA.
4.5 Effects of flanking sequences on trans-activation

The Myc/Max complex can function as a transcriptional activator. To determine whether Myc action \textit{in vivo} is affected by the difference in binding specificity for CCACGTGG and TCACGTGA described above, I analysed the effect of Myc activity on reporter plasmids with various sites upstream of a CAT gene derived from pPml-tkCAT (Fig. 4.8). I tested the effect of either a single half-site change (TCACGTGG; called Twt) or a double half-site change (TCACGTGA; called TwtA) from the wild type (CCACGTGG; called wt) as well as a site with a mutant core (CCACGTAG; called GTA). Two systems were available (described in chapter 3) for testing the trans-activation potential of Myc: transient transfection in REFs and activation of MycER cells. I tested the modified reporters in both systems.

I co-transfected 3 μg of these reporters into REFs with either 5 μg of BJ3 or BJ3-Myc with 3 μg of J4-LacZ for normalisation. The normalised CAT values are shown in Fig. 4.9. It is clear that trans-activation is greatly reduced by even a single half-site change and is almost completely abrogated by the double half-site change.

I stably co-transfected the reporters and the puromycin-resistance gene into Rat-1 MycER cells. This enabled me to measure the rate of expression from the reporters in the presence and absence of active Myc. I pooled approximately 1,500 puromycin-resistant colonies for each reporter. Exposure of the pools to OE resulted in an increased rate of CAT synthesis only in the pool with the wt sequence while the negative control (GTA) and both Twt and TwtA failed to mediate such an increase (Fig. 4.9).

The DNA binding preferences of the Myc protein as determined \textit{in vitro} are therefore reflected in the behaviour of the protein \textit{in vivo} and
Figure 4.8  Reporters with modified flanking sequences

The plasmids shown were derived from pPml-tkCAT by replacement of the *HindIII* - *SalI* fragment with synthetic oligonucleotides to give the sequences shown above. The only differences between the four reporters are shown in bold type. The reporters were sequenced to ensure no errors had been introduced.
wt: C CAC GTG G
Twt: T CAC GTG G
TwtA: T CAC GTG A
GTA: C CAC GTA G

Figure 4.8 Reporters with modified flanking sequences
Chapter Four DNA Binding Specificities

Figure 4.9  Effect of flanking sequences

A  In transient transfections

Subconfluent REFs in E4 + 10% serum were transfected with the indicated reporter, J4-LacZ and either BJ3-Myc or BJ3 as described and assayed for CAT and β-galactosidase activity. Shown is the fold activation (normalised CAT value with BJ3-Myc divided by that with BJ3).

B  In stably transfected cells

Rat-1 MycER cells stably transfected with the indicated reporter were arrested and treated in triplicate with either 17β-oestradiol (OE) or ethanol (EtOH) for 17 hours. The fold activation with OE compared to EtOH is shown.
Figure 4.9 Effect of flanking sequences
A In transient transfections  B In stably transfected cells
it is likely that the actual target genes of Myc will fit the constraints identified in the site selection.

4.6 Discussion

In this chapter I have investigated the preferred DNA binding sites of the Myc/Max and Max/Max complexes.

An extended binding site for each of the Myc/Max and the Max/Max complexes has been determined using full length *in vitro* translated proteins. Both complexes selected a 12-mer containing the hexanucleotide core CACGTG from a random oligonucleotide pool. The sequences selected by Myc/Max form a much more restricted set than those for Max/Max since the former discriminates at the -1 position relative to the hexanucleotide core while the latter does not.

The Myc/Max dimer fails to recognise the sequence TCACGTGA *in vitro* since this sequence fails to compete for the Myc/Max complex in band shifts, while competing efficiently for the Max/Max complex. Further, neither TCACGTGG nor TCACGTGA mediates expression from the minimal tk promoter in response to activation of MycER or co-transfection of Myc into REFs, while the sequence CCACGTGG does. It remains to be determined whether the other forms of Max have the same DNA binding specificity as the Max2 form used here however the discrimination by Myc/Max against T at position -1 is likely true with all forms of Max expressed in cells since it is apparent *in vivo*.

This discrimination against TCACGTGA by Myc/Max but not Max/Max is not simply a difference in the half-site preference of Myc as opposed to Max. Rather it probably reflects a difference in the way the two complexes contact DNA and may in turn be reflected in a difference in *in vivo* targets. If it were due to a half-site preference then one would expect a 12.5% frequency of T at the -1 position for sequences selected by
Myc/Max dimers: half of the time this position would be contacted by Myc (0% T) and the other half by Max (25% T) giving an overall frequency of 12.5%. It is clear from Fig. 4.5 that this is not the case. Sites with a single Myc-incompatible half-site are strongly discriminated against: only 1 is present out of 76 selected sequences. This idea is further supported by the result that a single half-site change (sequence Twt) is sufficient to abolish CAT expression in response to MycER and that there is no difference between Twt and the change in both half-sites (TwtA). Overall these results suggest that the proteins do not recognise half-sites independently of one another. Confirmation of this prediction must await resolution of the structure of DNA-bound Myc/Max dimers, for comparison with the solved structure of the DNA-bound Max/Max br-hlh-lz domain. Interestingly, this is consistent with a report that Max and Myc bend DNA in opposite directions while Myc/Max bends DNA in the same direction as Myc (although this result is now in doubt), as well as a second report showing that Max DNA binding is affected by phosphorylation while Myc/Max binding is not.

I also show that the preferred binding sites of Myc/Max and Max/Max complexes are very similar at the flanking positions -2 and -3 where purines are strongly favoured. Moreover it is clear from the sequences shown (Fig. 4.5) that certain residues are particularly disfavoured. These are T and C at the -2 position and C at position -3. According to these rules and the preferences at the -1 position, only 8% of CACGTG-containing sequences are likely to be high-affinity Myc/Max targets and 14% likely to be high-affinity Max/Max targets.

Interestingly, my binding consensus for Myc/Max dimers is in agreement with studies using in vitro translated truncated Myc protein, which most probably forms dimers with endogenous Max in the lysate. Additionally, bacterially expressed GST-Myc br-hlh-lz fusion
peptides\textsuperscript{139} and Myc expressed in Baculovirus or purified from Chinese Hamster Ovary cells\textsuperscript{357} all of which probably bind DNA as homodimers, also bind to similar sequences. Therefore the Myc homodimer seems to have binding preferences similar to those of Myc/Max. However, Myc homodimers are highly unlikely to exist in vivo\textsuperscript{147,312} and when formed are not functional in transcriptional activation\textsuperscript{283} and cellular transformation assays due to a decreased DNA binding efficiency.

There is a precedent for discrimination against a flanking 5'T or a 3'A being used to determine the specific targets for different transcription factors in yeast\textsuperscript{358}. Two unrelated br-hlh proteins (CPF1 and PH04) both recognise the sequence CACGTG, yet PH04 fails to substitute for a CPF1 null mutation in vivo since it cannot bind to the CPF1 target sites in which the CACGTG is flanked by a 5'T or a 3'A (or both). It has been suggested\textsuperscript{358} that inhibition of PH04 DNA binding by a flanking T is due to steric hindrance between a glutamic acid residue in the basic region and the methyl group of the T, while CPF1 has a smaller aspartic acid residue at this position. The validity of this hypothesis for Myc and Max remains to be demonstrated. However, in apparent agreement, Myc has an arginine residue with a very long carbon side-chain at the corresponding position, while Max has an alanine (amino acid position +3 according to Fisher's nomenclature\textsuperscript{359}). Both Mad and Mxi-1 have serine residues (only an -OH group larger than alanine) at this position and so we might expect that they would bind like Max rather than discriminating against T as Myc does. It will be interesting to test this prediction in the future.

In a similar fashion, the human E12 and E47 proteins discriminate against 5'T residues while their binding partner MyoD does not\textsuperscript{344}, and USF (which otherwise binds to the same CACGTG sequence as Myc) is not affected by a flanking 5' T\textsuperscript{334}, suggesting that this may be how the
targets for these two factors are distinguished; confirmation of this will await characterisation of their cellular binding sites. Thus discrimination between nucleotides flanking the core sequence-recognition motif may be a common regulatory mechanism in br-hlh containing transcription factors. Another pattern that is emerging from studies of transcription factors was first described by Berberich and Cole. In both the Myc/Max and Fos/Jun systems, only one protein is significantly induced (Fos, Myc) and this protein cannot form homodimers while the other constitutive protein (Jun, Max) can. In both cases DNA binding by the constitutive partner is regulated by phosphorylation of sites adjacent to the basic region. There is also a parallel between these phosphorylation sites and a domain in E12 that inhibits the DNA binding of homodimers but not heterodimers with MyoD.

Both Myc and Max are found in a variety of forms in cells and it remains to be seen whether the findings presented here will apply across the Myc family and to the various Max forms. Prochownik and VanAntwerp have looked at this area and found that in vitro translated L-Myc binds weakly to the CACGTG site with or without exogenous Max, and they suggest therefore that it may have a different preferred site or even another binding partner. They also find (in agreement with Berberich and Cole, 1992) that Max2 DNA binding activity is dramatically reduced by CKII phosphorylation or incubation with reticulocyte lysate (which contains CKII). We see reasonable DNA binding with in vitro translated proteins (and superior binding with Max2 compared with Max1) and can only presume that this would be further enhanced by de-phosphorylation.

Gene regulation by Max remains an open question. High levels of Max in transfections inhibit trans-activation (see also chapter 3) as well as cellular transformation by Myc. In mammalian cells, this
may be due to competitive displacement of Myc/Max from DNA sites by Max, either as a homodimer or as a heterodimer with one of its recently discovered alternative partners Mad and Mxi-1\textsuperscript{289,290} (see below). However, studies in yeast\textsuperscript{312} demonstrate that Max homodimers can indeed compete with Myc/Max \textit{in vivo}. Thus, as well as repressing the genes targeted by Myc/Max, the Max dimer, since it is less discriminatory, may repress genes that are not Myc targets but may be targets for other factors such as USF. It is also possible that genes repressed by Max/Max may be de-repressed on Myc expression when Max becomes complexed to Myc, although this is less likely given the high level of Max compared with Myc in cells. An additional level of control is revealed by the finding that Max/Max DNA binding activity but not that of Myc/Max is dramatically reduced by casein kinase II phosphorylation of Serine 11\textsuperscript{356}. This may be a mechanism by which Max/Max is displaced from the target sequences. Intriguingly, in PC12 cells, neither Max mRNA nor protein are detectable, while Myc is not only induced normally but can apparently also drive transcription\textsuperscript{364}. Whether there is therefore another Myc partner remains to be determined.

4.7 Addendum

Two further Max binding partners called Mad and Mxi-1\textsuperscript{289,290} were discovered after the work described in this and the preceding chapter was completed. Like Myc, Mad and Mxi-1 are br-hlh-lz proteins which fail to homodimerise but efficiently form heterodimers with Max and bind to CACGTG sequences. They do not associate with Myc or with other br-hlh, br-lz, or br-hlh-lz proteins. Both Myc/Max and Mad/Max dimers are favoured over Max homodimers; and, like Myc/Max but unlike Max/Max, Mad/Max binding to DNA is
unaffected by CKII phosphorylation. Expression of Mad or Mxi-1 represses Myc trans-activation\textsuperscript{289} and Ras co-transformation\textsuperscript{365}, and seems to be correlated with the differentiated state in which Myc levels are low\textsuperscript{290,291,366,367}. The levels of Max homodimers may therefore also be controlled by the levels of these proteins.

I decided to investigate whether Mad and Mxi-1 are involved in two processes in which Myc is down-regulated: growth arrest and differentiation.

I examined the expression of the Myc, Max, Mad and Mxi-1 proteins in murine erythroleukaemia cells (MEL cells) in which differentiation is correlated to Myc down-regulation\textsuperscript{105,165,174,177-179,368,369} and in MRC5 fibroblasts, a human fibroblast line which is not transformed and can be arrested easily by serum withdrawal in the absence of contact inhibition\textsuperscript{370}.

Fig. 4.10 shows that in differentiating MEL cells, Max expression is invariant while Myc is rapidly down-regulated (within 4 hours) before returning to the initial level from which it then decays as differentiation continues. Mxi-1 in contrast, is induced as the cells differentiate, being expressed at four times the level in differentiated cells compared with growing cells. Mad is undetectable in these cells. Fig. 4.11 shows that Mxi-1 expression also correlates with growth arrest in MRC5 fibroblasts, being four-fold induced in arrested cells compared with growing cells. Mad and Mxi-1 appear to be antagonists of Myc, adding another level of control to the Myc network, providing a sensitive switch controlling cell proliferation, differentiation and death, see Fig. 4.12. Their role seems to be to block Myc/Max complexes from target sites and also to repress Myc/Max target genes (or other genes to which they may bind)\textsuperscript{371}. This repression is apparently mediated through interaction with a homologue of the yeast Sin3 repressor. In view of this, the role of the Max
Figure 4.10  Expression of Myc, Max and Mxi-1 during MEL cell differentiation

Logarithmically growing cells (200,000 cells per ml) were triggered to differentiate with 5 mM hexa-methylene-bis-acetamide (HMBA) for the indicated times. The cells were harvested, lysed and mRNA was isolated. The RNA was separated on a denaturing gel and transferred to a Hybond N+ filter which was probed for Myc, Max, Mad, Mxi-1 and GAPDH. Shown in the figure are the levels of Myc, Max and Mxi-1 mRNA normalised to the level of GAPDH.
Figure 4.10 Expression of Myc, Max and Mxi-1 during MEL cell differentiation.
Figure 4.11  Mad and Mxi-1 expression in MRC5 cells

Cells were either grown at low density in 10% serum (growing), grown to 60% confluence and arrested for 48 hours in 0.5% serum (arrested) or arrested and then treated with 10% serum for 3 hours (arrested + serum). Cells were lysed and RNA prepared by the acid phenol method and analysed by northern blot. Shown is the level of Mad and Mxi-1 RNA normalised to the level of GAPDH.
Figure 4.11 Mad and Mxi-1 expression in MRC5 cells
Figure 4.12 Model for Myc regulatory network

- **Proliferation**
- **Apoptosis**
- **Trans-activation**
homodimer needs to be re-assessed. Possibly the Max homodimer blocks
target sites until the Myc heterodimer activates or the Mad heterodimer
represses. It may be interesting to repeat the site-selection protocol for
Mad and Mxi-1 to determine their preferred sites since, as with the initial
Max work, no preferred binding site has been determined.

The expression of Mad (or Mxi-1 depending on the cell type) in
association with differentiation is now well documented\textsuperscript{367,372}, although
there is little evidence so far for a specific effect of Mad in maintaining
the differentiated state. There is some evidence that Mad/Mxi-1 may
contribute to growth arrest however. We see an increase in Mxi-1
expression as human fibroblasts arrest and a corresponding decrease in
response to serum stimulation; Roy and Reisman\textsuperscript{373} see that Mad
expression accelerates growth arrest and Cerni et al.'s data suggest that
Mad inhibits growth in two ways: through repression of the Myc
pathway and also via a Myc-independent pathway mediated by the Mad
N-terminus. Two further proteins in the Mad/Mxi-1 family have also
been identified (Mad3 and Mad4\textsuperscript{374}) and it is likely that different
members of the family act in a tissue-specific manner.
Chapter Five

5 CYCLIN D1 INDUCTION

5.1 Introduction

Although Myc had been shown to act as a transcription factor\textsuperscript{312,326,337,346,375,376}, and it was therefore expected that it exerted most of its effects on cell proliferation by regulating the expression of target genes, no relevant cellular target had been found, although several candidate targets had been identified. For example, the \textit{\textalpha-prothymosin}\ gene was shown to be rapidly induced in response to Myc, but no CACGTG site had been found in the gene (although one was found subsequently, see chapter 7) and its function in relation to Myc's biological effects was unclear\textsuperscript{162} (see chapter 7 for further discussion). ECA-39 had been found to be similarly induced and does contain a CACGTG site but its biological role was also unclear\textsuperscript{377}. A number of other genes, including ornithine decarboxylase\textsuperscript{378} and Hsp-70\textsuperscript{378,379} are positively regulated in response to Myc but whether any of these were direct targets was unknown since there was no evidence of a \textit{fast} or \textit{protein synthesis-independent} response to Myc. In addition the expression of many genes is \textit{suppressed} by Myc. The best example of this is Myc itself (auto-regulation\textsuperscript{103}) which although rapid is not mediated by a CACGTG element\textsuperscript{380,381}, and others include NCAM (by N-Myc\textsuperscript{382}), collagens\textsuperscript{193}, histones\textsuperscript{195}, LFA-1\textsuperscript{192} and C/EBP alpha\textsuperscript{383}. Again it is possible that these are due to indirect effects since there is no evidence of rapid regulation nor for CACGTG sites mediating the effects.

My aim was to determine the genes through which the immediate effects of Myc, in particular proliferation, are mediated. None of the above genes were likely to have a direct role in promoting entry into the cell cycle. There are two means of identifying Myc targets: one can either devise a screen or test candidate genes individually. Since I wanted to
identify relevant target genes, I decided initially to test the most promising candidates.

5.2 Identification of Myc target genes

A Myc target gene might be expected to fall into the 'delayed early response' category i.e. those that are induced within a few hours of serum addition to quiescent cells and that require new protein synthesis for expression, suggesting that they are targets for the immediate early genes such as c-Myc. Examples included\textsuperscript{384}: HMGI-C, HMGI(Y), APRT, CHIP, MIF, T1, cyclin D1 as well as other cell-cycle genes.

Cyclin D1 was an excellent candidate, fulfilling all of the criteria I assigned: it is a delayed response gene\textsuperscript{218,384-387}, involved in oncogenesis (see Lammie and Peters, 1991\textsuperscript{388} for review), clearly involved in cell cycle control\textsuperscript{218,219,255,389,390} and, most interestingly, CSF-1 receptor activation induces both Myc and later cyclin D1 while a mutant (Phe 809) receptor fails to induce Myc or cell cycle progression but is rescued by ectopic Myc expression\textsuperscript{391,392}. For all these reasons, I decided to test whether cyclin D1 is regulated by Myc.

5.3 MycER activation induces cyclin D1

I was interested in identifying targets of Myc that are directly regulated. The MycER system (described previously in chapter 3) allows Myc activity to be rapidly increased in cells by the simple addition of 17\beta-oestradiol (OE) to the tissue culture medium. A direct target should respond rapidly to OE addition and so I decided to sample cells shortly after MycER activation.

A direct transcriptional target of Myc should also be regulated at the mRNA level without an intermediate step. I therefore included a cycloheximide control. This drug blocks new protein synthesis very
rapidly and so if any effect of Myc depends on the regulation of intermediaries, cycloheximide treatment should prevent it.

In order to obtain the best signal to noise ratio in the experiments, I decided to use cells in which endogenous Myc was at a minimum and therefore chose to use cells arrested in the absence of serum as my experimental system. This should also enable me to identify targets of Myc that are active in mediating entry into the cell cycle from the quiescent state. Experimentally, this also has the advantage that the cells are synchronous, and since MycER activation results in cell proliferation, as a simple control the cells can be routinely tested for S-phase entry by FACS analysis after an overnight application of hormone.

Since OE is applied as a solution in ethanol, ethanol alone was used as a negative control; as an additional control, I also used included normal Rat-1 cells and Rat-1 ΔMycER cells as well as the Rat-1 MycER cells (in each case both OE treated and ethanol treated).

I therefore grew MycER and control cells to confluence and arrested them by the complete removal of serum, leaving only insulin at 5 μg/ml as a survival factor (removal of serum in sparse cultures does not lead to a complete arrest). The arrest was verified after two days by FACS analysis prior to the experiment. Total RNA was prepared two and a half hours after treatment by CsCl centrifugation and analysed by northern blot shown in Fig. 5.1

It is clear that in the Rat-1 and Rat-1 ΔMyc ER cells, OE treatment has no effect on cyclin D1 RNA levels while in contrast, in MycER cells, cyclin D1 is clearly elevated in response to OE. Note that the base level of cyclin D1 is significantly lower in the Rat-1 MycER cells and as such any increase may actually be a de-repression rather than an activation – this point is discussed further in the discussion.
Figure 5.1  Induction of cyclin D1 but not cyclin A by MycER

Rat-1, Rat-1 ΔMycER or Rat-1 MycER cells were grown to confluence and arrested in 0% serum for 48 hours. These were then treated for 2.5 hours as indicated. OE: 2 μM 17β-oestradiol; EtOH: ethanol; Cyc: Cycloheximide 50 μg/ml. Cells were then harvested and 20 μg of total RNA loaded in each lane for northern blotting and hybridisation with the various probes as shown.
Figure 5.1 Induction of cyclin D1 but not cyclin A by MycER
As a further control, I probed the blot for cyclin A, which I did not expect to be a direct Myc target and up-regulated at such an early time-point, but rather a gene which is elevated later on in the cycle (an indirect target). Indeed, cyclin A levels are unaffected by OE treatment in all three cell types after a 2.5 hour treatment.

To determine the kinetics of induction, I repeated the experiment, including a time course following OE addition. I also included a second MycER clone (MycER-4) to confirm that cyclin D1 induction is not purely a clonal effect. Again the results show that cyclin D1 is induced following OE addition in both clones of the MycER cells only. The induction is rapid, occurring within one hour, and transient, declining after four and a half hours. Again the induction is refractory to cycloheximide Fig. 5.2A and B.

5.4 The mechanism and relevance of the cyclin D1 induction

To further characterise the induction of cyclin D1, I decided to investigate the mechanism of the induction using reporters containing the murine cyclin D1 promoter.

The murine cyclin D1 gene was cloned, and the intron-exon structure characterised, by Rosalind Smith who kindly provided reporter constructs. These consist of either a 4.5 kb or an 8.1 kb fragment of the D1 promoter (4.5 D1 and 8.1 D1 respectively) controlling the expression of the luciferase gene of pGL2-basic (Promega). The constructs contain the first (non-translated) exon of the cyclin D1 gene as well as a part of exon two. In both constructs, the translation start site of the cyclin D1 gene was mutated to allow translation of the luciferase gene only (Fig. 5.3).

I digested the cyclin D1 reporters with PmlII, an enzyme that recognises and cleaves the Myc binding site, CACGTG. This revealed the
Chapter Five

Cyclin D1 induction

Figure 5.2  MycER activation time course

A  Northern blot

Shown is a northern blot hybridised to Cyclin D1 and GAPDH. Cells were arrested for 48 hours in 0% serum + 5 μg/ml insulin and treated as indicated. From left to right the lanes are as follows. First 12 lanes: Rat-1 MycER cells (clone 3) induced for the indicated times in hours with either 2 μM 17β-oestradiol (number only) or 2 μM 17β-oestradiol and 50 μg/ml cycloheximide (number followed by C). Remaining 7 lanes: Indicated cell line treated with either ethanol (EtOH) or 2 μM 17β-oestradiol (OE) for 2.5 hours.

B  Graphical representation

Shown is the normalised Cyclin D1 expression in Rat-1 MycER cells derived from quantifying the hybridisations shown in Figure 4.2A above using a phosphor-imager and dividing the Cyclin D1 values by the GAPDH values.
Figure 5.2 MycER activation time course  

A Northern blot  

B Graphical representation
Figure 5.3  Cyclin D1 promoter reporter constructs

Constructs were constructed by cloning either 8.1 or 4.5 kb of the murine cyclin D1 promoter between the *KpnI* and *NheI* sites of pGL2-basic. The ATG site in exon I was destroyed in each case by digestion with *NcoI*. This modification was verified by sequencing.
Figure 5.3 Cyclin D1 promoter reporter constructs
presence of four such sites in the promoter (not shown), one of which was approximately 500 bp 5' from the transcriptional start, conserved in the human promoter (Ignacio Palmeiro, personal communication) and three more a further 3.5 kb upstream. This finding supported my interest in cyclin D1 as a Myc target.

My first experiment was to transiently transfect REFs with one of these reporters or pGL2-basic together with either BJ3-Myc or BJ3 and J4-CAT for normalisation of transfection efficiency. As can be seen from the results shown in Fig. 5.4, there is no detectable effect of Myc expression on the D1 promoter constructs (although these promoters are active, see Fig. 5.5A), while control transfections performed at the same time demonstrated trans-activation of a CACGTG containing reporter (M4MinCAT). This finding was initially surprising since I knew that a CACGTG site could be sufficient to mediate trans-activation by Myc, and it highlights the difference between actual cellular promoters and the artificial, small, simple constructs typically used for CAT assays. Nevertheless the results from the transient transfection experiments are consistent with the data from the northern blots in which levels of cyclin D1 RNA following activation of MycER increase rapidly then decline. By eleven hours post-stimulation, levels are no different to those prior to stimulation. As such it is not surprising that in a transient transfection where expression is assayed after 48 hours, the result is negative, particularly as luciferase activity is being assayed, and it has a very short half-life in vivo and is therefore representative of the expression level at the time of harvesting.

At this point there were several distinct explanations for the discrepancy between the northern blot and the transfections. Firstly the increase in cyclin D1 RNA may not be mediated by the cyclin D1 promoter or at least not by the region we have available to test. Since I
Figure 5.4  Effect of Myc co-transfection on cyclin D1 reporters

Subconfluent REFs in E4 + 10% serum were transfected with 3 µg of GL2-basic, 4.5 D1 or 8.1 D1 along with 1 µg J4-CAT for normalisation, or with 1 µg of MinCat or M4MinCAT along with 1 µg J4-luciferase for normalisation. In addition they were co-transfected with either BJ3-Myc or BJ3. Cells were harvested after 48 hours and assayed for CAT and luciferase activities. Shown is the fold activation of reporter expression with BJ3-Myc compared with BJ3.
Figure 5.4 Effect of Myc co-transfection on cyclin D1 reporters.
have over 8 kb of the cellular gene, it is unlikely that there are missing key regulatory sequences, but there is a possibility of a promoter-independent effect such as mRNA stabilisation or a Myc binding site in an intron for example. The second possibility is that the promoter constructs respond the same way as the endogenous promoter (as described above), and are up-regulated and then down-regulated again before they can be measured. To this end I tried to measure reporter activity rapidly (a few hours) following transfection but I was unable to obtain consistent results. The third possibility is that there is a difference in cyclin D1 regulation between rat and mouse (the murine promoter is being studied in rat cells) which I considered unlikely, or lastly that there is a difference between MycER and wild type Myc in their effects on cyclin D1. The effect of MycER is dependent on the integrity of the N-terminus of Myc however (since a deletion of amino acids 106-143 abrogates cyclin D1 induction) which makes this less likely.

The most likely explanation for the discrepancy between the transient transfections and the MycER time course was that in both cases, expression is regulated with similar kinetics. I therefore needed to examine the response of the promoter constructs shortly after Myc activation. Since transient transfection was not suitable for the measurement of trans-activation at early time points, I decided to use the MycER system once more. I transfected Rat-1 MycER cells as well as Swiss 3T3 MycER cells, with both the 4.5 D1 and 8.5 D1 constructs. I decided to include Swiss 3T3 cells because they are a mouse cell line and would control for any possible species difference between rats and mice, and also to confirm the results in another cell system. Transfections were performed with and without an additional 6 μg of REF genomic DNA to provide spacer DNA and prevent large concatamers of the promoter construct integrating together. In the event there was no discernible
difference between the results using the stable lines with spacer and those without.

The results (Fig. 5.5) show that exactly parallel to the induction of the endogenous gene, expression of the promoter constructs is elevated following OE treatment (but not EtOH treatment) in both the Rat-1 MycER and Swiss 3T3 MycER cells. Again the induction proved to be transient, disappearing after approximately 10 hours. The amplitude of the induction is much less than that seen for the endogenous cyclin D1 gene at the RNA level however, being only about two-fold.

At this point I was satisfied that studies on the endogenous gene paralleled those using promoter constructs to some extent. However, it still remained to be shown that cyclin D1 induction was mediated by the CACGTG elements and also that wild type Myc and not only MycER could have a similar effect.

Previous studies had shown that the hormone-binding domain of the oestrogen receptor domain has multiple functions, including hormone-binding\textsuperscript{397}, dimerisation\textsuperscript{398} transcriptional activation (via a subdomain called Taf-II)\textsuperscript{399} and a cis-acting "inactivation" function, which may result from the ability of this domain to bind the Hsp\textsubscript{90} molecule in a hormone-dependent manner\textsuperscript{400}. In the context of the MycER chimaera, the inactivation function blocks the association of MycER with Max unless ligand is bound\textsuperscript{315}. Moreover, not all functions of the hormone-binding domain are activated by all ligands that bind to it (for example see Fawell, 1990\textsuperscript{401}), in particular transcriptional activation by the hormone-binding domain is specifically activated by OE, but not by 4-hydroxy tamoxifen (OHT)\textsuperscript{402} although both block "cis-inactivation". As such MycER bound to OE will have two trans-activation regions: the Myc N-terminus and the oestrogen receptor Taf-II domain\textsuperscript{399}; it is therefore possible that any effect of MycER is mediated at
Figure 5.5  Cyclin D1 reporters are induced by MycER activation

A  Rat-1 MycER

Rat-1 MycER cells stably transfected with the indicated reporter were arrested for 48 hours in 0% serum + 5 μg/ml insulin at confluence and treated for 195 minutes with either ethanol (EtOH) or 2 μM 17β-oestradiol (OE). Shown is the luciferase activity from triplicate samples normalised to protein concentration.

B  Swiss 3T3 MycER

Swiss 3T3 MycER cells stably transfected with the indicated reporter were arrested for 48 hours in 0% serum + 5 μg/ml insulin at confluence and treated with 2 μM 17β-oestradiol for the indicated times. Shown is the fold increase in luciferase activity following activation.
Figure 5.5: Cyclin D1 reporters are induced by MycER activation.
least in part by Taf-II. Since OHT still releases MycER to associate with Max but does not lead to activation of the ER domain, it is a specific agonist of MycER\textsuperscript{314,315} and can be used instead of OE to stimulate MycER without activating Taf-II.

To demonstrate that MycER proteins were indeed functional after induction with 4OHT, FACS analysis was performed in serum-starved cells before and after stimulation with either OE or OHT. The results from these experiments (Fig. 5.6) show that the addition of either ligand leads to cell cycle induction by MycER proteins. I therefore harvested RNA from cells treated with OHT or OE and compared the induction of cyclin D1.

As shown in Fig. 5.7A, cells treated with OHT do not induce cyclin D1 mRNA levels although those treated with OE do. Furthermore, when MycER cells transfected with the cyclin D1 promoter constructs are exposed to OHT, there is no immediate effect on expression (Fig. 5.7B) although as expected, OE treatment transiently increases expression, consistent with the expression of endogenous cyclin D1. The activation of cyclin D1 is therefore likely to involve activation of both the ER and Myc trans-activation domains of the MycER fusion protein and cyclin D1 is most probably not a physiological target of Myc.

5.5 An inducible system for mammalian cells

As an additional safeguard, I wanted to devise a means to test the response of cyclin D1 and other genes to wild-type Myc rather than rely upon the activation of the MycER chimaera. To do this successfully clearly requires the rapid induction of Myc protein.

A number of systems had been used for the conditional expression of genes in mammalian cells but in the past had proved generally unsuitable for Myc (for successes see\textsuperscript{403-405}). The
Figure 5.6  FACS analysis of Rat-1 MycER cells in response to ethanol, 17β-oestradiol or 4-hydroxy tamoxifen

Confluent Rat-1 MycER cells were arrested for 48 hours in 0% serum + 5 μg/ml insulin and then treated as indicated for 17 hours prior to FACS analysis. Shown in the left panels is the proportion of cells plotted against DNA content. The right panel displays the proportion of cells in the S and G2 phases of the cell cycle following each treatment.
Figure 5.6 FACS analysis of Rat-1 MycER cells in response to ethanol, 17β-oestradiol or 4-hydroxy tamoxifen
Figure 5.7 Differential induction of MycER by 17β-oestradiol and 4-hydroxy tamoxifen

A Endogenous cyclin D1 expression

Rat-1 MycER cells were grown to confluence, arrested 48 hours and treated as shown prior to harvesting by the ‘acid phenol’ method for preparing total RNA. 20 µg of RNA were loaded in each lane of a denaturing gel and analysed by northern blot with cyclin D1 and GAPDH probes. Shown is cyclin D1 expression normalised to GAPDH derived from a quantification of the bands using a phosphor-imager.

B Cyclin D1 promoter activity

Rat-1 MycER 8.1-D1 cells were grown to confluence, arrested 48 hours and stimulated as shown. Shown is the mean luciferase activity normalised for protein concentration in triplicate extracts from cells harvested after the indicated times. The error bars represent the standard error of the means.
Figure 5.7 Differential induction of MycER by 17β-oestradiol and 4-hydroxy tamoxifen. 

A. Endogenous cyclin D1 expression

B. Cyclin D1 promoter activity
metallothionein promoter for example\textsuperscript{406-408} which has been widely used is rather 'leaky' i.e. even in the absence of the conditional signal, significant expression occurs. Since Myc exerts its effects at levels of only a few thousand molecules per cell, a useful system must be able to shut expression down efficiently. The tetracycline system\textsuperscript{409} is reported to allow control over up to five orders of magnitude with fairly rapid kinetics of activation. I therefore attempted to use this system for the inducible expression of Myc. I infected Rat-1 cells with a retrovirus driving expression of a tetracycline-controlled trans-activator (TTA) and pooled resistant cells. I then cloned the human c\textit{-myc} gene downstream of a promoter regulated by TTA and transfected this into the TTA-expressing cells. Forty clones were picked, expanded and tested for apoptosis in the absence of serum when tetracycline was removed, a simple and sensitive diagnostic test for Myc activity\textsuperscript{136}. Unfortunately no positive clones were identified.

At this point I decided to use an alternative system shown in Fig. 5.8 (the GalER system\textsuperscript{285}). The system consists, like the tetracycline system, of two parts. The first part is a retroviral construct with a neomycin-resistance gene, driving expression of a conditional trans-activator. The conditional trans-activator is in this case the Gal-4 protein's DNA binding domain fused to both the viral activator VP16 and to the hormone binding domain of the human oestrogen receptor (GalERVP or VPGalER depending on the order of the domains in the fusion protein; note only GalERVP is shown in Fig. 5.8). According to M. Busslinger (personal communication) GalERVP gives greater magnitude of induction but VPGalER gives tighter control of expression. The second part is a promoter (GC-promoter) which contains four Gal-4 DNA binding sites for the trans-activator to bind. The conditional trans-activator is analogous to MycER: in the presence of hormone, it can bind
Figure 5.8  Inducible system

Shown are the GC vector into which a cDNA may be introduced to provide inducible expression and pMV-7 GalERVP which expresses the GalERVP protein and also confers G418 resistance in eukaryotes.
Restriction sites:
* signifies a unique cloning site

B BamHI
C ClaI
H HindIII
P PstI
Pv PvuII
RI EcoRI
RV EcoRV
S SalI
Sm SmaI

Figure 5.8 Inducible system
to the Gal-4 sites in the GC promoter and drive transcription. To generate cells that express a particular gene when hormone is applied, cells are infected with the trans-activator and resistant cells pooled. These are then transfected with either a plasmid consisting of the gene of interest under the control of the GC promoter or with the GC promoter alone.

I infected Rat-1 cells with either MV-GalERVP or MV-VPGalER and pooled resistant colonies (over 2,000 in each case). I cloned the human c-myc gene into the GC-promoter construct to give GC-Myc and co-transfected either 10 µg of it or 10 µg of the empty GC-promoter into each of the two pools of cells along with 1 µg of a hygromycin-resistance gene (J6-Hygro). We pooled the resulting GC-empty cells to use as negative controls in later experiments and picked individual clones from the GC-Myc plates.

I expanded the clones, and tested each for apoptosis following OHT application. Of the 23 GalERVP-driven clones tested, four were positive, clones GV Myc-3, 5, 11 and 14. Of the clones from the VPGalER experiment none of the 15 tested were positive.

To verify that Myc is in fact being induced, I prepared RNA from each clone, grown to confluence, serum starved for 48 hours and treated with OHT or EtOH for eight hours. Clones 5 and 11 both induced Myc RNA in response to OHT (not shown).

For a system to be useful in identifying target genes, it needs to induce Myc rapidly, similar to the MycER system. To determine the kinetics of induction, I stimulated confluent, serum starved plates of clones 5 and 11 with OHT for increasing times, as well as cells growing in 10% serum which were treated with OHT or EtOH for two hours. The cells were harvested and total RNA prepared and then analysed by northern analysis. I probed the blot for Myc and as can be seen for the
more responsive clone 5 (Fig 5.9C), after only half an hour, Myc is strongly induced, peaking after four hours. This speed of response was considered to be sufficient to enable further experimentation. Moreover, the control of expression seemed tight as the cells survived the withdrawal of serum and Myc expression is practically undetectable in the absence of OHT although the exact number of Myc molecules produced was not measured. The induction seemed to work equally well in the presence or absence of serum.

To verify that the cells are induced to proliferate in response to Myc, Clone 5 cells were grown up, arrested for 48 hours, treated with OHT or serum and subjected to FACS analysis. The results (shown in Fig. 5.9A & B) show that the cells enter the cell cycle in response to OHT or serum although the percentage that do so is not as high as with MycER cells. These cells therefore induce Myc RNA and both die by apoptosis and enter the cell cycle in response to Myc expression and I considered them suitable for use in identifying Myc target genes.

To verify that cyclin D1 is not up-regulated by Myc, I probed the blot shown in Fig. 5.9 for cyclin D1 as well as GAPDH for normalisation. The results are shown in Fig. 5.10. Although Myc is induced strongly (nearly 15-fold), there is no effect on cyclin D1 expression as expected, confirming that wild type Myc does not induce this cyclin.

5.6 Discussion

Cyclin D1 is not a transcriptional target of Myc but Myc may well interact with the cyclin D1 promoter. Our results show that the MycER protein activated by OE is capable of trans-activating cyclin D1. This induction has been reported by Daksis et al. with very similar results. In addition, these authors show that the effect is transcriptional and that
Figure 5.9  GV-Myc5 cells induce Myc and enter the cell cycle

A  FACS analysis

FACS analysis of cells grown to confluence, serum starved for 48 hours and treated for 24 hours with either 10% serum, OHT or left untreated. Shown is the proportion of cells plotted against DNA content.

B  Graphical representation

Shown is the proportion of cells in the S and G2 phases of the cell cycle following each treatment from part A above.

C  Myc induction

Cells were either (left two lanes) growing exponentially in 10% serum and treated with EtOH or OHT for 2 hours or (right six lanes) grown to confluence, serum starved 48 hours and treated with OHT for the indicated time. They were then harvested and RNA prepared by the 'acid phenol' method and analysed by northern blot. Shown is the hybridisation to the human c-Myc fragment from BJ3-Myc.
Figure 5.9 GV-Myc5 cells induce Myc and enter the cell cycle
Figure 5.10  Cyclin D1 is not induced in Rat-1 GV-Myc 5 cells

Shown is a quantification using a phosphor-imager of the northern blot in Fig. 5.9, probed for Cyclin D1, Myc and GAPDH. Cells were either (left two pairs of columns) growing exponentially in 10% serum and treated with EtOH or OHT for 2 hours or (right six pairs of columns) grown to confluence, serum starved 48 hours and treated with OHT for the indicated time. Shown are the relative expression levels of cyclin D1 and Myc normalised to the level of GAPDH, compared with the uninduced state.
Figure 5.10 Cyclin D1 is not induced in Rat-1 GV-Myc 5 cells.
protein levels also increase. MycER when activated by OHT it is not capable of inducing cyclin D1 expression. Furthermore, the Myc transactivation domain (amino acids 106-143) is required for OE-dependent trans-activation. The effect is specific to cyclin D1 and we know that MycER can trans-activate from CACGTG sites\textsuperscript{312,326,337,346,375,376} (and also chapter 3) and that the cyclin D1 promoter contains such sites, which suggests that the Myc DNA binding domain may also be required although I have not tested this. The effect is also dependent on the Myc N-terminus. A likely explanation therefore is that the Myc portion of MycER recognises the cyclin D1 promoter, while the Myc transactivation domain acts in concert with the activated Taf-II domain of the oestrogen receptor to stimulate transcription.

One of the reasons I initially considered cyclin D1 as a Myc target was data on signal transduction by the CSF-1 receptor (CSF-1R). In NIH 3T3 cells, CSF-1R activation leads to tyrosine kinase activity, binding to PI-3 kinase and induction of Fos, JunB and Myc. Cells expressing a CSF-1R mutant (tyrosine 809 to phenylalanine)\textsuperscript{411} fail to induce Myc but are normal in the other responses to CSF-1 listed above. These cells fail to grow in semi-solid medium, cannot grow in the absence of serum and do not induce cyclin D1. Enforced Myc expression restored the ability to grow\textsuperscript{391} and studies in macrophages\textsuperscript{392} indicate that cyclin D1 induction is downstream of CSF-1R activation and of Myc. However, it has subsequently become clear that the CACGTG sites in the cyclin D1 promoter which are conserved in mouse and man\textsuperscript{410,412-414} are not in the serum-responsive part of the promoter and this region in fact responds to Jun rather than Myc\textsuperscript{413,414}.

An interesting observation is that the basal level of cyclin D1 expression is greatly reduced in MycER expressing cells. This phenomenon has been further studied by Eilers's group\textsuperscript{315} who showed
that Myc expression (both wild type and MycER) represses cyclin D1 transcription. Interestingly, the effect does not require Myc Box II (amino acids 128-143) or the leucine zipper of Myc, and is independent of OHT addition in MycER cells, suggesting that dimerisation with Max and DNA binding are not in fact required. It seems instead to operate by Myc competing for TFII-I with core promoter bound USF. Thus, some effects of Myc on transcription may not operate via classical DNA-binding and trans-activation, but rather via the formation of protein complexes. The evidence for this is that Myc can heterodimerise with both TFII-I\(^4\) and YY-I\(^4\) which are capable of binding to and activating transcription from the Adenovirus major late promoter\(^4\) as well as TATA-less promoters\(^4\) and the Adeno-associated virus type 2 P5 promoter\(^4\) respectively, through the initiator (Inr) sequences in each case. Myc prevents activation by USF (probably as a dimer with TFII-I) of the Adenovirus major late promoter Inr and it has been shown in an \textit{in vitro} system that Myc represses this promoter by interfering with the TFII-I/TFII-D complex. Since Myc interacts with TFII-I, the simplest explanation is a competition for this factor.

One model for the activation of cyclin D1 by MycER is that the MycER protein is tethered to the cyclin D1 promoter by interaction with another protein (such as YY-I). Following OE stimulation, the Taf-II domain trans-activates transiently before Myc is complexed to Max, is removed from the cyclin D1 promoter and binds to its own targets. Whether either the repression of cyclin D1 by Myc or its presence on the cyclin D1 promoter is relevant to Myc function is still unclear.

That Myc, which is known to promote cell cycle progression, should repress a cyclin seems contradictory. However, cyclin D1 expression does not correlate exactly with proliferation. Although it is induced by growth factors\(^3\)\(^8\)\(^4\)\(^6\), it is also induced at senescence\(^4\)\(^2\)
(when Myc is down regulated) and is induced in response to the tumour repressor, Rb\cite{421,422}. What is more, cyclin D1 overexpression can be antiproliferative, for example it is very difficult to obtain cyclin D1 overexpressing clones\cite{423}. Cyclin D1 expression is slightly different in various cell types but in rat fibroblasts, peaks following serum stimulation and then decreases as cells cross the G1/S boundary. This down-regulation and/or exclusion of the protein from the nucleus is probably a requirement for progress through the cell cycle\cite{218,219,237,424} and is a possible point of Myc action. It is also possible that in Myc overexpressing cells, selection acts in favour of those with reduced levels of cyclin D1 as constitutive expression of Myc results in decreased levels of cyclin D1. In NIH 3T3 cells in contrast, Myc induces cyclin D1 translation via upregulation of eIF-4E and repression is not observed\cite{425}. Marhin et al.\cite{426} find that Myc alone is not sufficient to repress cyclin D1 but that in conjunction with the loss of Rb, cyclin D1 expression and kinase activity are repressed. Despite these findings, cyclin D1 does promote proliferation in many circumstances. D-type cyclins can rescue cells arrested by Rb overexpression\cite{220,421,427} and can shorten G1\cite{219}. Cyclin D1 may also be required for G1 to S progression since microinjection of cyclin D1 antibodies or antisense arrests cells in G1\cite{218}. Cyclin D1 is also linked to cancer; for example it is found to be overexpressed in parathyroid adenomas\cite{428}, B-cell lymphomas\cite{429}, squamous cell carcinomas\cite{430}, breast and oesophageal cancers (see Hall and Peters, 1996\cite{431} or Motokura and Arnold, 1993\cite{412} for review). Although I failed to detect cooperation between activated Ras and cyclin D1 in a REF transformation assay (not shown), such a result has been reported by Lovec et al.\cite{423}; the resulting cells being immortalised, anchorage independent and able to form fibrosarcomas in nude mice.
Chapter Five

Cyclin D1 induction

A better understanding of the relationship between cyclin D1 and Myc will have to await delineation of the precise role for cyclin D1 in the cell cycle; it is clear now that it acts as a complex with cdk-4 or cdk-6 and is able to phosphorylate Rb (reviewed in Peters, 1994 and Sherr, 1994) in the G1 phase of the cycle. The outstanding question is whether cyclin D1 is required for Myc-dependent proliferation. The same techniques that demonstrated that cyclin D1 is required for G1/S progression (antisense and antibody injection) in normal fibroblasts could be applied to MycER cells to address this.
Chapter Six

6 CYCLIN E INDUCTION

6.1 Introduction

Although cyclin D1 was shown not to be a direct Myc target, I felt that the principle of screening genes by northern blot shortly after Myc activation was still valid because the most likely mechanism for Myc action remained the transcriptional regulation of target genes.

Myc rapidly induces the proliferation of quiescent cells, suggesting that one point of Myc action is in early G1. Myc also maintains the proliferation of growing cells; in particular, withdrawal of Myc after progression past the restriction point causes arrest in the next G1 phase. Although Myc also has effects at other stages of the cycle (for example apoptosis in response to the withdrawal of survival factors can occur at any point in the cell cycle), it seemed likely that one important effect of Myc was to act directly on the cell cycle machinery prior to the restriction point in G1. This is further supported by work with a heterozygous c-myc cell line\textsuperscript{434} showing that a reduction in the amount of Myc slowed the transition from G0 to G1 and on into S-phase.

The cell cycle machinery is complicated with several levels of control and extreme cell type specificity yet with a core of cdk's and cyclins common to most cells. Of this core, an obvious candidate for regulation by Myc was cyclin E. This cyclin is involved early in G1 and is essential for G1/S progression\textsuperscript{229,236,237,292,435}, and like cyclin A, is found at elevated levels in Myc-expressing cells\textsuperscript{436}.

6.2 Cyclin E RNA is induced by MycER activation

To determine whether cyclin E is rapidly regulated at the RNA level in response to Myc, I decided to probe a northern blot of RNA from activated MycER cells for cyclin E expression.
Chapter Six  Cyclin E induction

Rat-1 MycER cells were grown to confluence, arrested in 0% serum, 5 µg/ml insulin and treated with either OE or OHT prior to harvesting for northern analysis of cyclin E expression (Fig. 6.1A). In both cases expression was elevated although the induction was greater with OE (approximately double). Neither Rat-1 cells nor Rat-1 ΔMycER cells, included as controls, showed the induction, and the basal level of expression was similar in all three cell lines. I therefore considered the induction of cyclin E RNA to be a bona fide response to Myc unlike that of cyclin D1.

Cyclin E is expressed at low levels and I therefore conducted further experiments using polyA* RNA instead of total RNA (which gives better results, see Fig. 6.1B).

To be sure that the induction of cyclin E is not an artefact caused by the presence of the oestrogen receptor domain in MycER, I wanted to test the response of cyclin E to wild-type Myc. I therefore tested the GV Myc-5 line and the control cell population (GV con: transfected with the GC-promoter without the Myc gene inserted) for cyclin E expression. Fig. 6.2 shows that cyclin E is induced following OHT treatment, in both growing and arrested GV Myc-5 cells but not in GV con cells. Thus, cyclin E expression is induced by wild-type Myc. It was not however proved that the induction was either direct or relevant to Myc-induced proliferation.

6.3 The mechanism of cyclin E induction

To test whether Myc has a direct effect on cyclin E expression, I investigated whether inhibition of protein synthesis would block the induction. I used a panel of three protein synthesis inhibitors (anisomycin, emetine and puromycin) and prepared mRNA from arrested MycER cells treated with either ethanol or OHT, or with ethanol
Figure 6.1 Cyclin E RNA induction by MycER

A Oestradiol and OHT induction of cyclin E

Rat-1 MycER cells were grown to confluence, arrested for 48 hours and treated as shown prior to harvesting by the ‘acid phenol’ method for preparing total RNA. 20 μg of RNA were loaded in each lane of a denaturing gel and analysed by northern blot with cyclin E and GAPDH probes. Shown is cyclin E expression normalised to GAPDH derived from a quantification of the bands using a phosphor-imager.

B OHT induction of cyclin E

Rat-1 MycER cells were grown to confluence, arrested for 48 hours and treated with OHT for the indicated times. Cells were lysed, mRNA extracted and 10 μg loaded on a denaturing gel for northern analysis. The filter was probed for cyclin E and GAPDH.
Figure 6.1 Cyclin E RNA induction by MycER
A Oestradiol and OHT induction of cyclin E
B OHT induction of cyclin E
Figure 6.2  Induction of cyclin E by wild-type Myc

A  Northern blot

GV-Myc-5 and GV-Con cells were grown either to confluence and arrested for 48 hours in 0% serum + 5 μg/ml insulin (arrested) or grown at low density in 10% serum. Cells were treated with either OHT or ethanol (Et) for 3 hours. Cells were harvested and poly A⁺ RNA isolated, run on a denaturing gel and transferred to a membrane. 3.6 μg were loaded into lanes 1 and 2; 10 μg into lanes 3 and 4; 8.3 μg into lanes 5 and 6; 10 μg into lanes 7 and 8. The filter was hybridised sequentially to human cyclin E and rat GAPDH probes and exposed to film.

B  Quantification

The bands were quantified using a phosphor-imager and the fold induction with OHT compared with ethanol is shown.
Figure 6.2 Induction of cyclin E by wild-type Myc
A Northern blot B Quantification
or OHT as well as one of the inhibitors. I analysed the RNA by northern blot and hybridisation to cyclin E and GAPDH probes. In each case, the inhibition of protein synthesis failed to block the induction of cyclin E by OHT (Fig. 6.3). This suggests a direct effect of Myc on cyclin E RNA induction.

The increased level of cyclin E RNA in response to Myc could be due to increased transcription or to stabilisation of pre-existing cyclin E mRNA. Although increased transcription seemed likely since Myc is a transcription factor, an increased cyclin E mRNA half-life was possible as it had been demonstrated that cyclin E is regulated at the level of mRNA stability in response to Fos\(^{438}\).

To investigate the rate of RNA degradation, actinomycin D\(^{439,440}\) can be used to block transcription of new RNA. Molecules of RNA already existing at the time of actinomycin D treatment are unaffected and degrade as normal. Detection of RNA at increasing times after treatment will therefore reveal a decrease, the rate of which can be compared between cells growing in different conditions. I wanted to compare the rate of degradation in arrested Rat-1 MycER cells in the presence or absence of OHT.

I therefore grew Rat-1 MycER cells to confluence, arrested them for 2 days in the absence of serum and treated them with either OHT or ethanol (as a negative control) for 2 hours prior to actinomycin D treatment. Cells were then harvested at increasing times thereafter, poly A\(^+\) RNA prepared, and analysed for cyclin E and GAPDH by northern blot. Unlike a normal northern blot, the level of GAPDH probe that hybridises is not proportional to the amount of mRNA loaded. It can be seen from Fig. 6.4A that the level of GAPDH apparently increases over time – this is because GAPDH has a longer than average half-life: it is in fact the proportion of GAPDH that is increasing (approximately 10 \(\mu\)g of
Figure 6.3  Cyclin E induction does not require protein synthesis

Rat-1 MycER cells were grown to confluence, arrested for 48 hours and treated with protein synthesis inhibitors and either EtOH (-) or OHT (+) for 3 hours, harvested and poly A+ RNA isolated. 10 µg of mRNA were analysed by northern blot. The filter was hybridised to cyclin E and GAPDH probes and exposed to film. Control: no drug; Aniso.: anisomycin 50 µg/ml; Emetine: emetine 50 µg/ml; Puro.: puromycin 50 µg/ml.
Figure 6.3 Cyclin E induction does not require protein synthesis
Figure 6.4  Cyclin E induction is due to increased transcription rather than RNA stabilisation

A  Actinomycin block

Rat-1 MycER cells were grown to confluence, arrested for 48 hours and treated with either OHT or EtOH for 2.5 hours. Cells were then treated with actinomycin-D and RNA prepared at the indicated times and analysed by northern blot with the indicated probes.

B  Nuclear run-on

Rat-1 MycER cells were grown to confluence, arrested for 48 hours and treated with either OHT or EtOH for 2.5 hours. Cells were then lysed to release entact nuclei for run-on analysis against the indicated probes.
Figure 6.4 Cyclin E induction is due to increased transcription rather than RNA stabilisation
A Actinomycin block B Nuclear run-on
mRNA were loaded in each lane). As such the rate of decrease of cyclin E normalised to GAPDH is a measure of the relative degradation rate of cyclin E compared to GAPDH.

Figure 6.4A shows that the rate of degradation of cyclin E is essentially identical in OHT treated and control (ethanol treated) cells although the level of cyclin E is elevated in the OHT treated cells as expected. Thus MycER is not regulating the rate of cyclin E RNA degradation.

To test whether MycER can induce cyclin E transcription, the activity of the endogenous cyclin E gene was measured in the presence or absence of OHT by nuclear run-on assay. I isolated nuclei from Rat-1 MycER cells treated for 2 hours with either OHT or ethanol, and RNA synthesis of already initiated transcripts was continued in vitro in the presence of radioactive nucleotides. RNA was purified and hybridised to cyclin E, cyclin A or GAPDH probes. Fig. 6.4B shows that the rate of transcription is elevated in OHT treated cells, while as a control, neither GAPDH nor cyclin A transcription is affected.

6.4 MycER activation as a model for mitogenesis

To determine whether the Myc-induced increase in cyclin E RNA levels leads to an increase in the levels of cyclin E protein, I prepared protein samples following stimulation with OHT for analysis by western blot. In addition, to investigate the relevance of cyclin E induction to Myc-dependent cell cycle entry, I extended the experiment to include time points at which the cells would be passing through S-phase and beyond. To compare the changes in protein level with position in the cell cycle, I also prepared FACS samples at a series of times after OHT treatment. Finally, to compare the changes during MycER-driven entry
Chapter Six

Cyclin E induction

into the cell cycle with the behaviour of the cells in response to serum, I also performed these experiments in parallel with serum-treated cells.

It can be seen from Fig. 6.5 that with both OHT and serum, the majority of cells (over 60%) progress into S-phase and beyond. In both cases the response is rapid and the majority of cells enter S-phase after about 8-12 hours. The cells treated with serum subsequently arrest in G1 after completing one cycle. The MycER cells continue to cycle although further comparison between the two populations is impossible as apoptosis in the MycER cells becomes significant and the monolayer is disrupted.

Shown in Fig. 6.6 is a western blot performed using a cyclin E antibody. Similar to the result for mRNA levels, protein levels increase markedly (about 3-fold) following stimulation with OHT. A similar increase of cyclin E protein level is apparent following serum stimulation although levels fall after the cells complete one cycle. In contrast, after MycER activation with OHT, the elevated cyclin E level is maintained. This is consistent with our observations of the cells' behaviour: the OHT treated cells continue cycle while the serum treated cells do not. I also probed the western blot with an antibody to cdk-2. Unlike cyclin E, levels are essentially unchanged following treatment with either OHT or serum but do increase slightly as cells progress through S-phase (after about 12-24 hours).

6.5 Implications of cyclin E induction

Cyclin E expression and protein levels are upregulated by Myc but do they play a role in mediating Myc's effects, in particular mitogenesis? To address this I used the inducible GalER expression system again to generate Rat-1 cells in which exogenous human cyclin E expression is dependent on the presence of OHT in the medium. The
Figure 6.5  FACS analysis of MycER cells

A  FACS profiles

FACS analysis of Rat-1 MycER cells grown to confluence, serum-starved for 48 hours and treated for the indicated time with either serum or OHT. Shown is the proportion of cells plotted against DNA content.

B  Graphical representation

Shown is the proportion of cells in the S+G2 phases of the cell cycle at increasing times following stimulation, calculated from the profiles in part A above.
Figure 6.5 FACS analysis of MycER cells A FACS profiles B Graphical representation
Rat-1 MycER cells were grown to confluence, arrested for 48 hours and treated with either OHT or serum for the indicated times. Cells were harvested and 15 μg of protein loaded onto a gel for western analysis with anti cyclin E and cdk-2 antibodies.
Figure 6.6 Cyclin E and cdk-2 protein levels after induction by serum or OHT
cyclin E gene from UHG-cyclin E was excised and cloned into the GC-vector (creating GC-cyclin E). Rat-1 cells expressing GalER-VP16 were co-transfected with GC-cyclin E and J6-Hygro, hygromycin-resistant colonies picked and these were then tested by western blot for induction of human cyclin E using HE12, a human-specific cyclin E antibody. From an initial ten colonies screened, in two (E6 and E8), human cyclin E was expressed upon the addition of OHT (Fig. 6.7).

I arrested the E6 cells and tested them by FACS analysis for the ability to enter the cell cycle and found that they could not, while as a positive control, MycER cells did (not shown). Interestingly however, in the absence of insulin, the induction of cyclin E expression induced apoptosis in the E6 cells in a similar manner to that observed with OHT-treated MycER cells. Fig. 6.8 shows the results of an experiment in which E6 cells were transferred to medium without serum and filmed. Apoptosis was assayed (by Chris Gilbert) by reviewing the film frame by frame and scoring apoptotic deaths. After 72 hours all the cells treated with OHT had died while the controls were almost confluent. Myc therefore induces cyclin E expression and cyclin E induces apoptosis. The most likely hypothesis is therefore that Myc-induced apoptosis is mediated by the transcriptional activation of the cyclin E gene. Myc-induced mitogenesis is not mediated solely by cyclin E trans-activation however.

6.6 Cyclin E activity

Since cyclin E expression does not induce cell cycle entry, it was possible that cyclin E is not involved in Myc-induced proliferation, but this seemed unlikely in view of cyclin E's role in the G1/S transition\textsuperscript{229,236,237,292,435}. The second possibility was that cyclin E protein is required but is not sufficient. Cyclin E acts by binding to cdk-2 and
Individual hygromycin and G418-resistant colonies were ring cloned and expanded. Cells were grown to confluence, arrested and treated with either ethanol (-) or OHT (+) for 3 hours. Lysates were assayed for the presence of human cyclin E by western blot using a human cyclin E-specific antibody (Pharmingen HE12).
<table>
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<tr>
<th>Clone number</th>
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<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
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<tr>
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<td></td>
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</tr>
</tbody>
</table>

**Figure 6.7** Identification of clones expressing inducible human cyclin E
Figure 6.8  Cyclin E induces apoptosis

E6 cells were seeded onto glass cover slips in E4 + 10% serum at low density. After 24 hours of growth, the cover slips were removed into medium containing 0% serum or 0% serum + OHT for filming.

A  Photo-micrographs
Photographs were taken at the indicated times

B  Graph of apoptotic deaths
Shown is a graph of cumulative apoptotic cell deaths (assessed by reviewing the film frame by frame with the aid of Chris Gilbert).
Figure 6.8 Cyclin E induces apoptosis 
A Photo-micrographs
B Graph of apoptotic cell deaths
forming a protein kinase complex. I decided therefore to assay cyclin E-dependent kinase activity in response to MycER activation and also to exogenous cyclin E expression.

To analyse cyclin E-dependent kinase activity requires a reliable assay and a kinase target. Although the actual in vivo targets for cyclin E-dependent kinase includes Rb, histone H1 is frequently used for in vitro experiments. This substrate is found to be phosphorylated by a range of cdk/cyclin complexes in a similar manner to that seen in vivo.\(^{441-444}\). I therefore set up a kinase assay, and optimised the conditions (Fig. 6.9). As a positive control, I used 2 µl of whole cell extract. This resulted in a strong band co-migrating with histone H1.

Kinase activity from protein samples immuno-precipitated with anti-cyclin E is detectable and is abolished by incubating the antibody beforehand with its cognate immunogenic peptide (lanes 5 and 6), demonstrating specificity. The assay is sensitive to both the amount of substrate and the amount of labelled ATP (lanes 3, 4 and 5) but is not sensitive to the ATP concentration (lanes 1, 2 and 5).

To compare the induction of cyclin E protein levels with kinase activities, I performed kinase assays using the same cell lysates previously used for the western shown in Fig. 6.6. The result is shown in Fig. 6.10; kinase activity increases rapidly (7-8 fold) following both OHT or serum treatment. As seen with the western and consistent with the behaviour of the cells, the level of kinase activity is maintained by OHT but falls away in the serum treated cells after the cells enter S-phase (from 12 hours post-treatment). Interestingly, the kinase activity is not directly proportional to the protein level: with OHT, kinase activity continues to increase after 6 hours by which time the protein level is already at its maximum. With serum, kinase activity declines well after the protein level has already fallen, see especially the 12 hour time point.
Figure 6.9 Validation of immuno-precipitation / H1 kinase assay

Rat-1 MycER cells were grown to confluence in four 9 cm dishes, harvested in 4 ml of RIPA buffer and divided into 250 µl aliquots. Aliquots 1-6 were immuno-precipitated with cyclin E antibody before proceeding with the kinase assay using the parameters shown in the table. Samples 7 and 9 are negative controls using lysis buffer only and sample 8 is a positive control with 2 µl of cell extract (3 µg protein).
<table>
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<td>1</td>
<td>cyclin E</td>
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<td>lysis buffer only</td>
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**Figure 6.9** Validation of immuno-precipitation / H1 kinase assay
Figure 6.10  Cyclin E kinase activity in Rat-1 MycER cells

A  Induction of cyclin E kinase activity by OHT and serum

The same lysates used in figure 6.7 were immuno-precipitated with cyclin E antibody and a kinase assay performed with 5 μg of histone H1 and 5 μCi of labelled ATP. The resulting gel was exposed to film and also quantified using a phosphor-imager.

B  Comparison of protein level with kinase activity

Shown is a graphical representation of protein levels and kinase activity from the same samples. The kinase assay was quantified using a phosphor-imager and the western from Figure 6.6 was quantified by densitometry.
Figure 6.10 Cyclin E kinase activity in Rat-1 MycER cells
A Induction of cyclin E kinase activity by OHT and serum
B Comparison of protein level with kinase activity
Chapter Six

Cyclin E induction

at which kinase activity is at a maximum while protein levels are at a minimum. It is also evident (despite the inaccuracies of densitometry used to quantify the protein levels) that the kinase activity increases to a greater extent than does the protein level. Taken together it appears that kinase activity is greatly increased by both OHT and serum treatments but that kinase activity is only partially determined by the protein level. Given these results, it seemed likely that there is a secondary effect of Myc and of serum on cyclin E apart from increasing the protein level, that leads to activation of kinase activity.

Since I did not see cell cycle progression in response to human cyclin E expression in the arrested E6 cells, I attempted to determine whether the cyclin E expressed in these cells was kinase-active and, importantly, whether it could be activated. I performed kinase assays in E6 cells, both growing and arrested, in response to either OHT or serum or both (Fig. 6.11A & B). It is clear that the presence of cyclin E alone is not sufficient for kinase activity, but serum does activate the protein, proving that human cyclin E is capable of being activated in rat cells. This separates the induction of cyclin E kinase activity into two distinct steps: the induction of cyclin E protein levels and the activation of the cyclin E complex. Both take place in response to MycER activation.

There is therefore a difference in the role of cyclin E in mediating two Myc-dependent pathways: apoptosis and mitogenesis. My hypothesis was that cyclin E is a transcriptional target of Myc and that the concomitant increase in protein level is sufficient for apoptosis but not for cell cycle entry. Another Myc-dependent step activates cyclin E-associated kinase activity which may then be sufficient for cell cycle entry.
Figure 6.11 Cyclin E protein is not sufficient for kinase activity

A Kinase assay in E6 cells

Cells were either grown to confluence, arrested for 48 hours in 0% serum + 5 μg/ml insulin or grown in 10% serum at low density, and treated as indicated. Lysates were then precipitated with a human specific cyclin E antibody (Pharmingen HE67) and assayed for histone H1 kinase activity.

B Kinase assay and western in E6 cells

Cells were arrested for 48 hours in 0% serum + 5 μg/ml insulin and treated as indicated. Lysates were then precipitated with HE67 and assayed for histone H1 kinase activity (lower panel) or analysed by western blot using HE12 (upper panel). The experiment was performed by Ignacio Perez-Roger.
Figure 6.11 Cyclin E protein is not sufficient for kinase activity.
A Kinase assay in E6 cells B Kinase assay and western in E6 cells
6.7 Mechanism of cyclin E activation

I have defined two early effects of Myc: the trans-activation of cyclin E transcription and the activation of cyclin E-associated complexes. This activation may also operate through the trans-activation of a Myc target gene or through another pathway such as the interaction of Myc with other cellular proteins (such as p107). To identify the molecular mechanisms involved, I decided to determine how kinase activity is increased.

The kinase activity of cyclin/cdk complexes is regulated at four levels (for review, see Morgan, 1995\textsuperscript{275}): the expression level and hence binding of the cyclin partner, CAK phosphorylation of a conserved threonine in the cdk (required for kinase activity), de-phosphorylation of a conserved threonine-tyrosine pair (required for kinase activity) and the binding of a cyclin dependent kinase inhibitor (cdi) which inhibits kinase activity.

The activation of cyclin E-dependent kinase activity by Myc is most likely due therefore to a change in the proteins associated with cyclin E. Cyclin E preferentially binds to cdk-2 as its kinase partner and I found that the level of cdk-2 is essentially invariant following MycER activation and that the proportion of cdk-2 in the lower band (the CAK-phosphorylated form)\textsuperscript{445} is also constant (shown above in Fig. 6.6). The cdi’s bind to cyclins and inhibit their associated kinase activity. I therefore analysed the levels of the known cyclin E-associated inhibitors, the cdi’s p27\textsuperscript{Kip1} and p21\textsuperscript{Waf1} (p27 and p27 respectively hereafter), in the cells after stimulation (see Sherr and Roberts, 1995\textsuperscript{446} for review).

p21 is undetectable in Rat-1 MycER cells although it is readily detected in other rat cell lines (not shown). p27 is density-dependent in Rat-1 cells, expressed at very high levels at confluence compared with low density cells (Fig. 6.12A). The p27 level does respond to either OHT
Chapter Six

Cyclin E induction

Figure 6.12  p27 levels

A  p27 levels depend on cell density

Rat-1 MycER cells were grown in the conditions indicated prior to harvesting and western analysis with a p27 antibody. 15 μg of protein were used. Sparse High Serum: cells grown in E4 + 10% serum at about 30% confluence; Dense High Serum: cells grown in E4 + 10% serum to full confluence; Dense Arrested: cells grown in E4 + 10% serum to full confluence after which serum was replaced with 5 μg/ml insulin for 48 hours.

B Western blot following serum or OHT treatment

Rat-1 MycER cells were grown to confluence and arrested for 48 hours before being treated as shown and harvested. t=0: initial time point; S + ‘x’: serum treatment for ‘x’ hours; O + ‘x’: OHT treatment for ‘x’ hours. Western blot for p27 using 5 μg total protein. This experiment was performed by Ignacio Perez-Roger.

C Cyclin E associated protein following serum or OHT treatment

The lysates from part B above were used. 200 μg of protein were immuno-precipitated with cyclin E antibody prior to western analysis except P: immuno-precipitated with cyclin E antibody pre-blocked with peptide and W: 15 μg total protein loaded. This experiment was performed by Ignacio Perez-Roger.
Figure 6.12 p27 levels A p27 levels depend on cell density B Western blot following serum or OHT treatment C Cyclin E-associated protein following serum or OHT treatment
or serum however (Fig. 6.12B), being down-regulated after about 12 hours. Interestingly, p27 is maintained at this low level by MycER activation but in response to serum, returns to the level prior to induction after 30 hours (as the cells complete the cycle).

To determine if the down-regulation of p27 could be responsible for the activation of cyclin E-dependent kinase activity, I determined whether p27 was present in a complex with cyclin E. I therefore immuno-precipitated cyclin E and assayed the precipitate by western for p27. As shown in Fig. 6.12C, the p27 protein is in fact bound to cyclin E and the levels correlate well with the levels of protein seen in the p27 western (Fig. 6.12B). The down-regulation of p27 is not a rapid process however, starting after about 8 hours post-treatment and taking about 12 hours to be completed. This correlates reasonably well with the late phase of induction of kinase activity which takes 12 hours to reach its maximum but not with the early phase (the first 8 hours). In the serum-treated cells, p27 reappears as the cells complete the cell cycle and cyclin E kinase activity decreases. In the OHT treated cells, there is no reappearance of the inhibitor and kinase activity.

I therefore propose a model (Fig. 6.13) in which Myc down-regulates p27, resulting in the activation of cyclin E-associated kinase activity. Whether this is by transcriptional regulation or another mechanism remains to be determined. There may be another Myc-dependent mechanism that activates cyclin E-dependent kinase activity in the first few hours following OHT application.
Figure 6.13 Model of Myc action through cyclin E
6.8 Discussion

I have attempted to identify the early changes in gene expression following MycER activation and have found that cyclin E transcription is induced, leading to accumulation of cyclin E mRNA.

I have looked at the levels of cyclin E protein following MycER activation and find that they increase (about three-fold) in line with expectations from the mRNA data. Surprisingly however the kinase activity increases considerably more (at least seven-fold) and does not correlate with the protein level. I generated cells in which cyclin E expression is conditional (E6 cells), and show that cyclin E expression in the absence of serum does not lead to increased kinase activity in, agreement with Ohtsubo et al. This suggests a two-step model for Myc action in which Myc both induces cyclin E expression and activates cyclin E complexes. I was able to partly identify the mechanism of activation as p27 down-regulation although it remains to be shown that p27-free cyclin E complexes and not p27-bound complexes are responsible for the kinase activity. The p27 down-regulation is slow in comparison with the increase in cyclin E-dependent kinase activity and a third Myc-dependent step may control the initial increase observed.

To determine the importance of the cyclin E induction to Myc action, I investigated the effect of expressing exogenous cyclin E. Cyclin E expression alone was not sufficient to drive S-phase entry but surprisingly was sufficient to induce apoptosis even though the cyclin E produced did not form kinase-active complexes. This result provides the first link between Myc activity, the cell cycle machinery and apoptosis. I propose the following hypothesis for the mechanism of Myc action in which Myc induces cyclin E protein levels and hence apoptosis and also activates cyclin E/cdk-2 complexes by down-regulating p27, thereby driving the cell cycle.
A recent report\textsuperscript{448} shows, in agreement with our results, that in a heterozygous \textit{c-myc} cell line that grows more slowly due to reduced levels of Myc, the earliest effect during the transition from quiescence into the cell cycle is a delay in cyclin E induction. They also find that D-type cyclin expression is unaffected, consistent with our findings (this chapter and chapter 5), and that growth factor secretion and receptor expression are normal. Steiner \textit{et al.} report\textsuperscript{449} that activation of MycER leads initially to an increase in cyclin E-associated kinase activity and later to an increase in cyclin D1-associated kinase activity. The mechanism for kinase activation involves a Myc-dependent change in the cyclin E complexes and these complexes can be further activated by cdc-25a.

A number of questions remain to be answered before we have a complete understanding of the Myc/cyclin E pathway. Firstly, cyclin E as a relevant transcriptional target of Myc is an excellent opportunity to study Myc trans-activation. It will be interesting to investigate whether the Myc/Max complex binds to the cyclin E gene and to identify the binding sites and associated proteins. The promoter has recently been cloned and interestingly, in neither the human\textsuperscript{450} nor the murine\textsuperscript{451} promoter have CACGTG sites have been described. Instead cyclin \textit{E} gene transcription appears to be under the control of E2F-family transcription factors\textsuperscript{450-452}. Whether there is a link between E2F and Myc-dependent regulation or whether Myc can induce \textit{cyclin E} transcription in an E2F-independent manner will be interesting to explore.

It remains to be determined how p27 is down-regulated but this may be via the transcriptional regulation of another target gene or some other mechanism. It would be interesting to examine the response of the p27 promoter to Myc: is it rapidly down-regulated? Is this refractory to
protein synthesis inhibitors? If not can we identify the factors that mediate the down-regulation? Are they Myc targets?

The induction of apoptosis by cyclin E protein in the absence of kinase activity is also intriguing. The effect is blocked temporarily by the application of insulin in the same way as Myc-induced apoptosis. The finding is consistent with a recent report that kinase activity is not required for Myc-induced apoptosis. The mechanism is not at all clear although there have been reports of cell cycle proteins such as E2F causing apoptosis. One approach may be to characterise the domains of cyclin E that are required for apoptosis using a series of mutants. In particular, since kinase activity is not required, is binding to a cdk necessary? One substrate for cyclin E (as a complex with cdk-2) is Rb although since cyclin E is required for cell cycle progression even in the absence of Rb, it must also act via another mechanism which may or may not involve kinase activity. It would be interesting to determine if cyclin E protein can induce cell cycle progression in the absence of serum in Rb-deficient cells; if so is this by the same mechanism that causes apoptosis?

Given that Myc induces kinase-active cyclin E complexes and cell cycle progression, perhaps the most fundamental question is whether activated cyclin E complexes are sufficient to induce S-phase entry. Possibly the creation of a constitutively active cyclin E/cdk-2 fusion protein or of a cyclin E protein that prevents p27 binding and its expression from the inducible system would clarify the situation. An alternative approach might be the use of cells from a p27 (and p21) knock-out mouse or micro-injection of p27 antibodies or antisense into the inducible cyclin E cells. It is certain that significant progress into the mechanisms of Myc function can be made from studies of these questions.
Chapter Seven

7 DISCUSSION

7.1 Introduction

The aim of the work presented in this thesis was to investigate how Myc controls a cell's fate. It was already known that Myc expression is sufficient to drive entry into the cell cycle\textsuperscript{162} and that Myc is rapidly induced by mitogens\textsuperscript{151,152}; also that Myc expression in certain conditions results in cell death by apoptosis\textsuperscript{136} and that in many cell types Myc prevents differentiation\textsuperscript{105,175-179}. At the time this work began, the Max proteins had been discovered, the structure of Myc and Max proteins was known and the relationship between structure and function was becoming clear. Myc had all the hallmarks of a transcription factor but no target genes were known.

In this work, I have further characterised the mechanisms of Myc action by demonstrating transcriptional activity in mammalian cells and by determining the preferred binding sites for the Myc/Max and Max/Max complexes. I have identified cyclin E but not cyclin D1 as being a target for regulation by Myc at two levels: transcriptional activation of the \textit{cyclin E} gene and activation of cyclin E-associated kinase activity which operates partly via the down-regulation of p27. Cyclin E expression is sufficient to induce apoptosis but not cell cycle entry.

7.2 Trans-activation

The data in chapter 3 demonstrate that Myc can activate transcription in a sequence-specific manner from a CACGTG site. This activation is dependent on N-terminal (putative trans-activation) domains, a basic DNA binding domain and also requires dimerisation with Max.
The level of trans-activation seen in mammalian cells (about 4 fold) is rather low especially when compared with the levels seen in the yeast system of 50 to 200 fold. This could be due to the lower levels of Myc expression achieved in the mammalian system or because of interactions with other factors. The level of trans-activation is dependent upon the number of available CACGTG binding sites however as evidenced by the superior results obtained with M4MinCAT (4 repeats of CACGTG) compared with pPml-tkCAT (one CACGTG).

Max overexpression is able to reduce the level of Myc-dependent trans-activation, presumably by competing for Myc/Max sites (although see below). That Myc requires Max for trans-activation in yeast, trans-activation in mammalian cells (this work), transformation and apoptosis is demonstrated with the aid of reciprocal Myc and Max mutants that recognise each other but not their wild-type counterparts. In the light of these findings that Myc requires Max for all its biological activities, one expected that this might be universally true. Surprisingly some of the first genetic effects of Myc to be described seem not to depend on Max (ornithine decarboxylase induction, cyclin D1 suppression, possibly also interactions with other proteins including TFII-I, Rb, p107, p130 and TBP); whether this means that these effects are not important for the biological effects of Myc previously described remains to be determined. Somewhat strangely, in the rat PC12 cell line no Max expression can be detected and it remains to be seen how Myc acts in these conditions.

A large body of evidence (and also my results, chapter 3) suggest that Max homodimers act as repressors (either passive or active) but one paper reports trans-activation by Max homodimers in yeast cells. This effect requires not only the basic, helix-loop-helix and leucine zipper domains as would be expected for dimerisation and DNA
binding, but also requires amino acid sequences lying outside this minimal region. One possible explanation for the discrepancy is that Max homodimers can trans-activate but that they do so much more weakly than the other factors normally bound to the target CACGTG site in mammalian cells. Hence, upon Max expression, factors such as Myc/Max, TFE3, TFEB or USF which trans-activate more strongly are displaced by the weaker Max/Max complex with an apparent negative effect. Whether trans-activation by Max is relevant in vivo remains to be determined. Max expression is certainly able to abrogate trans-activation by Myc/Max\textsuperscript{312,323,326,337,346} (and also my results, chapter 3), but how important this is in vivo is now uncertain given the discovery of new Max partners (Mad and Mxi-1, see below). It has been suggested that the role of Max may be simply to maintain the chromatin structure of target genes in an available conformation so that upon Myc expression, transcription is rapidly increased or upon Mad/Mxi-1 expression, transcription is rapidly decreased.

7.3 DNA binding

In chapter 4, I present the results of a binding-site selection performed for both the Myc/Max and Max/Max complexes using full length in vitro translated proteins. Both complexes selected sequences containing the hexanucleotide CACGTG from a random pool. The Myc/Max complex selected a more restricted set of sequences than did the Max homodimer and this is reflected in the in vivo effect (trans-activation) of Myc. Specifically, a 5' T or 3' A residue flanking the hexanucleotide core sequence prevents Myc/Max binding and trans-activation. A subsequent study by Fisher and Goding\textsuperscript{466} that also examined the effect of flanking sequences agrees with these results. The conclusion is that of the many genes containing a regulatory CACGTG
site, only a subset will be potential Myc/Max targets while a larger set will be regulated by Max. This may well be a common mechanism for controlling precisely which of the large family of transcription factors is able to activate a particular gene; for example DNA binding by E47 but not by the related MyoD protein is inhibited by a T residue 5' to the CACNTG core sequence\(^{344}\), and similarly, PHO4 but not CPF-1 binding is inhibited by a T residue 5' to the CACGTG core\(^{358}\).

### 7.4 The Myc network

The control of cell growth, differentiation, death and senescence is not merely dependent upon the presence or absence of Myc protein. Additional partners for Max have been discovered (Mad\(^{289}\), Mxi-1\(^{290,291}\), Mad-3 and Mad-4\(^{374}\); I shall use the term 'MAD' herein to refer to any of these) which appear to act in opposition to Myc. In a variety of systems it is apparent that as cells move from a proliferating to a non-proliferating state, the predominant Max complex shifts from Myc/Max to MAD/Max\(^{289-291,366,367,372,373,467}\) (see also addendum to chapter 4). The MAD/Max complexes can bind to the same sequences as Myc/Max and Max/Max and, unlike Max/Max, they actively repress transcription via an interaction with the mammalian homologue of the yeast Sin-3 protein\(^{371,468}\). Both Mad and Mxi-1 form ternary complexes with Max and Sin-3, mediated by their N-terminal domains. Since Sin-3 is a transcriptional repressor, the likelihood is that MAD/Max targets Sin-3 to Myc target genes and represses them.

The model suggested is therefore that in growing cells Myc is expressed and all Myc molecules are bound to Max. These Myc/Max complexes activate transcription of certain target genes. Max homodimers may, depending on their phosphorylation state, also bind to target genes but do they not activate transcription significantly, if at
all. These target sites include the Myc/Max sites as well as other sites not recognised by Myc/Max that may be the targets for other transcription factors. When Myc expression falls, Max/Max complexes will replace Myc/Max and they may function to maintain the chromatin structure in a conformation available for future Myc/Max or mad/Max binding. As cells differentiate or arrest, MAD levels rise and MAD/Max dimers now compete for the target sites, repressing transcription. Whether this repression is necessary for maintenance of the differentiated or arrested state is still unclear.

7.5 Possible Myc targets

The second and most important aim of this work was to determine the target genes through which Myc exerts its effects on the cell cycle. In chapters 5 and 6, I describe the results of the search, in which cyclin E was finally identified as being transcriptionally regulated by Myc (discussed in the following section). During this time, a number of reports have described the identification of other possible target genes.

The earliest Myc target to be proposed was α-prothymosin. This protein is found in the nuclei of mammalian cells in large amounts (over 17 million copies per cell in rapidly growing myeloma cells) and it is down-regulated in arrested cells although levels are invariant during the cell cycle. Further, α-prothymosin seems to be required for progress through the cell cycle since reduced expression with antisense oligonucleotides causes arrest. Eilers et al. first reported an up-regulation in response to activation of MycER in 1991, and expression correlates quite well with Myc expression in both growing, differentiating and cancer cells. In 1994, Gaubatz et al. cloned the rat α-prothymosin gene and found a CACGTG site in the first intron that
mediates trans-activation by Myc. These results are questioned however by Mol et al.\textsuperscript{476} who have observed no effect of Myc or of dominant negative Max on the endogenous or transfected α-prothymosin genes, or of the CACGTG sequences present in the promoter on α-prothymosin expression.

The next gene to be suggested as a Myc target was the ornithine decarboxylase (ODC) gene\textsuperscript{477}. ODC is the first enzyme in the synthesis of polyamines, and it is another gene essential for proliferation. ODC is up-regulated upon stimulation of arrested cells with serum and the mechanism is cell-type specific, being transcriptional in some cells (Swiss 3T3) and post-transcriptional in others (T lymphocytes)\textsuperscript{478}. The ODC gene contains two evolutionarily conserved CACGTG putative Myc binding sites in intron 1, and these mediate Myc-dependent transcription of reporter constructs. Interestingly the dimerisation motifs of Myc are not required for this activity, suggesting that the Myc/Max dimer is not mediating the effect. Possibly Myc acts in association with other proteins to trans-activate ODC\textsuperscript{460} or to regulate transcriptional attenuation\textsuperscript{479}, although Pena \textit{et al.} found that both Myc and Max are bound to the ODC gene\textsuperscript{460,481} and Tobias \textit{et al.}\textsuperscript{482} show that Myc/Max trans-activates ODC while Max represses. There is also a possible role for ODC in cell transformation\textsuperscript{483} and Myc-dependent apoptosis\textsuperscript{484} as well as proliferation, making it an intellectually satisfying Myc target although further characterisation is required.

The ECA39 gene was discovered in 1992 by screening subtracted libraries from transgenic Myc-expressing mice\textsuperscript{377}. This is a gene previously found amplified in a teratocarcinoma cell line and found to have a CACGTG site 3' to the transcription start site. This site is required for Myc binding and for Myc-dependent trans-activation in COS cells. The role of ECA39 is unknown but expression is normally down-
regulated in differentiating COS cells and this is blocked by exogenous Myc expression. Whether ECA39 is involved in mediating any of the observed effects of Myc is unknown.

The p53 protein has also been suggested as a Myc target\textsuperscript{376,485}. Myc is able to trans-activate the \textit{p53} promoter in transfection experiments and Max is able to repress. While p53 is unlikely to be involved in proliferation because its effects are usually growth arresting\textsuperscript{486-489}, it may be relevant to Myc-induced apoptosis. Specifically, Hermeking and Eick\textsuperscript{318} have shown that p53 mediates Myc-dependent apoptosis.

### 7.6 Cyclin D1 as a Myc target?

In early 1992, the D type cyclins (D1, D2 and D3) had been identified but their function was not yet characterised. The regulation in macrophages of cyclin D1 in response to CSF-1 first indicated the possibility of a link with Myc\textsuperscript{392}. In these cells cyclin D1 is induced as a delayed-early gene and its level is maintained throughout the cell cycle as long as CSF-1 is present. This parallels Myc expression in the same cells. In NIH 3T3 cells Myc, Fos, JunB and, slightly later, cyclin D1 are induced following CSF-1 activation. Mutation of the CSF-1 receptor still allows Fos and JunB induction but not Myc. The exogenous expression of Myc\textsuperscript{391} or of cyclin D1\textsuperscript{490} restores the growth factor response. Cyclin D1 can be thought of as a delayed-early response gene and is essential for progression into S-phase. It acts by phosphorylating Rb, hence inactivating it\textsuperscript{218,424,491}.

Cyclin D1 is not a transcriptional target of Myc. I show in chapter 4 that cyclin D1 is an oestrogen-dependent target of MycER and that activation requires the N-terminus (amino acids 106-143) of Myc. The activation is most probably mediated by CACGTG sites in the cyclin D1
promoter and the induction can be mimicked using reporter constructs. The induction is dependent on 17β-oestradiol and is not observed with 4-hydroxy tamoxifen, an analogue which binds to the oestrogen receptor but fails to activate a trans-activation domain in the oestrogen receptor part of MycER. I infer from this that native Myc may interact with the regulatory elements of the cyclin D1 gene but that it fails to activate expression. This may therefore tell us something of the way Myc interacts with promoters, but tells us little of the mechanism by which Myc drives the cell cycle.

It is interesting to consider the reported effects of Myc on cyclin D1 in the light of data describing an interaction of TFII-I with Myc. The requirement for transcriptional initiation (of RNA polymerase II dependent genes) is a TATA box and/or an initiator element (Inr). TFII-I binds specifically to the Inr417,418 and supports basal transcription. TFII-I is also able to bind to E-box sequences and to USF417 and Myc415 proteins. Myc specifically inhibits TFII-I-dependent but not TFII-A-dependent transcription in a dose-dependent manner by interfering with the formation of TFII-I/TBP complex formation. A possible explanation for the effect of MycER on cyclin D1 (which does have an Inr sequence) could be that MycER represses transcription through binding to TFII-I (presumably in a Max-independent manner since the effect is not hormone dependent) but that oestradiol addition activates the latent trans-activation (Taf-II) domain and causes activation either by disrupting the Myc/TFII-I interaction directly or, since there are E-boxes in the promoter, by simple trans-activation. Myc can also interact with YY-I416 which also binds to Inr sequences419 and so a similar mechanism could apply to this protein.

Numerous other interactions between Myc and cellular proteins have been reported of which those with TBP (the TATA box binding
protein), Rb461 and p107 are probably the most relevant. Myc is able to abrogate (in certain circumstances) the arrest caused by Rb492 or p107462,463 whereas other oncogenes (such as Ras, Fos, Jun) are unable. Binding of p107 to the Myc trans-activation domain also significantly decreases Myc trans-activation suggesting that p107 may operate to suppress growth by titrating out Myc. Myc binds to TBP464 which is a key component of TFII-D, via sequences that overlap with those that mediate Rb binding. This further suggests that Rb-family proteins prevent Myc trans-activation by occluding the TBP interaction site.

7.7 Cyclin E as a Myc target

Apart from the D-type cyclins discussed above, the other regulator of progression through the G1/S boundary is cyclin E, which like cyclin D1, plays a role in the phosphorylation of Rb.

Cyclin E expression is rapidly induced in arrested Rat-1 cells in response to both wild-type Myc and activation of MycER by OHT. The induction is transcriptional in nature and refractive to protein synthesis inhibitors suggesting a direct effect. The amount of cyclin E protein rises to a level similar to that seen in serum-stimulated cells and is maintained after the cells proceed through the cell cycle. Additionally, cyclin E-dependent histone H1 kinase activity is induced, again to a level similar to that seen with serum treatment. Since cyclin E expression alone is not sufficient to induce kinase activity, Myc must also activate cyclin E complexes.

Cyclin E is not sufficient to drive Rat-1 cells into cycle (although kinase-active cyclin E complexes may be) but it is sufficient to cause apoptosis. As such, cyclin E is a Myc target that mediates some if not all of Myc's biological effects. The mechanism of this induction of apoptosis is as yet undetermined but there have been recent reports linking cell
cycle proteins (cdk-1\textsuperscript{493} and especially E2F\textsuperscript{454,459,494-496}) with cell death. It is likely that E2F and cyclin E mediate apoptosis via the same pathway since they form an autoregulatory loop\textsuperscript{452} in which E2F-1 expression strongly induces cyclin E. It is interesting that cyclin E kinase activity is not required for apoptosis and therefore a mechanism in which protein interactions rather than phosphorylation mediate apoptosis is likely to be responsible.

7.8 Mechanism and relevance of cyclin E kinase activation

I see that in agreement with other data\textsuperscript{229}, an increase in the level of cyclin E alone does not lead to an increase in the level of cyclin E-associated kinase activity. MycER activation increases both cyclin E levels and kinase activity and this is most probably via p27 down-regulation.

In particular, the down-regulation of p27 does not correlate perfectly with the increase in kinase activity, and cyclin E kinase activity becomes elevated while p27 levels in the cells remain high. This could be because of a lack of free p27, however, experiments in cells with both inducible human cyclin E and MycERTM (an improved version of MycER\textsuperscript{497} with an inactive Taf-II domain) show that cyclin E protein synthesised during periods of MycER activation fails to bind p27. These cyclin E molecules are kinase active and are bound to the CAK-phosphorylated form of cdk-2\textsuperscript{2498}. This change is due to an alteration in the binding properties of free p27\textsuperscript{2498,499}. Work in Martin Eilers's laboratory\textsuperscript{449} has demonstrated a Myc-dependent activation of both cyclin E and cyclin D1 kinase activities. This is dependent on the trans-activation, DNA binding and dimerisation domains. In agreement with my work, Steiner et al. observe a decrease in p27 protein levels and they
also find that cdc25a is required for maximal activation of cyclin E-
associated kinase activity.

Galaktionov et al.\textsuperscript{500} have shown that cdc25a is a transcriptional

target of Myc that can also induce apoptosis. The activation does not
require new protein synthesis indicating it is a direct target, and there
are Myc binding sites in the gene. Cdc25a (reviewed in Hoffmann,
1994\textsuperscript{501}) most probably acts in G1 by activating cyclin E/cdk2
complexes\textsuperscript{502,503}. These results complement those reported herein and
together suggest that Myc acts to stimulate G1 kinase activity in a
number of ways: the transcriptional induction of cyclin E, blocking of
p27 binding to cyclin E, the transcriptional induction of cdc25a and the
down-regulation of p27.

I report that cyclin E protein can induce apoptosis and that this
does not require kinase activation. This is consistent with a report\textsuperscript{453}
showing that cyclin E and D1 kinase activities are sufficient for G1-S
progression in response to Myc, but that kinase activity is not required
for apoptosis since the microinjection of cd1’s does not affect apoptosis.
Interestingly, cdc25a can also induce apoptosis and indeed seems
necessary for Myc-induced death. How this operates is unknown and,
since cdc25a is believed to act by stimulating kinase activity (which is not
required for apoptosis), a possible mechanism is not obvious.

7.9 Future perspectives

The data presented in chapters 3, 4, 5 and 6 above show that Myc
can trans-activate and bind to certain DNA sequences (while Max binds
to related ones) and that Myc acts at least in part by up-regulating cyclin
E. This information along with data from the large number of other
laboratories working on this field over the past few years raises many
questions. It would be interesting to know whether the Mad-family
proteins (MAD) have similar DNA binding specificities to Max/Max or to Myc/Max and whether they are therefore likely to act exclusively at Myc/Max target genes. The question of whether MAD expression is necessary to maintain the differentiated state is also of interest. One approach might be to express dominant negative Max mutants (for example with a deleted basic region) to prevent MAD/Max complexes from acting. This could be achieved with the GalER inducible system for example, which would enable expression at various points during differentiation. The priority, however, still remains the full characterisation of the target genes of the Myc network and the molecular mechanisms by which they act, whether by interaction with TFII-I, TBP, Sin-3 or other proteins.

Cyclin E and now cdc-25a are certainly the most provocative Myc targets as they both link Myc expression to cell cycle progression and also to apoptosis. The means of inducing both expression of these genes and cyclin E-associated kinase activity need to be investigated. Whether expression is induced by a Max-dependent means is one essential and relatively straightforward experiment (a MycER with a mutated dimerisation domain would be a first step) given that this is apparently not the case for ODC induction or for repression of cyclin D1. Since interaction with Max is essential for the biological effects of Myc, activation of a relevant Myc target gene would be expected to require Myc/Max interaction. Characterisation of the cyclin E promoter and the identification of the Myc site of action (by footprinting for example) might be a second step. CACGTG sites have not been found in the cyclin E promoter which appears to be controlled predominantly by the E2F family of transcription factors. Does Myc in fact act in the classical manner for a transcription factor (i.e. binding to a DNA motif
and interacting with the transcriptional machinery) at any of its target sites?

A fuller understanding of the mechanism by which cyclin E complexes are activated is also a promising route for understanding Myc function. Identification of all the proteins complexed to cyclin E throughout the cell cycle would be of great benefit. Better characterisation of cyclin E-induced apoptosis would also be of interest; does it operate via a p53 dependent mechanism? Is it E2F dependent? Does it require interaction with cdk-2 or with cdc25a? Finally, is a kinase-active cyclin E/cdk-2 complex sufficient to drive cell cycle entry or even the entire cycle – as Myc is? This last point is technically more difficult to address but should be possible with properly designed cdk-2 and cyclin E mutants. If active cyclin E is not sufficient, then what else does Myc do? Are ODC, α-prothymosin, ECA-39, cdc25a etc. able to provide the extra signal? There is no doubt that these questions will be addressed in the near future and the link between Myc and the cell cycle will be forged.
Retroviruses

Rous sarcoma virus: a function required for the maintenance of the transformed state

RNA species obtained from clonal lines of avian sarcoma and from avian leukosis virus

A physical map of the Rous sarcoma virus genome

Size and genetic content of viral RNAs in avian oncovirus-infected cells

DNA related to the transforming gene(s) of avian sarcoma viruses is present in normal avian DNA

Nucleotide sequences related to the transforming gene of avian sarcoma virus are present in DNA of uninfected vertebrates

DNA sequences homologous to vertebrate oncogenes are conserved in Drosophila melanogaster

Enemies within: the genesis of retrovirus oncogenes

Cellular oncogenes and retroviruses

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Human-tumor-derived cell lines contain common and different transforming genes

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Stage-specific transforming genes of human and mouse B- and T-lymphocyte neoplasms

A transforming gene present in human sarcoma cell lines


Consequences of myc invasion of immunoglobulin loci: facts and speculation

Onc gene amplification in promyelocytic leukaemia cell line HL-60 and primary leukaemic cells of the same patient

Homogeneously staining chromosomal regions contain amplified copies of an abundantly expressed cellular oncogene (c-myc) in malignant neuroendocrine cells from a human colon carcinoma

Amplification and enhanced expression of the c-myc oncogene in mouse SEWA tumour cells

Association of multiple copies of the N-myc oncogene with rapid progression of neuroblastomas

Elevated expression of the c-myc oncoprotein correlates with poor prognosis in head and neck squamous cell carcinoma

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Cloning and characterization of different human sequences related to the onc gene (v-myc) of avian myelocytomatosis virus (MC29)

Identification and nucleotide sequence of a human locus homologous to the c-myc oncogene of avian myelocytomatosis virus MC29

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Myc and Max Homologs in *Drosophila*

Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes

Human N-myc gene contributes to neoplastic transformation of mammalian cells in culture

N-myc can cooperate with ras to transform normal cells in culture

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G1 phase progression: cycling on cue

Effects of c-myc expression on proliferation, quiescence, and the G0 to G1 transition in nontransformed cells

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Ornithine decarboxylase activity is critical for cell transformation

Ornithine decarboxylase is a mediator of c-Myc-induced apoptosis

Transactivation of the human p53 tumor suppressor gene by c-Myc/Max contributes to elevated mutant p53 expression in some tumors

Evidence for a second cell cycle block at G2/M by p53

DNA damage triggers a prolonged p53-dependent G1 arrest and long-term induction of Cip1 in normal human fibroblasts

Growth arrest induced by wild-type p53 protein blocks cells prior to or near the restriction point in late G1 phase

p53 functions as a cell cycle control protein in osteosarcomas

Rescue of defective mitogenic signaling by D-type cyclins

Cyclin D1 is dispensable for G1 control in retinoblastoma gene-deficient cells independently of cdk4 activity

Abrogation by c-myc of G1 phase arrest induced by RB protein but not by p53

p34cdc2 and apoptosis

Deregulated transcription factor E2F-1 expression leads to S-phase entry and p53-mediated apoptosis

Deregulated expression of E2F-1 induces S-phase entry and leads to apoptosis
E2F-1:DP-1 induces p53 and overrides survival factors to trigger apoptosis

A modified oestrogen receptor ligand-binding domain as an improved switch for the regulation of heterologous proteins

Myc activation of cyclin E/cdk-2 kinase involves induction of cyclin E gene transcription and inhibition of p27Kip1 binding to newly formed complexes

Growth arrest by the cyclin-dependent kinase inhibitor p27Kip1 is abrogated by c-Myc

Cdc25 cell cycle phosphatase as a target of c-myc

The role of cdc25 in checkpoints and feedback controls in the eukaryotic cell cycle

Roles of active site residues and the NH2-terminal domain in the catalysis and substrate binding of human Cdc25

Cell cycle regulation of CDK2 activity by phosphorylation of Thr160 and Tyr15
Myc activation of Cyclin E/Cdk2 kinase involves induction of cyclin E gene transcription and inhibition of p27kip1 binding to newly formed complexes.

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Running title: Myc activation of CycE/Cdk2
Induction of the Myc-oestrogen receptor fusion protein (MycER) by 4-OH-tamoxifen (OHT) leads to the activation of Cyclin E/Cyclin-dependent kinase 2 (CycE/Cdk2) complexes followed by the induction of DNA synthesis. As CycE/Cdk2 activity is essential for G1/S transition, we have investigated the mechanism by which Myc can activate CycE/Cdk2. This is likely to involve at least two Myc-dependent steps: the activation of cyclin E gene transcription followed by accumulation of cyclin E mRNA in a protein synthesis-independent manner and the inhibition of p27Kip1 association with CycE/Cdk2 complexes containing newly synthesised CycE. Our results also indicate that lack of p27Kip1 binding leads to accelerated CAK phosphorylation of CycE-bound Cdk2.

Keywords: cell cycle; Cdk2; cyclin E; Myc; p27
Introduction

The proto-oncogene c-myc is a key regulator of cell proliferation and apoptosis (reviewed in Amati and Land, 1994; Henriksson and Lüscher, 1996; Marcu et al., 1992). c-myc encodes a basic-helix-loop-helix-leucine zipper transcription factor (Myc) that dimerises with Max (Blackwood and Eisenman, 1991; Prendergast et al., 1991) and binds to DNA in a sequence-specific manner (Blackwell et al., 1990; Prendergast, et al., 1991). Myc/Max heterodimers activate transcription (Amati et al., 1992; Kretzner et al., 1992) and are required for Myc-induced cell transformation, cell cycle progression and apoptosis (Amati et al., 1993a; 1993b).

Myc is rapidly induced by growth factors (reviewed in Kelly and Siebenlist, 1986) and is required for mitogenic signalling by the colony-stimulating factor (CSF) (Roussel et al., 1991) and platelet derived growth factor (PDGF) receptors (Barone and Courtneidge, 1995). Constitutive expression of Myc prevents exit from the cell cycle as well as differentiation (Henriksson and Lüscher, 1996; Marcu, et al., 1992) and induces apoptosis in the absence of survival cytokines (Evan et al., 1992; Harrington et al., 1994). Moreover, Myc activity is sufficient to drive resting cells into the cell cycle (Eilers et al., 1991). Although several genes have been identified that are regulated by Myc (Bello-Fernandez et al., 1993; Benvenisty et al., 1992; Galaktionov et al., 1996; Gaubatz et al., 1994; Reisman et al., 1993), none of these genes has been clearly implicated in Myc-induced progression from the G1 into the S phase of the cell cycle.
G1 progression is controlled by the activities of the cyclin-dependent kinase complexes cyclinD/Cdk4 (or Cdk6) and cyclinE (CycE)/Cdk2 (for reviews see (Draetta, 1994; Sherr, 1994). Cyclins D and E are essential for G1/S progression of higher eukaryotic cells (Baldin et al., 1993; Knoblich et al., 1994; Ohtsubo et al., 1995; Quelle et al., 1993) and when overexpressed are able to shorten the G1 interval (Ohtsubo and Roberts, 1993; Quelle, et al., 1993; Resnitzky et al., 1994; Wimmel et al., 1994) indicating that both cyclins are rate-limiting for G1/S transition. Myc-induced G1/S progression involves induction of CycE/Cdk2 activity several hours prior to the activation of cyclin D/Cdk4 complexes (Steiner et al., 1995). This rapid activation of CycE/Cdk2 in response to Myc suggested a close relationship between Myc function and CycE/Cdk2 activity. Regulation of CycE/Cdk2 activity occurs at multiple levels involving the synthesis of the subunits, assembly, phosphorylation-dephosphorylation and the association of inhibitory proteins such as p21Cip1 and p27Kip1 (reviewed in Morgan, 1995; Sherr and Roberts, 1995). To explore how Myc can induce CycE/Cdk2 activation we set out to identify the earliest Myc-sensitive events in this process.

Results
Myc-induced CycE/Cdk2 activation occurs within a small fraction of complexes

In order to follow the kinetics of Myc-dependent CycE/Cdk2 activation we used Rat1 fibroblasts expressing a regulatable fusion protein between Myc and the hormone-binding domain of the human oestrogen receptor (MycER) (Eilers et al., 1989; Evan, et al., 1992; Solomon et al., 1995). In agreement with Steiner et al., 1995 we found that when MycER was activated by 4OH-tamoxifen (OHT),
CycE-dependent kinase activity was rapidly induced. Upon exposure of confluent serum-free cultures to OHT for four and eight hours, we observed 60% and maximal induction of CycE-dependent kinase activity, respectively (Figure 1A, bottom panel and data not shown). We also detected a small but reproducible increase in the steady state levels of CycE protein during this induction (Figure 1A, top panel). Moreover, the cells entered S-phase after 12 hr of Myc activation (not shown).

The activity of CycE-dependent kinase complexes can be regulated via the binding of the cell cycle inhibitors p21\textsuperscript{Cip1} or p27\textsuperscript{Kip1}. Rat1 cells, however, do not express detectable levels of p21\textsuperscript{Cip1} (not shown). We therefore investigated the p27\textsuperscript{Kip1} levels bound to CycE at various times after Myc activation by immunoblotting following immunoprecipitation with CycE-specific antibodies. Although the kinase activity was found to be increased after 4 hr, the amounts of Cdk2 and p27\textsuperscript{Kip1} co-precipitated with CycE remained unaltered up to 8 hr after Myc activation (Figure 1B). Thus, either p27\textsuperscript{Kip1} is not implicated in the early phase of CycE/Cdk2 activation, or kinase activation occurs only within a small and in this experiment undetectable fraction of complexes. We therefore wanted to explore the nature of the complexes giving rise to Myc-induced CycE/Cdk2 activity.

First we wanted to determine the proportion of CycE/Cdk2 complexes that is bound to p27\textsuperscript{Kip1} after Myc activation. A p27\textsuperscript{Kip1}-specific antiserum was used for immunoprecipitation of a lysate derived from MycER cells after 4 hr of exposure to OHT. As compared to a control incubated with normal rabbit serum,
virtually all of the p27\textsuperscript{Kip1} could be depleted from the lysate (Figure 2, top panel). Subsequent immunoprecipitation of both lysates with a CycE-specific antiserum followed by immunoblotting indicated that all CycE-associated non-CAK-phosphorylated Cdk2 (corresponding to upper band) and about 80% of the faster migrating, CAK-phosphorylated form of Cdk2 (corresponding to lower band; (Gu et al., 1992) had been removed by the p27\textsuperscript{Kip1}-specific antiserum (Figure 2, middle panel). Nevertheless, the CycE-specific kinase activity precipitated by the CycE-specific antiserum remained nearly unaffected by the depletion of the p27\textsuperscript{Kip1}-bound CycE/Cdk2 complexes (Figure 2, bottom panel). Moreover, no kinase activity was found associated with p27\textsuperscript{Kip1}-specific immunoprecipitates (not shown). Thus upon Myc activation the bulk of CycE/Cdk2 complexes expressed in Rat1 cells remains bound to p27\textsuperscript{Kip1} and inactive. Only approximately 10% of the CycE/Cdk2 complexes appear p27\textsuperscript{Kip1}-free and contain solely CAK-phosphorylated Cdk2. It is this small fraction which is likely to be responsible for the Myc-induced kinase activation.

**Myc induces accumulation of cyclin E mRNA independent of protein synthesis**

In response to Myc the activation of CycE-dependent Cdk2 activity may be due to an elevation in the concentration of p27\textsuperscript{Kip1}-free CycE/Cdk2 complexes. This can be achieved by a decrease in p27\textsuperscript{Kip1} binding and/or an increased rate of CycE/Cdk2 association. Upon Myc activation there is a small increase in the levels of CycE protein (see Figure 1A). Activation of CycE/Cdk2 activity requires Myc to function as a transcription factor (Steiner, et al., 1995). Moreover, cyclin E mRNA levels have been reported to be elevated
10 hr after Myc stimulation (Jansen et al., 1993) and to be 50% lower in cells hemizygous for Myc (Hanson et al., 1994). We therefore examined the expression levels of cyclin E mRNA shortly after Myc activation. Cellular RNA from Rat1 MycER cells was isolated at various times after application of OHT to confluent cultures in the absence of serum and analysed by RT-PCR. We observed a rapid increase in the levels of cyclin E mRNA already 1 hr after Myc activation, reaching a three to four-fold induction after approximately 8 hr. During this period the levels of GAPDH mRNA remain unaltered (Figures 3A and 3B). Similar results were obtained using additional amplification cycles (not shown), indicating that the DNA amplification had not reached saturation. Nuclear run on analysis performed with nuclei harvested 2.5 hr after Myc activation shows that the induction of cyclin E mRNA occurs at least in part at the level of transcription, while GAPDH gene transcription remains unaffected. In addition, cyclin A gene transcription is not induced 2.5 hr after Myc activation (Figure 3D). This is expected, as the Myc-dependent induction of the cyclin A gene requires cyclin-dependent kinase activity (Rudolph et al., 1996). As shown by Northern analysis, the Myc-induced accumulation of cyclin E mRNA occurs in the presence of various protein synthesis inhibitors (Figure 3E). Moreover, cyclin E mRNA levels are not altered in response to OHT in the parental Rat 1 cells (not shown).

Recently the cdc25A gene encoding a Cdk-specific phosphatase was reported to be a direct target of Myc (Galaktionov, et al., 1996). Since Cdc25A can activate CycE/Cdk2 complexes in vitro (Steiner, et al., 1995), we wanted to explore whether cdc25A mRNA was
induced by Myc in our cell system. We therefore carried out a similar experiment as described above for cyclin E mRNA and determined the levels of cdc25A mRNA in the same RNA samples. Our results show that there is an induction of cdc25A mRNA in response to Myc. However in comparison to the response of cyclin E mRNA the induction of cdc25A mRNA is slow and only becomes significantly elevated after 8 hr (Figures 3A and 3B). Importantly however, there is no significant induction of cdc25A mRNA after 4hr, when CycE/Cdk2 kinase has already become active. Thus, although we cannot rule out some contribution of Cdc25A regulating CycE/Cdk2 activity, due to the slow induction kinetics it seems unlikely that Cdc25A plays a major role in the early activation of CycE/Cdk2 kinase by Myc. In addition, we did not detect any alteration in the cellular Cdc25A protein levels following Myc activation (Figure 3C).

Myc decreases p27^Kip1 binding to CycE/Cdk2 complexes containing newly synthesised CycE

In order to test the role of cyclin E gene transcription in kinase activation we constructed Rat1-derived cell lines in which the synthesis of the human Cyclin E (hCycE) protein is inducible. For this purpose we used a regulatable system (Braselmann et al., 1993) comprising the oestrogen-dependent transactivator GalER-VP16 and a human cyclin E cDNA under the control of a promoter containing Gal4 DNA binding sites. Rat1 cells infected with a retrovirus containing GalER-VP16 were transfected with the Gal4-dependent hCycE expression plasmid. Several cell clones showing OHT-dependent induction of hCycE as detected by an antibody specific for hCycE were isolated. Induction of hCycE was found to be
rapid and could be readily detected after 4 hr with equilibrium being reached after approximately 8 hr. Clone E6 is shown as a representative example (Figure 4A). Using the hCycE-specific antibody no kinase activity can be observed in absence of hCycE, confirming the specificity of the antibody (Figures 4A and 4B). Induction of hCycE in serum-free medium is not sufficient to induce kinase activity (Figure 4B; see also (Ohtsubo, et al., 1995; Resnitzky and Reed, 1995), although over a 16 hr period the degree of CAK-phosphorylation of hCycE-associated Cdk2 increased to similar levels as seen with the endogenous rat CycE (compare Figures 4C and 1B). Nevertheless, the transfected hCycE protein is functional as in the presence of serum it becomes associated with active kinase complexes (Figure 4B; see also (Ohtsubo, et al., 1995; Resnitzky and Reed, 1995).

Although the stimulation of CycE synthesis is not sufficient to induce CycE/Cdk2 activity, the Myc-induced increase in CycE synthesis may play a role in the activation of CycE/Cdk2 by boosting the fraction of activatable complexes. We therefore examined the fate of CycE/Cdk2 complexes containing newly synthesised CycE molecules in the presence and absence of active Myc. The GalER-dependent hCycE expression in Rat1 cells permitted us to perform such experiments, since following OHT induction we were able to specifically immunoprecipitate hCycE which was exclusively synthesised during the period of hormone induction. We thus infected these cells with the pBabe puro retrovirus (Morgenstern and Land, 1990a) encoding MycER™ (Littlewood et al., 1995) or the empty control virus and selected polyclonal populations of infected cells as well as several MycER™ infected
clones. As expected, only the cells in which MycER™ was activated with OHT were stimulated to enter the cell cycle in the absence of mitogens (Figure 5A). To investigate the status of hCycE/Cdk2 complexes polyclonal pools expressing either hCycE alone or hCycE together with MycER™ as well as two cell clones expressing both proteins were analysed 6 hr after exposure to OHT. While the same amount of hCycE was expressed under all conditions (Figure 5B), high levels of hCycE-specific kinase activity were only detectable in cells expressing active MycER™ (Figure 5C). In the absence of active Myc, Cdk2 and p27Kip1 associate with the newly synthesised hCycE (Figure 5D). Moreover, Cdk2 bound to this complex is predominantly in its non-CAK-phosphorylated inactive form (upper band; Figure 5D, top panel). In contrast, hCycE complexes immunoprecipitated from cells in which Myc had been activated contain only little p27Kip1 (Figure 5D, bottom panel). In addition, the associated Cdk2 appears to be predominantly in its active CAK-phosphorylated form (lower band; Figure 5D, top panel). Such alterations can only be observed when analysing CycE molecules newly synthesised in the presence of active Myc but not when examining the bulk of cellular CycE (see Figure 1B). Thus, because of the different behaviour of resident and newly formed CycE complexes in response to Myc activation and the concomitant Myc-induced accumulation of cyclin E mRNA, the Myc-dependent increase in CycE/Cdk2 kinase activity is likely to involve at least two steps: the stimulation of CycE/Cdk2 complex formation by inducing cyclin E gene transcription and the loss of p27Kip1 binding to the newly formed complexes. Moreover, the latter also appears to permit an increased rate of CAK-dependent phosphorylation of Cdk2. This is consistent with the capability of p27Kip1 to physically
interfere with CAK-induced activation of Cdk2 and Cdk4 (Kato et al., 1994; Polyak et al., 1994b).

**Myc prevents association of p27Kip1 with CycE/Cdk2 complexes**

The decreased binding of p27Kip1 to newly synthesised CycE/Cdk2 may be due to a Myc-dependent modification of the CycE/Cdk2 complexes or to an alteration in the binding properties of p27Kip1. Therefore we challenged active CycE-dependent kinase complexes from proliferating cells or from cells expressing activated MycER (in the absence of mitogens) with boiled Rat1 cell extracts in which p27Kip1 is released from intra-cellular complexes (Hengst et al., 1994; Polyak et al., 1994a). In both cases CycE/Cdk2 activity was completely inhibited (not shown), indicating that Myc-induced CycE/Cdk2 complexes are not resistant to inhibition by p27Kip1. Next we wanted to test whether the ability of p27Kip1 to bind to CycE/Cdk2 is regulated by Myc. For this purpose we prepared native extracts from MycER cells at various times after OHT application, since in such extracts p27Kip1 activity appears to reflect only the pool of non-complexed freely available p27Kip1. These extracts were used to challenge a target extract from proliferating E6 cells expressing hCycE-dependent kinase activity. The extract from cells in which MycER is inactive inhibits hCycE/Cdk2 activity two-fold. However, upon activation of Myc this inhibitory activity diminishes to become undetectable after 10 hr (Figures 6A, top panel and 6C). This regulation correlates with decreasing amounts of p27Kip1 entering hCycE/Cdk2 complexes after MycER cell extracts are added (Figure 6A, bottom panel). At the same time the overall levels of p27Kip1 in the MycER cell
Discussion

Mechanism of CycE/Cdk2 activation by Myc

We have analysed CycE/Cdk2 activation by Myc during the early phase of kinase induction. At most 10% of the CycE/Cdk2 complexes contribute to the induction of kinase activity when 60% of maximal kinase activity is observed 4 hr after Myc activation. During this time, the bulk of CycE/Cdk2 complexes remains bound to p27\textsuperscript{Kip1} and inactive. Newly synthesised CycE is likely to play a role in CycE/Cdk2 activation. Myc induces \textit{cyclin E} gene transcription and a rapid accumulation of \textit{cyclin E} mRNA in a protein synthesis-independent manner. We thus followed the fate of newly formed CycE/Cdk2 complexes in cells in which the synthesis of human (h) CycE can be rapidly induced via the chimeric transactivator GalER-VP16. In confluent mitogen-free cultures newly synthesised hCycE associates with non-CAK-phosphorylated, inactive Cdk2 and p27\textsuperscript{Kip1} indicating that p27\textsuperscript{Kip1} is in excess over hCycE/Cdk2 complexes. In contrast, Myc activation inhibits p27\textsuperscript{Kip1} binding to complexes containing newly synthesised hCycE. Moreover, the proportion of CAK-phosphorylated Cdk2 in these complexes...
increases and hCycE-associated kinase is activated. This is consistent with a dual role of p27Kip1 as an inhibitor of cyclin-dependent kinase activity and CAK-phosphorylation (Kato, et al., 1994; Polyak, et al., 1994b). We also show that the inhibition of p27Kip1 binding to CycE/Cdk2 is induced with similar kinetics as the induction of cyclin E mRNA accumulation. Since in response to Myc activation the bulk of cycE behaves differently from the newly synthesised molecules, our findings are consistent with a model in which Myc induces CycE/Cdk2 activation by stimulating cylin E gene transcription and inhibiting the binding of p27Kip1 to newly formed CycE/Cdk2 complexes. As a consequence CycE-bound Cdk2 undergoes accelerated CAK phosphorylation and kinase activation (Figure 7).

The role of Myc-dependent inhibition of p27Kip1

The Myc-dependent inhibition of p27Kip1 binding to the newly synthesised complexes appears to play an important role for kinase activation, as the induction of cyclin E mRNA is not sufficient to induce CycE/Cdk2 kinase activity in the absence of serum factors. This is strongly supported by our observations that CycE/Cdk2 complexes bound to p27Kip1 are inactive and Myc-dependent inhibition of p27Kip1 binding to CycE/Cdk2 complexes follows Myc-activation with similar kinetics as the accumulation of cyclin E mRNA. Moreover parallel experiments by Vlach et al. (1996) show that Myc can rescue a growth arrest induced by p27Kip1 over-expression. Similarly, a p21Cip-dependent growth arrest in response to Raf activation can be blocked by Myc in murine fibroblasts (A. Sewing and H. L. in preparation). Thus, the ability of
Myc to override cell cycle inhibitors such as p27\textsuperscript{Kip1} and p21\textsuperscript{Cip1} appears to be a major aspect of its function.

Our results are consistent with the idea that the inhibition of p27\textsuperscript{Kip1} association may involve sequestration of non-complexed p27\textsuperscript{Kip1} by a Myc-induced factor (Vlach \textit{et al.}, 1996), as during the time course of our experiments the overall concentration of p27\textsuperscript{Kip1} remains constant. However, since we are unable to biochemically distinguish between free and complexed p27\textsuperscript{Kip1}, alternative mechanisms of regulation such as post-translational modification of free p27\textsuperscript{Kip1} cannot be excluded.

Steiner \textit{et al.} (1995) reported that Myc-induced activation of CycE/Cdk2 activity correlates with the breakdown of large pre-existing complexes containing CycE and Cdk2 and proposed that this breakdown may be due to a release of p27\textsuperscript{Kip1} from such complexes. However, the activation of newly synthesised CycE/Cdk2 complexes by inhibition of p27\textsuperscript{Kip1} binding and accelerated CAK phosphorylation does not obviously require such a mechanism. Nevertheless, we have noted that from approximately 10 hr after Myc induction the overall cellular levels of p27\textsuperscript{Kip1} as well as its levels in CycE/Cdk2 complexes decrease (not shown). This suggests a role of p27\textsuperscript{Kip1} release and degradation (Pagano \textit{et al.}, 1995; Steiner, \textit{et al.}, 1995) during later phases of Myc-induced G1/S progression. In light of the decreased half-life of active as compared to inactive CycE/Cdk2 complexes (Clurman \textit{et al.}, 1996), p27\textsuperscript{Kip1} release from CycE/Cdk2 may contribute to maintaining high levels of CycE/Cdk2 activity. The short half-life of active CycE/Cdk2 complexes may also in part be responsible for the difference...
between the three to four-fold induction of *cyclin E* mRNA and the small induction of endogenous Cyclin E protein observed in our experiments.

**Involvement of Myc-induced transcription**

Since the Cdk2 phosphatase gene *cdc25A* has been reported to be a Myc target (Galaktionov, *et al.*, 1996), we also tested the induction of *cdc25A* mRNA by Myc in our cell system. However, compared to the Myc-dependent inductions of *cyclin E* mRNA and CycE/Cdk2 activity the response of *cdc25A* mRNA levels is slow. While after four hours of MycER induction *cyclin E* mRNA is induced to 75% and CycE/Cdk2 activity to approximately 60%, the levels of *cdc25A* mRNA are not significantly elevated at this time. Moreover, an increase in Cdc25A protein levels cannot be detected. Thus although we cannot exclude some contribution of Cdc25A in regulating CycE/Cdk2 activity, we have no evidence to suggest a major role of Cdc25A in the initial induction of CycE/Cdk2 kinase activity following Myc activation. The observations that CycE-dependent Cdk2 activity immunoprecipitated from cells with active Myc can be super-induced by Cdc25A *in vitro* (Steiner, *et al.*, 1995) and that overexpression of Cdc25A does not rescue a p27^Kip1^-induced growth arrest (Vlach, *et al.*, 1996) support this view.

Myc activates *cyclin E* gene transcription and leads to an accumulation of *cyclin E* mRNA independent of protein synthesis. However, no high affinity Myc consensus DNA binding sites have been found in either the human (Geng *et al.*, 1996) or the murine (Botz *et al.*, 1996) *cyclin E* genes. Instead *cyclin E* gene transcription appears to be under control of E2F family transcription factors.
Whether there may be a link between E2F and Myc-dependent regulation or whether Myc can induce cyclin E gene transcription in an E2F-independent manner will be interesting to explore.

Materials and methods

Cell culture

Rat1 fibroblasts expressing steroid hormone-inducible fusion proteins of the human c-Myc protein and the hormone binding domain of the oestrogen receptor, MycER (Eilers, et al., 1989) or MycER™ (Littlewood, et al., 1995) and the chimeric transcription factor GalER-VP16 (Braselmann, et al., 1993) were cultivated in phenol red-free Dulbecco's modified Eagle's medium supplemented with 10% charcoal-stripped foetal calf serum (FCS). Before induction of the chimeric proteins with 200 nM of 4-hydroxy-tamoxifen (OHT) the cells were grown to confluency and kept in serum-free medium in the presence of 5 μg/μl of insulin for 48 hours.

Rat1 cells expressing OHT-inducible Cyclin E

Rat1 cells expressing the chimeric transcription factor GalER-VP16 (Braselmann, et al., 1993) were generated via retroviral infection. Subsequently these cells were transfected with a vector, pGC-HE, containing the human cycE cDNA derived from pCycE/L UHG (Sewing et al., 1994) under the control of a GAL4-dependent promoter (Braselmann, et al., 1993). The plasmid pJ6-hygro (Morgenstern and Land, 1990b) was co-transfected with pGC-HE in a molar ratio of 1:10 and colonies resistant to 100 μg/ml of
Hygromycin B (Calbiochem) were isolated. To generate cells expressing inducible hCycE together with OHT-dependent Myc the clone E6 was infected with the retrovirus pBabepuro MycER™ (Littlewood, et al., 1995). Colonies resistant to 5 µg/ml puromycin (Sigma) were pooled (E6MTR P) or isolated as single cell clones (E6MTR 19 and E6MTR 21). A polyclonal population of E6 cells infected with the empty pBabepuro virus and selected under the same conditions served as a control (E6B).

**FACS analysis**

For cell cycle analysis, quiescent cells were stimulated with OHT and 20 hr later were trypsinised and fixed in 70% ethanol, stained with propidium iodide (10 µg/ml) and analysed with a Becton Dickinson FACScan.

**Immunoblotting, immunoprecipitation and kinase activity**

Cells were lysed in 50 mM Tris-HCl, pH 8, 150 mM NaCl, 1% Triton, 1 mM DTT, 10 mM NaF, 0.5 mM Na₃VO₄, 18 µg/ml Aprotinin and 100 µg/ml PMSF. Cellular debris was removed by sedimentation and protein concentration was determined using the BioRad DC protein assay. Cellular proteins (10 to 30 µg) were resolved by SDS-PAGE, transfered onto Immobilon P membranes (Millipore) with a Genie Blot II (Idea Sci.), blocked with PBS, 0.1% Tween 20 and 5% skimmed milk powder and probed with the following rabbit polyclonal antibodies (from Santa Cruz Biotechnology): rat CycE, M-20 (sc-481), human CycE, C-19 (sc-198), p27Kip1, C-19 (sc-528), Cdk2, M2 (sc-163) and Cdc25A, 144 (sc-097). Filters wer washed three times for 10 min in PBS, 0.1% Tween 20. Proteins were
detected using horseradish peroxidase conjugated secondary antibodies (goat anti-rabbit IgG Fc, Pierce) and visualized using the enhanced chemiluminescence system (Amersham). For immunoprecipitations, 200 µg of protein were incubated with 1 µg of the appropriate antibody and Protein A-Sepharose beads (4 Fast Flow from Pharmacia Biotech.) for two hours at 4°C, washed four times with lysis buffer and resolved by SDS-PAGE followed by immunoblot analysis. For kinase assays, immunoprecipitates were washed twice more with kinase buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 1 mM DTT) and reactions were performed in 30 µl of kinase buffer containing 15 µg Histone HI, 30 µM ATP and 3 µCi [γ-3²P]ATP (Amersham), for 20 min at 30°C. Radiolabelled Histone HI was resolved by SDS-PAGE and quantified with a phosphorimager. For the experiment described in Figure 6, proliferating E6 cells were treated with OHT for 12 hr before they were harvested in lysis buffer. From these extracts hCycE-dependent kinase activity can be immunoprecipitated using hCycE-specific antibodies. 200 µg of protein from this extract was combined with 300 µg of protein from native extracts from Rat1 MycER cells which had been rendered quiescent and then treated with OHT for various times. The mixed extracts were incubated for 20 min at 30°C. Then hCycE complexes were immunoprecipitated with specific antibodies and analysed for kinase activity and composition as described above.

**RT-PCR**

Total cellular RNA was prepared according to (Belyavsky et al., 1989) with slight modifications: cells were lysed in 400 µl of G-CN solution (for a 10 cm petri dish of confluent cells). After vortexing thoroughly, 37.5 µl of 1 M Na-Acetate (pH 4.0) and 800 µl of
phenol:chloroform (5:1) were added and the mixture was incubated on ice for 10 min. After centrifugation (20 min at 4°C), the RNA was precipitated with 2.7 volumes of ethanol, washed with 70% ethanol, air dried and resuspended in water. cDNA was synthesised using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT, BRL) in the buffer provided by the manufacturer in a final volume of 22 µl at 37°C for 1 hour in the presence of 1 µg of oligo d(T)_{12-18} (Pharmacia Biotech.), 1 mM each dNTP (Pharmacia Biotech) and 1 µl of RNasin (Promega), with 2 µg of denatured RNA as template. The cDNA was then used as template for PCR amplification using native Pfu DNA polymerase and the buffer provided by the manufacturer (Stratagene) in a final volume of 50 µl containing 200 mM each dNTP, 0.5 µg of each primer and 3 µCi of [α-32P] dCTP (Amersham). Denaturation, annealing and extension steps were at 94°C, 52°C and 72°C, respectively, for one min each. The number of cycles was 18, 22 and 26 to ensure that the reaction had not reached saturation. The primers used were:

GAPDH5', CGTCTTCACCACCATGGAGA
GAPDG3', CGGCCATCACGCCACAGTIT
CycE5', GAGGTCTGGAGGATCATGTT
CycE3', CTGCATCAACTCCAACGAGG
Cdc25A5', CCAGTGAGGGAGATGTCC
Cdc25A3', TCATTGCAAGCCTATCTCG.

The amplified products were resolved on a native 4% polyacrylamide gel in TBE buffer and quantified using a phosphorimager.
Nuclear run-on analysis and hybridisation probes

Preparation of nuclei, elongation reaction, RNA isolation, hybridisation and filter washes were carried out exactly as described by (Roberts and Bentley, 1992). All probes were isolated cDNA fragments. They were denatured and 1µg/slot was blotted onto Genescreen (Dupont). The DNA was cross-linked to the filter by exposure to UV light for 2 minutes. Cyclin E cDNA was derived as a 1.25 kb Xba I/Sac II fragment from pGEX 3XP (Sewing, et al., 1994). Cyclin A cDNA was isolated as a 1.5kb EcoR I fragment (lacking 3'-untranslated sequences) from pBJ4cycA and was originally derived from pCyc A (Pines and Hunter, 1990). GAPDH cDNA was prepared as a 1.5kb EcoR I fragment from PRLC GAP (Tso et al., 1985).

Northern analysis

Poly(A)^+ RNA was prepared using the 'Fast Track' protocol according to the manufacturer's instructions (Invitrogen). For Northern analysis the RNA was electrophoresed through a 0.8% agarose-formaldehyde gel. The RNA was transferred to a HybondN+ membrane (Amersham) and hybridised in QuickHyb according to the manufacturer's instructions (Stratagene). Probes were labelled with [α-^32P] dCTP by random priming of the same cyclin E and GAPDH cDNA fragments described above.

Acknowledgements

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References
Figure legends

Figure 1. Myc induces CycE-dependent kinase activity without apparent changes in the complex composition. Quiescent Rat1 MycER cells were stimulated with OHT and harvested at the indicated times (hours). (A) Immunoblot of CycE (top panel); CycE-dependent kinase activity was measured using histone H1 as a substrate (bottom panel). (B) CycE-specific immunoprecipitates were analyzed by immunoblotting for Cdk2 (top panel) and p27Kip1 (bottom panel); L, lysate; P, immunoprecipitation carried out with a previously peptide-blocked antibody. Similar results were obtained in multiple experiments. A representative example is shown. The arrows indicate the positions of the bands corresponding to the non-CAK-phosphorylated (upper band) and CAK-phosphorylated (lower band) forms of Cdk2 (Gu, et al., 1992).

Figure 2. Immunodepletion of p27Kip1. Cell lysates were prepared 4 hr after OHT stimulation and immunoprecipitated twice with p27Kip1 antibodies (Depleted) or with normal rabbit serum (Control). The efficiency of the depletion was monitored by immunoblotting of p27Kip1 (top panel). The lysates were then incubated with CycE antibodies and analyzed for co-immunoprecipitation of Cdk2 (middle panel) and CycE-dependent kinase activity (bottom panel). The arrows indicate the positions of the bands corresponding to the non-CAK-phosphorylated (upper band) and CAK-phosphorylated (lower band) forms of Cdk2 (Gu, et al., 1992).

Figure 3. Myc induces accumulation of cycE mRNA. (A) Total RNA was prepared from Rat1 MycER cells at the indicated times (hours)
after OHT addition and analyzed by RT-PCR using primers specific for *cycE*, *cdc25A* and GAPDH. (B) Phosphorimager quantification of the experiment shown in A. (C) Cdc25A protein levels remain constant after Myc activation: Cdc25A protein was analyzed by immunoblotting of Cdc25A immunoprecipitates; L, lysate; P, peptide-blocked antibody. (D) Nuclear run-on analysis: quiescent Rat1 MycER cells were exposed to OHT or EtOH (as a solvent control) for 2.5 hr. Nuclei were prepared for nuclear run-on assay and the same amounts of *in vitro*-synthesised [$\alpha$-32P] UTP-labelled RNA were hybridised against the indicated probes. (E) Myc induces accumulation of CycE mRNA independently of protein synthesis: quiescent Rat1 MycER cells were treated with no drug (control) or 50 $\mu$g/ml of protein synthesis inhibitors (anisomycin, emetine or puromycin) and either EtOH (-) or OHT (+) for 3 hr. Subsequently, poly(A)$^+$ RNA was isolated and 10$\mu$g of RNA/lane were analysed by Northern blotting and hybridised with the indicated probes.

**Figure 4.** Induction of CycE is not sufficient to induce kinase activity. (A) Human CycE (hCycE) expression in E6 cells treated with OHT for the indicated times (hours) was monitored by immunoblotting. (B) hCycE-dependent kinase activity requires the presence of serum (FCS): quiescent E6 cells were treated with FCS and/or OHT for 12 h and hCycE-dependent kinase activity was measured using histone H1 as a substrate. (C) CAK-phosphorylation of Cdk2 in hCycE/Cdk2 complexes was monitored by immunoblotting of hCycE-specific immunoprecipitates with Cdk2 antibodies; L, lysate. The arrows indicate the positions of the bands corresponding to the non-CAK-phosphorylated (upper band) and CAK-phosphorylated (lower band) forms of Cdk2 (Gu, *et al.*, 1992).
Figure 5. Myc activation causes alterations in the composition of hCycE complexes and induces kinase activity. (A) FACS analysis of E6 cells infected with empty pBabe puro (E6B) or with pBabe puro MycER™ (E6MTR P) in the presence and absence of OHT. Cells were harvested 20 hr after stimulation (B) The expression of hCycE was monitored by immunoblotting. (C) hCycE-dependent Histone HI kinase activity was measured. (D) hCycE-specific immunoprecipitates were analysed by immunoblotting for the presence of Cdk2 (top panel) and p27^Kip1 (bottom panel). In B, C and D cells were treated with OHT for 6 hr before being harvested and analysed. E6MTR P is a polyclonal population of E6 cells infected with the retrovirus pBabe puro MycER™; E6MTR 19 and E6MTR 21 are two independent clones derived from E6MTR P; E6B is a polyclonal population of E6 cells infected with the empty pBabe puro vector. The arrows in D indicate the positions of the bands corresponding to the non-CAK-phosphorylated (upper band) and CAK-phosphorylated (lower band) forms of Cdk2 (Gu, et al., 1992).

Figure 6. Myc inhibits p27^Kip1 binding to CycE complexes. (A) Cell extracts were prepared from Rat1 MycER cells which had been rendered quiescent and then treated with OHT for the times indicated (hours). These extracts were mixed with the target extract (T) containing active hCycE-dependent kinase (see Materials and methods). Following incubation, hCycE-specific immunoprecipitates were analysed for histone HI kinase activity (top panel) and immunoblotted to reveal the degree of p27^Kip1 co-immunoprecipitation (bottom panel); T, target extract alone. (B) The levels of p27^Kip1 in the Rat1 MycER extracts were monitored by
immunoblotting. (C) Phosphorimager quantification of the kinase assay shown in (A). (D) Immuno-depletion of p27^Kip1. Cell lysates were prepared from arrested MycER cells and immunodepleted with normal rabbit serum (IgG) and an anti-p27^Kip1 serum (α-p27^Kip1) as described in the legend to Figure 2. The levels of p27^Kip1 in the depleted extracts were determined by immunoblotting (top panel). The depleted lysates (L-IgG and L-α-p27^Kip1) were then mixed with the target extract (T) and hCycE-dependent histone H1 kinase activity was determined as described above (bottom panel).

**Figure 7.** Model of CycE/Cdk2 activation by Myc. Myc stimulates cycin E gene transcription, therefore allowing increased synthesis of Cyclin E protein. At the same time Myc also inhibits the binding of p27^Kip1 to newly formed CycE/Cdk2 complexes which undergo accelerated CAK phosphorylation and kinase activation.
Fig. 1.

A

0 1 4 8

CycE

Histone HI

B

L P 0 1 4 8

CycE IP

Cdk2

Kip1

p27
Fig. 2.

A  
Control Depleted

B  
p27 Kip1
CycE IP
Cdk2
Histone HI

C  

D  
OHT
DMSO
CycE
CycA
GAPDH

E  
Control Ancon. Estet. Patern. + OHT
CycE
GAPDH
Figure 4.

A

B

C

D

Histone HI

hCycE

Cdk2

0 4 8 12 16 20

- - + + OHT
- + - + FCS

L 0 4 8 12 16 20

hCycE IP

Cdk2

Histone HI
A

E6B  E6MTR P
- OHT
+ OHT

B

Uninduced  E6B  E6MTR P  E6MTR 19  E6MTR 21

HCycE

C

E6B  E6MTR P  E6MTR 19  E6MTR 21

Histone HI

D

Lysate  E6B  E6MTR P  E6MTR 19  E6MTR 21

hCycE IP  Cdk2  p27 Kip1
Fig. 7.

Diagram showing interactions between Myc, Cdk2, OycE, OycE/Cdk2, p27, Kip1, OAK, and phosphorylation (P).
Distinct DNA binding preferences for the c-Myc/Max and Max/Max dimers

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ABSTRACT

The transcription factor c-Myc and its dimerisation partner Max are members of the basic/helix-loop-helix/leucine-zipper (bHLH-Z) family and bind to the DNA core sequence CACGTG. Using a site-selection protocol, we determined the complete 12 base pair consensus binding sites of c-Myc/Max (RACCACGTG-GTY) and Max/Max (RANCACGTGNTY) dimers. We find that the c-Myc/Max dimer fails to bind the core when it is flanked by a 5’T or a 3’A, while the Max/Max dimer readily binds such sequences. Furthermore, we show that inappropriate flanking sequences preclude transactivation by c-Myc in vivo. In conclusion, Max/Max dimers are less discriminatory than c-Myc/Max and may regulate other genes in addition to c-Myc/Max targets.

INTRODUCTION

The c-Myc (Myc) protein is known to be a key regulator of cell proliferation, differentiation and apoptosis (1–4). Myc and its partner protein Max dimerise via helix-loop-helix-leucine zipper interactions and bind the core DNA sequence CACGTG (5–15; for reviews, 16, 17). Two major max mRNA splice variants encode the proteins Max1 and Max2, which are equally abundant in cells and differ by the presence of a nine amino-acid insert preceding the basic domain in Max2 (5, 6). Other variants have been described, in which the carboxy-terminal is truncated in Max1 and Max2 (5, 6). Max proteins form homodimers which bind to the same DNA sequence (5, 7, 8, 11, 12, 20), whereas Max proteins form homodimers which bind to the DNA sequence (5, 7, 8, 11, 12, 20). Myc alone neither forms homodimers nor binds DNA except at very high concentrations in vitro (7, 12 and references therein).

It was recently demonstrated that Myc is a transcriptional regulator (7, 13, 21–25). Dimerisation with Max is required for all tested biological activities of Myc, including transactivation of promoters containing Myc/Max binding sites (7), cooperative transformation of primary cells (14) as well as induction of cell-cycle progression and apoptosis in non-transformed cells (25b). Furthermore, these activities all require the Myc amino-terminal transactivation domain (4, 26, 27). Thus, the Myc/Max dimer is a transcription factor responsible for the biological activities of Myc.

METHODS

Proteins

Myc and Max2 proteins were synthesised as previously described (12). Synthesis was controlled for by immunoprecipitating 35S labelled proteins with the appropriate antibody followed by SDS gel electrophoresis. DNA binding activity of unlabelled protein was monitored in a trial mobility-shift assay using labelled wt1 oligonucleotide (see below).

Oligonucleotides and probes

Double stranded probes were prepared by annealing synthetic oligonucleotides and were end-labelled with T4 Polynucleotide Kinase with 32P γ-ATP under standard conditions. The double-stranded wt12 probe (12) (derived from the Ems sequence; 33, 35) is composed of wt1: 5’-TCGACOCCGACCACGTGGTCGCCCTC-3’ and wt2: 5’-TCGAGAGGGACCACGTGGTCGCGCG-3’. For site selection, we used the following oligonucleotides (34): Primer F: 5’ GTGTCAGTTGACTGAATT-CCCTC-3’, Primer R: 5’ CAGGTCAGTTCCGCGAT-
CCTGTCG 3', Random Oligonucleotide R76: 5' CAGGT-

\text{Binding site selection and mobility shift assays}

Binding site selection was carried out as described (34), by immunoprecipitation of DNA sequences bound to Myc/Max and Max/Max complexes. Immunoprecipitation was carried out as described (12) using the peptide specific antisera PM (anti-Myc) (36) and MX (anti-Max) (12). Each antibody is specific for its corresponding protein or Max alone was exposed to a random oligonucleotide pool. Bound oligonucleotides were then co-immunoprecipitated with either Myc or Max-specific antibodies, as appropriate. This permitted the isolation of DNA sequences bound to Myc/Max or Max/Max complexes respectively (12), whereas oligonucleotides that do not efficiently bind these dimers are not precipitated. The selected DNA was amplified by PCR and used for a subsequent round of selection. Repeated rounds of selection and amplification were performed until specific binding to the selected oligonucleotide pool was evident. Specific DNA binding was measured after each round by immunoprecipitating radiolabelled amplified probe with the Myc or Max-specific antibodies in the presence and absence of the respective cognate immunogenic peptide, and selection was continued for one round after recording a significant level of peptide-sensitive immunoprecipitation (five rounds with Max, eight with Myc/Max). As a positive control we performed immunoprecipitations (with and without peptide) using radiolabelled wt12 probe which specifically binds to both Myc/Max and Max/Max complexes (12). In addition, a parallel selection was performed with unprogrammed reticulocyte lysate to control for any endogenous DNA binding activity, and no enrichment was observed.

\text{RESULTS}

\text{Binding site selection}

We determined the DNA binding preferences of the Myc/Max and Max/Max dimers using a previously described selection strategy (34). We used the Max2 form for these studies (hereafter termed Max) since it binds DNA more efficiently than Max1 both as a homodimer and as a heterodimer with Myc (12) (B.A. and H.L., unpublished data).

A mixture of \textit{in vitro} translated full-length Myc and Max proteins or Max alone was exposed to a random oligonucleotide pool. Bound oligonucleotides were then co-immunoprecipitated with either Myc or Max-specific antibodies, as appropriate. This permits the isolation of DNA specifically bound to Myc/Max or Max/Max complexes respectively (12), whereas oligonucleotides that do not efficiently bind these dimers are not precipitated. The selected DNA was amplified by PCR and used for a subsequent round of selection. Repeated rounds of selection and amplification were performed until specific binding to the selected oligonucleotide pool was evident. Specific DNA binding was measured after each round by immunoprecipitating radiolabelled amplified probe with the Myc or Max-specific antibodies in the presence and absence of the respective cognate immunogenic peptide, and selection was continued for one round after recording a significant level of peptide-sensitive immunoprecipitation (five rounds with Max, eight with Myc/Max). As a positive control we performed immunoprecipitations (with and without peptide) using radiolabelled wt12 probe which specifically binds to both Myc/Max and Max/Max complexes (12). In addition, a parallel selection was performed with unprogrammed reticulocyte lysate to control for any endogenous DNA binding activity, and no enrichment was observed.

\text{Myc/Max and Max selected sequences}

To determine the binding site preferences of the two dimers, we cloned and sequenced the DNA molecules selected in the above experiments. To preserve low affinity sites, oligonucleotides from the T3 or T7 primers using the Sequenase kit (United States Biochemicals).

Mobility shift assays were performed as described in (12) and complexes were quantified on a Molecular Dynamics phosphor imager using ImageQuant v. 2.0. The background values, obtained with unprogrammed reticulocyte lysate, were subtracted from all others.

\text{Cell culture and transfection}

\text{Reporter constructs and CAT assays}

We made CAT reporter constructs by replacing the HindIII–PstI fragment of pBLCAT2 (39) with the sequence 5' AGCTTGGCG-ACCAGCTGTTGGCACCACCTTGCAATATGAGGTC-ACGGTTGTTGGCCTCGAACCAGGACCACCCTCGCAG 3' to yield CoreG. The plasmids CoreG, TcoreG and GTA differ only as indicated in Figure 5A. We assayed CAT enzymatic activity in lysates of transfected cells by liquid scintillation using standard procedures (40).

\text{RESULTS}

\text{Binding site selection}

We determined the DNA binding preferences of the Myc/Max and Max/Max dimers using a previously described selection strategy (34). We used the Max2 form for these studies (hereafter termed Max) since it binds DNA more efficiently than Max1 both as a homodimer and as a heterodimer with Myc (12) (B.A. and H.L., unpublished data).

A mixture of \textit{in vitro} translated full-length Myc and Max proteins or Max alone was exposed to a random oligonucleotide pool. Bound oligonucleotides were then co-immunoprecipitated with either Myc or Max-specific antibodies, as appropriate. This permits the isolation of DNA specifically bound to Myc/Max or Max/Max complexes respectively (12), whereas oligonucleotides that do not efficiently bind these dimers are not precipitated. The selected DNA was amplified by PCR and used for a subsequent round of selection. Repeated rounds of selection and amplification were performed until specific binding to the selected oligonucleotide pool was evident. Specific DNA binding was measured after each round by immunoprecipitating radiolabelled amplified probe with the Myc or Max-specific antibodies in the presence and absence of the respective cognate immunogenic peptide, and selection was continued for one round after recording a significant level of peptide-sensitive immunoprecipitation (five rounds with Max, eight with Myc/Max). As a positive control we performed immunoprecipitations (with and without peptide) using radiolabelled wt12 probe which specifically binds to both Myc/Max and Max/Max complexes (12). In addition, a parallel selection was performed with unprogrammed reticulocyte lysate to control for any endogenous DNA binding activity, and no enrichment was observed.

\text{Myc/Max and Max selected sequences}

To determine the binding site preferences of the two dimers, we cloned and sequenced the DNA molecules selected in the above experiments. To preserve low affinity sites, oligonucleotides from the penultimate as well as the final rounds of selection were taken (Figures 1 and 2).
Both the Myc/Max and the Max/Max complexes selected sequences containing the previously described hexanucleotide CACGTG (73% and 90% for Myc/Max in the penultimate and final rounds respectively, and 55% and 91% for Max/Max). To investigate the base composition at positions flanking the core we must allow for the fact that the orientation of the selected molecules is indeterminate since the core sequence CACGTG is a palindrome. To account for this we considered all half-sites from the core; thus, —1 refers to the position adjacent to the core, —2 to that two residues away and so on.

Surprisingly the two complexes exhibited very different preferences at the —1 position. Of the sequences containing the CACGTG motif, only one of the 76 selected by Myc/Max had a T residue at this position, demonstrating that the TCAC half site is strongly disfavoured. In contrast, the Max homодimers show no significant preference at this position.

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**Figure 2.** Nucleotide sequence of DNA molecules selected by the Max/Max complex. See Figure 1 legend for details.

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**Figure 3.** Consensus sequences for positions flanking the CACGTG core in binding sites selected by the Myc/Max and Max/Max complexes. The compilation is from the sequences shown in Figures 1 and 2. The sequences containing a CACGTG from the core; thus, —1 refers to the position adjacent to the core, —2 to that two residues away and so on.

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**Figure 4.** Myc/Max but not Max/Max discriminates against TCACGTGA. In vitro translated Myc and Max proteins were incubated together with both the radiolabelled probe CGCCGACACGTGGTCCCTC and increasing amounts of either identical unlabeled oligonucleotide or a similar oligonucleotide differing only at the underlined positions, as indicated. Myc/Max and Max/Max complexes were resolved by gel electrophoresis as previously described (12). The amount of radioactive probe in each complex was quantified and normalised to the amount of probe in the same complex in the absence of competitor.

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Both complexes have a clear preference for purines (R) at positions —2 and —3. The —2 position is especially well defined: A is the preferred residue but a G is allowed while either pyrimidine (Y) is strongly disfavoured. At —3, the preference appears less strong and only a C is significantly disfavoured. The consensus binding site of the Myc/Max dimer is RACC-
ACGTGTY, while the consensus for Max homodimers is RANCACTGNYT. Neither complex shows any preference for particular bases at the -4 position (Figure 3) nor at positions further out.

Among the sequences lacking a CACGTG, the only obvious feature was a CACATG core found in three out of 52 sequences selected by Max, none of them in the final round of selection (Figure 1). Max/Max and Myc/Max dimers can bind to this sequence in gel retardation experiments, although with lower affinity than for the CACGTG sequence (11 and data not shown). Moreover, multiple CACGTG sites can mediate Myc-induced transactivation (21). Computer analysis of those sequences lacking either CACGTG or CACATG motifs failed to reveal any recurrent feature other than AT-rich sequences similar to those brought down by the unprogrammed lysate control (data not shown).

Specifically, the sequence TCTCCTA reported as being associated with Myc (41, 42) was not found.

To confirm the difference in binding preference of the Myc/Max and Max/Max complexes, we analysed the DNA binding activities of these complexes in gel retardation assays as previously described (12) and performed competition experiments. When using a radiolabelled CCACGTGG probe, a 100-fold excess of TCACGTGA was only able to compete weakly for the Myc/Max complex in comparison with competition by the CCACGTG sequence itself, while both sequences competed efficiently for the Max/Max complex (Figure 4). Moreover, when using TCACGTGA as a probe, we only detect the Max/Max complex and not the Myc/Max complex (data not shown).

Sequences flanking the hexanucleotide core affect transactivation by Myc/Max in vivo

We next wanted to determine whether the difference in binding specificity of Myc/Max for CCACGTGG (termed CoreG) and TCACGTGA (termed TorreA) in vitro may be reflected in Myc/Max dimer activity in vivo. Therefore, we assessed the ability of Myc to activate transcription from promoters driven by these sequences. We measured Chloramphenicol Acetyl Transferase (CAT) expression from reporter plasmids (Figure 5A) in which the minimal Herpes Simplex Virus-Thymidine Kinase promoter was linked to either CoreG, TorreA, the single half-site change TorreG, or the non-binding core mutant CCACGTA (12) (termed GTA). We stably co-transfected the reporters with a puromycin resistance gene into Rat-1 cells expressing MycER, a β-oestradiol-inducible Myc-oestrogen receptor fusion protein (37) and pooled approximately 1500 puromycin resistant colonies for each reporter. Exposure of the transfected cells to β-oestradiol results in an increase in the rate of CAT synthesis in the pool containing the CoreG reporter, while the GTA, TorreG and TorreA reporters were not significantly transactivated by MycER (Figure 5B).

**DISCUSSION**

We report the complete dodecameric DNA binding sites for the Myc/Max and Max/Max complexes derived from selection-amplification experiments with a random oligonucleotide pool and full-length in vitro-translated Myc and Max2 proteins. We find that the binding preferences of the two complexes are distinct, although they both select the same CACGTG core. The sequences selected by Myc/Max form a much more restricted set than those for Max/Max since the former discriminates against T residues at the -1 position relative to the hexanucleotide core while the latter does not. It remains to be determined whether the other forms of Max have the same DNA binding specificity as Max2. However, the discrimination against T at position -1 by Myc/Max heterodimers is most likely true with all forms of Max expressed in cells, since Myc activates reporter genes containing CoreG, but not TorreG or TorreA sequences in vivo (see Figure 5).

One might expect that a difference in DNA site preference between the Myc/Max and Max/Max complexes would be due to a difference in the half-site preference of Myc compared to Max. If this were the case then sequences with a T 5' to the hexanucleotide core (or an A 3') such as TCACGTGG (or CCACGTGA) should still be efficiently recognised since there would still be one half-site recognised by Myc. Only sequences with both a 5' T and a 3' A which exclude Myc from both sides should be discriminated against. However in the sequences selected by Myc/Max, those with a single Myc-incompatible half-site are strongly discriminated against (only 1 out of 76). Furthermore, the TorreG reporter is no more able than the TorreA reporter to mediate Myc-driven transcription in vivo. These results suggest that the proteins in a dimer do not recognise half-sites independently of one-another. Confirmation of this prediction must await resolution of the structure of DNA-bound Myc/Max dimers, for comparison with the solved structure of the DNA-bound Max/Max bHLH-Z domain (20).
We also show that the preferred binding sites of Myc/Max and Max/Max complexes are very similar at the flanking positions -2 and -3, where purines are strongly favoured. Moreover, it becomes clear from the sequences shown in Figure 3 that certain residues are particularly disfavoured. These are T and C at the -2 position and C at position -3. According to these rules only 8% of CACGTG containing sequences are likely to be high affinity Myc/Max targets and 14% likely to be high affinity Max/Max targets.

Interestingly, our binding consensus for Myc/Max dimers is in agreement with studies with in vitro translated truncated Myc protein (33), which most probably forms dimers with endogenous Max in the lysate (12). Additionally, bacterially expressed GST-Myc bHLH-Z fusion peptides (31) and Myc expressed in Malin in the lysate (12). Additionally, bacterially expressed GST-Myc binding efficiency (all of which probably bind DNA as homodimers) also bind to when formed, are non-functional in transcriptional activation (7) -2 position and C at position -3. According to these rules only -2 and -3, where purines are strongly favoured. Moreover, it cannot bind to the CPFl target sites in which the CACGTG is less discriminatory, may also repress genes not regulated by Myc.

REFERENCES

SHORT REPORT

Expression of cyclin D1 mRNA is not upregulated by Myc in rat fibroblasts

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Conflicting results have been published regarding the regulation of cyclin D1 mRNA in rat fibroblasts expressing a hormone-regulated Myc protein, MycER. We confirm that activation of MycER with oestrogen rapidly induces cyclin D1 mRNA, even in the presence of cycloheximide. However, we show that this is an artefact resulting from an oestrogen-activated transcriptional activation domain in the oestrogen receptor part of the MycER chimaera. First, addition of 4-hydroxy-tamoxifen (4OHT), which does not activate this domain, allows association of MycER with Max and induces cell proliferation in serum-starved Rat-1-MycER cells without affecting cyclin D1 mRNA levels or the activity of D1 promoter-luciferase constructs. Second, Rat-1 cells expressing a mutant MycER with a hormone-binding domain that still binds 4OHT but no longer binds oestrogen, are driven into the cell cycle in response to 4OHT but fail to up-regulate cyclin D1 mRNA. Finally, Rat-1 cells in which wild-type human c-Myc expression can be induced, also progress into the cell cycle without increased D1 mRNA expression.

Keywords: cyclin D1; Myc; oestrogen receptor; tamoxifen

The proto-oncogene c-myc is a key regulator of cell proliferation and death; for review see (Amati and Land, 1994). c-myc encodes a nuclear transcription factor (Myc) that dimerises with Max (Blackwood and Eisenman, 1991; Prendergast et al., 1991), binds to DNA of the sequence CAC(G/A)TG (Blackwell et al., 1990; Kerkhoff et al., 1991; Prendergast and Ziff, 1991; Solomon et al., 1993) and activates transcription (Amati et al., 1992; Kretzner et al., 1992). Further proteins have been identified that associate with Max, termed Mad and Mxi (Ayer et al., 1993; Zervos et al., 1993). In contrast, high expression of Mad or Mxi is often found in quiescent and differentiating cells (Ayer et al., 1993; Blackwood et al., 1992; Zervos et al., 1993). Max homodimers and Mad/Max or Mxi/Max heterodimers also bind to CAC(G/A)TG but act as repressors of transcription (Amati et al., 1992; Kretzner et al., 1992; Ayer et al., 1995; Schreiber-Agus et al., 1995).

Myc is rapidly induced by growth factors and its ectopic expression is sufficient to drive cells from quiescence into the cell cycle (Eilers et al., 1991). Several genes have been identified that are regulated by Myc (Benvenisty et al., 1992; Bello-Fernandez et al., 1993; Reisman et al., 1993; Gaubatz et al., 1994); however the targets that mediate Myc’s mitogenic effects have not been found.

Progress through the mammalian cell cycle is regulated by complexes between cyclins and cyclin-dependent kinases (cdk’s), for review see (Sherr, 1994). Expression of genes encoding these compounds is often low in quiescent cells and is in itself regulated in a cell-cycle or growth-factor dependent manner (Sherr, 1994). Therefore, these genes are candidates for mediating Myc action.

Activation of MycER, a chimaera between Myc and the hormone-binding domain of the human oestrogen receptor (Eilers et al., 1989) in confluent Rat-1A cells strongly induces cyclin A mRNA and to a lesser degree cyclin E (Jansen-Dürr et al., 1993). Similar results have been obtained with Rat-1A and Rat-6 cells that constitutively express Myc (Jiang et al., 1993; Hoang et al., 1994). Fibroblasts in which one copy of the endogenous Myc gene has been deleted, show a delay in the induction of both cyclin E and cyclin A mRNAs in response to growth factors (Hanson et al., 1994).

Conflicting results have been obtained regarding the regulation of cyclin D1 mRNA by Myc. Repression of cyclin D1 mRNA by constitutive Myc or MycER expression has been observed in Balb/c-3T3 and Rat-1 cells (Jansen-Dürr et al., 1993; Philipp et al., 1994) and in rat embryo fibroblasts (Lovec et al., 1994), although the mechanism remains unclear. Another report however has shown a rapid increase of cyclin D1 transcription in response to activation of MycER in serum starved Rat-1-MycER cells (Oaksh et al., 1994). We have reproduced these findings (not shown) and confirm that the addition of oestrogen to these cells results in a rapid but transient increase in cyclin D1 mRNA which is unaffected by pre-treatment with cycloheximide. The effect is not seen in control Rat-1 cells nor in cells expressing a biologically inactive MycER (Stone et al., 1987; Eilers et al., 1989) in which residues 106–143 of Myc are deleted (not shown).

These data strongly suggest that the regulation of cyclin D1 in these cells is mediated by a direct interaction between MycER proteins and regulatory elements of the cyclin D1 gene.

Previous studies have shown that the hormone-binding domain of the oestrogen receptor has multiple functions, including hormone-binding (Kumar et al., 1986), dimerization (Fawell et al., 1990a), transcriptional activation (Webster et al., 1988) and a cis-acting ‘inactivation’ function, which may result from the ability of this domain to bind the Hsp90 protein in a hormone-dependent manner (Picard et al., 1988). In the context of the MycER chimaera, the inactivation

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function blocks the association of MycER with Max unless ligand is bound (Philipp et al., 1994). Not all functions of the hormone-binding domain are activated by all ligands that bind to it (e.g. Fawell et al., 1990b). In particular, transcriptional activation by hormone-binding domain is specifically activated by oestrogen, but not by 4OHT (Berry et al., 1990) although both block 'cis-inactivation'. Since 4OHT still releases MycER to associate with Max but does not allow transcriptional activation by the ER domain, it is a specific agonist of MycER (Eilers et al., 1989; Philipp et al., 1994). To demonstrate that a particular gene is induced by Myc, it is therefore of paramount importance to demonstrate that it is induced in

Figure 1  Effects of oestrogen and 4OHT on MycER function and cyclin D1 expression in Rat-1-MycER cells. (A) Association of Myc and Max in Rat-1 MycER cells after treatment with 4OHT or oestrogen. Cell lysates were immunoprecipitated with a polyclonal anti-Max antibody (91/3; kind gift of B Lüscher) in the absence (NP40) or presence (RIPA) of non-ionic detergents. Precipitates were resolved by SDS-PAGE, blotted and probed with a monoclonal anti-Myc antibody 9E10 (Evan et al., 1985). (pre; preimmune serum; Max: anti Max antibody; oes: 17β-oestradiol).

B FACSCAN analysis of Rat-1-MycER cells. Cells were starved in 0% PCS for 48 h and treated with either 4OHT, oestrogen or left untreated for 17 h. Cells were trypsinised, washed in PBS, fixed in 70% ethanol, washed again and treated with 1 mg ml⁻¹ RNase for 15'. Cells were then resuspended in 50 μg ml⁻¹ propidium iodide and analysed on a FACStar (Becton Dickinson). (C) Cyclin D1 expression in Rat-1 MycER cells treated with 4OHT or oestrogen. Cells were grown to confluence, serum starved for 60 h in medium containing 0.1% PCS and stimulated with either oestrogen or 4OHT. Shown is the ratio of cyclin D1 to GAPDH mRNA from duplicate samples as determined by quantification (using a phosphorimager) of a Northern blot from RNA isolated after the indicated times. (D) A stably integrated cyclin D1 promoter fragment is activated by the addition of oestrogen, but not 4OHT in Rat-1-MycER cells. Constructs consisting of either an 8.1 kb BamHI/Nhel fragment or a 4.5 kb Xbal/Nhel fragment of the murine cyclin D1 promoter (Smith et al., 1995) cloned into the Kpnl site of pGL2-Basic (Promega) were obtained from R Smith and C Dickson; the ATG downstream of the transcription start site was mutated by digestion with Ncol followed by Mung Bean nuclease to allow translation of luciferase only. Rat-1-MycER cells were co-transfected by calcium phosphate co-precipitation with 10 μg of the 8.1 kb D1 promoter construct and 1 μg of J6-Puro (Amati et al., 1993). Following puromycin selection, resistant cells were pooled, grown to confluence, serum-starved for 48 h and then treated with either oestrogen or 4OHT. Shown is the mean luciferase activity normalised for protein concentration in triplicate extracts from cells harvested at the indicated times. The error bars represent the standard error of the means.
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Response to 4OH T in cells carrying the MycER chimaera (Eilers et al., 1991) or preferably that it is induced by wild type Myc. We now report that cyclin D1 fails these critical tests.

Two controls were used to demonstrate that addition of 4OH T activated Myc function in the MycER chimaeras: first, we made use of the observation that the attached hormone-binding domain blocks dimerization of Max with MycER in the absence of hormone (Philipp et al., 1994). MycER protein was present in anti-Max immunoprecipitates from cells that had been pre-treated with either oestrogen or 4OH T, but not in ones from control cells (Figure 1A). No MycER proteins were detected in precipitates with pre-immune serum or with anti-Max antibodies in the presence of ionic detergents (RIPA buffer) that have been shown to disrupt the association between Myc and Max (Blackwood et al., 1992). Taken together, these results document a specific association between MycER and Max after the addition of either oestrogen or 4OH T. To demonstrate that MycER proteins were indeed functional after induction with 4OH T, FACSCAN analysis was performed in serum-starved cells before and after stimulation with either oestrogen or 4OH T. The results from these experiments (Figure 1B) show that the addition of either ligand leads to cell cycle induction by MycER proteins.

To analyse whether the trans-activational activity of the ER domain contributes to cyclin D1 regulation, RNA was prepared from serum-starved Rat-l-MycER cells before, and at several time points after, stimulation with either oestrogen or 4OH T. Duplicate samples were prepared and analysed by Northern blotting. Figure 1C shows a quantification of the amount of cyclin D1 mRNA relative to an internal standard (GAPDH) from such an experiment. The data show that induction of cyclin D1 mRNA was observed only in response to oestrogen and not to 4OH T. These observations demonstrate that the transcription activation function of the ER domain contributes significantly to the induction of cyclin D1 mRNA in MycER cells. Essentially identical results were obtained in density-arrested Rat-lA-MycER cells and serum-starved Balb-c/3T3 cells (P Steiner and ME, unpublished).

To explore whether the effects of MycER on Cyclin D1 are due to regulation at the promoter level, we introduced an 8.1 kb cyclin D1 promoter-luciferase reporter into Rat-l cells. The results (Figure 1D) show that addition of oestrogen, but not 4OH T, led to a specific transient increase in luciferase activity within three hours. Similar results were obtained with a 4.5 kb cyclin D1 promoter construct (not shown). Taken together with the previous results, these data suggest that Myc can target the transactivation domain of the oestrogen receptor to the cyclin D1 promoter.

To confirm that upregulation of cyclin D1 mRNA is due to the hormone-binding domain of MycER, we made use of a cell line expressing a MycER mutant, termed MycER* (kind gift of Trevor Littlewood) in which the hormone-binding domain carries a glycine to arginine substitution at position 525 that renders it unable to bind oestrogen while leaving its affinity for 4OH T essentially unaltered (Danielian et al., 1993; Littlewood et al., 1993). Immunoprecipitations from Rat-l-MycER* cells showed that MycER* was
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Figure 3 Cyclin D1 is not induced in Rat-1 GV-Myc5 cells. (A) FACSCAN of cells grown to confluence, serum starved for 48 h and treated with either serum (10%), 4OH T or left untreated for 24h. (B) Phosphorimager quantification of a Northern blot probed for cyclin D1, Myc and GAPDH from cells grown to confluence, serum starved for 48 h and treated with 4OH T for the indicated time. Rat-1 cells were infected with MV-GalERVP (a retrovirus which expresses GalERVP) (Braselmann et al., 1993), G418 resistant cells were pooled and co-transfected with I6-puro and the same plasmid into which the human c-Myc cDNA downstream of this promoter and generated cell controls in which Myc expression is induced by 4OH T. Although the cells progress into the cell cycle response to Myc expression (Figure 3A), cyclin D1 mRNA is not regulated (Figure 3B). Control cells lacking the Myc cDNA are unaffected by 4OH T (not shown).

Our results indicate that the induction of cyclin D1 mRNA by MycER requires the transcriptional activation functions of both the hormone binding domain of the oestrogen receptor and the Myc portion of the MycER chimera. This induction is resistant to the previous addition of cycloheximide, suggesting that no additional protein synthesis is required to mediate the effect. Thus, we infer that native Myc may interact directly with regulatory elements of the cyclin D1 gene, but fails to activate expression of cyclin D1. Instead, expression of Myc and MycER in the absence of hormone leads to a constitutive repression of cyclin D1 mRNA levels (Jansen-Dürr et al., 1993; Lovec et al., 1994; Philipp et al., 1994). Whether the oestrogen-induced activation of cyclin D1 transcription by MycER and the repression by Myc are due to similar or distinct mechanisms remains unclear. The data also argue strongly against a simple model in which the growth factor-induced increase in cyclin D1 mRNA is mediated by Myc protein. This is supported by the observation that deletion of one copy of the endogenous c-myc gene delays serum-induced expression of cyclins E and A, but not cyclin D1 (Hanson et from BJ3-Myc3 (Amati et al., 1993) had been cloned (GC-Myc). Individual GC-Myc colonies were isolated and screened for conditional Myc expression by assaying for induction of apoptosis in 4OH T treated, serum starved cells. Positive clones were then assayed for progress into the cell-cycle in response to 4OH T. A positive clone, number 5 was used in further studies
In addition, the growth-factor responsive portion of the cyclin D1 promoter does not contain an E-box element and disruption of the E-box in the cyclin D1 promoter has no effect on the growth factor mediated induction of cyclin D1 mRNA (Herber et al., 1994).

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