Synergistic Regulation
of
cdc2 and Cyclin A
Gene Transcription
by
Co-operating Oncogenes

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Abstract

Co-operative transformation of Schwann cells and rat embryo fibroblasts by oncogenic ras and the viral oncoproteins SV40 large T, or Adenovirus E1A is accompanied by synergistic regulation of specific cell cycle regulatory genes. For example, in Schwann cells, ras inhibits proliferation, and as a prerequisite for the growth arrest, inhibits expression of cell cycle regulators such as \textit{cyclin A} and \textit{cdc2}. In contrast, in cells fully transformed by \textit{ras} and large T (or E1A) the same cell cycle regulators are over-expressed.

In transfection experiments it was shown that \textit{ras} and E1A regulate transcription of the \textit{cdc2} and \textit{cyclin A} genes. In proliferating cells the activities of the \textit{cdc2} and \textit{cyclin A} promoters are suppressed up to ten-fold by the \textit{ras} oncogene. E1A can activate the same promoters in mitogen-starved cells. Moreover, together \textit{ras} and E1A synergistically stimulate both promoters in contact-inhibited cells. All three types of regulation are mediated by distinct promoter elements which are conserved in both \textit{cdc2} and \textit{cyclin A} promoters and are sufficient to confer the oncogene-induced responses. Possible mechanisms involved in the regulation through these elements are investigated and discussed.

The synergistic regulation of cell cycle gene transcription by co-operating oncogenes not only provides a link between growth control signals and the cell cycle, but also shows that distinct oncogene-induced signals converge on common targets. Such signal integration processes may at least in part explain the synergistic nature of oncogene co-operation in cell transformation.
Acknowledgements

-And I expect that little bundle of yours is all you’ve got in the world?

DOSTOEVSKY, THE IDIOT¹

It has been five years now since I ran away from Russia with my "little bundle" and have set my feet into the lab. It is now very difficult for me to find the right words to express how happy and exciting my life has been ever since. It might not look as such for the uninitiated: overall, it consisted of little but cell cultures, medium changes and acrylamide gels (about a dozen a day). However, for me it was definitely more exciting then even the sudden collapse of my fossil empire state. I owe my happiness to the people around me, and knowing them has become my most precious life asset.

I am most grateful to Hucky, who made it all possible and showed unprecedented patience in teaching me some useful bits of science and even more useful bits of general wisdom. Special thanks to Colleen, Hannah and Rubin, who were, together with Hucky, the dear hosts and friends at my first home in England.

I am grateful to all the on- and off-coming members of the lab, Ed, Mary, Shaun, Carolyn, Bruno, David Solomon, Frank, Susan, Sharon, Alison and Andreas for support, advice, criticisms, probes, medium changes, coffee from the downstairs vending machines, many hours of joy and lots of happy memories. I must take a Russian Orthodox stand and publicly apologise for all the trouble I caused these dear people: mostly, radioactive spills and unauthorised mess. I will leave in the lab all my drawings. I hope that they will occasionally boost the moral of the next generation. I also want Frank, my dear and gentle bench neighbour, to inherit all the junk he will find useful at

my work place (By the way, Frank, it has a couple of lucky charms hidden in it. So, I would think, it is worth a look...).

Finally, I thank Harriet, my dear wife, and our parents for their love and unlimited support in my weird life with the science on the doorstep.
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Abbreviations

AMV  Avian Myeloblastosis Virus
bp   base pair
BSA  Bovine Serum Albumin
CBF  Core Binding Factor
°C   degrees Centigrade
CDI  Cyclin-Dependent-Kinase Inhibitor
Cdk  Cyclin-Dependent Kinase
259 CM Conditioned Medium from IN/259 Cells
cm   centimeter
cpm  counts per minute
DMEM Dulbecco's Modified Eagle's Medium
DMS Dimethyl Sulphate
DMSO Dimethyl Sulfoxide
DNA Deoxyribonucleic Acid
DTT Dithiothreitol
(d)NTP (2'-Deoxy)Nucleotide 5'-Triphosphate
(d)ATP (2'-Deoxy)Adenosine 5'-Triphosphate
(d)CTP (2'-Deoxy)Cytosine 5'-Triphosphate
(d)GTP (2'-Deoxy)Guanosine 5'-Triphosphate
dTTP 2'-Deoxy Thymidine 5'-Triphosphate
EDTA Ethylenediaminetetraacetic Acid (Disodium Salt)
EGF  Epidermal Growth Factor
EGTA Ethylene Glycol Bis(β-aminoethylether)N,N,N',N'-tetraacetic Acid
EMSA Electromobility Shift Assay
ERE E1A Response Element
EtBr Ethidium Bromide
<table>
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<tr>
<td>FCS</td>
<td>Fetal Calf Serum</td>
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<tr>
<td>g</td>
<td>gram</td>
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<tr>
<td>GAP</td>
<td>GTPase activating protein</td>
</tr>
<tr>
<td>GDS</td>
<td>Guanine Nucleotide Dissociation Stimulator</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-Hydroxyethyl)-1-Piperazine Ethanesulfonic Acid</td>
</tr>
<tr>
<td>hr</td>
<td>hour(s)</td>
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<tr>
<td>HSV</td>
<td>Herpes Simplex Virus</td>
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<tr>
<td>kb</td>
<td>kilobase</td>
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<tr>
<td>MAPK</td>
<td>Mitogen-Activated Protein Kinase</td>
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<tr>
<td>Mo-MuLV</td>
<td>Moloney Murine Leukemia Virus</td>
</tr>
<tr>
<td>M.W.</td>
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<tr>
<td>n</td>
<td>nano</td>
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<tr>
<td>NP40</td>
<td>Nonionic Detergent P-40</td>
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<td>ONPG</td>
<td>O-Nitrophenyl-b-D-Galactopyranoside</td>
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<td>p</td>
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<td>Polyethylene Glycol</td>
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<td>PIPES</td>
<td>1,4-Piperazinediethane Sulfonic Acid</td>
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<td>PLL</td>
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<tr>
<td>PMSF</td>
<td>Phenylmethylsulphonyl Fluoride</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>RNA</td>
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<td>RTK</td>
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<tr>
<td>Sarkosyl</td>
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<tr>
<td>sec</td>
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<td>w/v</td>
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Chapter 1

Introduction
1.1 Multistep Tumorigenesis and Oncogene Co-operation

There is persuasive evidence that cancer arises as a result of a multistep process, which involves the accumulation of multiple genetic alterations, including chromosomal translocations, gene amplification, point mutations and deletions (Rabbitts, 1989). These genetic changes cause activation or inactivation of oncogenes and tumor suppressor genes, respectively (Bishop, 1991; Marshall, 1991; Cooper, 1990).

The first evidence that different oncogenic lesions can co-operate in multistep tumorigenesis was the finding that single oncogenes were unable to fully transform primary rodent cells. Instead, certain combinations of cellular (ras and myc), or cellular and viral (ras and Adenovirus E1A) oncogenes were required to induce tumorigenic proliferation (Land, 1983; Ruley, 1983).

Oncogene co-operation has also been observed in vivo. In transgenic mice expressing two co-operating oncogenes under the control of tissue-specific promoters the incidence of tumours is greatly enhanced when compared with mice bearing a single oncogene (Sinn, 1987; Hanahan, 1989). A similar effect can be observed in mice lacking two tumor suppressor genes such as Rb (retinoblastoma) and p53 (Williams, 1994). The causal involvement of multiple oncogenes in the step-like progression of cancer was demonstrated in reconstituted organs (Thompson, 1989). In these experiments single oncogenes introduced into few cells of the reconstituted organ were able to induce distinct types of premalignant lesions, i.e. dysplasia, or hyperplasia. In contrast, when introduced in combination the oncogenes primarily induced carcinomas.

Oncogene cooperation can also be observed with RNA and DNA tumour viruses. A number of acutely transforming avian retroviruses have acquired a pair of cooperating oncogenes, i.e. AEV-ES4 (oncogenes v-erbA and v-erbB); E26 (v-myb+ets); and MH2 (v-mil/raf and v-myc) (Palmieri, 1989).
Inactivation of one of the two genes results in loss of tumorigenicity. For example, truncation of the ets element in the gag-myb-ets gene of the E26 virus ablates the erythroid transformation potential of the virus (Nunn and Hunter, 1989). Moreover, the studies of the transforming proteins of DNA tumor viruses also strongly support the idea that oncogene cooperation is an essential feature of oncogenesis. Some of the first evidence for the ability of oncogenes to cooperate came from the demonstration that complete morphological transformation by adenovirus requires the combined action of the E1A and E1B viral proteins (Houweling, 1980; van der Elsen, 1983). In another early example of co-operation, polyomavirus large T and middle T antigens were shown to be both required for the transformation of primary rat embryo fibroblasts (Rassoulzadegan, 1982).

1.2 Co-operating Oncogenes and their Functions

The products of cellular oncogenes and their normal counterparts, the proto-oncogenes, are crucial elements of the cellular signalling network regulating cell proliferation and differentiation. They resemble growth factors, growth factor receptors, adaptors and GTP-binding proteins, cytoplasmic protein kinases and transcription factors. Many of the proto-oncogenes are directly implicated in mitogenic responses as highly conserved elements of the cellular signalling network and ultimately control a specific set of genes (Hunter, 1991; Lewin, 1991).

The products of DNA tumor virus oncogenes act by disableing cellular tumor suppressors through direct physical interactions. The gene products of tumour suppressor genes are negative regulators of cellular proliferation. They exert their function either, like Rb, through interaction and inactivation of transcription factors involved in cell cycle control, or like p53, through
activation of cell cycle inhibitory genes and activation of programmed cell
death (Hollingsworth, 1993; Hunter, 1993; Hinds and Weinberg, 1994; El-
Deiry, 1993; Clarke, 1993).

Most frequently, combinations of co-operating oncogenes consist of a
membrane-bound or cytoplasmic signal transducer, such as ras, and a nuclear
protein involved in transcriptional control, such as myc or EIA (Hunter,
1991). To begin to understand the molecular basis of oncogene co-operation,
the functions of the genes involved have to be considered. Within the
framework of this thesis, it is relevant to concentrate on ras and EIA as
paradigmatic examples.

1.3 The Ras Signal Transduction Pathway

The three human ras genes encode four highly related proteins (H-, N-, K4A- and K4B-ras) which exhibit high affinity for guanine nucleotides and
function as molecular switches that cycle between the active GTP- and the
inactive GDP-bound form. Oncogenic ras proteins are locked in the GTP-
bound form by point mutations and are constitutively activated (Barbacid,
1987). The ras proteins operate as critical downstream targets of mitogen-
activated tyrosine kinases (McCormick, 1993; Feig, 1993), and in turn, the
signals from activated ras are mediated, at least in part, by a cascade of
serine/threonine kinases (Roberts, 1992), which ultimately controls the
activities of transcription factors (Sistonen, 1989; Pulverer, 1991).

Ras proteins are key mediators of signals that are transmitted from stimulated
growth factor receptors (e.g., the EGF and PDGF receptors) and nonreceptor
tyrosine kinases (e.g., Lck), as well as from some G protein-regulated
serpentine receptors (Satoh and Kaziro, 1992). The first evidence for such an involvement was the finding that anti-ras neutralizing antibodies, which are potent blockers of ras function, were also shown to block the mitogenic activity of EGF or PDGF (Mulcahy, 1985; Kung, 1986) or the transforming activities of the v-src or v-fms tyrosine kinase oncogenes (Smith, 1986). Similarly, a dominant negative ras (Ras17N) protein, which antagonizes endogeneous ras function, was also capable of blocking the mitogenic stimulation of receptor tyrosine kinases (RTKs) (Szeberenyi, 1990; Medema, 1991; Schwighoffer, 1993; Cai, 1990). Further support for the transmission of tyrosine kinase signals through ras was provided by a number of observations showing that both the transient stimulation of tyrosine kinases by growth factors, or cellular transformation by constitutive activation of tyrosine kinase oncogenes, lead to elevated levels of ras-GTP (Satoh, 1992).

Ras proteins possess intrinsic GTPase and GDP/GTP exchange activities. However, these activities are too low to account for the rapid and transient GDP/GTP cycling that occurs during mitogenic stimulation. Instead, a complete model for ras function includes regulatory proteins that control the rate of GTP/GDP cycling (Downward, 1992; Sato and Kaziro, 1992; Bourne, 1990b). These regulatory proteins include GTPase activating proteins (GAPs) which stimulate hydrolysis of bound GTP to GDP (Downward, 1992; Hall, 1992; Bollag, 1992; McCormick, 1991), and guanine nucleotide exchange factor proteins (GDS) such as Sos (Bowtell, 1989; Chardin, 1993) which promote the replacement of bound GDP with GTP.

The rapid and transient elevation of ras-GTP levels triggered by various extracellular stimuli is believed to occur via either inhibition of GAP activity or stimulation of GTP exchange factors (Torti, 1992; Downward, 1990; Li,
Activation of \textit{ras} GDS, i.e. Sos, after extra-cellular stimulation involves adaptor proteins such as Grb2 (Rozakis-Adcock, 1992; Buday, 1993). In unstimulated cells Grb2 is believed to exist as a cytoplasmatic complex with Sos: the two \textit{src} homology 3 (SH3) domains of Grb2 recognize proline rich SH3 binding motifs present in the C-terminus of SOS. Upon ligand stimulation, the Grb2 associates with the autophosphorylated tyrosine kinase (TK) via its SH2 domain and consequently recruits SOS to the plasma membrane. Since the GDP/GTP exchange activity of SOS is not regulated during this translocation event, Grb2 mediates activation of \textit{ras} by TKs through regulating the association of SOS with membrane-bound \textit{ras} (Egan, 1993; Lowenstein, 1992; Simon, 1993; Buday, 1993; Gale, 1993; Li, 1993; Olivier, 1993). In addition to Grb2, a second SH-2-containing protein, designated SHc, has also been implicated in linking activated tyrosine kinases with \textit{ras} (Pelicci, 1992).

\textit{Ras} activates a cascade of serine/threonine kinases which transmits tyrosine kinase-induced signals to the nucleus. The first member of this cascade is the Raf-1 proto-oncoprotein which has been placed downstream of \textit{ras} by a variety of observations. First, activated \textit{ras} can result in hyperphosphorylation of Raf-1 in the absence of mitogenic stimuli (Morrison, 1988). Subsequently, it was shown that a dominant negative Raf-1 mutant protein could block the transforming activity of oncogenic \textit{ras} (Heidecker, 1992), as well as transcription activation from \textit{ras} response elements (Bruder, 1992). Conversely, a dominant negative \textit{ras} (17-Asn) could block the ability of a variety of activated RTKs to activate Raf-1 (Wood, 1992). Genetic studies in \textit{D.melanogaster} have implicated both \textit{ras} and Raf proteins as critical downstream elements required for the signalling from torso and sevenless receptor tyrosine kinases (Dickson, 1992, Sprenger, 1993). Similarly, a Raf homologue has been identified recently as the downstream
component of Ras signalling in C.elegans (Han, 1993). Finally, Raf-1 has recently been shown to complex with activated Ras proteins (Moodie, 1993; Hallberg, 1994), which translocate Raf-1 to the cell membrane. Here it becomes activated via a yet unknown mechanism (Leevers, 1994).

Raf-1 functions as an activator of mitogen-activated protein kinases (MAPKs). In fact, it may itself act as a MAPKKK, to directly activate the dual specificity MAPK kinases (or MEKs), which in turn activate the MAP kinases (or Erks; Kyriakis, 1992; Liaw, 1990; Adams, 1992). Evidence for the order of this cascade comes from both biochemical and genetic studies. For example, both MAPKK and MAPKs are constitutively activated in Raf-transformed NIH 3T3 cells (Dent, 1992). Furthermore, a bacterially expressed v-Raf phosphorylates and activates MAPKK in vitro (Dent, 1992). MAPKKs have recently been cloned from a variety of sources and shown to be able to phosphorylate MAPKs in vitro on tyrosine as well as threonine residues (Wu, 1993; Crews, 1992a; Crews, 1992b; Seger, 1991). Both phosphorylations are required to activate the serine/threonine kinase activity of MAPK.

The signalling cascade of serine/threonine kinases as identified in mammalian cells is remarkably well-conserved in other organisms (Sprague, 1992). For example, a MAPK cascade which mediates the pheremone-stimulated, G protein controlled mating response has been found in S. cerevisiae and S.pombe (Leevers, 1992; Sprague, 1992; Errede, 1993a). STE7 and byr1 are yeast homologs of MAPKK, while FUS3/KSS1 and spkl are the MAPK homologues in these two organisms, respectively (Errede, 1993b; Elion, 1993). Similar MAPK cascades have also been implicated in receptor tyrosine mediated signalling pathways in C.elegans and D.melanogaster (Ahn, 1992; Tsuda, 1993).
Although the signalling pathway that connects tyrosine kinaes, via ras, to the MAPK kinase cascade has been described as linear, it is apparent that such a linear pathway is an oversimplification. For example, MAPK is not always activated in ras-transformed cells (Gupta, 1992), and at least some G protein mediated signalling from serpentine receptors can activate MAPK in a Ras-independent fashion (Gallego, 1992). Further complexity is demonstrated by the recent isolation of a MEK kinase which may trigger MAPK activation by a pathway which is distinct from the Raf-1 mediated pathway (Lange-Carter, 1993). Another complexity of the MAPK cascade arises from the observation that MAPKs can also phosphorylate Raf-1 kinase, suggesting the existence of a negative feedback mechanism regulating the MAPK cascade (Robbins, 1993).

Ultimately, MAPK phosphorylates and activates nuclear transcription factors known to be involved early responses to ligand-mediated receptor activation such as c-Jun (Pulverer, 1991; Baker, 1991;), and the ets-family protein p62TCF (elk-1) (Gille, 1992; Treisman, 1994). Transcriptional activation by these phosphorylated transcription factors appears to depend on a physical link to the transcriptional preinitiation complex which is provided by adaptor molecules, such as the CREB binding protein (CBP) (Chrivia, 1993; Arias, 1994). These adaptor proteins include p300, a protein known to be targeted by the Adenovirus E1A oncoprotein (Eckner, 1994; Arany, 1994; see also below).

1.4 Adenovirus Transforming Protein E1A

The early region 1 (E1) of adenoviruses consists of two transcriptional units, E1A and E1B, which are both required for adenovirus-induced transformation
of primary rodent cells (Flint, 1980). E1A is a multi-functional protein. One of its activities is to target and inactivate the tumour suppressor gene \( Rb \) (retinoblastoma; Whyte, 1988). E1B binds and inactivates \( p53 \), another tumour suppressor gene product (Sarnow, 1982). The E1A region alone can immortalize primary rodent cells, but for complete morphological transformation E1B is required in addition (Houweling, 1980; van der Elsen, 1983). The E1B gene encodes two major proteins: the 21 kD and 55 kD tumour antigens, both of which are independently capable of cooperating with the E1A gene products in transformation (Bernards, 1986; Barker and Berk, 1987). In transformed cells the E1A gene is transcribed into two differently spliced mRNAs (12S and 13S) (Berk and Sharp, 1978). These mRNAs code for two proteins the larger of which contains an additional domain (Perricaudet, 1979). Comparison of E1A proteins from different adenovirus serotypes has led to the identification of three conserved regions (CR) (Kimelman, 1985). Mutational analysis has demonstrated that these conserved regions correspond to three major functional domains within the E1A protein (Kimelman, 1986).

The CR1 and CR2 domains are involved in the transforming capacity of E1A (Lillie, 1986; Schneider, 1987; Jelsma, 1989). On the other hand, CR3, which is present only in the larger 13S mRNA products, is not necessary for transformation or immortalisation of primary cells (Lillie, 1986; Moran, 1986; Schneider, 1987). Of the conserved regions required for transformation, CR1 is able to trigger DNA synthesis upon adenovirus infection of quiescent primary baby rat kidney cells, but for cellular proliferation complimentation with CR2 is required (Zerler, 1987; Moran and Zerler, 1988). Although, transformation requires in general both CR1 and CR2, some experiments indicate an independent role of CR1 and CR2. For example, E1A mutants in CR1 or CR2, together with \( ras \) still can induce foci in primary rat
embryo fibroblast cultures (Svensson, 1991) and CR1, but not CR2, is sufficient to maintain cellular proliferation at the non-permissive temperature in ts-SV40-large T-immortalized rat embryo fibroblasts (Riley, 1990).

In addition to the Retinoblastoma protein (p105Rb) the CR1 and CR2 domains interact with a number of other cellular regulatory proteins, including p130 and p107 (structurally related to Rb), p58cyclinA and p33cdk2 protein kinase, as well as p300, p90, p80, p50, p40, p28 (Egan, 1988; Whyte, 1988; Giordano, 1989; Pines and Hunter, 1990; Giordano 1991a; Giordano, 1991b; Herrmann, 1991; Kleinberg and Schenk, 1991; Svensson, 1991; Tsai, 1991).

Rb, p107 and p130 inhibit the activity of of certain transcription factors, such as the members of the E2F family, through physical interaction in a cell cycle-dependent manner (Chellappan, 1991; Shirodkar, 1992; Cobrinick, 1993). In turn, El A disrupts these complexes through formation of more stable interactions with Rb, p107 and p130 and constitutively activates these transcription factors (Moran, 1993; Nevins, 1994). Moreover, the cyclin-dependent kinase 2 (p33cdk2)/cyclinA complex, a key regulator of the cell cycle (Tsai, 1991), is found to be associated with El A (Tsai, 1991, Pagano, 1992), probably through its interaction with p107 (Devoto, 1992; Ewen, 1992; Faha, 1992).

Together with the CR1 domain, the N-terminus of El A is involved in binding a nuclear protein of 300 kD (p300) (Egan, 1988; Subramanian, 1988; Whyte, 1989; Rikitake and Moran, 1992; Eckner, 1994). Presumably this interaction also plays a role in cell transformation, since N-terminal mutations in El A that disable binding to p300 also block El A transforming activity (Subramanian, 1988; Whyte, 1989). Moreover, binding of El A to p300 induces G0/G1 exit, blocks differentiation and inhibits certain transcriptional
enhancer elements (Moran, 1993). p300 is highly related to the transcriptional coactivator CBP (Chrivia, 1993), which links members of the CREB/ATF family of transcription factors with the general transcription factor TFIIID (Kwok, 1994).

The transforming function of CR1 in established cell lines also has been associated with the ability of E1A to alter the expression of several growth factor-inducible genes, like collagenase, stromelysin, JE, c-myc, c-jun and junB (van Dam, 1989; Offriga, 1990; van Dam, 1990). Although c-jun has been shown to interact with CBP (Arias, 1994), it remains unclear whether such regulation may involve p300 or related factors.

1.5 Oncogene Co-operation and Regulation of the Cell Cycle

Investigation of oncogenes has provided seminal insight into their function. However, so far little has been learned about how these genes cooperate in multi-step tumorigenesis. This is due to the fact that rather than cumulative, cooperation of oncogenes is mainly of synergistic nature. Analysis of such mechanisms requires approaches where the action of single oncogenes can be directly compared with the effects of multiple oncogenes. Appropriate experimental systems have been established in rat Schwann cells (Ridley, 1988) and REF52 cells, an established cell line from rat embryo cells (Hirakawa and Ruley, 1988). In both systems ras and the simian virus 40 (SV40) oncogene large T (or adenovirus E1A) are required together to induce full transformation. Similar to E1A and E1B, SV40 large T antigen, also binds and inactivates both tumour suppressors Rb and p53 (Ludlow, 1989; Sarnow, 1982).
When acting together, \textit{ras} and E1A (or SV40 large T) co-operate in Schwann and REF 52 cells inducing cellular growth in soft agar and proliferation in absence of exogenous mitogens, while the viral oncoproteins alone only reduce growth factor requirements. In contrast, oncogenic \textit{ras} alone, induces growth arrest in these cells. Thus, the presence of the co-operating viral oncogene fundamentally alters the cellular response to activated \textit{ras}. In fact, \textit{ras} alone acts as a growth suppressor, while only in presence of SV40 large T or E1a the cells become sensitive to the oncogenic potential of \textit{ras} (Ridley, 1988; Hirakawa and Ruley, 1988).

The nature of the \textit{ras}-induced growth arrest was studied in Schwann cells, expressing oncogenic \textit{ras} together with the temperature-sensitive mutant of SV40 large T, \textit{tsA58} (Tegtmeyer, 1975). By shifting the cells to the non-permissive temperature of 39°C, cells expressing \textit{ras} without functional SV40 large T were found to reversibly arrest in G1 and G2 phases of the cell cycle. Instead, cells harbouring the \textit{tsA58} gene alone continued to proliferate. These and protein microinjection experiments indicate that oncogenic \textit{ras} can block the onset of both DNA synthesis (S phase) and mitosis (M phase) in normal Schwann cells (Ridley, 1988).

The entry into S and M phases of the cell cycle is controlled in all eukaryotes by a mechanism which is based on a family of highly conserved serine/threonine protein kinases. Activation of the catalytic subunits, the cyclin-dependent kinases (Cdks), requires association with cyclins, the regulatory subunits of the kinase complexes. Distinct kinase complexes become active at different checkpoints during the cell cycle. The succession of these checkpoints enables the cell to control its proliferation in accordance with the extra-cellular stimuli and with the successful accomplishment of the previous stages of the cycle (Nurse,
The Cdks are regulated by the available cyclin concentration and by various phosphorylation events on the catalytic subunit (Hartwell and Weinert, 1989; Murray, 1992). The cyclin-Cdk complexes can also be negatively regulated by inhibitory proteins, termed cyclin-dependent-kinase inhibitors (CDIs). These proteins have been shown to exert their function by direct binding to the active cyclin-Cdk complexes (Serrano, 1993; Harper, 1993; Gu, 1993; Pines, 1993).

The first Cdk, *cdc2*, was identified in the fission yeast *S. pombe*, as a gene required for progression from G1 to S, and G2 to M phases of the cell cycle, as there is only one Cdk gene in yeast (Nurse and Bissett, 1981; Lee and Nurse, 1988). In mammalian cells various related Cdks act at different stages of the cell cycle. The human *cdc2* gene was first isolated through its ability to compensate a deficiency in yeast *cdc2* (Lee and Nurse, 1987), and later has been shown to encode the catalytic subunit of the major protein kinase active at the transition from G2 to mitosis (Pines and Hunt, 1987; Murray and Kirshner, 1989; Th'ng, 1990). Cdk2, which is approximately 65% identical to *cdc2*, is mostly active in late G1 and during S phase (Fang and Newport, 1991; Tsai, 1993). Experiments using frog cell-free extracts indicate that Cdk2 but not *cdc2* is required for DNA replication (Fang and Newport, 1991). Other members of the family, Cdk4 and Cdk5 are thought to act in mid to late G1.

In higher eukaryotes the members of the cyclin family can be broadly subdivided into G1 (termed, *D* and *E*) and mitotic (termed, *A* and *B*) cyclins. The respective cyclins are selectively or maximally expressed during the indicated stages of the cell cycle and bind preferentially to distinct Cdk proteins (Pines, 1993). Both cyclins *A* and *B* interact with *cdc2*, forming the
complexes which are active in late G2 phase and are able to induce oocyte maturation or mitosis in a Xenopus cell-free system (Swenson, 1986; Pines and Hunt, 1987; Minshull, 1989; Murray and Kirschner, 1989). However, cyclin A is not only involved in the G2/M transition, since in human cells, disruption of cyclin A function inhibits DNA replication (Girard, 1991; Pagano, 1992; Zindy, 1992). Consistent with these results cyclin A but not cyclin B has been shown to form a kinase complex with Cdk2 during late G1 and S phase (Tsai, 1991). The mammalian G1 cyclins, designated D1, -2, -3 and E, were isolated through their ability to complement conditionally defective CLN function in S. cerevisiae (Xiong, 1991; Lew, 1991; Koff, 1991). The mouse cyclin D genes are more highly related to their human counterparts that to one another (Inaba, 1992), implying that they are not functionally redundant. Moreover, the D type cyclins are differentially expressed in various cell lineages (Matsushime, 1991; Kiyokawa, 1992; Cocks, 1992; Motokura, 1992; Ajchenbaum, 1993). Cyclin D binds to at least three members of the Cdk family: Cdk2, Cdk4 and Cdk5, although preferentially to Cdk4 (Matsushime, 1992; Xiong, 1992). When growth factor-deprived cells are restimulated to enter the cell cycle, the D-type cyclins generally appear earlier in G1 than cyclin E, although in continuously proliferating cell population their levels may oscillate only minimally (Matsushime, 1991). In contrast, cyclin E expression is periodic and peaks at the G1/S transition when it forms active complexes with Cdk2 (Lew, 1991; Koff, 1991; Koff, 1992). Cyclin E forms complexes with p107, a protein related to Rb, and with the E2F transcription factor (Lees, 1992), and has been implicated in the initiation of DNA replication (Dulic, 1992).

Analysis of the ras-induced G1 and G2 arrest in Schwann cells revealed alterations in the expression of certain key cell cycle regulators. For example,
the protein and mRNA levels of \textit{cyclin A} and \textit{cdc2} are suppressed in the arrested cells. In contrast, in cells expressing \textit{ras} and functional large T, \textit{cyclin A} and \textit{cdc2} protein and mRNA levels are overexpressed, while in cells expressing large T alone their expression is not induced. Moreover, it has become clear that at least the suppression of \textit{cyclin A} expression is causally involved in the ras-induced cell cycle arrest. Expression of exogenous cyclin A can rescue cells expressing \textit{ras} alone from G2 arrest and in the presence of phorbol esters permits cells to reenter full cycling (C. Barth, in preparation). Therefore, the regulation of essential cell cycle regulatory genes by cooperating oncogenes appears as an important facet of cell transformation. Furthermore, the synergy of \textit{ras} and viral oncogenes may be reflected in their synergy to activate \textit{cdc2} and \textit{cyclin A} expression. Since signalling pathways induced by oncogenes ultimately control the transcriptional regulation of target genes, \textbf{cell cycle regulatory genes may be a target for co-operating oncogenes.} Therefore, the regulation of \textit{cdc2} and \textit{cyclin A} gene transcription by \textit{ras} and E1A was investigated in this thesis.
Chapter 2

Materials and Methods
2.1 Materials

2.1.1 Cell Lines

HeLa S3 (Puck et al, 1956)
Rat 1 (Penn, 1990)
10 tsT Schwann cells (Ridley, 1988)
10r3 Schwann cells (Ridley, 1988)
secondary rat embryo fibroblasts (sREFs) (Land, 1983)
REF52 (Hirakawa and Ruley, 1988)

2.1.2 Media, sera and supplements

Low glucose DMEM (Dulbecco's modified Eagle's medium) containing 1 mg/ml glucose and 0.11 mg/ml sodium pyruvate was used for Schwann cells.
High glucose DMEM containing 4.5 mg/ml glucose without sodium pyruvate was used for all other cell types.
Foetal calf serum (FCS) was obtained from Globepharm Ltd.
Trypsin contained 2.5 mg/ml trypsin and was mixed 1:4 with versene containing 0.2 mg/ml EDTA.
Forskolin (Calbiochem) was solubilised at 25 mM in ethanol, and stored at 120°C.
Hygromycin B was solubilised at 100 mg/ml in 10 mM Hepes pH 7.9 and stored at 4°C.
2.1.3 Bacterial Strains and Genotypes

DH5α  F-, φ80lacZ deltam15, recA1, endA1, gyrA96, thi-1, hsdR17 (rK-, mK+), supE44, relA1, deoR, D(lacZYA-argF) U169
NM538  F-, supF, hsdR (rK-, mK+), trpR, lacY

2.1.4 Reagents and Equipment

Acetyl CoA  Sigma
Acids Acetic (glacial)  FSA
Acrylamide and N,N'-methylene bisacrylamide solution: Northumbria Biologicals Ltd.
Agarose (SeaKem)  FMC BioProducts
Agarose (SeaPlaque)  FMC BioProducts
Ammonium persulfate:  Bio-Rad
Antibiotics:
   Ampicillin (Na salt)  Sigma
   Hygromycin B  Sigma
Kanamycin  Sigma
Puromycin  Sigma
Aquasol  Du Pont
BactoAgar  Difco
Boric acid  FSA
BSA, Fraction V  Boehringer Mannheim
Buffers
   HEPES
   PIPES
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<td>BDH</td>
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<tr>
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<td>Sigma</td>
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<td>Chloroform</td>
<td>FSA</td>
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<tr>
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<td>Sigma</td>
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<td>Sigma</td>
</tr>
<tr>
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<td>FSA</td>
</tr>
<tr>
<td>EGTA</td>
<td>Sigma</td>
</tr>
<tr>
<td>EtOH</td>
<td>James Burrough</td>
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<td>Pharmacia</td>
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<tr>
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<td>BDH (deionised with Bio-Rad AG 501-X8 ion-exchange resin)</td>
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<tr>
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<tr>
<td>Molecular weight markers</td>
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\( \lambda \) Hind III: BRL

\( X174 \) Hae III: BRL

1 kb ladder: BRL

pBR322 Msp I: BRL

NaCl  \quad BDH

NaOH  \quad BDH

Nylon filters: Hybond-N  \quad Amersham

Needles and syringers  \quad Sabre

Nonidet NP-40  \quad Sigma

Nucleotides, deoxynucleotides, and dideoxynucleotides triphosphates:

Pharmacia

Oligonucleotides  \quad ICRF Clare Hall Services Unit

ONPG (O-nitrophenyl-\( \beta \)-D-Galactopyranoside) Sigma

Parafilm  \quad American National Can

Petri dishes  \quad Sterilin

PEG  \quad Koch Light

Phenol, pH 7.0  \quad BRL

Phenol, pH 5.0  \quad BRL

Polyvinylpyrrolidone  \quad Sigma

Protease Inhibitors  \quad Sigma

PMSF

Aprotinin

Protein Assay Mix  \quad BioRad

RNAsin  \quad Promega

SDS  \quad Sigma

Sucrose  \quad Sigma

TEMED  \quad BioRad

Tissue culture dishes  \quad Falcon

Triton X-100  \quad Sigma
2.1.5 Radiochemicals

The following radiochemicals were obtained from Amersham International plc:
1. Redivue (α\textsuperscript{32}P)dATP, aqueous solution 3000 Ci/mmol
2. Redivue (γ\textsuperscript{32}P)dATP, aqueous solution >5000 Ci/mmol
3. (α\textsuperscript{32}P)UTP, aqueous solution 800 Ci/mmol
4. (1-\textsuperscript{14}C) Acetyl-coenzyme A, in 0.01M sodium acetate buffer pH 6.0 56mCi/mmol

2.1.6 Enzymes

Calf Intestinal Alkaline Phosphatase  Boehringer
Tobacco Acid Pyrophosphatase  Sigma
AMV Reverse Transcriptase  Pharmacia
Taq DNA polymerase  Boehringer
DNA polymerase I, Klenow Fragment  Pharmacia
Proteinase K  Boehringer
Restriction endonucleases  BRL, Boehringer, New England Biolabs, Pharmacia
RNase A  Sigma
RNase T1  Sigma
2.1.7 Stock Solutions

**AMV Reverse Transcription Buffer, 10x:**

- 500 mM Tris-HCl pH 8.5
- 60 mM MgCl₂
- 400 mM KCl
- 10 mM DTT
- 10 mM each dNTP

**Ca²⁺-Transfection Buffer, 2x:**

- 50 mM HEPES, pH 7.1
- 280 mM NaCl
- 1.5mM Na₂HPO₄

**Chloroform/Isoamyl Alcohol:**

24:1 (v/v) mixture of chloroform:isoamylalcohol

**Denhardt's Solution, 100x:**

- 2% (w/v) BSA (bovine serum albumin)
- 2% (w/v) Ficoll
- 2% (w/v) PVP (polyvinyl pyrrolidone)
**β-Galactosidase Buffer, 2x:**

- 120 mM Na$_2$HPO$_4$.7H$_2$O
- 80 mM NaH$_2$PO$_4$.H$_2$O
- 1.33 mg/ml ONPG
- 2 mM MgCl$_2$
- 2.7 ml/l β-mercaptoethanol.

**Guanidin-Based Denaturation Buffer:**

- 4 M guanidin tio-izocyanate
- 25 mM sodium citrate pH 7.0
- 0.1 M β-mercaptoethanol
- 0.5% sarcosyl

**LB Agar:**

LB medium, supplemented with 1.5% w/v bacto agar

**LB Medium, per liter:**

- 10 g Bacto-tryptone
- 5 g yeast extract
- 10 g sodium chloride
Adjusted to pH to 7.5 with 5N sodium hydroxide

**Ligation Buffer:**

- 50 mM Tris-HCl pH 7.8
- 10 mM MgCl$_2$
- 10 mM DTT
- 1 mM ATP
**Lysis Buffer:**

0.1% Triton X-100  
0.25 M Tris-HCl, pH 8.0

**PBS:**

100 mM NaCl  
4.5 mM KCl  
7 mM Na$_2$HPO$_4$  
3 mM KH$_2$PO$_4$

**Phenol:**

 contained 0.1% (W/v) 8-hydroxyquinoline and was euilibrated with TE pH 8.0

**Sequencing Buffer, 10x:**

100 mM KCl  
100 mM (NH$_4$)$_2$SO$_4$  
200 mM Tris-HCl pH 8.8  
50 mM MgSO$_4$

**SM, per liter:**

NaCl 5.8g,  
MgSO$_4$ 7H$_2$O 2g,  
1M Tris HCl pH 7.5 50 ml,  
2% gelatin 5ml  
Sterilized by autoclaving
SSC, 20x:

17.53% (w/v) sodium chloride
8.82% (w/v) sodium citrate

Adjusted to pH to 7.0 with sodium hydroxide

Stop/Loading Dye Solution:

0.3% Xylene Cyanol FF
0.3% Bromphenol Blue
0.37% EDTA in deionised formamide

TAE:

40 mM TrisOAc,
2 mM EDTA

TBE:

89 mM Tris base,
89 mM boric acid,
2 mM EDTA

TE:

10 mM Tris pH 7.5;
1 mM EDTA

TM Buffer:

20 mM Tris-HCl, pH 8.0
2 mM MgCl₂

Top Agar:

LB medium, supplemented with 0.6% w/v bacto agar
Transfection Buffer I:
30mM KOAc
50 mM MnCl2
100 mM KCl
10 mM CaCl2
15% v/v glycerol

Transfection Buffer II:
10 mM Na-MOPS pH 7.0
75 mM CaCl2
10 mM KCl
15% v/v glycerol

PCR buffer, 10x:
100 mM Tris-HCl pH 8.3,
15 mM MgCl2,
500 mM KCl

2.1.8 Proteins and Antibodies

Sp1 protein (Promega), 50 ng/μl is prepared from HeLa cells infected with recombinant vaccinia virus containing a full-length Sp1 cDNA (Kadonaga, 1987).

The Ets-1 and Elf-1 proteins were kindly provided by Dr J. Ghysdael, Institute Curie, Orsay, France. The proteins were produced in baculovirus infected SF9 cells from a chicken Ets-1 cDNA clone (Bosselut, 1990) and a human Elf-1 cDNA clone (Thompson, 1992).
Rabbit polyclonal **anti-**Sp1 **antiserum "2892-E"** and its pre-immune control "2892-I" was a kind gift of Dr S.Jackson, Wellcome/CRC Institute, Cambridge.

2.1.9. Cloning Vectors

**EMBL3** is a λ-based 28.7 kb vector with the cloning SalI site for the replacement fragments of up to 20 kb (Frischauf, 1983).

**pBJ3, -4, -9Ω**

The pBR322 backbone of the vectors pJ3Ω and pJ4Ω (Morgenstern and Land, 1990b) was exchanged with a pBluescript backbone, as described in Amati, (1993). To obtain pBJ9, the RSV LTR promoter fragment, Schmidt-Ruppin D strain (Gorman, 1982), was excised at NdeI and HindIII sites from the RSV LT (Jat, 1986) and cloned in exchange of the SV40 early promoter at the PvuII/HindIII sites of pBJ3Ω.

**Blueskipt KS+ Stratagene**

**pBLCAT2**

A CAT reporter construct driven by the HSV tk promoter (Luckow and Schutz, 1987)

**pΔtk**

A promoter-less derivative of pBLCAT2. The HSV tk promoter was excised with BamHI and BglIII.
2.1.10 Oligonucleotides

Synthetic oligonucleotides were used for cloning, PCR amplification and primed cDNA synthesis as well as for gel retardation assays. The sequences of the oligonucleotides, along with their ICRF synthesis reference numbers are indicated below: Sequences of mutated sites and the binding sites within the gel retardation probes are highlighted in bold font.

The \textit{cdc2} Promoter Oligonucleotides

\textit{Primers for sequencing and truncation mutagenesis:}
246cdc2 #23442  
AAAGCATGCTCGTCCTGACTAGAAACAGTAGG  
240cdc2 #23442  
AAAGCATGCGCTGACTAGAAACAGTAGGACGACA  
226down #23443  
AAGCATGCAGTAGGACGACACTCTGCCGACTGGA  
213down #23441  
AAAGCATGCAGTAGGACGACACTCTGCCGACTGGA  
198down #23444  
AAAGCATGCAGTAGGACGACACTCTGCCGACTGGA  
180cdc2 #26996  
AAAGCATGCGGCTGGACCTCTGTGCTGTGAGAGGCCTCAGTTGG  
165cdc2 #26995  
AAAGCATGCCCCTCTCTAGCTCGTGTGCTGTGCAGTTGG  
155cdc2 #26997  
AAAGCATGCTTTTCTCTAGCTCGTGTGCTGTGCAGTTGG  
145cdc2 #26998  
AAAGCATGCCCCTCTCTAGCTCGTGTGCTGTGCAGTTGG
135cdc2 #26999
AAAGCATGCCTCTTTCTTTCGCGCTCGAGC
125cdc2 #27000
AAAGCATGCGCCGCTCTAGCCACCCGGAA
SacII(cdc2) #26994
ATTATTCGCGGCGGCGCAGC
SphI(cdc2) #26993
GCATGCCTCTCTATATAATTTAACATC

**Primers for site-directed mutagenesis of the RIE:**

mRIcdc2/down #27138
GTAGCTTAAAGATTCCGCTGACTAGACTAG
mRIcdc2/up #27139
CTAGTCAGCGGAAATCTAAGCTACTGTTCGC

**Primers for site-directed mutagenesis of the Ostrowski element:**

m(Ostrowski)/down #27140
CCTCGGTCCGCTGACCTAAAGACGAGG
m(Ostrowski)/up #27141
CTACTGAAAGTTTAGCTGCAGCGGACGAGG

**Primer for site-directed mutagenesis of Goblin elements:**

m(1, 2) cdc2 #29645
CTTCCCGGGATTGAATTCGCGGAAGAAGAAGGAAAGGGAAGGATTGAATTCAAAAAGCAGGAG
The cyclin A Promoter Oligonucleotides

Primers for sequencing and truncation mutagenesis:

primer S1 #26161
CGCAGGCGCGAGGAGGTT

primer S2 #25363
CGAAGAACAGGCGC

primer S3 #25362
AGTAGTCAGGTGCAC

primer S4 #25365
GTCCCAGAGCTAAAG

primer S5 #25361
TTTGGGTGCCCAGC

primer S6 #25364
GCAGAGATCGCGGCG

cycA-1 #27130
TGTGAGCTCCGTGTAAATAATTTATG

cycA-2 #27131, 42117
GCTCCGGGGATGGACAGCGGGATC

cycA-2a #40539
GCTCCGGGGATGGAC

cycA-7
GGAGTGGACAGCGGGAT

cycA-35
CAGCGGATCAGGCCTGC

cycA-2R #42281
ATCAGCGCTGCAGCGCC

cycA360 #31663
AAAGCATGCGTTTCCTTTGGTTTACCCTTC
cycA390 #31661
AAAGCATGCTTTGTTTCTCCCTGCCGA

Primers for site-directed mutagenesis of RIE:
mRIcycA/down #27134
CTGCGCCTTTCGCAACCTAAGCTTCCCTGCCGA
mRIcycA/up #27135
GCTCAAGACCACGCAGGAAGCTTAGGTTGCAAGGCAG

Primers for mutagenesis of the CBF site:
m(CBF)/down .#27136 ....
CCTCGGCCCTGCAACAATCTTGAGCTGGTGAGCG
m(CBF)/up .#27137 ....
GCTCAAGATTGTGCAGGCGCGAGGGAGTTGCG

Primers for site-directed mutagenesis of Goblins elements:
mAcycA/up #33302
GCTGCCGAAAATTAGAATTGGTATAGTTAGGGCCCGACGCTT
m(A, B)cycA/down #33301
CGAAATTCTATTTTCCGCAGCGTGAAATCTATTTTAGCCAGTTTTCTCCCTC
CTGCCG
mAcycA/down #33300
CGAAATTCTATTTTGCGCAGCTTTCTCCCGCCACGCCAGTTTTCTCCCTC
CTGCCG
mBcycA/down #33305
CGGCAGCGTGAATTCTATTTTAGCCAGTTTTCTCCCTCCTGCGG
mBcyCA/up #33306

GAATTACGCTGCGGCGGCTAGGATTTAGGGCCCGACGCTT

Oligonucleotides for Cloning and Bandshift Analysis

ets-1 #42167-8

5'-AGCTTGTTACGCTTCGCGCATGCT
3'----.ACCATGGCAAGGCCACGTACGAGATC

elf-1 #42165-6

5'-AGCTTGTTACGCTTCGCGCATGCT
3'----.ACCATGGCAAGGCCACGTACGAGATC

RIE(cdc2) #42071-2

5'-AGCTTGTTACGCTTCGCGCATGCT
3'----.ACCATGGCAAGGCCACGTACGAGATC

m246 #33322-3

5'-AGCTTGTTACGCTTCGCGCATGCT
3'----.ACCATGGCAAGGCCACGTACGAGATC

m240 #33320-1

5'-AGCTTGTTACGCTTCGCGCATGCT
3'----.ACCATGGCAAGGCCACGTACGAGATC

m1RIE(cdc2) #42069-70

5'-AGCTTGTTACGCTTCGCGCATGCT
3'----.ACCATGGCAAGGCCACGTACGAGATC
RIE(cycA) #42065-6
5' -AGCTTGGTACC GCCCTCCTCGGCCCTGCGTGGTGCATGCT
3' -.. ACCATGGGCAGGAGCCGGGACGCACCACGTACGAGATC

m1RIE(cycA) #42063-4
5' -AGCTTGGTACCGCGCaatCggCCTCGGGCTCGTGGTGCATGCT
3' -.. ACCATGGGCcgttACCGGGACGCACCACTACGAGATC

ERE(cdc2) #42062-1
5' -AGCTTGGTACC GC CGCCCTCCTCGGCGATGCT
3' -.. ACCATGGGCAGGAGCCGGGACGCACCACGTACGAGATC

G1 #42165-6
5' -AGCTTGGTACC GCTCTAGGCCACCCGCATGCT
3' -.. ACCATGGGCAGGAGCGGCGTACGAGATC

G2 #42167-8
5' -AGCTTGGTACC GCTCTAGGCCCCGCATGCT
3' -.. ACCATGGGCAGGAGCGGCGTACGAGATC

G3 #42169-70
5' -AGCTTGGTACC GCTCTCCCCACCCGCATGCT
3' -.. ACCATGGGA GAGGGGTGGGCGTACGAGATC

G4 #42171-72
5' -AGCTTGGTACC GCTCTCCCCACCCGCATGCT
3' -.. ACCATGGGA GAGGGGTGGGCGTACGAGATC
m1 #42161-2
5'-AGCTTGGTACCGaaagttCCACCCGCATGCT
3'-....ACCATGGGctttcaaaGGTGGCGTACGAGATC

m2 #42121-2
5'-AGCTTGGTACCGCTCTAGaataaaGCATGCT
3'-....ACCATGGCGAGATCttatttCGTACGAGATC

m3 #42119-20
5'-AGCTTGGTACCGCTCTAGCCtCCCGCATGCT
3'-....ACCATGGCGAGATCGGaGGGCGTACGAGATC

GC-I #43190-1
5'-AGCTTGGTACCGTTCTCCGCCCGCATGCT
3'-....ACCATGGCAAGAGGCGGGCGTACGAGATC

Sp1 #42171-2
5'-AGCTTGGTACCGCGCCCCGCCCCGCATGCT
3'-....ACCATGGCGCGGGGCGGGGCGTACGAGATC

Primers for PMPA Mapping

RACE #42169
GTGAAGCTTGGTACCTGACTCAGCATGCT

CAT1 #38988
GCCATTTGGATATATCAACGGTGG

CAT2 #38989
TATCCCATATCACCAGCTCACCGT
E1A primers

E1A #37356
ATGAGACATATTATCTGCCAC

E1A-SP6 #37355
GAATTGGATTTAGTGACACTATAGAATAATCGATCACCTCCGGTACAAGG

2.1.11 DNA Constructs

12S E1A Constructs:

pE1A, 2R/G, del2-11, del2-23, delCR1, del2-23/CR1, delCR2

All constructs expressing 12S E1A, either the wild type or the mutants, were assembled in the context of pBJ9Ω. All mutants were generated by the exchange of the HindIII/ClaI fragment of the wild type 12S E1A clone (van Dam, 1989) with the HindIII/ClaI fragments from E1A mutants described by Whyte, (1989), Pipas & Moran, (1991) and Schneider, (1987) (see also Fig. 6.5 and 7.9)

pEJ6.6

An expression construct, containing a 6.6 kb BamHI fragment encoding the activated c-Ha-ras gene from EJ-bladder carcinoma cells (Capon, 1983; Land,1983)

J4lacZ

A β-galactosidase expressing construct was generated by cloning a HindIII/BamHI fragment of the lac Z gene from CH110 (Pharmacia) into pBJ4Ω at the same sites.
**cdc2 Constructs:**

**cdc2CAT, ΔHindIcdc2, ΔBamH1cdc2, ΔPst1cdc2, ΔSph1cdc2, ΔSmaIcdc2 (110cdc2)**

A reporter construct of the 2.5 kb fragment cdc2 promoter and its 5'end truncation mutants in the context of pBLCAT2 were a kind gift from S.Dalton (Dalton, 1992)

**cdc2(mut E2F)-CAT**

The construct contains base substitution mutations of all three E2F sites, rendered non-functional, within the full-length cdc2 promoter in the context of cdc2-CAT (Dalton, 1992).


A set of 5'end truncation mutants in the context of the cdc2CAT reporter construct was generated by using PCR amplification (see section 2.2.23). Fragments were amplified with the SacII(cdc2) primer and one of the following primers: 240down, 226down, 213down, 198down, 180cdc2, 165cdc2, 155cdc2, 145cdc2, 135cdc2, 125cdc2. The fragments were cloned into pBLCAT2 at the SphI/Sac2 sites and verified by sequencing. The 5'end of the deletions correspond to the positions -240, -226, -213, -198, -180, -165, -155, -145, -135, -125 bp from the first reported start site in the promoter (Dalton, 1991).

**m(1, 2) cdc2**

The construct contains base substitution mutations of both Goblin elements within the context of the cdc2 promoter.
A fragment of the promoter amplified by PCR with the SphI(cdc2) and m(1, 2) primers was cloned into cdc2CAT between the SphI and SmaI sites and verified by sequencing.

**mRIc**dc2

The construct contains base substitution mutations of the ras inhibitory element (RIE) within the context of the cdc2 promoter. It was generated by recombinant PCR (see section 2.2.23). Two DNA fragments were generated in the first round of PCR amplification: one with the primers SphI(cdc2) and mRIcdc2/up, and the other with primers mRIcdc2/down and SacII(cdc2). The products of the first PCR round were then combined together and annealed/amplified with the primers SphI and SacII(cdc2). The final single product of amplification was cloned back into cdc2CAT construct between the SphI and SacII sites and verified by sequencing.

**m(Ostrowski)**

The construct contains base substitution mutations of the Ostrowski element in the vicinity of the ras inhibitory element (RIE) within the context of the cdc2 promoter. It was generated by recombinant PCR (see section 2.2.23). Two DNA fragments were generated in the first round of PCR amplification: one with primers SphI(cdc2) and m(Ostrowski)/up, and another with the primers m(Ostrowski)/down and SacII(cdc2). The fragments were then combined and annealed/amplified with the primers Sph and SacII(cdc2). The final product - a single fragment - was cloned into cdc2CAT construct between the SphI and SacII sites and verified by sequencing.
**Cyclin A Constructs:**

**Cyclin A cDNA**

An EcoRI fragment of human *cyclin A* cDNA cloned into pGEM4Z was a kind gift from Dr J. Pines (Pines and Hunter, 1990).

**pBl 12, 18, 21**

The constructs were obtained by recloning the inserts from EMBL3 human genomic clones #12, 18 and 21 into the SalI site of Blueskript KS+. The cloned fragments are 6.7kb, 6.5kb and 3kb in size, respectively.

**cyclinA-CAT**

The construct was generated by cloning the *cyclin A* gene specific 3kb SalI fragment from pBL21 into pAtk (see section 2.1.9).

In order to facilitate further cloning within the pAtk context, the Smal site downstream of the CAT reporter gene was destroyed by cutting with XmaIII, filling in the ends with DNA polymerase I, Klenow fragment, and then religating the blunt ends.

**5'end Truncations of the cyclin A Promoter: 450-, 390-, 360-cyclinA**

The 5'end deletions of the cyclinA promoter within the context of cyclinA-CAT were generated by using PCR. DNA fragments were amplified with the cycA2 primer and one of the following primers: A450, A390, A360, followed by cloning between the SphI and Smal sites into cyclinA-CAT.

The 5'ends of the truncations map at the positions -450bp, -390bp, and -360bp from the initiation codon.
mRIcyclinA
The construct contains a base substitution mutation of the ras inhibitory element within the context of the cyclin A promoter. It was generated by using recombinant PCR as described in section 2.2.23. Two DNA fragments were generated in the first round of PCR amplification by using primers cycA-1 and mRIcycA/up, or mRIcycA/down and cycA-2. The products of PCR amplification were then combined and annealed/amplified with primers cycA-1 and cycA-2. The final product - a single fragment - was cloned back into the cyclinA-CAT construct between the SacI and SmaI sites and verified by sequencing.

m(CBF)
The construct contains a base substitution mutation of the CBF binding site in the vicinity of the ras inhibitory element within the context of the cyclinA promoter. It was generated by using recombinant PCR as described in section 2.2.23. Two DNA fragments were generated in the first round of PCR amplification by using primers cycA-1 and m(Ostrowski)/up, or m(Ostrowski)/down and cycA-2. The products of PCR amplification were then combined and annealed/amplified with primers cycA-1 and cycA-2. The final product - a single fragment - was cloned back into cyclinA-CAT construct between the SacI and SmaI sites and verified by sequencing.

m(A, B)cyclinA
The construct contains base substituted mutations of both Goblins within the context of the cyclin A promoter. It was generated by recombinant PCR as described in section 2.2.23. Two DNA fragments were generated in the first round of PCR amplification: one with primers cycA-1 and mAcycA/up, or m(A, B)cycA/down and cycA-2. The fragments were then combined and annealed/amplified with primers cycA-1 and cycA-2. The final product - a
single fragment was then cloned back into the cyclinA-CAT construct between the SacI and SmaI sites.

**mAcyclinA**

The construct contains base substituted mutation of the first Goblin element within the context of the *cyclin A* promoter. It was generated by using recombinant PCR as described in 2.2.23. Two DNA fragments generated with primers cycA-1 and mAcycA/up, as well as with mAcycA/down, and cycA-2, and then combined and annealed/amplified with primers cycA-1 and cycA-2. The final product - a single fragment - was cloned into cyclinA-CAT construct between the SacI and SmaI sites and verified by sequencing.

**mBcyclinA**

The construct contains base substituted mutation of the second Goblin element within the context of the *cyclin A* promoter. It was generated by using recombinant PCR as described in 2.2.23. Two DNA fragments generated with primers cycA-1 and mBcycA/up, as well as with mBcycA/down and cycA-2 were combined and annealed/amplified with primers cycA-1 and cycA-2. The final product - a single fragment - was recloned into cyclinA-CAT construct between the SacI and SmaI sites and verified by sequencing.

**pEts C70**

A pBJ9Ω-based construct expressing an N-terminal truncation of chiken *ets-1* protein (Bosselut, 1990). The translated protein is 114 amino acids long, starts at the 70th amino acid of the full size *ets-1* and correspondes to the positions 2422-2764 bp of the chicken *ets-1* cDNA clone.
Minimal Constructs

RIE and m1RIE Reporter Constructs
The ras inhibitory element RIE and related sequences were cloned into reporter construct pBLCAT2 (see section 2.1.9) as double stranded oligonucleotides: RIEcdc2; m1RIEcdc2, RIEcycA; m1RIEcycA (see section 2.1.10). The correct orientation was ensured by using the Hind III and Xba I cloning sites. The Kpn I site present in the annealed oligonucleotides facilitated preliminary identification of the recombinants. All constructs were verified by sequencing.

ERE-CAT
The E1A inhibitory element (ERE) was cloned into reporter construct pBLCAT2 (see section 2.1.9) as a double stranded oligonucleotide ERE(cdc2) (see section 2.1.10). The correct orientation was ensured by using the Hind III and Xba I cloning sites. The Kpn I site present in the annealed oligonucleotide facilitated preliminary identification of the recombinants. The construct was verified by sequencing.

Goblin Reporter Constructs: G1-, G2-, G3-, G4-, m1-, m2-, m3-, GCI-, Sp1-CAT
The Goblin element and related sequences were cloned into promotorless reporter construct pΔtk (see section 2.1.9) between HindIII and XbaI sites as double stranded oligonucleotides: G1-4, m1, m2, m3, GC-I, and Sp1 (see section 2.1.10). The Kpn I site present in the annealed oligonucleotides facilitated preliminary identification of the recombinants. All constructs were verified by sequencing.
110cdc2-G1
The construct contains the Goblin element inserted into 110-cdc2 at the Sma I site, downstream from the CAT reporter gene.

2.1.12 RNase Protection Probes

E1A
The RNase protection probe for 12S E1A mRNA was synthesised with SP6 RNA polymerase on a PCR-generated template as described in section 2.2. 27. The PCR amplification of the template was performed with primers E1A and E1ASP6, the latter containing the minimal SP6 promoter sequence. The generated RNA probe is 392 bases long.

LacZ
A HindIII/PvuII fragment of the lac Z gene was subcloned from CH110 (Pharmacia) into Bluescript KS+ between the HindIII and PvuII sites of the polylinker. The RNA antisense probe generated with T7 polymerase is 372 bases long.

2.2 Methods

2.2.1 Tissue Culture Conditions

Cells were grown in humidified incubators in 10%CO2. Cells carrying the temperature sensitive SV40 large T antigen (10tsT and 10ras3 Schwann cells) were routinely maintained at 33°C, all other cells were grown at 37°C.
Unless specified, cells were maintained in Dulbecco's modified Eagle's media (DMEM) containing 2 µg/ml gentamycin and 100 µg/ml kanamycin. The medium was exchanged every 3 days. Schwann cells were maintained in low glucose DMEM containing 3% foetal calf serum (FCS), 10% 259 conditioned medium (see section 2.2.2) and 2 µM forskolin. The medium for 10r3 Schwann cells was exchanged every 2 days.

All Schwann cells were grown on poly-L-lysine-coated dishes. Poly-L-lysine (PLL) was solubilised in H2O to give a 0.4 mg/ml stock solution, and stored at -20°C. Dishes were incubated with 3 ml of PLL (13.3 µg/ml in H2O) for 10 min at room temperature, drained for 5 min, washed twice with sterile H2O and dried open in a sterile air-flow hood for 5-10 min.

All manipulations with living cells were performed in laminar flow hoods. Cells were passaged by first washing them in PBS and then briefly (1 min) incubating them with 1:4 v/v trypsin/versene. Detached cells were diluted with fresh media and replated. 10t Schwann cells were routinely split 1:3, the 10r3 line was split 1:8, all other cell types were split 1:20.

For storage and subsequent recovery, near-confluent 90 mm dishes were incubated with trypsin/versene, and resuspended in 3 ml of ice-cold 50% FCS/50% DMEM. Next, 0.3 ml of dimethylsulphoxide (DMSO) was added drop-wise with continuous mixing. Cells were then wrapped into paper tissue, stored at -70°C for 24-48 h, and later transferred into liquid nitrogen.

For the recovery, cells were thawed rapidly at 37°C, then slowly mixed with 10 ml of fresh warm media and plated out. After 2 hr incubation, the medium was replaced to dilute out the DMSO.
2.2.2 Growth of IN/259 cells and production of conditioned medium

IN/259 cells originate from a human glioma cell line. They were obtained from Dr M Noble (Ludwig Institute for Cancer Research, 91 Riding House St., London). They were maintained in low glucose DMEM and 10% FBS, fed every 2 days, and passaged by splitting 1:2. Once the cells reached confluency on a 150 mm dish, the medium was replaced with 20 ml fresh DMEM. Subsequently, every two days the conditioned medium (known as 259 CM) would be harvested and filtered through a 0.45 μ filter. The filter was prewashed with DMEM containing 0.03% BSA (to prevent non-specific binding of proteins to the filter).

259 CM was stored in 5 and 10 ml aliquots at -20°C and used within 10 days of harvesting.

2.2.3 Ca\(^{2+}\)- Transfection of REFs

Transfections procedures were based on the protocol by Wigler, (1979). Transient co-transfections always included J4LacZ, a plasmid expressing the lacZ gene, as a control for transfection efficiency.

Prior to transfection, cells were refed with 10 ml of medium containing 10% fetal calf serum. Each 9 cm dish of cells was transfected with up to 20 μg of plasmid DNA. The DNA was suspended in 500 μl of Ca\(^{2+}\)-Transfection buffer, followed by addition of 29 μl of 2.5 M CaCl\(_2\). After 20 min of incubation, once a white translucent precipitate was formed, the mixture was added dropwise to the cells upon continuous slow shaking of the plate. The precipitate was left on the cells for at least 16-20 hr. Then fresh 0.5% FCS DMEM was applied and the cells were further incubated for 30-48 hr before they were harvested for CAT and β-galactosidase assays. In case of the
transfection with ras alone, after removal of the precipitate the cells were maintained for 2-4 hr in 0.5% FCS DMEM before they were switched to 10% FCS. This procedure seems to prevent cell loss due to possible induction of apoptosis.

2.2.4 Schwann Cells Transfection

Transfection of Schwann cells in principle was performed following the same protocol as for REFs. Two days prior to transfection, confluent Schwann cells were split 1:3 onto 9 cm dishes and fed 3% FCS, 10% 259 CM, 2 μM forskolin. One hour before transfection the cells were refed. For the drug selection of stable clones, the precipitate was removed after the 6 hr incubation, cells were washed twice in PBS at 37°C, and left to grow in their normal medium for two days. Two days post-transfection, cells were split 1:3, grown for another two days and then subjected to drug selection with hygromycin B at 100 μg/ml. Resistance to hygromycin B was conferred by the co-transfected pBabe Hygro construct (Morgenstern and Land, 1990b).

2.2.5 Protein Extraction for CAT/β-Galactosidase Assays

Cells were washed twice with PBS. Ten ml of TM buffer was added to each plate and cells were allowed to swell for 5 min. The buffer was carefully discarded and 150 μl of Lysis Buffer was added drop-wise on to the plates. The plates were swirled gently and incubated for 3-5 min (until only nuclei were visible under the microscope).
The lysates were scraped off the plates with rubber policmen and were transferred to Eppendorf tubes.

All collected lysates were frozen on dry ice. The extracts, were then freeze/thawed trice, vortexed and spun on a microcentrifuge for 3 min. The supernatants were transferred to fresh Eppendorf tubes and stored frozen at -70°C.

2.2.6 CAT Assay

Thirty μl aliquots of the extracts (see section 2.2.5) were heated at 68°C for 15 min, cooled down to room temperature and added to a fresh mix of 1 μl chloramphenicol stock solution (5 mg/ml), 2 μl ¹⁴C acetyl CoA (54 mCi/mmole), 1.6 μl acetyl CoA stock solution (5 mg/ml), 7.5 μl 1 M Tris-HCl pH 8.0 and 38.5 μl water.

The reaction was incubated at 37°C for 1-4 hr. At the end of incubation the reaction tubes were transferred into ice. Two hundred μl of ice-cold ethyl acetate was added to the tube, vortexed vigorously for 30 sec and spun in a microcentrifuge for 3 min. From the phase separated mixture, 150 μl of the upper organic phase was collected and transferred to a scintillation vial containing 5 ml Aquasol (Du Pont).

A blank control with untransfected extracts was used as a negative control. As a positive control, 0.025 units of purified CAT were added to untransfected extracts. The upper limit of the linear range of the assay was estimated at 15,000 cpm. Samples with higher degree of conversion were diluted in accordance with the linear range of the signal.
2.2.7 β-Galactosidase Assay

One hundred and fifty μl aliquots of the cell extracts (see section 2.2.5) was mixed with an equal volume of 2xβ-Galacosidase Buffer: 120 mM Na₂HPO₄·7H₂O, 80 mM NaH₂PO₄·H₂O, 1.33 mg/ml ONPG (O-nitrophenyl-b-D-Galactopyranoside, Sigma), 2 mM MgCl₂, 2.7 ml/l β-mercaptoethanol.

The reaction was incubated at 37°C till it obtained lemon yellow colour and stopped with 500 μl of 1 M Na₂CO₃.

After spinning the mixture for 10 min at 13000 rpm on a microcentrifuge, the supernatant was transferred to a microcuvette (Kartell). The optical density was measured at 420 nm against an untransfected cell extracts control.

2.2.8 Random Priming

The protocol is based on DNA synthesis on a chosen template primed with random hexamer oligonucleotides (pdN₆, Pharmacia) (Feinberg and Vogelstein, 1983). The method yields radioactive products with a specific activity of at least 5x10⁸ cpm/μg.

DNA template (200 ng) was combined with 1μg of random primers (0.5 μM final concentration) in 10 μl of water. The mix was heated for 3 min in a boiling water bath and then chilled on ice. Subsequently, 3 μl of 10xKlenow Buffer (Boehringer), 1μl of 10 mM dCTP, dGTP, dTTP, 5 μl of ³²P-dATP (3000 Ci/mmol, 10uCi/ul), and 10 units of DNA polymerase I (Klenow fragment) were added and the total volume of the reaction was adjusted to 30 μl.

The reaction was incubated at 37°C for 1hr and then stopped by adding EDTA at a final concentration of 5mM.
The synthesised probe was separated from unincorporated nucleotides by double precipitation with isopropanol in 2.5 M NH₄OAc, as described in section 2.2.18. One μg of glycogen was added as a carrier to facilitate precipitation as a carrier.

Prior to hybridisation, the probe was dissolved in 100 μl of water, boiled for 5 min, chilled on ice and added to the hybridisation solution.

To minimise radiolysis, the probe was always used within 24 hr after the synthesis.

2.2.9 Plating and Maintaining Bacteriophage λ

Fresh plating cells were prepared by inoculating a colony of the NM538 strain into 20 ml of LB Medium, containing 0.2% maltose, and 10 mM MgSO₄. The cells were grown into the stationary stage, pelleted and resuspended in 10 ml of SM buffer.

A phage dilution in 200 μl SM buffer was mixed with 600 μl of fresh NM538 plating cells. After 15 min of incubation at 37°C, 7.5 ml of LB top agar (40°C) were quickly added to cells, swirled to insure good mixing, and then poured over the plates with LB agar. Once the top agar layer was set, the plates were transferred to 37°C. Within the next 6-12 hr the plaques became clearly identifiable on the bacterial lawn. The titres of phage clones were reduced with each round of purification, from 200,000 to 200 plaques per plate.

Plaques considered to be positive were picked up with a Pasteur pipette, as a plug of LB top agar, and put into 1 ml of SM buffer with a drop of chloroform. The phage was eluted by shaking for 2 hr, then limiting dilutions were played out, as described above, for further purification.
2.2.10 Plaque Blotting and Hybridisation

A nylon membrane (Hybond N) was carefully placed on the LB top agar surface of a plate. To avoid the distortion of the fragile top agar layer, the plates were usually pre-cooled at +4°C prior the blotting procedure. After one minute the membrane was removed from the agar surface and placed for 7 min, plaque side up, on a pad of absorbent filter paper soaked in denaturation solution (1.5 M NaCl, 0.5 M NaOH). For the following renaturation, the filter was transferred on a pad of absorbent paper soaked in neutralising solution (1.5 M NaCl, 0.5 M Tris-HCl pH 7.2, 0.001 M EDTA). After 3 min the filter was washed in 2xSSC, transferred on dry filter paper and air dried. Dry filters were baked in the oven for 2 hr at 80°C.

Filters prepared for hybridisation were first pre-hybridised for 1 hr at 65°C in 6xSSC, 5xDenhardt's solution, 0.5% SDS in presence of denatured sonicated non-homologous salmon sperm DNA at 20 μg/ml final concentration.

The radiolabeled DNA specific probe, at an average specific activity of 10^8 cpm/μg, was denatured by boiling for 5 min, then chilled on ice and added to the hybridisation mixture, with the final concentration not exceeding 100 ng/ml.

Hybridisation was performed in a sealed plastic bag with 5 ml of solution for each 100 ml² of membrane surface. Filters were left to hybridise in a shaking waterbath, at 65°C, for at least 12 hr.

After hybridisation the filters were washed twice in 2xSSC at 65°C for 15 min. The third wash usually was performed in 2xSSC, 0.1% SDS for 30 min. For a high stringency wash, the filters were rinsed in 0.1xSSC, at room temperature, for 10 min. Finally, filters were wrapped in Saran Wrap and autoradiographed at -70°C with an intensifying screen.
2.2.11 λ Phage DNA Preparation

Infection
A single colony of the NM538 bacterial strain was inoculated into 100 ml of LB Medium. After overnight incubation at 37°C, the culture was centrifuged at 4000g for 10 min and the bacterial pellet was resuspended in 12 ml of SM.
An estimated amount of $5 \times 10^8$ bacteriophages was added per $10^{10}$ cells, and incubated at 37°C with intermittent shaking. (A single plaque yields on average $10^7$ infectious phage particles, while a small-scale liquid culture lysis should have a titre of approximately $10^{10}$/ml. For the estimation of the cell number, it was assumed that $1\text{OD}_{600}=8 \times 10^8\text{cells/ml}$)
An infected aliquot was then added to 500 ml warm LB medium and incubated at 37°C with vigorous shaking. With an optimal ratio of bacteriophages to bacterial cells during the infection, full cell usually occurred after 12 hr of incubation. After lysis 10 ml of chloroform was added to the culture followed by additional half an hour incubation and shaking.

Purification
The lysed cultures were cooled to room temperature, and pancreatic DNase and RNase were added to a final concentration of 1 μg/ml. The digestion was allowed to proceed for 30 min at room temperature. Solid sodium chloride was added to a final concentration of 1 M and the solution was left on ice for 1 hr. All debris was removed by centrifugation at 11, 000g for 10 min at 4°C. Supernatant was transferred and mixed with PEG
6000 solution (to a final concentration 10% w/v). The mix was left on ice for at least 1 hr to allow precipitation of the phage. The particles were recovered by centrifugation at 11,000g for 10 min at 4°C. The supernatant was discarded and the pellet was resuspended in SM. An equal volume of chloroform was added to the suspension. The organic and aqueous phases were separated by centrifugation at 1600g for 15 min. After centrifugation, the aqueous phase containing the bacteriophage was recovered and the particles were pelleted by centrifugation at 25,000 rpm for 2 hr in a Beckman SW27 rotor. After discarding the supernatant, the pellet was resuspended in 1 ml SM by shaking the samples overnight at 4°C.

**Extraction of DNA**

EDTA was added to the suspension of purified phage particles to a final concentration of 20 mM, followed by proteinase K to a final concentration of 50 μg/ml and SDS to 0.5%. The suspension was mixed by inverting the tube several times and was incubated for 1 hr at 65°C. An equal volume of equilibrated phenol was added to the tube, mixed thoroughly and spun at 1600g for 5 min. The aqueous phase was extracted once again with phenol/chlorophorm, followed by three rounds of dialysis overnight against 1000-fold volumes of TE.

**2.2.12 Quantification of Nucleic Acids**

Concentration and purity of nucleic acid solutions was assessed on the basis of their absorbance at 260 and 280 nm. One unit of optical
density A260 corresponds to 50 µg/ml for dsDNA, 40 µg/ml for RNA or 33µg/ml for ssDNA (including oligonucleotides). With maximal UV absorbance of proteins at 280 nm, the ratio A260/A280 is indicative of the extent of protein contamination in nucleic acid solutions. Ratios of 1.7 and over were considered acceptable.

2.2.13 Plasmid DNA Preparation

The following protocol exploits advantages of simple alkaline lysis followed by selective precipitation of supercoiled DNA in presence of polyethylen glycol. This method proved to yield large quantities of plasmid DNA suitable for various applications, such as transient transfection of mammalian cells, in vitro transcription, sequencing and cloning. 50 ml of LB medium with transformed bacterial culture was grown overnight under ampicillin selection at 37°C, with vigorous shaking.

The cells were spun down at 3,000g for 5 min and resuspended in 5 ml of 50 mM glucose, 25 mM Tris-Cl pH 8.0. 10 ml of 200 mM NaOH, 1% w/v SDS was added. The mix was gently swirled and left on ice for 10 min.

Next, 7.5 ml of 5 M KOAc pH 4.8 was added, followed by gentle vortexing and another 5 min of incubation on ice. After the incubation, the mix was spun down at 3500g for 10 min, the supernatant was collected and incubated with RNaseA (at a final concentration of 20 µg/ml) for 20 min at 65°C.

Following the RNase A treatment, an equal volume of isopropanol was added to the mix and a precipitate was formed on dry ice, within 20 min. The precipitate was spun down at 3500g for 10 min, washed in 70% ethanol and resuspended in 0.5 ml of TE. The TE solution was transferred
into an Eppendorf tube and spun in a mini-centrifuge to remove the debris. An equal volume of the 13% PEG(M.W. 800), 1.6 M NaCl was added to the TE solution and the mix was incubated on ice for 1 hr. Once a translucent precipitate became visible the suspension was spun in a microcentrifuge for 5 min. The pellet was washed with 70% ethanol, dissolved in 200 μl TE and reprecipitated with addition of 20 μl of 3M NaOAc and 2.5 volume of ethanol. The pellet was washed in 70% ethanol and dissolved in 200 μl TE. The concentration of plasmid DNA was determined as described in Section 2.2.12.

2.2.14 Plasmid DNA Miniscale Preparation

Individual colonies were picked off the LB agar plates and grown in 3 ml selective BHI media at 37°C overnight. One ml of the overnight culture was spun in an Eppendorf tube, the supernatant was discarded and the pellet was resuspended in 100 μl of 50 mM glucose, 25 mM Tris pH 8.0, 10 mM EDTA. 200 μl of 200 mM NaOH, 1% v/v SDS was added with gentle mixing followed by incubation on ice for 10 min. 150 μl ice-cold 5 M KOAc, pH 4.8, was added to the tube, mixed again, and then incubated for 5 min on ice. Cellular debris was pelleted in a microcentrifuge for 3 min and the supernatant was transferred into a fresh Eppendorf tube. RNase A was added to a final concentration of 2 μg/ml and the tube was incubated at 68°C for 15 min. Following the incubation, the sample was mixed with equal volume of isopropanol and left on ice for 10 min, for the DNA precipitate to form. The DNA was pelleted in a microcentrifuge for 5 min, washed in 70% ethanol and dissolved in 100 μl TE. For analytical
restriction digests. 3 μl of the miniprep DNA solution usually sufficed for a single reaction.

2.2.15 Preparation and Transformation of Bacteria

A single colony was picked from a fresh overnight LB agar plate into 3 ml of LB media and grown overnight at 37°C. 100 ml of prewarmed LB was inoculated with 1 ml of overnight culture and left to grow at 37°C under vigorous shaking. Once the density of the cell culture reached OD$_{600}$=0.6, the flask was transferred into ice and rapidly cooled down. The bacteria were pelleted in a Beckman J6 centrifuge at 4.2k rpm for 15 min, +4°C, and the supernatant was discarded. The pellet was resuspended in 50 ml sterile, ice-cold Transfection Buffer I by gentle swirling on ice. After 15 min incubation on ice, the bacteria were pelleted again, the supernatant discarded and the pellet resuspended in 10 ml sterile, ice-cold Transfection Buffer II by gentle swirling on ice. Aliquots of 0.1 ml were frozen on dry ice and stored at -70°C.

Plasmid DNA was transformed into bacteria by combining 50 μl of transformation competent bacteria, thawed on ice, with few μl containing up to 50 ng DNA. The bacteria were incubated on ice for 30 min, heat shocked for 5 min at 37°C and returned into ice for 1 min. One ml of LB medium was added and the cells were incubated at 37°C for 45-60 min. The bacteria were spun out of suspension at 3,000g for 1 min and the pellet was resuspended in 100 ml of LB medium. It was then plated on LB agar plates containing ampicillin at the concentration of 100 μg/ml. Plates were incubated at 37°C overnight to let the colonies grow to a visible size and then transferred to +4°C for short term storage.
2.2.16 Agarose Gel Electrophoresis

DNA molecules within the range of 0.1-20 kb length were resolved on agarose gel matrixes. Agarose powder was dissolved at concentrations of 0.5 to 2% w/v in 1xTAE or 1xTBE buffers, melted by heating in a microwave oven, and cast in a gel tray with a teflon comb. Once set, the gel was submerged in a electrophoresis tank filled with the TAE or TBE buffer and the comb was carefully removed.

DNA samples and markers were mixed with 1/10th volume of loading dye: 50 mM EDTA, 100 MM Tris 8.0, 50% v/v glycerol, 0.4% w/v bromphenol blue and loaded into the wells. They were electrophoresed at a constant voltage of 5 V/cm. For visualisation of the DNA, ethidium bromide was added to the buffer and the gel (EtBr, up to 0.01 μg/ml), so that resolution of DNA bands could be monitored under 254 nm UV light during the corse of the electrophoresis. Gels were photographed using Polaroid instant print film type 57 or 55.

For preparative purposes, DNA was resolved in low melting point (LMT) brands of agarose in 1xTAE buffer. DNA bands were visualised and excised with a razor blade under long wave UV light. The excised slices of agarose containing DNA were melted at 68°C and diluted with 200 μl TE. Most enzymatic reactions with DNA, such as ligation, are unaffected by the presence of low concentrations of LMT agarose.

2.2.17 Phenol Extraction

Aqueous nucleic acid solutions were deproteinised by extractions with phenol and chloroform. An equal volume of TE-saturated phenol was added, followed by vigorous mixing on a vortex mixer for 10 sec and
centrifugation at 10,000g for 3 min. The aqueous phase was transferred and reextracted with a 1:1 phenol/chloroform mix, followed by a final extraction with chloroform. After the extractions the nucleic acids were precipitated.

2.2.18 Nucleic Acid Precipitation

Nucleic acids were recovered from aqueous solutions by one of the following methods.

**Ethanol precipitation**

Nucleic acid solution was brought to 0.3M NaOAc pH 5.2, 20 µg glycogen or tRNA as a carrier, and 70% v/v ethanol. After incubating for 10 min on ice, the sample was spun at 10,000g for 5 min, the pellet was retained, washed in 70% ethanol, and dissolved in the appropriate aqueous solution.

**Ammonium acetate/isopropanol precipitation**

Nucleic acid solution was brought to 2.5M NH4OAc, 50% isopropanol and incubated for 5 min at room temperature, followed by spinning the sample under the same conditions as above. Under these conditions of precipitation, free nucleotides and short oligonucleotides remain in solution. The lesser protein denaturing effect of isopropanol also allows direct precipitation of DNA out of enzymatic reactions without substantial losses of DNA.
2.2.19 Restriction Digest

Analytical digests were performed using 0.5 to 1 μg of DNA and a 3 to 5 fold unit excess of enzyme in a total volume of 20 μl. All buffer conditions were as specified for each enzyme by New England Biolabs. If case of multiple restriction digests with incompatible buffer conditions, each of the digests was carried out separately, followed by phenol/chloroform extraction and ethanol precipitation.

For the digestion of DNA in presence of low melt agarose, the total volume of the reaction was increased to 100 μl, with the agarose concentration being less then 0.05%. The reaction was incubated for 6-12 hr.

2.2.20 Ligation

The DNA fragments used for ligation were prepared by modifying plasmid DNA with appropriate restriction endonuclease enzymes, as well as with DNA polymerase I, Klenow fragment, and T4 DNA polymerase. Standard preparations usually provided about 1 μg of an isolated DNA fragment in 50 μl LMT agarose. In a ligation of compatible and overhanging ends, 1 μl (20 ng) vector DNA was combined with 4 μl (100ng) insert DNA in 50 mM Tris pH 7.8, 10 mM MgCl₂, 1 mM ATP, 20 mM DTT, 0.2 μl T4 DNA ligase (6 Weiss units) in a total volume of 20 μl. DNA fragments excised from LMT agarose gels were melted at 65°C, cooled down to 37°C and added to the reaction. The final concentration of LMT agarose in a ligation reaction was kept at less then 0.4% w/v. Ligations were incubated overnight at 14-16°C and stopped by heating at
65°C for 5 min. A 5 μl aliquot was used to transform 100 μl competent bacteria.

To obtain blunt ends, 1 μl (10 units) of T4 DNA polymerase, along with 2 μl of 5mM dNTP stock solution, was added at the end of a restriction digest. After 15 min incubation at room temperature, the sample was heated at 65°C for 10 min. To fill in recessed 3' tails a similar procedure was carried out using E.coli DNA polymerase I, Klenow fragment.

In order to prevent self-ligation of DNA molecules 5'-phosphates were removed by Calf Intestinal Alkaline Phosphotase (CIP). 3 units per each μg of treated DNA were added to restriction digests. The reactions were incubated at 37°C for 30 min and then stopped with 50 mM EGTA and heat treatment at 65°C for 15 min. Subsequently, the DNA was separated on a LMT agarose gel.

For cloning oligonucleotides, they were 5'-phosphorylated using polynucleotide kinase and annealed to form double-strands with overhanging vector-compatible ends. 10 mmol of dsDNA was then directly added into ligation with gel purified CIP-treated vector DNA. A restriction site designed within the oligonucleotide sequence simplified preliminary identification of recombinant clones.

### 2.2.21 Standard PCR Amplification

PCR amplification was set up using 0.5-ml microfuge tubes and a Hybaid Thermal Reactor. Into a 100 μl reaction the following components were added: 10 μl 10x PCR buffer, each primer to 0.5 μM final concentration, 50 μl 5 mM dNTP stock solution, 50 ng-1μg template DNA (corresponds to 10^6 target molecules) and 2 units Taq DNA polymerase. The mixture
was overlayed with 50 µl of mineral oil and the tube was positioned in the programmed Hybaid Thermal Reactor.

The profile of temperature cycles, carried out by the Reactor, was as follows:

a) initial denaturation at 94°C for 3 min, followed by 15 to 35 cycles of
b) 1 min denaturation at 94°C,
c) 1 min annealing at lowest Tm for the two primers,
d) 3 min primer extension at 72°C.

The final extension was left to run for 5 min.

To assess the result of amplification, a 10 µl aliquot was run on a 1% agarose gel in presence of ethidium bromide.

2.2.22 Deletion mutagenesis

All promoter deletions used in the project were 5'-end truncations obtained by PCR. For each truncation, a specific PCR primer was designed. At its 3'-proximal part the primer was complementary to the promoter sequence. It also contained an extended sequence tag with a convenient restriction site at the 5'end. In combination with the cycA2 primer for the cyc A promoter, or the sacI(cdc2) primer for the cdc2 promoter, the designed primers were used in amplification of promoter sequences. The products of amplification were the truncated mutants of the promoters, with restriction sites at both ends. These sites were utilised for cloning the amplified fragments into reporter constructs. All products of PCR-based cloning were verified by DNA sequencing.
2.2.23 Recombinant PCR

The following protocol for mutagenesis of PCR products (Huguchi, 1990) was used for generating base substitution mutations within the full context of the cdc2 and cycA promoters. The first round of two PCR amplifications yields products that overlap over the site of mutation, introduced as part of the "inside" overlapping primers. Both of the primary products are used in a second round of PCR amplification carried out with a pair of "outside" primers. During the second round, one of the possible products of reannealing would be a heteroduplex with recessed 3' ends. Taq polymerase extends those ends, thereby creating a template that can be specifically amplified by "outside" primers.

The protocol consists of two rounds of standard PCR as follows. Two separate PCR reactions are carried out for the left and right fragments of the mutagenised template. The left fragment is amplified with the left "outside" primer and the right "inside" primer. Accordingly, the right fragment is enriched using the right "outside" and left "inside" primer. Ten μl of each PCR reaction is run on a 1% LMT agarose gel in presence of ethidium bromide. The bands are cut out and melted in 200 μl TE at 68°C. Two ml of each of the isolated fragments are combined together and used in the next round of amplification with a pair of "outside" primers. The final product of the amplification is purified on a LMT agarose gel and stored in TE solution.

The structure of all cloned PCR-based constructs was confirmed by DNA sequencing.
2.2.24 DNA Sequencing

DNA samples were sequenced with the Thermal Cycle Dideoxy DNA Sequencing Kit (New England Biolabs).
As little as 20 ng of template dsDNA was used in a single sequencing reaction. 20-25 cycles of repeated sequencing were performed with thermostable DNA polymerases CircumVent or Taq, as described in the Kit protocol.
The same protocol applied to sequencing products of PCR amplification. In this case, a PCR fragment was first gel purified in LMT agarose.
In a reaction, 20 ng of template DNA was combined with 1 pmol end-labeled sequence specific primer, 1.5 μl of 10xSequencing Buffer and the volume was adjusted to 14 μl with water. With the addition of 2 units of Vent DNA polymerase the mixture was aliquoted into four tubes, containing 3 μl deoxy/dideoxy solutions for each of the nucleotides (New England Biolabs).
Reactions were overlaid with a drop of mineral oil and incubated in a Thermal Cycler for 20 cycles of 1 min 95°C, 1 min 55°C, 1 min 72°C.
The reactions were terminated with addition of 4 μl Stop/Loading dye, and stored at -70°C.
The samples were heated at 85°C for 10 min and loaded in 2 μl aliquots on a denaturing PAGE.

2.2.25 Eukaryotic RNA Preparation

The protocol is based on the method developed by (P.Chomczynski, 1987).
Cells were washed with PBS and lysed on plates with 1.5 ml of denaturation buffer: 4 M guanidin tio-izocyanate, 25 mM sodium citrate pH 7.0, 0.1 M β-mercaptoethanol, 0.5% sarcosyl. The lysates were scraped off the plates with a rubber policeman. The following components were added to the lysates with proper mixing in the given order:
- 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).

The final mixture was kept on ice for 15 min and then spun down at 4000 rpm for 20 min at 4°C. The upper aqueous phase was transferred and mixed with an equal volume of cold isopropanol. After a 60 min incubation on dry ice, RNA was spun down at 4000 rpm for 20 min.

The pellet was resuspended in 200 µl of denaturation buffer, an equal volume of isopropanol was added and the RNA was stored as a precipitate at -70°C.

### 2.2.26 PMPA Mapping of the mRNA 5'-ends

The following protocol is based on the enzymatic removal of the methylated cap-site at the 5'-end of mRNA by tobacco acid pyrophosphatase, followed by the ligation of a primer to the exposed 5'-end phosphate (Fromont-Racine, 1993). Primer extension towards ligated 5'-ends produces templates that can be amplified by PCR. The specificity of PCR is confirmed by rounds of amplification with a number of nested sequence specific primers.

10 µg of total cellular RNA was treated with 2 units of tobacco acid pyrophosphatase (Sigma) for 30 min at 37°C in 10 µl of 50 mM NaOAc pH 6.0, 10 mM β-mercaptoethanol, 1 mM EDTA, 2 mM ATP. After the
treatment, RNA was extracted once with phenol/chloroform, precipitated with ethanol and used for ligation with 10 pmol of the unphosphorylated primer (RACE, see also section 2.1.9). The reaction was run for 16 hr at 17°C in 10 µl of 50 mM Tris-HCl pH 7.5, 10 mM MgCl₂, 20 mM DTT with 20 units of human placental ribonuclease inhibitor (Boehringer) and 3 units of T4 RNA ligase (Pharmacia). The reverse transcription reaction was performed as follows: 1 µl of the ligation reaction products was mixed with 10 ng of sequence specific primer, 1 µl of 60 mM MgCl₂, 1 µl of 10xAMV Reverse Transcription Buffer and 8 µl of water. The sample was incubated for 5 min at 95°C and then for 45 min at 42°C. One µl of the 5 mM dNTP stock solution and 3 units of AMV reverse transcriptase (Pharmacia) were subsequently added and the incubation was prolonged for 45 min at 42°C. The reaction was stopped by a 5 min incubation at 95°C. The DNA was amplified using the ligated primer and the sequence specific primer in 30 cycles of PCR (60 sec at 94°C, 2 min at 55°C, 3 min at 72°C). One µl of the products was amplified in the second round of PCR using the ligated primer and a nested sequence specific primer. The products were analysed on an agarose gel stained with ethidium bromide and finally on a denaturing acrylamide gel (using ³²P-labeled nested primer).

2.2.27 RNase Protection

RNA probes were transcribed in vitro using PCR-amplified DNA templates as described by (Yang, 1992). A DNA template for the RNA probe was amplified by PCR, with the antisense primer containing a 5'-end extention:
5'-GAATTGGATTTAGGTGACACTATAGAATAC. This sequence represents the minimal SP6 RNA polymerase promoter. PCR amplification was carried out as described in Section 2.2.21. Once completed, the amplified template was purified from nonincorporated free primers and dNTPs by isopropanol precipitation (Section 2.2.18).

One μg of the PCR-amplified template was used for the in vitro transcription reaction. The reaction mixture contained 40 mM Tris-HCl pH 7.5, 6 mM Mg2Cl, 2 mM spermidine, 10 mM NaCl, 10 mM DTT, 1 unit/μl RNasin, 500 μM of each ATP, CTP, GTP, 10 mM UTP as well as 30 μCi α32P-UTP. The reaction was initiated by addition of 10 units of SP6 RNA polymerase, followed by incubation at 40°C for 1 hr. Twenty units of DNase I was then added to degrade the DNA template in 45 min at 37°C.

The synthesised RNA was extracted with phenol/chloroform and precipitated with ethanol. Ten micrograms of total RNA and 300,000cpm of the antisense probe were mixed in 30 μl of hybridisation buffer containing 80% formamide, 0.4 M NaCl, 40 mM 1,4-piperazinediethane sulfonic acid (PIPES) pH 6.4, 1 mM EDTA. The RNA was denatured at 85°C for 5 min and then reannealed at 50°C overnight.

Next morning the mixture was chilled on ice and diluted with 350 μl of 10 mM Tris-HCl pH 7.5, 5 mM EDTA, 300 mM NaOAc. RNase A (7 μg) and RNase T1 (25 units) were added to each sample and the digestion was allowed to proceed for 45 min at 30°C. To stop the reaction, 10 μl of 20% SDS solution and 20 μg of proteinase K were added, followed by incubation for 30 min at 37°C. The samples were finally extracted with phenol, precipitated with ethanol, dissolved in 10 μl Stop/Loading Dye: 80% formamide, 2 mM EDTA, 0.1% bromphenol blue and run on the denaturing PAGE, along with size markers.
2.2.28 Eukaryotic DNA Preparation

One 90 mm dish of confluent cell culture was washed with PBS, scraped off into a 15 ml Falcon tube and treated with 2 ml of DNA lysis buffer: 40 μg/ml proteinase K, 100 mM Tris-Cl pH 8.0, 100 mM NaCl, 2 mM EDTA, 0.5% w/v SDS. The incubation lasted for at least 16 hr. The digested lysate was then extracted with phenol/chlorophorm and precipitated with isopropanol. The DNA precipitate was washed in 70% ethanol and resuspended in 1 ml TE. The yield was quantified by UV spectrophotometry.

2.2.29 Oligonucleotide manipulations

Oligonucleotides were synthesised on an Applied Biosystems Model 370 oligonucleotide synthesiser. Oligonucleotides were resuspended at 1 mg/ml in TE and purified from incomplete strands using denaturing PAGE. Typically, 10 μl of DNA was combined with an equal volume of deionised formamide. This was then heated at 95°C for 5 min, chilled on ice and run on a 10% denaturing PAGE.

Following electrophoresis, the gel was transferred between two sheets of Saran Wrap and exposed to 254 nm UV light against the white screen (usually, white paper towelling). Under these conditions the separated bulk of DNA could be identified as a shadowed band. The band was excised from the gel, and the oligo was eluted by overnight incubation in 0.5 M NH4OAc, 10 mM MgCl2, 1 mM EDTA at 37°C and then ethanol precipitated. Oligonucleotides which were used for cloning and gel retardation assays were annealed by combining 10 μg of each strand in 20 μl TE, 10 mM
MgCl$_2$. The sample was heated to 90°C for 5 min and then slowly cooled down to room temperature over several hours.

### 2.2.30 Oligonucleotide Probe Preparation

Single strand oligonucleotide probes were 5'-phosphate labelled by polynucleotide kinase. 50 to 100 ng DNA was combined in a reaction mix containing 66 mM Tris 7.5, 10 mM MgCl$_2$, 50 mCi $^{32}$P-ATP 3000-5000 Ci/mmole, 15 mM DTT, 10 units T4 PNK, in a total volume of 10-20 µl, and incubated at 37°C for one hour.

To generate double stranded probes, equimolar quantities of the labeled complementary oligonucleotides were annealed and then purified on a native PAGE as described in Section 2.2.30.

### 2.2.31 Nuclear Protein Extraction

Cells were washed in PBS and harvested by scraping them off the plates with rubber policmen. 5x10$^7$ to 1x10$^8$ cells were spun down in a single Eppendorf tube in a microcentrifuge at +4°C. The pellet was washed with PBS and spun again. The cells were resuspended in 1 ml of 10 mM Hepes pH 7.9, 1.5 mM MgCl$_2$, 10 mM KCl, 0.5 mM DTT, 0.5 mM phenylmethylsulphonyl fluoride (PMSF), and incubated on ice for 15 min allowing them to swell and lyse. The release of nuclei was checked by phase contrast microscopy.

Nuclei were pelleted by centrifugation at 250g and resuspended in 500 µl of 5 mM Hepes pH 7.9, 26% glycerol (v/v), 1.5 mM MgCl$_2$, 0.2 mM
EDTA, 0.5 mM DTT, 0.5 mM PMSF. NaCl was added to a final concentration of 500 mM and the extraction was left on ice for 30 min. Extracts were centrifuged at 13000 rpm in a microcentrifuge for 10 min. Supernatants were aliquoted and snap-frosen in dry ice/ethanol. Aliquots were stored at -70°C.

2.2.32 Electromobility Shift Assay (EMSA)

DNA-protein binding reactions for electromobility shift assays (EMSA) were performed in 20 µl of 20 mM Hepes pH 7.9, 30 mM KCl, 2 mM MgCl₂, 1 µM ZnSO₄, 10% glycerol, 0.5 mM DTT, 2 µg poly dAdT (Pharmacia). Two and a half µg of nuclear protein extract or 0.5 ng of purified protein combined with 2 µg BSA were added to the binding reaction, followed by 20 fmol of a ³²P-labelled oligonucleotide probe and an excess of unlabelled competitor. The reaction was incubated for 30 min at room temperature. When specified, 2 µl of pre-immune serum or Sp1 anti-serum were added along with the extracts at the onset of the binding reaction. To identify the DNA-protein complexes, the samples were loaded on native polyacrilamide gel as described in 2.2.33.

2.2.33 Native Polyacrylamide Gel Electrophoresis (PAGE)

Non-denaturing polyacrylamide gels were used for separation of protein:DNA complexes in EMSA. 5% w/v polyacrylamide gels (29:1 w/w acrylamide:N,N'-methylene bisacrylamide) of 2.5 mm thickness were
polymerased in 0.5xTBE with the addition of 0.1% w/v ammonium persulfate and 0.1% v/v TEMED.

Prior to loading the samples, the gels were pre-run at 10 V/cm for 1hr.
For optimal separation the bromphenol marker was run 3/4 of the gel length.

The gel was transferred onto a sheet of 3MM Whatmann paper, dried on a heated gel drier and autoradiographed with an intensifying screen at -70°C.

2.2.34 Denaturing PAGE

Denaturing PAGE was used for size-dependant separation of nucleic acids in DNA sequencing, RNA probe preparation, RNase protection and oligonucleotide purification. 6% to 20% w/v polyacrylamide gels (19:1 w/w acrylamide:N,N'-methylene bisacrylamide, 50% w/v urea, 1xTBE) were polymerased with the addition of 0.1% v/v ammonium persulfate, 0.1% v/v TEMED. Gels were run at constant power of 60 Watt (50 mWatt/cm^2) in order to maintain a surface temperature of 55°-60°C. If required, gels were subsequently fixed for 10 min in 10% v/v glacial acetic acid, 10% v/v methanol and dried onto 3MM Whatman paper using a heated (80°C) gel drier.

In preparative experiments fragments were identified either by autoradiography or by UV shadowing and then cut out from the gel with a razor blade.
2.2.35 Protein Quantification

Protein content was measured using a BioRad Protein Assay kit following recommendations of the manufacturer.

Five-fold stock assay mix was diluted with water.

1-5 μl extract was added to 1 ml assay mix in a disposable plastic cuvette, mixed well, incubated for 5 min and measured at A595. A standard curve for 1 to 40 μg gamma-globulin (BioRad) was used to calibrate the readings.
Chapter 3

Ras-Dependent Regulation of the cdc2 Promoter in Schwann Cells
3.1 Introduction

As mentioned in Chapter 1, rat Schwann cells exemplify one of the model systems, in which oncogene co-operation has a clear effect on the regulation of the cell cycle (Ridley, 1988).

Schwann cells are the glial cells of the peripheral nervous system. They can be purified and maintained proliferating \textit{in vitro} for many generations as a homogeneous population of cells, without spontaneous differentiation (Bunge and Bunge, 1984).

Co-operation of oncogenic \textit{ras} and the DNA tumour virus transforming proteins - SV40 large T antigen, or Adenovirus protein E1A, is required for full transformation of Schwann cells. With the introduction of these oncogenic activities, the cells change their morphology and reduce their doubling time from about 2 days to 12 hr. The cells also change their growth factor requirements: forskolin and 259 CM (see section 2.1.1 and 2.1.2) become dispensable for the sustained growth of the transformed cells. Finally, the cells are able to form foci and grow in a soft agar. In contrast, \textit{ras} alone induces a cell cycle arrest in the G1 and G2 phases of the cell cycle, while cells expressing large T alone exhibit reduced growth factor requirements (Ridley, 1988).

The opposite proliferative behaviour of Schwann cells expressing \textit{ras} and SV40 large T (or E1A) together, as compared with cells expressing \textit{ras} alone suggested cell cycle regulation as a possible target for co-operating oncogenes. Indeed, protein and mRNA levels of cyclin dependent kinase \textit{cdc2} and its regulatory subunit \textit{cyclin A} appeared to be both overexpressed in presence of \textit{ras} and active large T. However, in \textit{ras} -arrested cells, both \textit{cdc2} and \textit{cyclin A} expression was found suppressed. Moreover, the demonstration that overexpression of exogenous \textit{cyclin A} in these cells permits an escape from the G2 arrest suggests that the suppression of \textit{cyclin A} gene expression
is causally involved in the ras-mediated cell cycle arrest (C. Barth, in preparation). Thus, cell cycle regulatory genes may be targets on which signals controlled by ras and SV40 large T converge.

In mammalian cells, transcription of the cdc2 gene has been demonstrated to be restricted to proliferating cells. Moreover, it appears to be cell cycle regulated as the promoter is shown to be active only in late G1, S and G2 phases of the cycle (Dalton, 1992).

To test whether co-operating oncogenes can regulate transcription of the cdc2 gene, the cdc2 promoter was introduced into Schwann cells, expressing oncogenic ras and/or temperature-sensitive SV40 large T.

3.2 Regulation of the cdc2 Promoter in Schwann Cells Expressing Oncogenic Ras and SV40 Large T

Initially, the Schwann cell line 10r3, expressing v-Ha-ras and the temperature sensitive mutant tsA58 of SV40 large T (Ridley, 1988), was analysed for its ability to regulate the activity of the cdc2 promoter. In this cell line the effects of ras and SV40 large T or ras alone can be studied by cultivation of the cells at either the permissive (33°C) or non-permissive (39°C) temperature for tsA578. At 33°C the cells are transformed and proliferate rapidly. In contrast, after 24 hr at 39°C the culture undergoes cell cycle arrest (Ridley, 1988). The 10r3 cells were co-transfected using the Ca-transfection method (see Section 2.2.4) with 5 μg of cdc2-CAT, a chloramphenicol acetyltransferase (CAT) reporter gene driven by the 2.5 kb fragment of the human cdc2 promoter (Dalton, 1992), 3 μg of J4lacZ, a lacZ reporter gene driven by the long terminal repeat of the Moloney murine leukaemia virus (Mo-MuLV LTR) (Morgenstern and Land, 1990a), and 2 μg of the pBabe Hygro plasmid, conferring resistance to hygromycin (Morgenstern and Land, 1990b). Stably
transfected colonies were selected with hygromycin, as described in Section 2.2.4. The polyclonal cell populations derived exhibited detectable levels of β-galactosidase and CAT activities.

To assess whether the cdc2 promoter can be regulated by the oncogenes, the CAT activity from cells kept in permissive and non-permissive conditions for tsA578 was monitored. Twenty-four hours after plating of sub-confluent cultures, half of the cultures was shifted to 39°C, while the rest was left at 33°C. At the time of the temperature shift, as well as 12 and 24 hr later, aliquots of cells were lysed and assayed for β-galactosidase and CAT activities (see Sections 2.2.6 and 2.2.7) (Figure 3.1). Within the 24 hr time course, the activity of the lacZ gene, expressed as per mg of protein content, did not change at either temperature (Fig. 3.1a). In contrast, the CAT activity driven by the human cdc2 promoter was reduced by 50% in cells shifted to 39°C within 24 hr, while it remained unaffected at 33°C (Fig. 3.1b). Given the high stability of the CAT protein, these results suggest that the temperature shift induces a rapid suppression of cdc2 promoter activity prior to establishment of the cell cycle arrest.

3.3 Regulation of the cdc2 Promoter in Schwann Cells Expressing SV40 Large T Alone

In order to test whether the effect on the cdc2 promoter described in section 3.2 may be due to the ras oncogene, the cdc2 promoter reporter construct was introduced into 10tsT Schwann cells. 10tsT cells express temperature-sensitive mutant tsA58 of SV40 large T alone and represent the parental clone for 10r3 Schwann cells. Unlike 10r3, the 10tsT Schwann cells proliferate at both 33°C and 39°C (Ridley, 1988).
The *cdc2* reporter construct, *cdc2*-CAT, and the β-galactosidase expression vector, J4lacZ, were transiently co-transfected into 10tsT cells. As a control, the same constructs were co-transfected into 10r3 cells as well. The cells were transfected for 20 hr at 33°C, followed by 12 hr incubation at the same temperature. The cultures were then trypsinised, split 1:3 and replated. Subsequently, the cells were incubated for further 12 hr at 33°C to allow full attachment, before half of the cultures were shifted to 39°C. At the time of the shift, as well as twelve and twenty four hours thereafter, triplicate samples were assayed for β-galactosidase and CAT activities (Fig 3.2). Similar to the experiment described in section 3.2, the levels of β-galactosidase activity did not change after temperature shift to 39°C in transiently transfected 10r3 and 10tsT Schwann cells.

In transiently transfected 10r3 cells, the CAT activity driven by the *cdc2* promoter is reduced by almost 50% after 24 hr of incubation at 39°C as observed for the stably transfected cells. In contrast, in 10tsT cells, incubation at 39°C did not affect the level of the CAT activity.

These results indicate that the suppression of the *cdc2* promoter at 39°C in 10r3 cells is associated with the activity of oncogenic ras, rather than due to the inactivation of SV40 large T or the change in temperature.

### 3.4 Conclusions

Comparing the reporter activities, driven by the *cdc2* promoter in 10r3 and 10tsT Schwann cells, it became evident that the *cdc2* promoter can be transcriptionally controlled by *ras* and *tsT*.

As the experiments showed, oncogenic *ras* alone can induce suppression of *cdc2* promoter activity in Schwann cells, which precedes the ras-induced cell
cycle arrest. This supports the idea that the inhibition of cell cycle gene expression by ras is causally involved in the arrest.

In presence of ras and active SV40 large T together the cdc2 promoter is highly active in 10t3 cells, reflecting its dependence on both oncogenes. This result is compatible with the notion that SV40 LT can rescue cells from the ras-induced cell cycle arrest (Ridley, 1988).

Taken together, cell cycle gene promoter regulation by co-operating oncogenes may be an important mechanism underlying the contrasting cellular responses to oncogenic ras alone or the combination of ras and SV40 LT. To investigate the molecular basis of this mechanism, it becomes necessary to investigate through which elements the cdc2 promoter is regulated by co-operating oncogenes.
Fig. 3.1 Regulation of the *cdc2* promoter in pools of stably transfected 10r3 Schwann cells.

(A) β-galactosidase activity, driven from the stably transfected J4lacZ construct, and (B) CAT activity, driven from the stably co-transfected cdc2CAT construct, measured at different times of incubation at 39°C and 33°C.

All measured activities were normalised against the protein content of the cellular extracts. The standard deviation of the mean values is indicated by error bars.
Fig. 3.2 Regulation of the *cdc2* promoter in 10r3 and 10tsT Schwann cells.

10r3 and 10tsT Schwann cells were transiently co-transfected with 10 μg of
*cdr2CAT(C, D)* and *J4lacZ(A, B)* constructs. Following the transfection, the
cells were shifted to 39°C (B, D) or left at 33°C (A, C) and then assayed for
their β-galactosidase and CAT activities at different times points after the
shift.

All measured β-galactosidase activities were normalised against the protein
content of the cellular extracts. All measured CAT activities were normalised
against the β-galactosidase activities of the same cellular extracts. The
standard deviation of the mean values is indicated by error bars.
Chapter 4

Isolation and Characterisation of the Human *Cyclin A* Promoter
4.1 Introduction

The data presented in the previous chapter indicate that the cdc2 promoter is transcriptionally regulated by the co-operating oncogenes ras and tsT in Schwann cells. A similar pattern of regulation is suggested for the cyclin A gene, since its mRNA is overexpressed when both oncogenes are active and suppressed by ras alone. Importantly, suppression of cyclin A mRNA appears to be causally involved in the ras-induced cell cycle arrest, since ectopic cyclin A expression can rescue the cells from cell cycle arrest in presence of phorbol esters (C.Barth, in preparation). Thus, analysis of the transcriptional regulation of the cyclin A gene may provide important clues to understanding how the oncogenes can induce uncontrolled growth by their co-operative action.

In this chapter the isolation and characterisation of the human cyclin A promoter is described.

4.2 Screening

A human placenta DNA library, constructed in the phage vector λEMBL3 (Frischauf, 1983), was utilised to isolate the cyclin A promoter. The library was a kind gift of Dr A.-M. Frischauff, ICRF. The average size of the clones in the library was estimated to be about 20 kb.

Screening for a specific genomic fragment requires a library of adequate representation. A simple estimate shows that for a human genomic library with an average size of cloned fragments close to 20 kb, $7.6 \times 10^5$ clones will contain the sequence of interest with a probability of 99%. Therefore, a total number of $8.5-9.0 \times 10^5$ plaques was plated on four 245x245x20 mm Falcon dishes. The screening was performed by repeatedly using two
radiolabeled probes (see section 2.2.8 for the labelling protocol). One probe represented 2200 bp of the full length cyclin A cDNA clone (EcoRI-EcoRI fragment) (see section 2.1.11), the other one contained the first 297 bp from the 5' end of the same clone (EcoRI-Bgl I fragment). Plaque blotting and hybridisation with the probes was performed as described in section 2.2.10. Clones that gave positive hybridisation signals with both probes were selected and purified through two extra rounds of screening. Finally, three clones (## 21, 18, 12) were purified to homogeneity. They all contained genomic sequences highly homologous to the 5' proximal part of the cyclin A cDNA.

4.3 Physical Mapping and Partial Sequence Analysis of the Clones

The three genomic λ phage clones, selected for their homology with the cyclin A cDNA, were grown in preparative quantities and their DNA was extracted, as described in section 2.2.11. The inserts were excised with the Sal I restriction enzyme and subcloned into the Bluescript KS vector (Stratagene), generating pBl 21, 18 and 12. All three clones were then subjected to restriction and sequence analysis, following the protocols from section 2.2.19 and 2.2.24. As shown in Fig.4.1, restriction mapping of the clones shows that they overlap, suggesting a common origin for all three cloned fragments. Sequence analysis with primers specific to the cDNA clone confirmed that all three genomic clones contained sequences representing the 5'end of the cyclin A gene. The 1 kb fragment, shared by the clones between the Hind III and SmaI sites 5' proximal to the first codon of the open reading frame (ORF), was fully sequenced on both strands (Fig. 4.2).
4.4 Activity of the Cyclin A Promoter

In order to test the promoter activity of the isolated 5' proximal region of the human cyclin A gene, a reporter construct containing the 3 kb fragment A (Fig.4.1) upstream of the chloramphenicol acetylase gene, cyclinA-CAT, was generated (Fig.4.3). The construct was transiently transfected into proliferating secondary rat embryo fibroblasts (sREFs) and the cells were assayed for CAT activity, following transfection (see sections 2.2.3, 2.2.5-7 for the protocols). As shown in Fig.4.4, cells transiently transfected with CyclinA-CAT obtain a high chloramphenicol acetylation activity, comparable with the levels of activity driven from the constitutively active HSV tk promoter. Thus, on the basis of the transfection experiments, the cloned fragment A of the cyclin A gene exhibits a potent promoter activity.

4.5 Mapping of the Transcription Start Sites

To identify the transcription start sites within the endogenous human cyclin A promoter as well as the cyclinA-CAT construct, a pyrophosphatase-mediated PCR amplification (PMPA) technique of mapping, based on the method developed by Fromont-Racine et al, (1993), was applied (for the protocol see section 2.2.26). The method is based on the enzymatic removal of the methylated caps from mRNA with tobacco acid pyrophosphatase, followed by ligation of a primer (RACE primer, see section 2.1.10) to the exposed phosphorylated 5' ends of mRNA. cDNA was then synthesised with a sequence specific primer from the treated mRNA and used as a template in 3 rounds of PCR amplification with RACE and distinct nested sequence-specific primers (Fig.4.5).
The positions for the transcription start sites were mapped by the PMPA method with RNA from proliferating HeLa cells and RNA from the HeLa cells transiently transfected with 10 µg of the cyclin A reporter construct, cyclinA-CAT (see section 2.1.11). Ten pmol of the RACE primer (see section 2.1.10) was ligated, in accordance with the protocol, to the 5' ends of 1 µg of mRNA, following the treatment with tobacco acid pyrophosphatase. As a control, all the following amplification steps were also performed on aliquots of mRNA which were not treated with pyrophosphatase.

In the next step, cDNA was synthesised on the ligated mRNA templates using the CAT2 primer specific for the CAT reporter gene (see section 2.1.10) on the RNA from the transfected cells, or the cyclinA-2a primer specific for the human cyclin A promoter (see section 2.1.10) on the RNA from the non-transfected cells (Fig.4.5). After the first round of PCR amplification with a set of nested sequence specific primers (CAT1 and cyclinA-7, respectively) the products of amplification corresponding to the transfected and endogeneous promoters differed in size (data not shown). In the second round, the PCR products from the first round were amplified using the same sequence-specific primer, cyclinA-35 (see section 2.1.10), generating products of similar size (see below).

For precise mapping, a sequence-specific primer, cyclinA-2R (see section 2.1.10), was labelled with $^{32}$P (for labelling see protocol in section 2.2.30) and used in the third round of single-stranded PCR amplification. The products of this reaction were resolved on a denaturing polyacrylamide gel (see section 2.2.34) next to the promoter sequence, originating from the same $^{32}$P-labelled primer (see section 2.2.24.). The radioautograph (Fig.4.5) shows that amplification by PCR is dependent on treatment of the mRNA with pyrophosphatase. Untreated RNA from transfected and non-transfected cells failed to provide templates for sequence-specific PCR amplification. Thus, the amplified DNA fragments identify the 5' ends of mRNA transcripts.
Moreover, transcription start sites of the endogenous and the transfected promoters appear to be very similar. According to the adjacent sequencing ladder, the two most abundant fragments have the same mobility and map at positions -283 and -204 from the first codon of the ORF of the gene. Considering the length of the RACE primer ligated to the 5'end of the amplified template, the start sites represented by the fragments map to positions -254 and -175 relative to the start of the ORF of the cyclin A gene (Fig. 4.5 and 4.2).

Taken together, the PMPA mapping shows that, in HeLa cells, the endogenous as well as the cloned 3 kb fragment of the cyclin A promoter initiate transcription at the same set of multiple transcription start sites.

### 4.6 Structure of the Cyclin A Promoter

As it becomes evident from the DNA sequence (Fig. 4.2), the cyclin A promoter does not contain a TATA-box and is GC-rich. This is not unusual as a number of cell cycle regulatory genes such as cdc2 (Dalton, 1992), cyclin D (Motokura and Arnold, 1993), cyclin E (R. Weinberg, personal communication) also lack this element. Moreover, multiple transcription start sites in the cyclin A promoter appear to be another common feature shared with other TATA-less promoters, e.g. ets-1 and ets-2 (Jorcyk, 1991; Mavrothalassitis, 1990; Roeder, 1991; Weis and Reinberg, 1992).

A number of regulatory elements are present within the first 1 kb of the cyclin A promoter. They include binding sites for transcription factor Sp1 (Kadonaga, 1986) (positions -140, -383, -413), two sites similar to the E2F binding consensus (Mudryj, 1990) (positions -214, -80); a double motif TGCCT as a potential binding site for the p53 protein (Kern, 1991) (position -192), an inverted CCAAT box as a potential CTF binding site (McKnight and
(Tjian, 1986; Santoro, 1988) (position -307), and an ATF/CREB site (Lee, 1987) at the position -325 (Fig. 4.2).

4.7 Discussion

Three genomic clones, representing 5' proximal fragment of the human cyclin A gene, were isolated from a human placenta genomic library. When linked to the reporter gene, the cloned 3 kb fragment exhibited a strong promoter activity, detected in rat embryo fibroblasts.

Mapping of the transcription start sites of the endogenous promoter and the cloned promoter fragment in HeLa cells, revealed multiple 5' ends, with two major sites at positions -254 and -175 of the cyclin A promoter.

In its structure the cloned promoter is highly GC-rich and contains binding sites for several transcription factors, such as Sp1, E2F, CTF and CREB/ATF. However, it lacks both the TATA-box motif and known initiator consensus sequences, which commonly define the transcription start sites (Roeder, 1991; Zawel and Reinberg, 1992; Weis and Reinberg, 1992).

Taken together, these results confirmed the nature of the 3 kb cloned genomic fragment as containing the cyclin A gene promoter.

A recent report has confirmed the structure of the human cyclin A promoter described above (Henglein, 1994). In this report, transcriptional initiation was mapped on the endogenous promoter in different types of cells by means of S1-analysis. Apparently, all attempts to map the sites by the alternative means of RNase protection or primer extension were unsuccessful (Henglein, 1994).

In our experience, mapping of the start sites on the GC-rich part of the cyclin A promoter by means of RNase protection also proved very evasive (data not shown; D. Parkinson, personal communication). By means of PMPA technique the promoter start sites were easily detected and mapped. However,
as one of its drawbacks, the PMPA method may not necessarily adequately represent all the start sites and even may select through PCR amplification for the shorter products. According to Henglein et al, (1994), the multiple pattern of transcription initiation sites observed on the *cyclin A* promoter depends on the type of cells tested. For example, in Raji cells the major start sites were mapped at more then 10 positions: -303, -297, -287, -282, -279, -278, -273, -272, -264, -254 ; while in HL60 cells 90% of transcription starts from position -254 upstream from the ORF. In the PMPA mapping experiment described in section 4.5, the same position -254 was identified as the first major start site for the *cyclin A* promoter in HeLa cells.

Henglein et al, (1994) also provide the first evidence that the *cyclin A* promoter is only active in proliferating cells and that upon mitogenic stimulation transcription of the gene is activated in late G1 phase.

The responses of the *cyclin A* and *cdc2* promoters to co-operating oncogenes are investigated in the following chapters.
Fig.4.1 Restriction map of genomic human *cyclin A* clones.

The clones pBl 21, 18 and 12 contain overlapping portions of the *cyclin A* gene. The shared sequences are aligned. The position corresponding to the AUG start codon is indicated.

The *cyclin A* cDNA probes, used for screening the DNA library, are shown at the bottom of the scheme (filled bars).
Fig. 4.2 DNA sequence of the human *cyclin A* promoter.

The sequence represents the HindIII-SmaI fragment C, 5' proximal to the position corresponding to the start codon of the human *cyclin A* gene. The ATG codon is highlighted in bold font; consensus binding sites for known transcription factors and Hind III and Sma I restriction sites are indicated. Asterisks mark two major transcription start sites mapped in HeLa cells.
**HindIII**

5'...**AGCT** TTGTATATCC TTATATATAT ATATAAATAT AAAAAATTGT
TAAAGGCAG TATAGTTAAG AGAGTTTTAT TTTAATAAGG TCATATGTTT
TTTACTATGT TTAAAAACT TTACCTCTGA AAGGAACATA ATTATAATCTA
GGTCACCTAGA AGTCATTGTG GTTTTTTTGT GTTGGCCACA GCTTGGGGAA
AAATAGAAA AAATTAATTG ATGTATTGAT ATTTGTATAC ACTGCAATAAT
ATTATATAT ACAGTATTTG AAGGTGCCAT CTAAATTAA TTGcATCTTC
ATTAGAAAAG ATAAAAAACGA TAAACCAACAA TTTCTGTATA CTAGATAAAA
GCCCACTGTA GATGACATTA CAGTC7ACT AGACGTATTA CTATGTGAGC
TCGGTGTTAA ATCAATTTATG CACATATTAT ATAcTCTACAA GCCATATGAC
CGTAGTTTAT ATGCCTCTATT AACACATAAG AAAACGGAAA ATC GGAGATA
CTAAAAAACG TGCCCCAGAT TTGAACCTT TGGAAAAAGT CACTTAAGCT
AACAGGACTT CCAGAGCTAA AGGCCTGGCA ACCAAAATG ATAGTCGCCA
AATGTTTAAAT CCGTAAAAAT CTGAGTTCG ACACTTTGGGAC
AGCCCTGTAC ACTAGGTCGC TCAGCTTAAA ATATCAGGAA GCCTCGGGCC
CTAAATCCTTA CCTCTCCCCA CCCCGCGACG GTTTCTCCCCC GCCAGGCGCAG

**Sp1**

TTTGTTTCTC CCTCCTGCGC GCCCCTGCTC AGTTTCTTTT

**CREB/ATF**

TCACCTGCCG TGGACCTGTC GCCGTGAAATG AGCTCAAGGC GCGAGCGCCTT

...**CTF**

TCATGTGTTCC ATTTCAATAG GCAGCAGGATC TGGAAACTGC CAAACAGCG

**E2F**

CGCTCGGCGG GGGCGCCCTG GCGATCTCTG GCGACGGCCACG

* p53

CTGGGCAGTG CGCGCGCGCC CTTTCCGCAA CTCCTCGGAC CTCCTGCTGT

**Sp1**

CTTACCTGGA GAGCGCTGGC GCGGCGCGGT GTAGGCTGCG TGGGCCTGGG

**E2F**

ACCGCGCGCT ACCACTATT TGCTGGGCGG TGGCGCTGAG TGGCGCTGAG

**SmaI**

GCAGAGTGCA CGCTGCTTGG CCGCGCGGAC TGATCCCGCC GTCCACCTCC GCCAGG

+1

CAGTCAGG ...3'
Fig. 4.3 Physical map of the human cyclin A promoter reporter construct.

The reporter construct contains the HindIII-Smal fragment A of the human cyclin A gene cloned into the pΔtk vector (see section 2.1.9).
Fig.4.4 Activity of the human *cyclin A* promoter in transiently transfected cells.

The cyclinA-CAT reporter construct was transiently transfected into growing rat embryo fibroblasts. Positive and negative controls were provided by the Herpes Simplex Virus tk promoter in pBLCAT2 and the promoterless pΔtk constructs (see section 2.1.9). The efficiency of transfection was controlled by co-transfection with the *lacZ* expressing construct J4lacZ (see section 2.1.11). The CAT activity values obtained were normalised against the corresponding β-galactosidase activities. The standard deviation of the mean values is indicated by error bars.
The diagram illustrates the relative CAT activity for different constructs:

- **CyclinA-CAT**
- **p Δtk**
- **pBLCAT2**

The **Cyclin A Promoter** is indicated with a black bar. Below it are the constructs:

- **CyclinA-CAT**
- **p Δtk**
- **pBLCAT2**

The diagram shows the relative CAT activity for each construct, with bars representing the activity levels.
Fig. 4.5 Mapping the transcription start sites of the *cyclin A* promoter by PMPA.

**A.** Autoradiograph of the PMPA mapping, in alignment with the sequence of the promoter. PMPA was applied with (lanes 1, 2) or without (lanes 3, 4) pyrophosphatase treatment on RNA from subconfluent HeLa cell cultures (lanes 1, 3) and HeLa cells transfected with the cyclinA-CAT construct (lanes 2, 4).

**B.** Schematic representation of the PMPA technique as applied to the transfected and endogenous *cyclin A* promoters.
**A**

1st primers:
- cycA-2a
- CAT2
- cycA-2a
- CAT2

Treatment:
- pyrophosphatase
- pyrophosphatase

**B**

- tobacco acid pyrophosphatase
  - p-5' mRNA ORF p-5' mRNA CAT
  - T4 RNA Ligase
  - mRNA ORF mRNA CAT
  - AMV Reverse Transcriptase
  - mRNA cDNA cycA-2a mRNA CAT2
  - 1st PCR
  - mRNA cDNA cycA-7 mRNA CAT1
  - 2nd PCR
  - mRNA cDNA cycA-35
  - Labeling
  - cycA-2R

**cyclin A Promoter**

- P^{32} cycA-2R

Sequencing
Chapter 5

Regulation of the *cdc2* and *cyclin A* Promoters by Oncogenic *Ras*
5.1 Introduction
As described in chapter 3, oncogenic ras regulates promoter activity of the \textit{cdc2} gene in Schwann cells. Depending on the absence or presence of its co-operating partner SV40 large T, ras either suppresses, or contributes to the activation of the \textit{cdc2} promoter. Like Schwann cells, secondary rat embryo fibroblasts (sREFs) (Land, 1983) and the established REF52 cell line (Hirakawa and Ruley, 1988) can only be fully transformed by ras in combination with SV40 large T or Adenovirus E1A, while ras alone is insufficient to do so. Extending the analogy with Schwann cells, ras also has been shown to induce cell cycle arrest in REF52 cells (Hirakawa and Ruley, 1988).

Moreover, ras regulates the levels of the \textit{cdc2} and \textit{cyclin A} mRNA expression in a very similar manner: these levels are suppressed in the ras-arrested cells and are elevated in cells transformed by co-operative action of ras and LT (Barth, in preparation). Similar to the \textit{cdc2} promoter the \textit{cyclin A} promoter has been shown to be active only in proliferating cells and upon stimulation of resting cells it is switched in the late G1 phase of the cell cycle (Henglein, 1994). Therefore, the \textit{cyclin A} promoter may also be a target for regulation by co-operating oncogenes.

In these chapter the responses of the \textit{cdc2} and \textit{cyclin A} promoters to ras alone was investigated. Secondary REFs and REF52 cells have been used for the analysis. Their main advantage for the study of ras--induced regulation is the absence of the conditional co-operating partner tsT present in 10r3 Schwann cells.
5.2 Regulation of the *cdc2* and *cyclin A* Promoters by Oncogenic Ras

In order to study the regulation of the *cdc2* promoter by oncogenic ras, the activity of the promoter was assayed in growing sREFs or REF52 cells in presence of increasing amounts of oncogenic ras. Cells were transiently co-transfected with 2.5 µg of the reporter construct, cdc2-CAT (Dalton, 1992; see section 2.1.11), and pEJ6.6, a construct expressing an activated c-Ha-ras gene (6.6 kb BamHI fragment), (Capon et al, 1983; Land et al, 1983; see section 2.1.11). Proportional to the dose of the pEJ6.6 plasmid, the activity of the *cdc2* promoter was suppressed up to 10-fold (Fig. 5.1). The efficiency of transfections was controlled with J4lacZ, a construct expressing lacZ gene under the control of Mo-MuLV LTR (see section 2.1.11). In the titration experiments, the total DNA concentration was kept constant by adding the appropriate amount of the corresponding empty vector.

In contrast to non-established cells and REF52 cells, there is a number of immortalised rodent cell lines, such as Rat1 and NIH 3T3, in which oncogenic ras alone is sufficient to induce full transformation of cells (Land, 1983) and therefore, does not appear to exhibit any inhibitory effects on cell growth. To test whether the *cdc2* promoter is sensitive to ras-induced suppression in Rat1 cells, these cells were also co-transfected with cdc2-CAT and pEJ6.6, as described above. As shown in Fig. 5.1c, within the same range of titration, pEJ6.6 does not affect the activity of the *cdc2* promoter in growing Rat1 cells.

Thus, the results from transient transfections with oncogenic ras suggest that the *cdc2* promoter can be suppressed by ras in sREFs and REF52 cells, but not in Rat1 cells. This is consistent with the phenotypical responses to ras, observed in these cells.

To address the regulation of the *cyclin A* promoter by oncogenic ras, a reporter construct cycA-CAT (see section 2.1.11) was transiently co-
tansfected with increasing amounts of pEJr6.6 into sREFs and REF52 cells. As shown in Fig. 5.1a, b, similarly to the cdc2 promoter, the cyclin A promoter is also efficiently suppressed by oncogenic ras in a dose-dependent manner.

Thus, both the cdc2 and cyclin A promoters appear to be suppressed by oncogenic ras in growing sREFs and REF52 cells.

5.3 Mapping the Ras Response

To investigate the mechanism of ras-induced promoter suppression, the sites conferring the response were mapped in both promoters.

A set of 5' proximal truncation mutants of the cdc2 promoter was generated (see section 2.1.11.) (Fig.5.2b). The ras response of each mutant was then tested in transient co-tranfection experiments with increasing amounts of the ras expression plasmid, pEJ6.6 (see section 2.1.10). The experiments show that the response to ras is retained in all mutants containing the first 246 nucleotides upstream from the first transcription start site (position -246) (Fig.5.2a). Deletion of six nucleotides to the position -240 abrogated the negative regulation by ras.

Thus, the nucleotides between positions -246 and -240 of the cdc2 promoter are required for the ras induced promoter suppression. In order to confirm the role of this site within the full context of the 2.5 kb fragment of the cdc2 promoter, a mutant mRIcdc2 (RI stands for Ras Inhibition) (see section 2.1.11.) was generated by recombinant PCR (see section 2.2.23). In the mutant, the nucleotide sequence CCTCGT between positions -246 and -240 is substituted by the sequence AAGATT. By testing the regulation of mRIcdc2 in a transient co-transfection with pEJ6.6 (Fig.5.2c), it was demonstrated that increasing amounts of ras did not suppress activity of the mutant promoter.
any more. Thus, the sequence motif CCTCG positioned 243 nucleotides upstream of the first start site proved to be necessary in mediating the negative response to ras.

Analysis of the 5'end truncation mutants of the cyclin A promoter (see section 2.1. and Fig. 5.3b) identified the first 360 bp of the promoter as sufficient for the ras-induced suppression (Fig. 5.3a). Comparing the DNA sequence of this part of the promoter with the ras inhibitory motif CCTCG from the cdc2 promoter revealed a highly conserved motif CCTCCTCGGCCCTG 175 bp upstream from the first codon of the open reading frame of the cyclin A gene (compare with CTTCCCTCGTCCGCTG from the cdc2 promoter, see Table 5.1). A base substitution mutation within this motif was then generated by means of recombinant PCR (see section 2.2.23). The resulting mutant mRIcyclinA contains the AAGCTT substitution of the CCTCG motif within the context of the 3 kb fragment of the cyclin A promoter (see section 2.1.11). In contrast to the wild type promoter, transient co-transfection of the mRIcyclinA mutant with pEJ6.6 did not yield any suppression by ras, but showed rather elevated activity as compared with the wild type promoter (Fig.5.2c).

Thus, the pentanucleotide CCTCG present in both cdc2 and cyclin A promoters is required for mediating the ras-induced suppression. It is also a part of the conserved motif C(c/t)TCCTCG(t/g)CC(g/.)CTG shared by both promoters. This motif was designated the Ras Inhibitory Element (RIE).

5.4. Function of the Ras Inhibitory Element (RIE)

To explore whether the RIE may also be sufficient to confer the ras-induced suppression, the activity of the motif was tested in a heterologous environment. Single RIEs from the cdc2 and cyclinA promoters were linked
to the Herpes Simplex Virus (HSV) tk promoter in pBLCAT2 (see section 2.1.9), generating RIE(cdc2) and RIE(cyclinA) (see section 2.1.11). The constructs were then transiently transfected into sREFs and tested for their responses to oncogenic ras (Fig.5a). The HSV tk promoter alone, when introduced into growing sREF, leads to a substantial expression of the chloramphenicol acetylase gene, which is not affected by co-transfection of EJras6.6. However, by linking a single RIE site from the cdc2 or cyclin A promoters to this reporter, expression of the CAT gene is considerably reduced. Co-transfection of pEJ6.6, a construct expressing an activated c-Ha-ras gene (Land, 1983), further suppresses the activity of the RIE-tk promoter, almost to background levels. Thus, introduction of a single RIE site renders the HSV tk promoter sensitive to ras-mediated suppression. This implies that the identified RIE sites are not only necessary, but also sufficient in conferring the ras-induced suppression of gene transcription in sREFs.

5.5. Sequence Specificity of RIEs

The newly identified RIE sites from the cdc2 and cyclin A promoters have been shown to be necessary and sufficient for suppression of the promoter activities in response to oncogenic ras. Table 5.1 shows the sequences of the RIEs from both promoters, aligned with the RIE-related consensus binding sites for several transcription factors, as they were identified by GeneWorks genetic manipulation software. The binding consensus core of the ets-family of transcription factors (Wasylyk, 1993) and sites similar to the GCF (stands for GC-rich Factor) binding consensus (Kageyama and Pastan, 1989) can be found in both RIEs. Moreover, an AP-1 related Ostrowski element (Owen,
1990), or a binding site for the core binding factor (CBF) (Kamachi, 1990) are located 3' of the *cdc2* and *cyclin A* RIEs, respectively.

*In vitro* binding studies demonstrate that different members of the *ets*-family display distinct DNA binding specificities, determined by the sequences flanking the TCC core. For example, the binding specificity of *ets-1*, a protein regulating the T cell receptor (TcR) α and -β enhancers (Ho, 1990; Gottschalk and Leiden, 1990) is defined by the consensus CTTCCGG (Fisher, 1991; Wotton, 1994); while, *elf-1*, a protein regulating the IL-2 enhancer (Durand, 1988), exhibits highest affinity to CTTCCTC (Wang, 1992). There are data to suggest that certain members of the *ets*-family can operate as downstream targets of *ras* (Pognonec, 1988; Lai and Rubin, 1992; reviewed in Wasylyk, 1993). Multiple evidence also suggest that *ets*-like proteins execute their function via cooperative binding with a number of other transcription factors, such as AP-1 (Wasylyk, 1990), SRF (Dalton and Treismann, 1992), FAP (Gutman, 1991), and core binding factor (CBF) (Wotton, 1994). In fact, both an AP-1 like Ostrowski element (Owen, 1990) and the consensus for CBF (Kamachi, 1990) are present in the vicinity of the RIEs in the context of the *cdc2* and *cyclin A* promoter, respectively (*Table 5.1*).

The TCC triplet, representing the binding consensus core for *ets*-related proteins, overlaps with the CCTCG sequence implicated in the suppression by *ras* (see mutants mRI in *Table 5.1* and section 5.3). To investigate the possibilities of *ets*-related protein involvement in the mechanism of RIE regulation, the minimal TCC core required for ets binding was mutated into CAA within the context of both RIE(cdc2) and RIE(cyclinA) constructs, generating m1RIE(cdc2) and m1RIE(cyclinA) (see section 2.1.11, *Fig.5.4B* and *Table 5.1*). Testing the mutants for their response to oncogenic *ras* in transient co-transfection revealed that mutation of the *ets*-binding site core in both cases abrogates the negative response to *ras* (*Fig.5.4C*).
To address the role of the Ostrowski element, it was mutated from TGACTAG into TGCAGCT within the full context of the cdc2 promoter in construct m(Ostrowski) (see section 2.1.11). The site-directed mutation did not affect the ras-mediated suppression (Fig. 5.5), suggesting that the Ostrowski element is irrelevant to the observed inhibition by ras (see Table 5.1).

The CBF binding consensus GTGG adjacent to RIE in the context of the cyclin A promoter, was tested also for its role and mutated into ACAA within the full promoter context in construct m(CBF) (see section 2.1.11). This mutation did not affect the regulation of the promoter by ras (Fig. 5.5). Thus, within the cyclin A promoter context CBF is unlikely to be implicated in the ras-dependent regulation through the RIE site.

5.6 RIE: Studies of in vitro Protein Binding

The possible implication of ets-like proteins in RIE function was further explored in DNA binding experiments with ets-1 and elf-1 proteins in vitro. Chicken ets-1 protein, expressed in baculovirus infected insect cells SF9 (see section 2.1.8), forms sequence-specific complexes with probes corresponding to the ets-1 binding consensus and the RIE sites from the cdc2 and cyclin A promoter with similar efficiency (Fig. 5.6A, B; Table 5.1; see also section 2.1.10). In contrast, the ras-insensitive mutants m240 and mRlcyclinA are neither capable of binding ets-1, nor efficient in competing with the wild type RIE binding site (Fig. 5.6). The mutant probe m246 also binds ets-1 and competes with the wild type RIE site, although this mutant contains a substitution within the minimal consensus binding core TCC. Presumably, the flanking sequences of the extended binding consensus in m246 are able to
confer binding specificity. Interestingly, this mutant is still functional in its response to ras within the full context of the promoter (Table 5.1).

The binding consensus for another ets-related protein, elf-1, has a perfect match with the RIE sequence of the cdc2 promoter (see Table 5.1). To test whether elf-1 is also able to bind the RIEs, in vitro binding by baculovirus produced human elf-1 (see section 2.1.8) was assayed as described above. As shown in Fig.5.7, elf-1 forms sequence-specific complexes with the probes corresponding to its own binding consensus and the RIEs from the cdc2 and cyclin A promoters (Fig.5.7). Similarly to the in vitro complexes with ets-1, the functional mutant probe m246 competes efficiently with the wild type RIEs, while the ras-insensitive mutant 240 does not.

Thus, the mutant analysis of the RIE provides good correlation between DNA binding of ets-family of proteins to RIEs and their biological function.

5.7 RIE: in vivo Analysis of the Dominant Negative Mutant of Ets-1

To test whether ets-like proteins can regulate the RIEs in vivo, the dominant negative mutant of ets-1 was assayed in transient co-transfection with the RIE(cdc2) reporter construct (Fig.5.8). The C-terminal 114 amino acids of the ets-1 protein lack any transactivation domains, but contains the conserved DNA binding domain sufficient for sequence-specific binding of the protein. It has been demonstrated that the isolated C-terminal part of ets can function as a dominant-negative mutant by abrogating ets-dependent regulation (Langer, 1992).

To test whether the dominant negative mutant of ets-1 can disturb the RIE-mediated regulation, a construct expressing the C-teminal 114 amino acids of ets-1, pEtsC70 (see 2.1.11), was transiently co-transfected with 2.5 µg RIE-CAT and 5 µg pEJ6.6 (see section 2.1.11) into sREFs. As shown in Fig.5.8,
introduction of pEts-C70 released suppression of the RIE(cdc2) reporter mediated through the RIE site. The result suggests that the DNA binding domain of ets-1 is able to bind RIE in vivo. By retaining its binding specificity, the C-terminus of ets-1 is likely to compete with the endogenous factors mediating the ras-induced suppression through RIE.

5.8 Conclusions

The ras induced suppression of the cdc2 and cyclin A promoters is mediated through conserved Ras Inhibitory Elements (RIE). In fact, each RIE is necessary and sufficient to confer this response. Within the RIE several binding motifs of known transcription factors can be found. Among them, a binding site for ets-like proteins is required for the biological activity of both RIEs. Two members of the ets-family with distinct DNA binding specificity, chicken ets-1 and human elf-1, appear to be able to bind RIE in vitro. Moreover, binding of both proteins correlates well with the biological activity of the RIEs. Further analysis suggests that the DNA binding domain of chicken ets-1 is able to target RIE sequences. These results indicate that ets-family proteins can regulate RIE sites. Nevertheless, it needs to be still proven that such regulation is relevant to the observed inhibition by ras.

Besides the ets-like transcription factors, RIEs bare considerable resemblance with the part of the binding consensus for GCF factor (Kageyama and Pastan, 1989). GCF has been cloned and characterised as a negative transcription factor involved in suppression of the β-globin gene. The GCF binding sites are present in the promoters of the EGF receptor and c-Ha-ras genes (Johnson, 1992). The factor is abundant in mammalian tissues, and its potential contribution to RIE regulation is currently under investigation.
The cell cycle arrest and suppression of *cyclin A* and *cdc2* gene transcription induced by activated *ras* alone stands in contrast to uncontrolled proliferation and overexpression of *cyclin A* and *cdc2* in presence of two co-operating oncogenes such as *ras* and E1A (or SV40 large T). Thus, the role of a viral oncoprotein, as a co-operating partner, may also be of critical importance for the regulation of the *cdc2* and *cyclin A* promoters, and it is examined in the following chapters.
Fig. 5.1 Regulation of the \textit{cdc2} and \textit{cyclin A} promoters by oncogenic ras.

Proliferating sREFs (A), REF52 (B) and Rat1a (C) cells were transiently co-transfected with 2.5 mg of cdc2-CAT (filled bars) or cyclin A-CAT (hatched bars) and increasing amounts of pEJ6.6 as indicated.

All measured CAT activities were normalised against the β-galactosidase activities expressed from the co-transfected J4lacZ construct (see section 2.1.11). The standard deviation of the mean values is indicated by error bars.
Fig. 5.2 Mapping of the ras response within the cdc2 promoter.

5' proximal truncation mutants of the cdc2 promoter (B) were assayed for their response to ras in transient co-transfections with pEJ6.6 (A).

A mutant of the full-length promoter, with base substitutions as indicated between positions -240 and -246 (B), was transiently co-transfected with pEJ6.6 and assayed for the suppression by ras (C).

All measured CAT activities were normalised against the β-galactosidase activities expressed from the co-transfected J4lacZ construct. The standard deviation of the mean values is indicated by error bars.
Fig. 5.3 Mapping of the ras response within the cyclin A promoter.

5'proximal truncation mutants of the cyclin A promoter (B) were assayed for their response to ras in transient co-transfections with pEJ6.6 (A).

A mutant of the full promoter, with base substitution at position -175, as indicated (B), was transiently co-transfected with pEJ6.6 and assayed for the suppression by oncogenic ras (C).

All measured CAT activities were normalised against the β-galactosidase activities expressed from the co-transfected J4lacZ construct. The standard deviation of the mean values is indicated by error bars.
A) **Figure A:**

![Graph](image)

**Relative CAT Activity**

- **pEJ6.6, µg:**
  - 0
  - 5
  - 10

B) **Figure B:**

**Cyclin A Promoter**

- -254
- -3kb
- -1kb
- -450
- -390
- -360

**CAT**

- -3kb
- -1kb
- -450
- -390
- -360

**mRNAcyclinA**

**CCTCTCCTCGGCCTG**

**CCTaagcttCCTG**

C) **Figure C:**

![Graph](image)

**Relative CAT Activity**

- **pEJ6.6, µg:**
  - 0
  - 5
  - 10

**- cyclinA-CAT**

**- mRNAcyclinA**
Fig. 5.4 Activity of the *ras* inhibitory elements (RIEs).

CAT reporter constructs, driven by HSV tk promoter under the control of single RIE sites or one of the indicated mutants (B), were transiently co-transfected with pEJ6.6 into sREFs and assayed for their response to *ras* (A and C).

All measured CAT activities were normalised against the β-galactosidase activities expressed from the co-transfected J4lacZ construct. The standard deviation of the mean values is indicated by error bars.
A) Relative CAT Activity

B) pEJ6.6, µg

(CTTCCCTCGTCCGCTGACTAG) RIE(cdc2)

(CCTCCTCGGCCCTGCGTGGT) RIE(cyclinA)

(CTgttTCGGCCCTGCGTGGT) mut1RIE(cdc2)

(CGttTCGGCCCTGCGTGGT) mut1RIE(cyclinA)

HSV tk CAT pBLCAT2

C) Relative CAT Activity

pEJ6.6, µg

- RIE(cdc2)
- m1RIE(cdc2)
- m1RIE(cyclinA)
Fig. 5.5 Mutant analysis of the \textit{cdc2} and \textit{cyclin A} promoter suppression by oncogenic \textit{ras}.

\textbf{A.} 2.5 \textmu g of the m(Ostrowski)CAT construct (see section 2.1.11), containing the mutation TGCAGCT of the Ostrowski element within the full context of the \textit{cdc2} promoter, was transiently co-transfected with the pEJ6.6 construct, expressing activated c-Ha-ras, into sREFs.

\textbf{B.} 2.5 \textmu g of the m(CBF)-CAT construct (see section 2.1.11), containing the mutation ACA of the CBF binding consensus within the full context of the \textit{cyclin A} promoter, was transiently co-transfected with the pEJ6.6 construct, expressing activated c-Ha-ras into sREFs.

All measured CAT activities were normalised against the \textbeta-galactosidase activities expressed from the co-transfected J4lacZ construct. The standard deviation of the mean values is indicated by error bars.
A) 

B) 

Cyclin A Promoter

CAT

mRlcyclinA

m(CBF)

-m(CBF)

-mRIcyclinA

pEJ6.6, µg
Fig. 5.6 Electrophoretic mobility shift analysis of the ras inhibitory sites and their mutants in presence of ets-1.

A. An oligonucleotide containing the ets-1 consensus binding was incubated in presence of the baculovirus produced chicken ets-1 (see section 2.1.8 and 2.2.32). The competitors used at 20-fold molar excess were the ets-1 cold probe (lane 3), RIEs of the cdc2 or cyclin A promoters (lane 4 and 7) and their mutants (lanes 5, 6 and 8). Lane 1 represents binding in the control SF9 cell extracts (see section 2.1.8).

B. Complexes with baculovirus produced chicken ets-1 were formed on the probes representing the ets-1 binding consensus (lane 1), RIEs of the cdc2 and cyclinA promoters (lane 2 and 5) and their mutants (lanes 3, 4 and 6).
Fig. 5.7 Electrophoretic mobility shift analysis of the *ras* inhibitory sites and their mutants in presence of *elf-1*.

Complexes were formed in SF9 cell extracts with baculovirus produced human *elf-1* (see section 2.1.8 and 2.2.32) and the probes representing either the *elf-1* binding consensus (lane 1), or the RIEs of the *cdc2* and *cyclin A* promoters (lane 3 and 7). The specificity of binding was assayed in competition with 20-fold molar excess of the cold probe representing *elf-1* binding consensus (lane 2), RIEs (lanes 5 and 8) and the 246 and 240 mutants of the RIE(cdc2) (lanes 4 and 6).
Fig. 5.8 Activity of the ras inhibitory element (RIE) in presence of the dominant negative mutant of ets-1.

2.5 μg RIE-CAT were co-transfected with 5 μg pEJ6.6, expressing activated c-Ha-ras, and increasing amounts of the plasmid pEts-C70, expressing the C-terminal 114 amino acids of chicken ets-1 (see section 2.1.11). All measured CAT activities were normalised against the β-galactosidase activities expressed from the co-transfected J4lacZ construct. The standard deviation of the mean values is indicated by error bars.
Table 5.1 Ras Response Elements and their mutants

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Response to Ras</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>cdc2:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RIE</td>
<td>CTTCCTCGTCCGCTGACTAG</td>
<td>yes</td>
</tr>
<tr>
<td>-246</td>
<td>aTgCCTCGTCCGCTGACTAG</td>
<td>yes</td>
</tr>
<tr>
<td>-240</td>
<td>aTTCgcatgCGCTGACTAG</td>
<td>no</td>
</tr>
<tr>
<td>mRI</td>
<td>CTTaagattCCGCTGACTAG</td>
<td>no</td>
</tr>
<tr>
<td>m(Ostrowski)</td>
<td>CTTCCCTCGTCCGCTGcagct</td>
<td>yes</td>
</tr>
<tr>
<td>m1RI</td>
<td>CTTcaatCCGCTGCTGACTAG</td>
<td>no</td>
</tr>
<tr>
<td><strong>cyclinA:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RIE</td>
<td>CCTCCTCGGCC.CTGCGTGGT</td>
<td>yes</td>
</tr>
<tr>
<td>mRI</td>
<td>CTTaagcttCC.CTGCGTGGT</td>
<td>no</td>
</tr>
<tr>
<td>m1RI</td>
<td>CCcaatCCGCC.CTGCGTGGT</td>
<td>no</td>
</tr>
<tr>
<td>m(CBF)</td>
<td>CCTCCTCGGCC.CTGCAaaat</td>
<td>yes</td>
</tr>
<tr>
<td><strong>DNA Binding Sites:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ets core</td>
<td>TCC..........................</td>
<td></td>
</tr>
<tr>
<td>ets-1</td>
<td>CTTCGG.........................</td>
<td></td>
</tr>
<tr>
<td>elf-1</td>
<td>CTTCCTC.........................</td>
<td></td>
</tr>
<tr>
<td>CBF</td>
<td>..................................GTGG.</td>
<td></td>
</tr>
<tr>
<td>Ostrowski</td>
<td>..................................TGACTAG.</td>
<td></td>
</tr>
<tr>
<td>GCF</td>
<td>..................CCTCggccNCTG....</td>
<td></td>
</tr>
</tbody>
</table>

*Positions conserved between the RIE from the cdc2 and cyclin A promoters are highlighted with bold font*
Chapter 6

Identification of E1A Response Elements in the cdc2 and cyclin A promoters
6.1 Introduction

Schwann cells and REFs transformed by co-operating oncogenes such as ras and SV40LT or E1A contain high levels of cdc2 and cyclin A mRNA (C. Barth, in preparation; D. Parkinson, unpublished). This is in contrast to cells expressing ras alone and indicates a dramatic switch in promoter regulation in the presence of two co-operating oncogenes.

In this chapter the contribution of a single viral oncogene to cdc2 and cyclin A promoter regulation is explored.

6.2 Activation of the cdc2 and cyclin A Promoters by 12S E1A

The Adenovirus E1A region encodes two splice variants, 12S and 13S, which are both equally transforming. Yet, E1A (13S) contains an additional domain, termed conserved region 3 (CR3). This domain is a potent broadly acting transcriptional activator, that directly binds to the TATA-binding protein (TBP), an essential component of the polymerase II preinitiation complex (PIC; Lee, 1991). However, the CR3 domain is not required for the cooperative transformation of primary cells (Lillie, 1986; Moran, 1986; Schneider, 1987), suggesting that transactivation by direct interaction with PIC is irrelevant for the transforming function of E1A. Similar to E1A (13S), SV40 large T has also been shown to activate transcription promiscuously through direct interaction with TBP (Gruda, 1993). Thus, the E1A (12S) protein can be regarded as the only viral oncoprotein suitable to study specific regulation of cdc2 and cyclin A promoters which may be relevant to cell transformation.

Given that cdc2 and cyclin A promoters are cell cycle regulated (Dalton, 1992; Henglein, 1994), their regulation by E1A (12S) was analysed in serum starved cells in transient co-transfections with a 12S E1A expressing plasmid, pE1A (see section 2.1.11). Twenty four hours before transfection,
subconfluent rat embryo fibroblasts were switched to 0.5% FCS DMEM. The cells were transiently co-transfected with pElA and either the cdc2-CAT, or the cyclinA-CAT reporter constructs (see section 2.1.11).

The results of the co-transfection experiments reveal a strong activation of reporter CAT activities in response to increasing amounts of pElA (Fig.6.1). Thus, both cdc2 and cyclin A promoters can be stimulated by 12S E1A.

6.3 Role of E2F Sites in the Activation of the cdc2 Promoter by E1A

The observed activation of the cdc2 promoter by 12S E1A is consistent with the previously reported role of the E2F binding sites in the transcriptional regulation of the cdc2 promoter. The E2F sites have been shown to mediate the retinoblastoma protein (Rb)-dependent suppression on the cdc2 promoter (Dalton, 1992). Since, E1A is able to bind Rb, disrupt the Rb-E2F complexes, and restore activity of the E2F transcription factor (reviewed in Nevins, 1994), it is expected to induce promoter activity in an E2F site-dependent manner.

The cdc2 promoter contains three potential E2F binding sites (Dalton, 1992). To test the role of these E2F sites in E1A-mediated activation of the promoter, a mutant cdc2 promoter construct, cdc2(mut E2F)-CAT, with all three E2F sites rendered non-functional (Dalton, 1992), was assayed in transient co-transfection with pElA in sREFs. As shown in Fig. 6.2, the loss of the E2F sites does not affect the ability of 12S E1A to activate the cdc2 promoter. These data indicate that the cdc2 promoter contains E1A-response elements distinct from the E2F sites.
6.4 Identification of the E1A Response Element (ERE)

In order to identify the regulatory elements mediating the E1A-response of the cdc2 and cyclin A promoters, a set of cdc2 and cyclin A promoter mutants (see section 2.1.11) was assayed in presence of the 12S E1A expressing construct, pE1A (Fig.6.3). Truncation mutants of the cdc2 promoter (see section 2.1.11) were transiently co-transfected with pE1A (see section 2.1.11) into rat embryo fibroblasts under the conditions described in section 6.2. All mutants retaining position -165 upstream from the first transcription start site (Dalton, 1992) remained E1A responsive, while truncation at position -155 rendered the promoter insensitive to E1A (Fig.6.3). Thus, the nucleotide sequence between the positions -165 and -155 contains a necessary component implicated in the E1A-dependent activation of the cdc2 promoter (Fig.6.3).

A similar approach to mapping an E1A-response element identified such a region between -390 and -360 bp upstream from the first codon of the cyclin A gene (Fig.6.3)

Sequence comparison of the two mapped promoter regions revealed a common conserved decameric sequence motif 5'-C.CCCTCCTGC, which subsequently is designated as E1A Response Element (ERE).

6.5 Analysis of the E1A Response Element (ERE)

Next, the ERE was tested for its ability to mediate the observed E1A response.

A single ERE was linked to the Herpes Simplex Virus (HSV) tk promoter in pBLCAT2 (see section 2.1.9), generating ERE-CAT (see section 2.1.11) The construct was tested for its promoter activity in absence and presence of 12S
E1A through transient co-transfections into sREFs (Fig. 6.4). Introduction of the single ERE site next to the HSV tk promoter suppresses the promoter. However, in presence of E1A the suppression imposed by the ERE site is released.

6.6 Domains of 12S E1A Required for Induction of the ERE

In the previous section it has been shown that 12S E1A was able to release ERE-mediated suppression of a heterologous promoter. The multifunctional nature of E1A is based on multiple protein-protein interactions involving different domains of the protein. To distinguish which domains are required for activation through the ERE, 12S E1A mutants were tested for their ability to activate the ERE-CAT reporter construct (Fig. 6.5). The mutants used have been documented for being expressed in sREFs at similar levels (Schneider, 1987).

Transient co-transfection of the ERE-CAT construct with each of the 12S E1A mutants shown in Fig. 6.5 demonstrated that the activation of the ERE requires an intact N-terminus and the conserved region CR1 of the E1A protein. In fact, all mutations tested, except a deletion in the conserved region 2 (del CR2), disabled the activation of the ERE by 12S E1A. Since the mutation in the CR2 domain impairs binding of Rb, p107 and p130 proteins (ref), this result is consistent with the observation that the activation of the cdc2 promoter by E1A does not require the E2F binding sites (see section 6.3).

However, as the activation of the ERE requires the N-terminus of the E1A protein, it is possible that another cellular protein is involved in this process. As mentioned in section 1.4, the N-terminal part of the E1A proteins is involved together with the CR1 domain, in the association to a nuclear protein.

6.7 Conclusions

12S E1A activates the *cdc2* and *cyclin A* promoters through a conserved sequence motif designated E1A Response Element (ERE) - CGCCCTCCTGC. Functional study of ERE reveals a potent inhibition mediated by the element in the heterologous context of the Herpes Simplex Virus tk promoter. 12S E1A re-activates the promoter and requires an intact N-terminus and the CR1 domain for this function.

The N-terminus of E1A binds p300, a protein implicated in the control of cellular proliferation (Moran, 1993; see Chapter 8). Thus, the regulation of the *cdc2* and *cyclin A* promoters through the EREs among others may involve the cellular protein p300.
Fig.6.1 Activation of the *cdc2* and *cyclin A* promoters by 12S E1A. Serum deprived semi-confluent rat embryo fibroblasts were transiently co-transfected with 2.5 μg of either the cdc2-CAT, or the cyclinA-CAT reporter construct (see section 2.1.11) and the indicated amounts of the pE1A construct, expressing 12S E1A (see section 2.1.11). Throughout the experiment the cells were kept in 0.5% foetal bovine serum (FCS). All measured CAT activities were normalised against the β-galactosidase activities expressed from the co-transfected J4lacZ construct (see section 2.1.11). The standard deviation of the mean values is indicated by error bars.
The graph shows the relative CAT activity (on the y-axis) against the amount of pE1A (on the x-axis) in micrograms (μg). The x-axis includes values of 0, 0.3, 0.6, 0.9, and 1.2 μg. The graph compares two conditions: cdc2CAT (hashed bars) and cyclinACAT (striped bars).
Fig.6.2 Activation of the cdc2 promoter by 12S E1A does not depend on the E2F binding sites. Two and a half μg of the mutant reporter construct, cdc2(mut E2F)-CAT, containing three modified E2F sites (Dalton, 1992), were assayed in transient co-transfections with pE1A in sREFs, under the conditions described in section 6.2. All measured CAT activities were normalised against the β-galactosidase activities expressed from the co-transfected J4lacZ construct. The standard deviation of the mean values is indicated by error bars.
cdc2 Promoter +1 CAT

Mutant E2F sites +1

- cdc2CAT
- cdc2(mut E2F)CAT
Fig. 6.3 Mapping the E1A Response within the *cdc2* and *cyclin A* Promoters. Two and a half μg of the 5' proximal mutants of the *cdc2* (A) and *cyclin A* (B) promoters (see section 2.1.11) were assayed for their response to 12S E1A in transient co-transfections with the indicated amounts of pE1A (see section 2.1.11).

All measured CAT activities were normalised against the β-galactosidase activities expressed from the co-transfected J4lacZ construct. The standard deviation of the mean values is indicated by error bars.
Fig.6.4 Activity of the E1A Response Element.

Two and a half μg of the CAT reporter construct, containing a single ERE site linked to the HSV tk promoter, was tested for its activity in presence of co-transfected one μg pE1A. The HSV tk promoter construct pBLCAT2 (see section 2.1.9) was used as a control.

All measured CAT activities were normalised against the β-galactosidase activities expressed from the co-transfected J4lacZ construct. The standard deviation of the mean values is indicated by error bars.
Fig.6.5 E1A domains required for the activation of the ERE.

A. One µg of the mutant 12S E1A expressing constructs (see section 2.1.11) was transiently co-transfected with two and a half µg of the ERE-CAT or the control reporter construct pBLCAT2 (see section 6.5).

B. Schematic representation of the expression constructs containing 12S E1A mutants (see section 2.1.11). Conserved regions 1 and 2 (CR1 and CR2) are indicated.

All measured CAT activities were normalised against the β-galactosidase activities expressed from the co-transfected J4lacZ construct. The standard deviation of the mean values is indicated by error bars.
Chapter 7

Co-operative Regulation
of the cdc2 and cyclin A Promoters
by
ras and 12S E1A
7.1 Introduction

In previous chapters it was demonstrated that transcription of the \textit{cdc2} and \textit{cyclin A} promoters is regulated by the oncogenes \textit{ras} and 12S E1A. Oncogenic \textit{ras} is able to suppress activity of the promoters in proliferating primary cells through the regulatory element RIE, while 12S E1A can activate the promoters in serum deprived cells through the distinct conserved regulatory element - ERE.

In this chapter the response of the \textit{cdc2} and \textit{cyclin A} promoters to the combination of \textit{ras} and 12S E1A is explored.

7.2 Activation of the \textit{cdc2} and \textit{cyclin A} Promoters by Co-operating Oncogenes

To study the regulation of the \textit{cdc2} and \textit{cyclin A} promoters by a combination of co-operating oncogenes, the activities of the reporter constructs cdc2-CAT (Dalton, 1992; see section 2.1.11) and cyclinA-CAT (see section 2.1.11) were analysed in transient co-transfection of secondary rat embryo fibroblasts and REF52 cells in presence of \textit{ras} and 12S E1A. To measure the effects of the co-operating oncogenes in absence of additional extracellular mitogenic stimuli, the cells were grown to saturation density and deprived of serum prior to transfection. Thus, different to the assay with E1A alone (see chapter 6), the following experiments were carried out with contact inhibited cells. Briefly, the cells were grown in 10% FCS DMEM until they reached confluency. To allow an additional cycle of proliferation, they were incubated with fresh 10% FCS medium for 24-30 hr. Then the serum was reduced to 0.5%. Forty hours later, the cells were co-transfected by using the Ca\textsuperscript{2+} precipitate method (for the protocol see section 2.2.3) with 2.5 \mu g of the cdc2-CAT
reporter construct, 7 μg of pEJ6.6, a construct expressing an activated c-Ha-ras gene (Land, 1988, see section 2.1.11), 0.3-10 μg of pE1A, a 12S E1A expressing construct (see section 2.1.11), and 3 μg of J4lacZ, a lac Z expressing construct (see section 2.1.11). The precipitate was left on the cells in 0.5% serum for 20 hrs, and then washed off with fresh 0.5% FCS medium. Fourtyeight hours after the wash the cells were harvested (section 2.2.5) and assayed for CAT (section 2.2.6) and β-galactosidase (section 2.2.7) activities.

The results of the transfection experiments indicate (Fig.7.1) that similarly to the endogeneous cdc2 promoter (Dalton, 1992) the activity of the transfected reporter construct is very low in resting cells. With the promoter activity being so low, the sensitivity of the CAT assay does not allow to detect the ras-induced inhibition of the cdc2 promoter (see chapter 5). Titration of pE1A within the range of 0.3-2.5 μg resulted in activation of the cdc2 promoter by 12S E1A alone up to two-fold. Further titration of pE1A up to 10 μg leads to decrease in expression for all transfected activities including the β-galactosidase control. This may be due to the ability of E1A to induce apoptosis in primary cells at low serum conditions (Evan, 1992). At the same time, co-transfection of pEJ6.6 and pE1A within the same range of concentrations resulted in a 5-7 fold activation of the cdc2 promoter.

Similarly to cdc2, co-transfection of 2.5 μg of the cyclinA-CAT reporter construct with 7 μg of pEJ6.6, 0.6 μg of pE1A and 3 μg of J4lacZ resulted in low basic activity of the transfected cyclin A promoter in absence and presence of single oncogenes and its activation by the combination of ras and 12S E1A (Fig.7.2).
7.3. Synergistic Promoter Activation by Co-operating Oncogenes

To explore the possibility that the activation of the $c\text{dc}2$ and $\text{cyclin} A$ promoters by $ras$ and 12S E1A may involve an induction of E1A expression by $ras$, 12S E1A mRNA levels were examined in transfected cells. Rat embryo fibroblasts were treated and co-transfected with pE1A (12S E1A), pEJ6.6(ras) and J4lacZ(lacZ) as described in section 7.2. Forty-eight hours after transfection, the cells were lysed in Guanidin-Based Denaturation Buffer and total cellular RNA was prepared for the RNase protection analysis (see section 2.2.25 and 2.2.27 for the protocols). The RNA samples were hybridized with the $^{32}\text{P}$-labelled anti-sense RNA probes corresponding to the 5'proximal part of 12S E1A and lacZ cDNA (see section 2.1.12). As shown in Fig. 7.3, the levels of lacZ mRNA, as indicated by the protected probe, are easily detected in RNA samples from cells transfected with J4lacZ (compare lanes 1, 2, 4, 5, 6 with lane 3). Co-transfection of $ras$ (compare lanes 1 and 2) does not affect the levels of the lacZ protected signal, implying that expression of control lacZ mRNA is not affected by ras. RNA from cells, co-transfected with pE1A, show a specific protection signal with the antisense E1A probe (lanes 3, 5 and 6). Most importantly, co-transfection of $ras$ (compare lanes 5 and 6) does not increase the level of the E1A probe protection. This shows that $ras$ does not affect the expression of 12S E1A mRNA from the pE1A construct.

Thus, the co-operative activation of the $c\text{dc}2$ and $\text{cyclin} A$ promoters by $ras$ and 12S E1A is likely to be a bona fide synergistic event.
7.4 Mapping the Co-operative Response

In a monolayer of density arrested rat embryo fibroblasts the co-operating oncogenes ras and 12S E1A synergise in activating the cdc2 and cyclin A promoters. To map the co-operative response on the cdc2 promoter, a set of 5′end truncation mutants (see section 2.1.11) was tested in transient co-transfection with ras and 12S E1A (Fig 7.4). The reporter constructs for each of the mutants were co-transfected with 7 μg of pEJ6.6 (activated c-Ha-ras), 1 μg pE1A(12S E1A) and 3 μg J4lacZ (LacZ) (see section 2.1.11). The experiment shows that the response maps between positions -125 and -110, containing the nucleotide sequence CTCTAGCCACCC. The presence of this sequence in the mutant 125cdc2 renders the constitutive core of the cdc2 promoter (positions -110 to + 75; Dalton, 1992) responsive to co-operative activation and also suppresses basic activity of the constitutive core in resting cells.

A direct repeat of the mapped sequence is present in the cdc2 promoter 30 bp upstream of position -125. This repeat (CTCTAGCCGCCC) is highly conserved and only varies in one position from the motif identified in the deletion analysis.

To ascertain the role played by the identified elements within the full context of the promoter, a mutant m(1, 2)cdc2 (see section 2.1.11) was generated by recombinant PCR (see section 2.2.23). The mutant contains base substitution mutations (GAATTCAATCCC) for both sequence motifs within the context of 2.5 kb of the cdc2 promoter (see Fig.7.4B). The mutant was tested for its response to the co-operating oncogenes in transient co-transfections of rat embryo fibroblasts, as described in section 7.2. As shown in Fig.7.4C, the mutant promoter, in contrast to the wild type, does not respond to the co-transfected ras and E1A and remains inactive in the transfected cells. In fact, even in mitogenically stimulated cells the activity of the mutant promoter
appears to be greatly reduced (Fig. 7.6). These data show that the identified sequence motifs are important elements of cdc2 promoter regulation in normal cells and are required for the synergistic activation by ras and 12S E1A. For the convenience of reference, the identified dodecamers were termed Goblins 1 and 2, reflecting the dangerous liaisons of these small elements.

To identify the elements responsible for the co-operative activation of the cyclin A promoter, a set of 5'end deletion mutants of the promoter (see section 2.1.11 and Figure 7.5) was first tested in a series of transient co-transfections, as described for the cdc2 promoter. As shown in Fig. 7.5, mutant analysis revealed that promoter constructs including 450 bp of sequence upstream of the ORF were activated by the co-operating oncogenes. However, when the promoter was truncated to position -390, it lost its response. Comparison of the DNA sequence between positions -450 and -390 of the cyclin A promoter with the cdc2 promoter identified two elements, A (CTCTCCCCACCC) and B (TTCTCCGCCC), as related to the previously described Goblins. There are 9 bp of unrelated sequence between the elements A and B in the cyclin A promoter, and 115 bp of unrelated sequence between B and the first reported start site (Heinglein, 1994) (see also Fig. 8.3). In comparison, in the cdc2 promoter Goblins 1 and 2 are separated from each other by 18 bp of unrelated sequence, and Goblin 1 is positioned 110 bp upstream of the first transcription start site (Dalton, 1992) (see also Fig. 8.2).

To ascertain the role of A and B elements, several mutants within the full context (3kb) of the cyclin A promoter were generated by recombinant PCR (see section 2.2.23). The mutants, designated mA-, mB- and m(A, B)cyclinA (see Fig. 7.5B and section 2.1.11), contain base substitutions (GAATTCTATTTT) in place of element A or B, and both A and B, respectively. The mutants were tested for their responses to the co-operating oncogenes in a set of transient co-transfections carried out as described above.
As shown in Fig. 7.5C, mutation of a single element reduced synergistic activation of the promoter in co-transfection with pEJ6.6 and pE1A. Nevertheless, the co-operative effect was eliminated only when both elements were modified. Thus, a pair of elements similar to the Goblins of the cdc2 promoter are required for co-operative activation of the cyclin A promoter by ras and E1A. In accordance with the nomenclature, the elements A and B were therefore designated Goblin 3 and 4, respectively. Unlike the cdc2 promoter, modification of both Goblins within the full context of the cyclin A promoter has only little effect on the promoter activity in normal cells in the presence of mitogenic stimuli (Fig.7.6).

7.5 Goblins Can Function as Enhancer Elements

To test whether the function of a Goblin as a regulatory element depends on its position, the Goblin element was removed from its normal context in the 125-cdc2 construct (see Figs. 7.4B and 7.7) and positioned downstream of the CAT reporter gene, generating the construct 110cdc2-G1 (see Fig.7.7A). Similar to 125cdc2, in transient co-transfection of 110cdc2-G1 with pEJ6.6 and pE1A, the construct exhibits suppression of the constitutive core promoter activity (-110 to +75) in resting cells and a strong co-operative response to ras and 12S E1A (see Fig.7.7B).

Thus, the Goblin element is able to impose co-operative regulation on the constitutive promoter core while positioned downstream of the reporter gene and therefore exhibits the features of an enhancer element.
7.6 Goblins Are Sufficient to Mediate Activation by Co-operating Oncogenes

To further investigate the regulatory functions of Goblins, they were compared with other documented enhancer elements. The comparison revealed similarity between Goblins and the tandem repeats of the 21 bp enhancers in the Simian Virus 40 promoter (SV40) (Gidoni, 1985). For example, the sequence of Goblin 4 (TTCTCCCGCCC) bears strong similarity with the first repeat of the 21 bp enhancer, termed GC-I box (TTCTCCCGCCC). This repeat plays the key role in activating early transcription from the SV40 promoter (Gidoni, 1985). Moreover, the 21p enhancers can mediate promoter activity in absence of a TATA-box element from multiple transcription start sites (Pugh and Tjian, 1991).

Given the features of the 21bp enhancers, the Goblins were tested for their ability to mediate transcriptional activation of a minimal reporter gene in response to ras and 12S ElA. A mutant construct, G1-CAT (see Fig.7.8A and also section 2.1.11), was generated by cloning an oligonucleotide G1, containing a single Goblin 1 element (see section 2.1.9), into the promoter-less reporter construct pAtk (see section 2.1.11) upstream from the CAT coding sequence. Transient co-transfection of G1-CAT with pEJ6.6 and pE1A revealed that this reporter construct was still highly sensitive to cooperative action by ras and 12S ElA, yielding 8-10 fold activation. In addition, the three other Goblins were also shown to mediate the response to ras and ElA within the same context of minimal reporter constructs G2-, G3, and G4-CAT (Fig.7.8).

However, at this stage it remains unclear whether the Goblin element exhibits features of a minimal promoter, or whether the observed response is mediated by the Goblin in conjunction with a non-identified cryptic promoter.
Nevertheless, it can be concluded that Goblins are regulatory elements sufficient to mediate synergistic activation in response to co-operating \( ras \) and 12S E1A in a heterologous environment.

### 7.7 Domains of 12S E1A Implicated in the Co-operative Activation

In synergistic activation of the \( cdc2 \) and \( cyclin A \) promoters in a monolayer of density arrested cells, E1A co-operates with \( ras \) and performs its function through Goblins - elements distinct from ERE (.The fact that two different response elements mediate promoter regulation by E1A alone and E1A and \( ras \) together implies that, possibly, different functions of E1A protein are involved in these two types of promoter regulation. As it has been mentioned before, multiple functions exercised by 12S E1A are mediated by separate domains of the protein and, therefore could be distinguished by mutant analysis.

The G1-CAT reporter construct, containing a single Goblin driving the CAT reporter gene (see section 2.1.1 and section 7.7), was transiently co-transfected into rat embryo fibroblasts along with 7 µg pEJ6.6, 3 µg CH110 and 1 µg of each of the 12S E1A mutant constructs (see section 2.1.11 and Fig.7.9A). As shown in Fig.7.13, a mutant of 12S E1A with the deletion of amino acids 2-11 is highly efficient in co-operative activation with \( ras \). On the other hand, mutants with the deletion of aminoacids 2-23 and/or CR1 are strongly impaired in their ability to activate the reporter construct.

Comparison of these data with the E1A mutant analysis of on the ERE site (see chapter 6) reveals clear difference: while the function of E1A mediated by ERE required the amino-terminal 11 amino acids (see Chapter 6), co-operation with \( ras \) is independent of this domain.
It is worth pointing out that the amino acids 11-23 and CR1, necessary for the co-operative activation through Goblins, are the only two essential domains for co-operative transformation of primary rat embryo fibroblasts by 12S E1A and \textit{ras} (S. Shellard, unpublished).

\section*{7.8 Sequence Specificity of Goblins}

To investigate sequence specificity requirements for Goblin, a set of mutant Goblin elements and related sequence motifs were tested for their ability to mediate co-operative activation by \textit{ras} and E1A.

The 3' half (CCA/GCCC) of the Goblin elements contains a sequence related to the GC-boxes (CCGCCC) of the SV40 promoter which binds the transcription factor Sp1 (Dynan, 1983; Gidoni, 1984). In vitro binding and protection analysis with Sp1 demonstrated that the third guanin plays crucial role and is required for specific binding of this transcription factor (Dynan and Tjian, 1983). The 5' half of the Goblin does not share any resemblance with any known binding consensus sequences.

The mutants generated for the functional assay of the Goblins represent base substitutions of the 5' half (mutant 1 - AAAGTTCCACC) leaving the GC-box intact, base substitutions of the 3' half (mutant 2 - CTCTAGAATAAA) and a point mutant of the guanin, implicated in Sp1 binding (mutant 3 - CTCTAGCCTCCC). The oligonucleotides representing the mutant sites, as well as the GC-I box of SV40 (TTCTCCGCC) and the extended Sp1 consensus (GCCCGGCC) binding sites were cloned into the minimal reporter context of pAtk, as described for Gl-CAT (see section 7.6 and 2.2.11), generating m1-, m2-, m3-, GC1- and Sp1-CAT, respectively.
As shown in Fig.7.9, in transient cotransfection with pEJ6.6 and pE1A neither of the mutants, nor the GC-I box and the Sp1 consensus site respond to cooperating oncogenes.

These results indicate that the Goblin-mediated response to ras and 12S E1A is sequence specific. Moreover, factors interacting with the GC-I box such as the Sp1 transcription factor alone are not sufficient to mediate the co-operative response.

7.9 In vitro Analysis of Goblin-Specific DNA Binding Complexes

To explore the DNA-protein interactions specific for the Goblin elements and to test whether they involve Sp1, cellular extracts were tested for specific DNA binding activities in electromobility shift assays (see section 2.2.32). Nuclear extracts were prepared from rat embryo fibroblasts (see section 2.2.31). Complexes were formed with 32p-labelled double stranded oligonucleotides, corresponding to Goblin 1(G1) and its point mutant 3 (m3) (see section 7.8 and section 2.2.10), and resolved on polyacrylamide gels (see section 2.2.34).

According to the radio autograph of the gel (Fig.7.11), two complexes of low electrophoretic mobility, designated A1 and 2, are formed on the Goblin probe in nuclear extract. Competition with the unlabelled Goblin probe in fifty-fold excess eliminated complex A1 and reduced complex A2 (lanes 2 and 3). On the other hand, similar competition with the point mutant m3 did not affect either of the complexes (lanes 4 and 5). Moreover, point mutant m3 as a labelled probe does not form the same complexes A1 and 2 in the nuclear extracts (lane 6). Altogether, the data suggest that nuclear extracts from rat embryo fibroblasts contain a DNA binding activity that is sequence specific for the Goblin element.
To test whether the Sp1 transcription factor (see section 7.7) may be involved in Goblin-specific protein interactions, the complexes described above were compared with the complexes formed on the extended Sp1 consensus site. As shown in Fig. 7.12, complexes B1, 2 and 3 formed with the Sp1 probe which have a different electrophoretic mobility than complex A1. Cross-competition with the unlabelled Sp1 and G1 probes (lanes 2, 3, 6, 7) shows that these complexes have different sequence specificities as well. At the same time, complex A2 is formed on both probes and is efficiently reduced in cross-competition. Challenge of the complexes with SP1-antibodies, kindly provided by Dr S.Jakson, MRC/Wellcome Institute, Cambridge, results in the elimination of complex B1(lanes 4 and 8). However, A2, the only complex which exhibits similar sequence specificity for the Goblin 1 and Sp1 sites, all the other complexes remain unaffected by the antibodies.

Finally, the binding of purified human Sp1 protein (Promega) to the Goblin element and the Sp1 consensus binding site was compared in electromobility shift assays (Fig. 7.13). Purified Sp1 forms a strong complex with its binding consensus probe Sp1, but only very weak interaction can be observed with the Goblin probe(lane 7). Moreover, the Sp1 complex is not cross-competed in presence of unlabelled G1 probe(lanes 5 and 6). These data indicate low specificity of \textit{in vitro} binding to the Goblin sequence for the Sp1 protein.

Thus, the data derived suggest that the Goblin-specific DNA binding complexes formed \textit{in vitro} are unlikely to contain the Sp1 transcription factor.

\section*{7.10 Conclusions}

Signals controlled by \textit{ras} and 12S E1A converge to activate transcription of the \textit{cdc2} and cyclin A promoters in synergistic manner. This activation of both promoters is mediated by pairs of conserved elements, termed Goblins.
Each of the Goblins is sufficient to mediate the co-operative response, and inactivation of both elements is required to render the promoters insensitive to the oncogenes. Functional analysis of Goblins allows to classify them as enhancer elements. Further studies in vitro indicate the presence of a nuclear DNA binding activity, specific for the Goblin elements.
Fig. 7.1 Activation of the *cdc2* promoter by oncogenic *ras* and 12S E1A.

Serum deprived, confluent rat embryo fibroblasts were transiently co-transfected with 2.5 μg of *cdc2*-CAT, 7 μg of pEJ6.6, expressing activated c-Ha-ras, and indicated amounts of the pE1A construct, expressing 12S E1A (see section 2.1.11).

All measured CAT activities were normalised against the β-galactosidase activities expressed from the co-transfected J4lacZ construct. The standard deviation of the mean values is indicated by error bars.
Fig. 7.2 Activation of the cdc2 and cyclin A promoters by oncogenic ras and 12S E1A.

Serum deprived, confluent rat embryo fibroblasts were transiently co-transfected with 2.5 µg of either the cdc2-CAT (A), or the cyclinA-CAT (B) reporter constructs (see section 2.1.11), 0.6 µg of the pE1A construct, expressing 12S E1A, and 7 µg of pEJ6.6, expressing activated c-Ha-ras (see section 2.1.11).

All measured CAT activities were normalised against the β-galactosidase activities expressed from the co-transfected J4lacZ construct. The standard deviation of the mean values is indicated by error bars.
Fig. 7.3 Expression of 12S E1A mRNA in sREFs is independent of oncogenic ras.

Secondary rat embryo fibroblasts (sREFs) were transiently co-transfected with 0.6 μg pE1A (12S E1A) (lanes 3, 5 and 6), 7 μg pEJ6.6(ras) (lanes 2 and 6), and J4lacZ(lacZ) (lanes 1, 2, 4, 5 and 6). RNA was isolated 48 hr after transfection and 5 μg of total cellular RNA was analysed by RNase protection with 32P-labelled anti-sense RNA probes corresponding to the 5’proximal part of 12S E1A and lacZ cDNAs. Both probes were added together to all samples. The indicated RNase-resistant products were separated on a 6% denaturing polyacrylamide gel and autoradiographed.
Transfected Plasmids:

- + - - - + pEJ6.6

- - + - + + pE1A

+ + - + + + J4lacZ
Fig. 7.4 Mapping of DNA sequences necessary for co-operative activation by ras and E1A of the cdc2 promoter.

5'proximal truncation mutants of the cdc2 promoter (B) were assayed for their co-operative response in transient co-transfections with pEJ6.6 and pE1A, as described in section 7.2 (A). The nucleotide sequence required for the response to co-operating oncogenes between -125/-110 and its direct repeat at position -145 are indicated (B). A full size promoter with base substitutions (see B) was also transiently co-transfected with pEJ6.6 and pE1A and assayed for co-operative activation (C).

All measured CAT activities were normalised against the β-galactosidase activities expressed from the co-transfected J4lacZ construct. The standard deviation of the mean values is indicated by error bars.
A) A graph showing relative CAT activity for different conditions:

- ras
- +E1A
- E1A+ras

Relative CAT Activity

-2500 -722 -165 -155 -135 -125 -110

B) Diagram of the cdc2 promoter and CAT region:

- cdc2 promoter
- CAT
- CTCTAGCCGCC
- CTCTAGCCACCC
- gaa11 catCCC

C) Graph showing relative CAT activity for different conditions:

- - cdc2CAT
- - m(1, 2)cdc2

- ras E1A ras+E1A
Fig.7.5 Mapping of DNA sequences necessary for co-operative activation by ras and E1A of the cyclin A promoter.

5'proximal truncation mutants of the cyclin A promoter (B) were assayed for their co-operative response in transient co-transfections with pEJ6.6 and pE1A, as described in section 7.2 (A).

The nucleotide sequences required for the response to co-operating oncogenes between -450 and -390 are indicated (B). A full size promoter with base substitutions at these positions (see B) was also transiently co-transfected with pEJ6.6 and pE1A and assayed for the co-operative activation (C).

All measured CAT activities were normalised against the β-galactosidase activities expressed from the co-transfected J4lacZ construct. The standard deviation of the mean values is indicated by error bars.
**A)**

Relative CAT Activity

-3kb - 1kb - 450 - 390 - 360

- ras
- +E1A
- E1A+ras

**B)**

**Cyclin A Promoter**

-254

A: TTCTCCCCCC

B: CTCTCCCCCC

mAcyclinA

mBcyclinA

m(A,B)cyclinA

gaattctatattt gaattctatattt

**C)**

- cyclinA-CAT
- mAcyclinA
- mBcyclinA
- m(A,B)cyclinA
Fig. 7.6 Activity of mutant promoters m(1, 2)cdc2 and m(A, B)cyclinA in transiently transfected proliferating cells.

The mutant reporter constructs m(1, 2)cdc2 (see section 7.4) and m(A, B)cyclinA were transiently transfected into growing rat embryo fibroblasts at high serum concentration (10%). The activity of the mutant promoters was compared with the transfected wild type cdc2 and cyclinA promoters (see section 2.1.11).

All measured CAT activities were normalised against the β-galactosidase activities expressed from the co-transfected J4lacZ construct. The standard deviation of the mean values is indicated by error bars.
Relative CAT Activity

- m(1, 2)/cdc2
- m(A,B) cyclinA
- cyclin A-CAT
- cdc2-CAT
Fig.7.7 Enhancer activity of the Goblin element.

Reporter constructs 125cdc2, 110cdc2 and 110cdc2-G1 (A, see also section 7.5) were assayed for the co-operative response in transient co-transfection with pEJ6.6 and pE1A, as described in section 7.2 (B).

All measured CAT activities were normalised against the β-galactosidase activities expressed from the co-transfected J4lacZ construct. The standard deviation of the mean values is indicated by error bars.
Fig. 7.8 Response of Goblin elements to single and co-operating oncogenes. Reporter constructs G1-, G2-, G3-, G4-CAT (A, see also section 7.6) were assayed for the co-operative response in transient co-transfection with pEJ6.6 and pE1A, as described in section 7.2 (B).

All measured CAT activities were normalised against the β-galactosidase activities expressed from the co-transfected J4lacZ construct. The standard deviation of the mean values is indicated by error bars.
A)

(CTCTAGCCACCC) □ G1-CAT
(CTCTAGCCGCCC) □ G2-CAT
(CTCTCCCCACCC) □ G3-CAT
(CTCTCCCCGCCC) □ G4-CAT

□ p Δtk
-110  +1

cdc2  CAT

□ 110-cdc2

B)

Relative CAT Activity

ras  E1A  E1A+ras
Fig. 7.9 Mapping of E1A domains required for the Goblin response to cooperating oncogenes.

A. The G1-CAT reporter construct (see section 7.7) was assayed for the cooperative response as described in section 7.2, in transient co-transfections into REFs with pEJ6.6 and each of the constructs expressing distinct 12S E1A mutants (see section 2.1.11).

B. Schematic representation of the expression constructs containing 12S E1A mutants (see section 2.1.11). Conserved regions 1 and 2 (CR1 and CR2) are indicated.

All measured CAT activities were normalised against the β-galactosidase activities expressed from the co-transfected J4lacZ construct. The standard deviation of the mean values is indicated by error bars.
A)

E1A Mutants: 

- - E1A delCR1 2R/G del2-23/CRI del2-23 del2-11 delCR2

ras: 

- + + + + + + + +

<table>
<thead>
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<th>Relative CAT Activity</th>
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B)

**RSV LTR**

<table>
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<tr>
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<tr>
<td>1</td>
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</tr>
<tr>
<td>80</td>
<td>120</td>
</tr>
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</tr>
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</table>

E1A (12S)

2R/G

del2-11

del2-23

delCR1(35-65)

del2-23/CRI

delCR2
Fig. 7.10 Mutant Analysis of the Goblin 1 element.

Reporter constructs G1-, Sp1-, SV40-, m1-, m2- and m3-CAT (A, see also section 7.8) were assayed for the co-operative response in transient co-transfection with pEJ6.6 and pE1A, as described in section 7.2 (B).

All measured CAT activities were normalised against the β-galactosidase activities expressed from the co-transfected J4lacZ construct. The standard deviation of the mean values is indicated by error bars.
### A)

<table>
<thead>
<tr>
<th>DNA Sequence</th>
<th>Symbol</th>
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<tbody>
<tr>
<td>(CTCTAGCCACCC)</td>
<td>G1-CAT</td>
</tr>
<tr>
<td>(TTCTCCGCCC)</td>
<td>SV40-CAT</td>
</tr>
<tr>
<td>(CGCCCCGGCCC)</td>
<td>Sp1-CAT</td>
</tr>
<tr>
<td>(aaagttCCACCC)</td>
<td>Gmut1-CAT</td>
</tr>
<tr>
<td>(CTCTAGaataaa)</td>
<td>Gmut2-CAT</td>
</tr>
<tr>
<td>(CTCTAGCtCCCC)</td>
<td>Gmut3-CAT</td>
</tr>
<tr>
<td>(CTCTAGCtCCCC)</td>
<td>p Δtk</td>
</tr>
</tbody>
</table>

### B)

- ras
- E1A
- E1A+ras

**Relative CAT Activity**

- 6
- 5
- 4
- 3
- 2
- 1
- 0
Fig. 7.11 *In vitro* binding of cellular proteins to Goblin sequences.

DNA-protein complexes were formed with nuclear extracts from sREFs with the $^{32}$P-labelled oligonucleotides (see section 2.1.9) corresponding to Goblin-1 (G1; lanes 1-5), and its biologically inactive point mutant, m3 (lane 6), as described in section 7.9. The complexes were resolved on a 5% polyacrylamide gel. The binding reactions were performed in presence of 20- and 50-fold excess of the unlabelled oligonucleotides G1 (lanes 2, 3) and m3 (lanes 4, 5).

The complexes were resolved on a 5% EMSA gel and autoradiographed. Sequence-specific complexes A1 and A2 are indicated.
Fig. 7.12 Comparison of Goblin and Sp1 DNA-protein complexes formed in vitro.

Complexes were formed in nuclear extracts from sREFs with the $^{32}$P-labelled oligonucleotides (see section 2.1.9) corresponding either to the Sp1 (Sp1; lanes 1-4), or Goblin 1 (G1; lanes 5-8) binding sites. Binding reactions were also performed in presence of unlabelled Sp1 (lanes 2, 7) and G1 (lanes 3, 6) probes in 50-fold excess.

Rabbit polyclonal antiserum against human Sp1 protein (2982-E) or the pre-immune control serum (2982-I) was added to the binding reactions as indicated.

The complexes were resolved on a 5% EMSA gel and autoradiographed. Goblin-specific complexes A1-2, described in section 7.9, and complexes B1-3, formed with the Sp1 probe, are are indicated.
1 2 3 4 5 6 7 8

probe

competitor, 50-fold

α-Sp1

2892-1
2892-1
2892-1
2892-1
2892-1
2892-1
2892-1
2892-1
2892-1

A1

A2

B1

B2

B3
Fig. 7.13 In vitro binding of the human Sp1 protein to the Sp1 DNA binding site and the Goblin (G1) element.

Complexes were formed with 5 ng of purified human Sp1 protein (Promega) (lanes 2-11) and $^{32}$P-labelled probes corresponding to either the Sp1 binding consensus (Sp1; lanes 1-6), or the Goblin (G1; lanes 7-11) (see section 2.1.9). Binding reactions were also performed in presence of 20- and 50-fold excess of unlabelled Sp1 (lanes 3, 4, 10, 11) and G1 (lanes 5, 6, 8, 9) probes.

The complexes were resolved on a 5% EMSA gel and autoradiographed.
<table>
<thead>
<tr>
<th>Competitor</th>
<th>Probe</th>
<th>Sp1 Protein</th>
</tr>
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<tbody>
<tr>
<td>Spl, 20-fold</td>
<td>Spl</td>
<td>++</td>
</tr>
<tr>
<td>Spl, 50-fold</td>
<td>Spl</td>
<td>++</td>
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<tr>
<td>G1, 20-fold</td>
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<td>G1, 20-fold</td>
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<td>Spl, 20-fold</td>
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<td>Spl, 50-fold</td>
<td>G1</td>
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Image: Gel with bands for Sp1 protein.
Chapter 8

Discussion
8.1 Oncogene Co-operation and Signal Integration

The transcriptional regulation of the *cdc2* and *cyclin A* promoters by co-operating oncogenes was analysed. This revealed a mechanism by which signals induced by *ras* and E1A converge and synergistically activate gene transcription independent from normal cell cycle control (Fig. 8.1). The co-operative activation is mediated by pairs of conserved enhancer elements, termed Goblins (Fig. 8.2 and 8.3), which are necessary and sufficient for the oncogenic response. In contrast to co-operative activation, transcriptional regulation of the same promoters by either of the oncogenes alone is mediated through distinct conserved elements. Consistent with the observation that oncogenic *ras* alone inhibits expression of *cdc2* and *cyclin A* genes in Schwann cells, *ras* has been found to suppress both *cdc2* and *cyclin A* promoters. This suppression is mediated through the conserved *ras* inhibitory elements (RIEs) (Fig. 8.1, 8.2 and 8.3) and may involve a member of the ets-family of transcription factors. At the same time, 12S E1A alone can activate the *cdc2* and *cyclin A* promoters through the conserved E1A response elements (EREs) (Fig. 8.1, 8.2 and 8.3), through a mechanism which may involve p300, a transcriptional coactivator implicated in the control of growth and differentiation. However, the activation by 12S E1A alone is observed only in absence of cellular contact inhibition. In a monolayer of highly confluent cells, 12S E1A alone remains inactive and requires co-operation with *ras* in order to activate the *cdc2* and *cyclin A* promoters through the Goblin elements.

Oncogene-specific regulation of *cdc2* and *cyclin A* gene transcription is likely to be important for the induced phenotypes. Co-operative promoter activation requires the same domains of E1A (11-23 aa and CR1; see section 7.7) that are required for co-operative transformation of REFS (S.Shellard, in
preparation). In addition, the suppression of cell cycle regulatory genes, such as cyclin A, is causally involved in the ras-induced cell cycle arrest in Schwann cells (C. Barth, in preparation; section 1.5). Moreover, since expression of both the cdc2 and cyclin A genes is essential for cell proliferation (Th'ng, 1990; Girard, 1991; Pagano, 1992; Zindy, 1992; Pines, 1993), it can be postulated that the activation of these genes by ras and E1A together is a necessary component of the programme through which cooperating oncogenes induce cell transformation. If so, ras-induced cell cycle arrest and ras and E1A-induced proliferation are directly linked to the transcriptional regulation of the cdc2 and cyclin A genes by these oncogenes. The fact that oncogene-controlled signals converge to regulate common targets by integrative processes may therefore be an essential principle at the basis of oncogene co-operation. Since the oncogenes regulate the cdc2 and cyclin A promoters through multiple response elements, at least part of the signal integration must take place at promoter level.

8.2 Ras-Induced Suppression

Analysis of the ras-induced regulation of the cdc2 and cyclin A gene transcription reveals inhibition of both gene promoters through the conserved Ras inhibitory elements, RIEs (Fig. 8.1, 8.2 and 8.3). As mentioned before, inhibition of cyclin A gene expression is required for the implementation of the ras-induced growth arrest in Schwann cells (C. Barth, in preparation). In fact, growth inhibition is a known feature of oncogenic ras. Besides sREFs and REF52 cells (Hirakawa and Ruley, 1988; see section 5.1), ras also induces growth arrest in Schneider cells from D. melanogaster (Johansen, 1989) and in the pheochromocytoma cell line (PC-12) ras-induced growth
arrest is followed by differentiation (Bar-Sagi and Feramisco, 1985; Muroya, 1992).

Further analysis of the ras -induced suppression of the cdc2 and cyclin A promoters indicates that members of the ets proto-oncogene family of transcription factors (reviewed in Seth, 1992; Macleod, 1992; Wasylyk, 1993) are able to interact with and regulate the RIE. In vitro binding assays (see section 5.6) show remarkable correlation between binding of several members of ets -family, ets-1 and elf-1 , to wild-type and mutant RIEs and their respective biological responses. Moreover, in vivo a dominant negative mutant of ets-1 is able to abrogate suppression mediated by the RIE.

All members of the ets -family share a conserved DNA-binding domain, that specifically interacts with sequences containing the common core sequence TCC. The specificity of DNA binding for different ets-related proteins has been shown to be determined by sequences flanking the core (see Wasylyk, 1993 for the review). Furthermore, ets -like proteins are also involved in protein-protein interactions that determine the stability of ternary complexes formed on the DNA and their biological activity. Although the ets domain is sufficient for specific DNA binding, a number of proteins like ets-1, ets-2, GABP α, SAP1 and elk1 appear to contain intra-molecular domains which inhibit DNA binding. In turn these domains are inactivated by association with accessory factors (Lim, 1992; Thompson, 1991; Dalton and Treisman, 1992; Janknecht and Nordheim, 1992). However, although an alternatively spliced isoform of ets-1 lacks this inhibitory domain (Jorcyk, 1991), activation by ets-1 of the T cell receptor α enhancer still requires association with a second unrelated factor (Ho, 1990), while activation of the polyoma virus enhancer also requires fos and jun (Wasylyk, 1990a).
Analysis of the RIE sites shows that the conserved sequence shared between the \textit{cdc2} and \textit{cyclin A} promoters extends for 10 bp from the TCC core. Mutant m246, with a single base substitution within the core and an intact flanking sequence on both sides, retains sensitivity to \textit{ras} and its ability to bind \textit{ets-1} and \textit{elf-1} in vitro. By introducing further mutations into the 5'-proximal part of m246 outside of the RIE consensus, binding to ets-like proteins must ultimately become impaired. Analysis of the \textit{ras} -response of such mutants may help to further establish the possible role of \textit{ets} -like proteins in the \textit{ras} -induced regulation. Moreover, they will also help to investigate the possible role of the GCF binding site which overlaps with the ets consensus binding motive. Due to the fact that the sequences flanking the TCC core determine the binding specificities of ets-related proteins, RIE sites may be used to identify the members of the protein family, which due to their high specificity are most likely to be involved in the \textit{ras} -induced regulation of the RIE element.

\textit{Ets} -related proteins have been implicated in the regulation of gene expression during a variety of biological processes, including growth control, transformation, T-cell activation and lymphocute differentiation, Drosophila development and Xenopus oocyte maturation. Although, generally the \textit{ets} proteins have been shown to activate transcription, dependent on promoter/enhancer context certain members of the ets family can act as transcriptional repressors. For example, \textit{ets-1} activates stromelysin 1 in HeLa cells (Wasylyk, 1991) but represses T cell receptor β enhancer in quiescent Jurkat cells (M.Owen, personal communication). Recent studies carried out with \textit{ras} -transformed fibroblasts implicate members of the \textit{ets} family as potential downstream targets of Ras (Bruder, 1992; Dudek, 1992; Langer, 1992). In another example, the \textit{ets} -related protein \textit{Yan} was shown to participate in negative control of eye development in \textit{D.melanogaster} (Lai
and Rubin, 1992). The protein is localized in nuclei of undifferentiated cells in the developing eye and appears to be strongly down-regulated in differentiating cells. Genetic analysis places Yan downstream from the sevenless (sev) receptor tyrosine kinase and Ras1, whose activation directs the precursor R7 cells towards differentiation. Loss-of-function mutations of the Yan gene also result in differentiation of supernumerary photoreceptors in the Drosophila eye, and suggests that Yan and Ras1 have opposing effects on neural development.

The described features of ets-like proteins suggest two possible models of the ras-induced regulation on the cdc2 and cyclin A promoters. First, oncogenic ras may induce an ets-like repressor specific for the RIE site (compare with the inhibition of the TCR β enhancer by ets-1). This may occur through phosphorylation-induced elongation of the protein half-life, as observed with ets-2 in T-cell activation (Fujiwara, 1990). In the second model, ras may prevent inactivation of a factor similar to the Yan protein, and result in RIE-mediated suppression. However, the fact that the dominant negative mutant of ets-1 abrogates RIE-mediated suppression supports the first model and suggests that active suppression is induced by oncogenic ras. As a next step, the identification of the factors specifically binding and regulating the RIE sites will help to elucidate the mechanism of ras-induced suppression.

8.3 E1A-dependent Activation

Analysis of the E1A-dependent regulation of the cdc2 and cyclin A gene transcription shows an activation of both gene promoters mediated by a distinct conserved E1A response element, ERE (see Fig.8.2 and 8.3).
Mutant analysis of 12S E1A indicates that the N-terminus of the protein is required for the regulation on the ERE site. This domain of E1A is linked with cell growth control and contains a binding site for a high molecular weight cellular protein, p300 (Egan, 1988). E1A mutants that retain the p300 binding site are sufficient to induce quiescent primary fibroblasts into S phase (Wang, 1991). Consistent with the analysis of mutant cdc2 and cyclin A promoters, this ability of E1A does not require gene activation through E2F binding sites (Raychaudhouri, 1991), suggesting that the amino-terminal domain of E1A may stimulate gene expression through promoter elements other than E2F sites.

Among the regulatory elements targeted by the E1A amino-terminal domain are GC-rich sequences from tissue-specific enhancers (reviewed in Moran, 1993), and a p300 DNA binding site selection experiment resulted in isolation of sequences with the following consensus: 5'-CACTCCC-3', although the cloned p300 protein has not been found to bind to DNA directly (Rikitake and Moran, 1992). Recent cloning of p300 (Eckner, 1994) revealed a structure highly related to CBP, a transactivator interacting with the members of the CREB/ATF family and the basal transcription factors of the preinitiation complex (Chrivia, 1993). Based on this similarity, it is suggested that interaction with E1A can abrogate transactivation by p300 and therefore result in the suppression of target genes. Indeed, the N-terminal domain has been implicated in the suppression of several tissue-specific enhancers (Rikitake and Moran, 1992). However, recent evidence also indicates that E1A, depending on its N-terminal residues, can activate the cellular hsp70 heat shock promoter through the TATA box sequence (Kraus, 1992). At present, the involvement of p300 in the E1A-dependent activation through the ERE site remains unclear. However, recent cloning of the protein permits to address this question directly. For example, genetic complementation of the
p300 interaction with the mutant 12S E1A may directly show involvement of p300.

**8.4 Co-operative Regulation**

As mentioned above, analysis of the transcriptional regulation of the *cdc2* and *cyclin A* gene by the co-operating oncogenes ras and E1A reveals a potent activation of both gene promoters. It is mediated by the directly repeated Goblin elements (see Fig.8.2 and 8.3). This activation takes place in serum deprived, contact inhibited cells. It appears, that under these conditions none of the previously described mechanisms of regulation by either ras, or E1A alone can be observed. Thus, co-operative activation of the promoters marks a distinct mechanism of regulation, induced by the synergistic action of the oncogenes.

Investigation of the Goblins, reveals an unusual role played by them in both the *cdc2* and *cyclin A* promoters. Site-directed mutagenesis of both Goblins renders the promoters insensitive to co-operating oncogenes. Moreover, these elements appear to be sufficient to mediate the co-operative activation in a minimal heterologous context. This, as well as similar positioning of the elements in relation to the transcription start sites in both *cdc2* and *cyclin A* promoters and visible similarity to the Sp1 binding sites, suggested that Goblins may exemplify minimal promoters sufficient to initiate RNA polymerase II transcription in response to co-operating oncogenes. Preliminary experiments indicate that co-operative activation of Goblins leads to a re-direction of transcription start sites. However, to become conclusive this finding needs further thorough investigation. Yet, further analysis of the
phenomenon may help to elucidate the mechanism of activation by co-operating oncogenes.

The role played by Goblins in the normal regulation of the \textit{cdc2} and \textit{cyclin A} promoters remains highly elusive. There are two pieces of evidence that might suggest possible involvement of Goblins in growth-dependent regulation of the promoters. First, site-directed mutagenesis of the elements in the context of the \textit{cdc2} promoter strongly impairs promoter activity in normal proliferating cells (see Figure 7.6). Second, introduction of a single Goblin element next to the constitutive core of the \textit{cdc2} promoter renders the construct, similar to the complete promoter, suppressed in resting cells (see Figure 7.4). These observations suggest that Goblin-specific regulation is likely to act during the cell cycle. However, in the context of the \textit{cyclin A} promoter the Goblins do not show the same features as in the \textit{cdc2} promoter. A recent study of the mitogenic activation of the \textit{cyclin A} promoter (D. Parkinson, personal communication) implicated a CREB/ATF site to be the major site involved in this response. While deletion of the Goblin elements show no effect, mutation of the CREB/ATF site abrogates activation of the \textit{cyclin A} promoter in response to serum. However, in presence of co-operating oncogenes a mutant promoter containing the Goblins in absence of the CREB/ATF site is still co-operatively activated (D. Parkinson, personal communication). These results suggest that mitogenic or cell cycle regulation of the promoters studied may involve multiple independent pathways. On the other hand, they provide evidence that the oncogenic Goblin-dependent pathway can be distinct from signalling pathways induced by mitogens.

As mentioned above, Sp1 binding sites and GC-boxes from the SV40 promoter share certain sequence similarity with the Goblins. However, further analysis shows that regulation of the Goblin elements requires a transcription
factor(s) different from Sp1. At present, the nature of the Goblin-specific factors remains unclear. In vitro studies detect a specific DNA-binding activity present in nuclear extracts. Recent cross-liking experiments (A. Sewing, personal communication) suggest that two polypeptides of 55 and 45 kD can specifically bind to Goblin sequences. The isolation and characterisation of these proteins will necessary to study the molecular mechanisms involved in the synergistic activation of $cdc2$ and $cyclin$ A promoters by co-operating oncogenes.
Fig. 8.1 Regulation of the *cdc2* and *cyclin A* promoters by co-operating oncogenes.

Schematic representation of the promoters and regulatory elements mediating responses to *ras* and 12S E1A. Regulatory elements RIEs (*ras* inhibitory elements), EREs (E1A response elements) and Goblins are indicated. Transcription factors possibly implicated in the responses are indicated next to the regulatory elements.
human cdc2 promoter

human cycA promoter
Fig. 8.2 Oncogene-dependent regulatory elements in the human $cdc2$ gene promoter.

The elements mediating response to oncogenes: $ras$ inhibitory element (RIE), E1A response element (ERE), and co-operative response elements, Goblins, are indicated within the sequence of the human $cdc2$ gene promoter. Two major transcription start sites are underlined (Dalton, 1992).
SphI

GCATGCCTCTCTATATATTAAATCTGATGTGAAAATATTTTAAAATTTAATAAAT
CATTTCAAATGTTTTATAATTGTATAATAAAACAAATGAAAGCACAGCGATATAAAATAA
TAATATTAAATTTCAAAATGAGGTAGAAACAAAGCACAGCGATATAAAATAA
ATTTTCCTTTTACATTTTTGAGGGCTCTTTTGAGTTTTGGATTTCCTTCTTAAG
GTCACATGAAATGTGTCTCTTTGAGCGCCAGCCGCAATCAGCATTAGAAAAAC
ATAACTATACACTCTCCTAACCCTAAGTATTTGAAGTGAAAGTAAATGGAATCTCGA
TGTAACACAGAGATACCTTTTTTGATGAGCTATTTTGAGTATAATAAATAATTTGAA
CTGTGCCAATGCTGGGAGAAAAATTTAAAAGAAGAACGGAGCGAACAGTAGCT
TCCTCGTCGCTGCTGACTAGAAACAGTAGACGACTCTCTCCGACTGGAGGAG
ROME
AGGTTGCGCTCGCACTCAGTTGGAGCCGCACTCTCTCCGACTGGAGGAG
ERE
GGCCCTTTTTTCTCTTTTCTGCGCTCTAGCCACCCGGGAAGGCCTGCCAGC
Goblin 1
GTAGCTGGCCTGATTTGCGCTTTTGAAGGCTACTAGGCAAACGTGCTGCCGCTGC
GCCGCCGCGGCCGCCGCCGCCGCGGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCC
SacII
Fig. 8.3 Oncogene-dependent regulatory elements in the human *cyclin A* gene promoter.

The elements mediating response to oncogenes: Ras inhibitory element (RIE), E1A response element (ERE), and co-operative response elements, Goblins, are indicated within the sequence of the human *cyclin A* gene promoter. Major transcription start sites are underlined (Henglein, 1994; see also section 4.5).
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