A thesis entitled

Regulation of E2F by Phosphorylation

Presented by

Lorna Josephine Morris

To

The University of Glasgow

For the degree of

Doctor of Philosophy

Division of Biochemistry and Molecular Biology

Institute of Biomedical and Life Sciences

University of Glasgow

Scotland

September 2000

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

To my mum and dad

Abstract

The E2F proteins are a family of transcription factors that function in regulating the G1 to S phase transition. The activity of E2F is regulated in a cell-cycle dependent manner by its physical interaction with members of the retinoblastoma protein (pRb) family, which are inactivated via phosphorylation by cyclin-dependent kinases (CDK), most notably cyclinD-CDK complexes in G1. This ensures that the activation of E2F-responsive genes is closely coupled with cell cycle position. In this study, the role of direct phosphorylation of E2F in the regulation of E2F activity is explored. CyclinE-CDK2 can directly phosphorylate E2F-5 *in vitro*, and phosphorylation is abolished by mutation of threonine 251 to alanine in the *trans*activation domain of E2F-5 (T251A), whilst mutation of serine 307 to alanine (S307A) did not affect phosphorylation. Evidence is presented that phosphorylation of E2F-5 by cyclinE-CDK2 facilitates transcription and cell cycle progression. Specifically cyclinE-CDK2 causes a significant increase in E2F-5 dependent transcription, which is not observed for T251A. In addition, S307A is compromised in its ability to activate transcription. Taken together the results suggest that phosphorylation of E2F-5 at distinct sites by cyclinE-CDK2 and an as yet unidentified kinase augments E2F-5 dependent transcription.

The p300/CBP family of proteins have been implicated as important co-activators for a variety of transcription factors, including E2F-1. Here it is shown that the binding of E2F-5 to p300 is stabilised upon phosphorylation of E2F-5 by cyclinE-CDK2, suggesting enhanced recruitment of p300 is mechanistically important for stimulating E2F-5 dependent transcription. Since *cyclin E* is a well-known E2F responsive gene and the results imply that E2F activity may be directly regulated by cyclinE-CDK2, an autoregulatory feedback loop may exist between E2F-5 and cyclin E

that drives the expression of E2F target genes, thereby promoting irreversible entry into S phase. Overall, these results define a novel level of control in the regulation of cell cycle progression by E2F.

Contents

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 5

CHAPTER 6

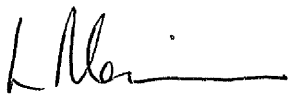
CHAPTER 7

CHAPTER 8

List of figures

Declaration

All the work presented within this thesis was performed entirely by myself and in no way forms part of any other thesis. The work was carried out while I was a graduate student at the Division of Biochemistry and Molecular Biology, Institute of Biomedical and Life Science, University of Glasgow, UK. I was under the supervision of Professor Nicholas B. La Thangue.



Lorna Josephine Morris

September 2000

Publications

The following publication was submitted during the course of the work presented in this thesis.

Morris, L., K.E. Allen, and N.B. La Thangue. (2000). Regulation of E2F transcription by cyclin E-Cdk2 kinase mediated through p300/CBP co-activators. *Nat. Cell Biol.* **2**:232-239.

Abbreviations

A	Adenine, Alanine or peptide A (angel)
α	Anti-(body)
ATF	Activating Transcription Factor
ATP	Adenosine 5'-triphosphate
β-gal	β-galactosidase
BSA	Bovine Serum Albumin
C	Cytosine or peptide C / Control peptide
CAK	Cyclin Activating Kinase
cAMP	Cyclic adenosine mono-phosphate
CBP	CREB binding protein
CDC-	Cell Division Cycle -
CDI	Cyclin-CDK Inhibitor
CDK-	Cyclin Dependent Kinase -
cDNA	Complementary deoxyribonucleic acid
CIP	Calf Intestinal Phosphatase
CREB	cAMP response element binding protein
Cyc	Cyclin
D	Aspartate or Peptide D
DHFR	Dihydofolate reductase
dH_2O	Distilled water
DMEM	Dulbecco's Modification of Eagles Medium
DNA	Deoxyribonucleic acid
DNA-PK	Double-stranded DNA-activated protein kinase
DOC	Deoxycholate
DP-	DRTF1 Polypeptide
DTT	Dithiothreitol
EC	Embryonal Carcinoma
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetra-acetic acid
E2F-	E2 factor

FACS	Fluorescence activated cell sorting
FCS	Foetal Calf Serum
FITC	Fluorescein isothiocyanate
F9EC	F9 Embryonal Carcinoma
G	Guanine
g	Gram or Gravities
G0	Quiescence
G1	Gap 1 phase
G2	Gap 2 phase
GST	Glutathione-S-transferase
GTP	Guanosine 5'-triphosphate
h	Hour
his	6 x Histidine tag
HEPES	N-[2-Hydroxethyl]piperazine-N'-[2-ethanesulfonic acid]
IB	Immunoblot
IP	Immunoprecipitation
IPTG	Isopropyl-ß-D-thiogalactopyranoside
KDa	Kilodalton
M	Molar or Mitosis
mg	Milligram
μg	Microgram
min	Minute(s)
ml	Millilitre
μl	Microlitre
mM	Millimolar
mm	Milimetre
μM	Micromolar
mRNA	Messenger RNA
n	Nano
ng	Nanogram
NP-40	Nonidet P40
NLS	Nuclear localisation signal
OD	Optical Density

P	Phosphate group
p55	DP-1 doublet as resolved by immunoblotting
PAGE	Polyacrylamide gel electrophoresis
pRb	Retinoblastoma susceptibility gene product
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline Tween (20)
PMSF	Phenylmethylsulfonyl fluoride
PKC	Protein Kinase C
p55L	Faster migrating half of DP-1 p55 doublet
s.d.	standard deviation
SDS	Sodium dodecyl sulphate
Sf9	*Spodoptera frugiperda*
S	serine
T	threonine
TAD	*trans*activation domain
TRITC	Tetramethylrhodamine isothiocyanate
(+/+)	Wild type
(+/-)	Heterozygous mutant
(-/-)	Homozygous mutant

Chapter 1

Introduction

1.1 The Cell Cycle

The mammalian cell cycle has been divided into four distinct phases, G1, S, G2 and M phase. DNA replication (S-phase) and cell division (M-phase) are separated by two gap periods (G1 and G2) during which cells are believed to assess their capacity to progress on to the next phase of the cell cycle. A newly divided cell in G1 phase has several choices. It can commit to DNA replication in preparation for a further round of mitosis, or it can become quiescent, differentiate or undergo apoptosis. Growth factors trigger signalling cascades which stimulate entry through G1. However at a specific point in G1, the commitment point, growth factors are no longer required and progression through the cell cycle then depends on the intrinsic workings of the cell cycle clock (Sherr, 1993).

1.1.1 Cyclin Dependent Kinases (CDKs)

Progression through the cell cycle is dependent on phosphorylation of key regulatory proteins by cyclin-dependent kinases (CDKs) which require binding of regulatory subunits known as cyclins as an initial step in their activation (Lees, 1995). Whilst the levels of cyclin proteins oscillate throughout the cell cycle, the levels of CDK remain relatively stable. The accumulation of cyclin subunits, displacement of cell cycle inhibitors and phosphorylation by the CDK-activating kinase (CAK) all

contribute to the periodicity of CDK activity during the cell cycle. Cyclin accumulation is determined by cell cycle-regulated transcription and proteolysis.

In vitro, cyclins are able to bind a large variety of CDKs (Pines, 1995). Most of the cyclin-CDKs phosphorylate the same basic consensus sequence *in vitro* (K/R)-S/T-P-X-(K/R) where the basic residues are preferred but not essential (Nigg, 1993; Pines, 1995). However *in vivo* only a handful of specific cyclin-CDK substrates have been described (Nigg, 1993; Pines, 1995).

1.1.2 Regulation of the G1-S transition by cyclin-CDKs

The transition from G1 into S phase is a key regulatory step in the mammalian cell cycle, which is controlled by the G1 cyclins and their associated kinases. G1 cyclins comprise D-type cyclins (D1, D2 and D3) and cyclin E. The levels of D-type cyclin mRNA accumulates in G1 and cyclin E mRNA accumulates somewhat later, peaking during late G1 and declining during early S phase (Sherr, 1993; Ohtsubo *et al.*, 1995). Destruction of G1 cyclins is important for resetting the balance of regulatory factors ready for the next cell cycle, such that the timing of subsequent transitions are not inappropriately advanced (Koepp *et al.*, 1999).

The mRNA of D-type cyclins accumulates in response to growth factors and because both the mRNA and proteins turn over rapidly, cyclin D levels decline precipitously when growth factors are withdrawn (Sherr, 1993). The importance of cyclin D in regulating progression through G1 has been highlighted by several experiments. Inhibition of expression of cyclin D1 causes cell cycle arrest before S phase and microinjection of cyclin D1 antibodies during G1 prevents fibroblasts from entering S phase (Baldin *et al.*, 1993). However antibody injection is ineffective in

preventing S phase entry when cells are injected in late G1, suggesting the function of cyclin D1 is to stimulate progression through G1 rather than promote the G1-S transition.

The D-type cyclins are known as growth factor sensors. When cyclin D1 is over-expressed cells enter S phase early and have a reduced dependence on growth factors (Quelle *et al.*, 1993). Mitogen stimulated activation of Ras triggers a signalling cascade which promotes transcription of the cyclin D1 gene via the activation of the extracellular signal-regulated protein kinases (ERKs), although the actual physiological target(s) that mediates this process has not been defined (reviewed in Sherr, 1993).

Members of the cyclin D family assemble with their catalytic partners, CDK4 and CDK6 and the resulting group of kinases are most recognised for their role in phosphorylating pRb, which plays an important role in the regulation of the G1/S transition (see Section 1.1.5).

The rise in cyclinE-CDK2 kinase activity which occurs at the end of G1 is necessary for entry into S phase in most cells (Koff *et al.*, 1992; Ohtsubo *et al.*, 1995). The transcription factors, Myc and E2F have been implicated in transcriptional regulation of the cyclin E promoter (Perez *et al.*, 1999; see section 1.2). Furthermore when Myc and Ras are overexpressed they cooperate to generate cyclinE-CDK2 activity and allow S-phase induction without the induction of cyclinD1-kinase activity (Leone *et al.*, 1997). Cyclin E regulates its own levels through autophosphorylation by cyclinE-CDK2 on threonine 380, which acts as a signal for its destruction by the proteosome (Won and Reed, 1996).

In vitro cyclinD1-CDK4 can phosphorylate pRb, whereas cyclinE-CDK2 has a much broader specificity being able to phosphorylate histone H1, pRb and the cyclin dependent kinase inhibitor, p27 (Sheaff *et al.*, 1997; Mittnacht, 1998). Recently the

consensus motif for phosphorylation by cyclinD1-CDK4 was shown to differ from that for phosphorylation by cyclinE/A-CDK2 (Kitagawa *et al.*, 1996). Thus differences in target sequence specificity may account for their ability to phosphorylate different substrates.

1.1.3 CDK-activating kinase (CAK)

The CDKs themselves are regulated by phosphorylation. The binding of a cyclin to a CDK causes a conformational change which makes threonine 160 in the CDK more accessible for phosphorylation by the CDK-Activating Kinase, CAK (Jeffrey *et al.*, 1995; Fisher and Morgan, 1994). Phosphorylation of this conserved threonine residue is critical for the activity of CDK complexes (Fisher and Morgan, 1994).

CAK is a multimeric enzyme complex composed of a distantly related cyclin-CDK pair, cyclin H-CDK7 and the assembly factor MAT1 (Serizawa *et al.*, 1995). CAK is also associated with the basal transcription factor TFIIH, suggesting it may have an additional direct role in the regulation of the basal transcription complex. It can phosphorylate the carboxy-terminal domain (CTD) of RNA Pol II (Serizawa *et al.*, 1995). Free CAK has a different substrate specificity to TFIIH associated CAK, suggesting that the association with TFIIH may convert if from an activator of CDKs to a kinase primarily responsible for regulating transcription (reviewed in Dynlacht *et al.*, 1997).

1.1.4 Cyclin dependent kinase inhibitors (CDI)

The CDK inhibitors (CDI) sequester CDKs to halt cell cycle progression in response to a variety of signals (Figure 1.1). In mammals there are two families of CDIs. The INK4 (inhibitors of CDK4) family of cyclin-dependent kinase inhibitors specifically inhibit the catalytic subunits of CDK4 and CDK6 by interfering with the formation of cyclinD dependent kinase complexes (Hall *et al.*, 1995). The family consists of four related polypeptides: p16 (*INK4a*), p15 (*INK4b*), p18 (*INK4c*) and p19 (*INK4d)* (Sherr and Roberts, 1999; Figure 1.1). The gene encoding p16 is known as the INK4a/ARF locus as it also encodes p19 ARF, from a separate promoter and using an alternative reading frame (Quelle *et al.*, 1995). ARF stabilises the tumour suppressor protein p53 and is required for p53 dependent growth arrest. Although signals leading to the synthesis of INK4 proteins are poorly understood the p16 inhibitor accumulates progressively as cells age and may be involved in the maintenance of cellular senescence.

The other group of cyclin dependent kinase inhibitors is the CIP/KIP/WAF family, which comprises three distinct gene products: p21, p27 and p57 (Sherr and Roberts, 1999; Figure 1.1). These polypeptides share an amino-terminal conserved region shown to be important for interaction with, and inhibition of cyclin-CDK complexes (Nakanishi *et al.*, 1995). They have a broader action than the INK4 proteins and are capable of interacting with and inhibiting all G1 cyclin-CDK complexes *in vitro* (Sherr and Roberts, 1999). They do not detectably associate with kinase subunits unless a cyclin is present (Hall *et al.*, 1995). Evidence for the importance of p27 in the inhibition of proliferation has been gained by inactivating p27 in mice (Nakayama *et*

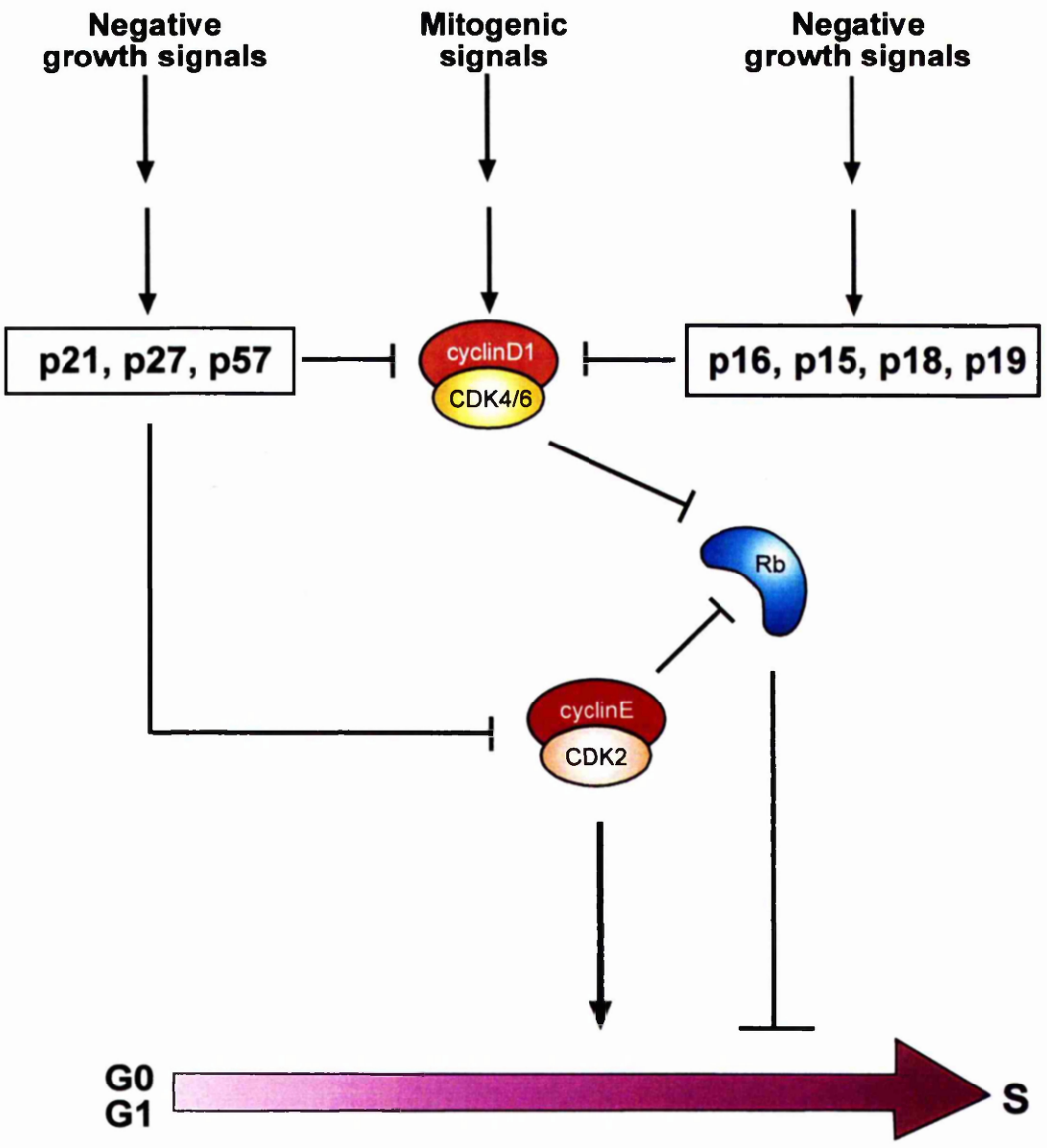


Figure 1.1 Control of the G1-S transition

D-type cyclins accumulate in G1 in response to mitogenic signals and complex with CDK4/6 forming a kinase reponsible for the initial phosphorylation of pRb. In late G1 cyclinE-CDK2 activity accumulates and is believed to complete the process of pRb phosphorylation and promote entry into S phase by an Rb-independent mechanism (see Section 1.4.1). Counteracting the cyclin-CDKs are two classes of CDKIs, which are thought to be induced by negative growth signals. (Adapted from Zhang, 1999)

al., 1996). $p27^{-/-}$ null mice are larger than control mice as a result of increased numbers of cells in all tissues and organs.

Recent studies have shown that the interaction of CIP/KIP proteins with cyclinD-dependent kinases *in vivo*, can cause activation of cyclinD1-CDK4 by promoting both its assembly and its nuclear localisation (La Baer *et al.*, 1997; Cheng *et al.*, 1999). Furthermore primary mouse embryonic fibroblasts (MEF) deficient in p21 and p27 have compromised cyclinD-dependent kinase activity, which can be restored to physiological levels by reintroduction of p21 or p27 into the cells (Cheng *et al.*, 1999). Sequestration of cyclinD-CDK complexes by p27 in proliferating cells is believed to contribute to the emergence of cyclinE-CDK2 activity during cell cycle progression (Sherr and Roberts, 1999). Moreover p27 has also been demonstrated to be a substrate for cyclinE-CDK2 and phosphorylation of p27 targets it for destruction by the proteosome (Sheaff *et al.*, 1997; Montagnoli *et al.*, 1999). Thus cyclinE-CDK2 can augment its own activity, enabling cells to transit from G1 into S phase.

Cell cycle arrest resulting from the activation of a CDI occurs in response to a variety of signals, including mitogen starvation, DNA damage and transforming-growth-factor-β (TGF-β). For example the p53 tumour suppressor protein induces the expression of p21 in response to DNA damage (Ko and Prives, 1996). Thus, p21 is the mediator of a 'checkpoint' which results in cell cycle arrest allowing for DNA repair. Furthermore mouse embryonic fibroblasts that lack p21 are significantly deficient in their ability to arrest in G1 in response to DNA damage (Deng *et al.*, 1995).

Cell cycle arrest induced by INK4 proteins may indirectly depend on an interaction of CIP/KIP family members with cyclinE-CDK2. For example, cell cycle arrest activated by TGF-β in Mv1Lu cells leads to an increase in p15 levels which

sequesters CDK4, thereby preventing the interaction of CDK4 with p27 and favouring the interaction of p27 with cyclinE-CDK2 (Reynisdóttir *et al.,* 1995). The cytoplasmic location of p15 ensures that the interaction of CDK4 occurs prior to contact with nuclear p27 (Reynisdóttir and Massagué, 1997). G1 arrest mediated by p16 also requires the indirect inhibition of cyclinE-CDK2 by p27 (Jiang *et al.*, 1998).

1.1.5 Regulation of pRb by G1 cyclin dependent kinases

The tumour suppressor gene, RB, is frequently found to be mutated in human tumour cells (Weinberg, 1995). These mutations frequently occur in a region of pRb referred to as the 'pocket' which is necessary for its negative growth regulating activity. The pocket binds to a number of proteins involved in the regulation of the cell cycle, including the E2F transcription factor, and several viral oncoproteins, such as adenovirus ElA (La Thangue, 1994).

One of the best studied mechanisms of negative growth control by pRb is through its ability to form a physical complex with E2F, an interaction which coincides with the inactivity of E2F target genes and cell cycle arrest (Dyson *et al.*, 1998; Lam and La Thangue, 1994). pRb activity is controlled principally through the regulated phosphorylation of pRb, which occurs as cells progress through the early cell cycle (Weinberg, 1995; Figure 1.1). During G1 of the cell cycle pRb is hypophosphorylated and in this state it binds to E2F. As cells progress from mid to late G1, pRb becomes increasingly phosphorylated and undergoes a conformational change, which leads to its dissociation from E2F. The rise in free E2F relative to the levels of E2F/pRb complexes leads to the activation of E2F target genes, the mechanisms of which are discussed in section 1.4.

The D-type cyclin-dependent kinase, composed of cyclin D1, D2 or D3 bound to CDK4 or CDK6, appears to be the CDK that is primarily responsible for initiating the phosphorylation of pRb (Hinds *et al.*, 1992; Hatakeyama *et al.*, 1994), whilst cyclinE-CDK2 is believed to be involved in completing the process of pRb phosphorylation at additional sites (Lundberg *et al.*, 1998; reviewed in Mittnacht, 1998; Figure 1.1). Different cyclin-CDK complexes preferentially phosphorylate pRb at distinct sites *in vitro*, suggesting they may exert distinct effects on pRb function *in vivo* (Kitagawa *et al.*, 1996; Zarkowska and Mittnacht, 1997).

Several lines of evidence indicate phosphorylation of pRb by cyclin-CDKs inactivates its growth-inhibitory activity. In the Rb-deficient cell line, SAOS2, ectopic expression of pRb causes cell cycle arrest and this is rescued by co-expression of cyclinE-CDK2, correlating with pRb phosphorylation (Hinds *et al.*, 1992). Furthermore G1 arrest induced by p16 requires a functional pRb (Lukas *et al.*, 1995). Another study revealed that expression of a dominant-negative Ras protein (Ras-N17) blocks the activation of cyclin D-CDKs. Ras-N17 blocked the induction of S phase in $Rb^{+/+}$, but not $Rb^{-/-}$ cells (Leone *et al.*, 1997). Thus the primary target of D-type cyclin-CDKs is pRb and a necessary step in the induction of cell cycle progression by cyclin D-CDKs is the phosphorylation of pRb.

There are 16 consensus phosphorylation sites in pRb and the contribution that individual sites play in the inactivation of pRb is controversial (Mittnacht, 1998). A recent study examined the effect of a panel of pRb phosphorylation site mutants on cell cycle progression and demonstrated that sites in the carboxy-terminus of pRb were critical for pRb inactivation (Chew *et al.*, 1998). Furthermore, overexpression of a phosphorylation-defective pRb in cells caused an S phase arrest suggesting pRb

phosphorylation is important for S phase completion in addition to its role in controlling entry into S phase.

1.2 The E2F pathway

The transcription factor E2F mediates the coordinated induction of a host of genes required for entry into S phase of the mammalian cell cycle. Binding sites for E2F have been identified in the promoters of genes that encode proteins required for DNA synthesis and replication of the genome, for example *DHFR, DNA polymerase α, cyclinE* and *cdc6* (Slansky and Farnham, 1996). The transcriptional activity of E2F is regulated during the cell cycle, which provides a means of coupling the induction of E2F-regulated-genes with cell cycle position.

1.2.1 E2F and DP Families

E2F is a heterodimer composed of an E2F protein and a DP protein (Lam and La Thangue, 1994; Wu *et al.*, 1996). To date there are six known E2F proteins, E2F-1 to E2F-6 and three DP proteins, DP-1 to DP-3 (Helin *et al.*, 1992; Kaelin *et al.*, 1992; Shan *et al.*, 1992; Girling *et al.*, 1993; Ivey-Hoyle *et al.*, 1993; Lees *et al.*, 1993; Beijersbergen *et al.*, 1994; Ginsberg *et al.*, 1994; Hijmans *et al.*, 1995; Ormondroyd *et al.*, 1995; Sardet *et al.*, 1995; Wu *et al.*, 1995; Zhang and Chellapan, 1995; Rogers *et al.*, 1996; Morkel *et al.*, 1997; Cartwright *et al.*, 1998; Trimarchi *et al.*, 1998). The E2F proteins share several discrete functional domains. All have a central DNA binding

domain and a heterodimerisation domain (Figure 1.2). With the exception of E2F-6, the carboxy terminus of each E2F protein contains the *trans*activation domain (TAD) and embedded within the TAD is a region involved in binding to pRb family proteins (Dyson, 1998; Figure 1.2). When E2F is bound to pRb it is converted from an activator to a repressor of transcription (Helin *et al.*, 1993a). However E2F-6 is thought to act solely as a repressor of transcription (Cartwright *et al.*, 1998; Trimarchi *et al.*, 1998). In this thesis the term E2F describes the heterodimer and the individual components are referred to by their appropriate number preceded by E2F or DP.

E2F-1, E2F-2 and E2F-3 have a number of structural and functional distinctions which place them in a separate subfamily to E2F-4 and E2F-5. The most striking difference is the presence of an additional 100 amino acids at the N-terminus of the E2F-1 subfamily (Figure 1.2). This region contains a cyclinA-CDK2 binding domain, the function of which is described in section 1.3 (Krek *et al.*, 1994). E2F-2 and E2F-3 have a high degree of similarity to E2F-1, particularly in their DNA binding domain (Ivey-Hoyle *et al.*, 1993; Lees *et al.* 1993). E2F-4 and E2F-5 are more similar to each other (80% similarity) than to other members of the E2F family (between 52% and 60% similarity) (Beijersbergen *et al.*, 1994; Ginsberg *et al.*, 1994; Hijmans *et al.*, 1995; Sardet *et al.*, 1995).

Recently, E2F-3 has been shown to be transcribed from two different start sites within the E2F-3 gene locus (Adams *et al.*, 2000). This produces two different transcripts. E2F-3a, the larger of the two products contains similar domains to E2F-1, whereas E2F-3b is truncated at the N-terminus resulting in a product lacking the

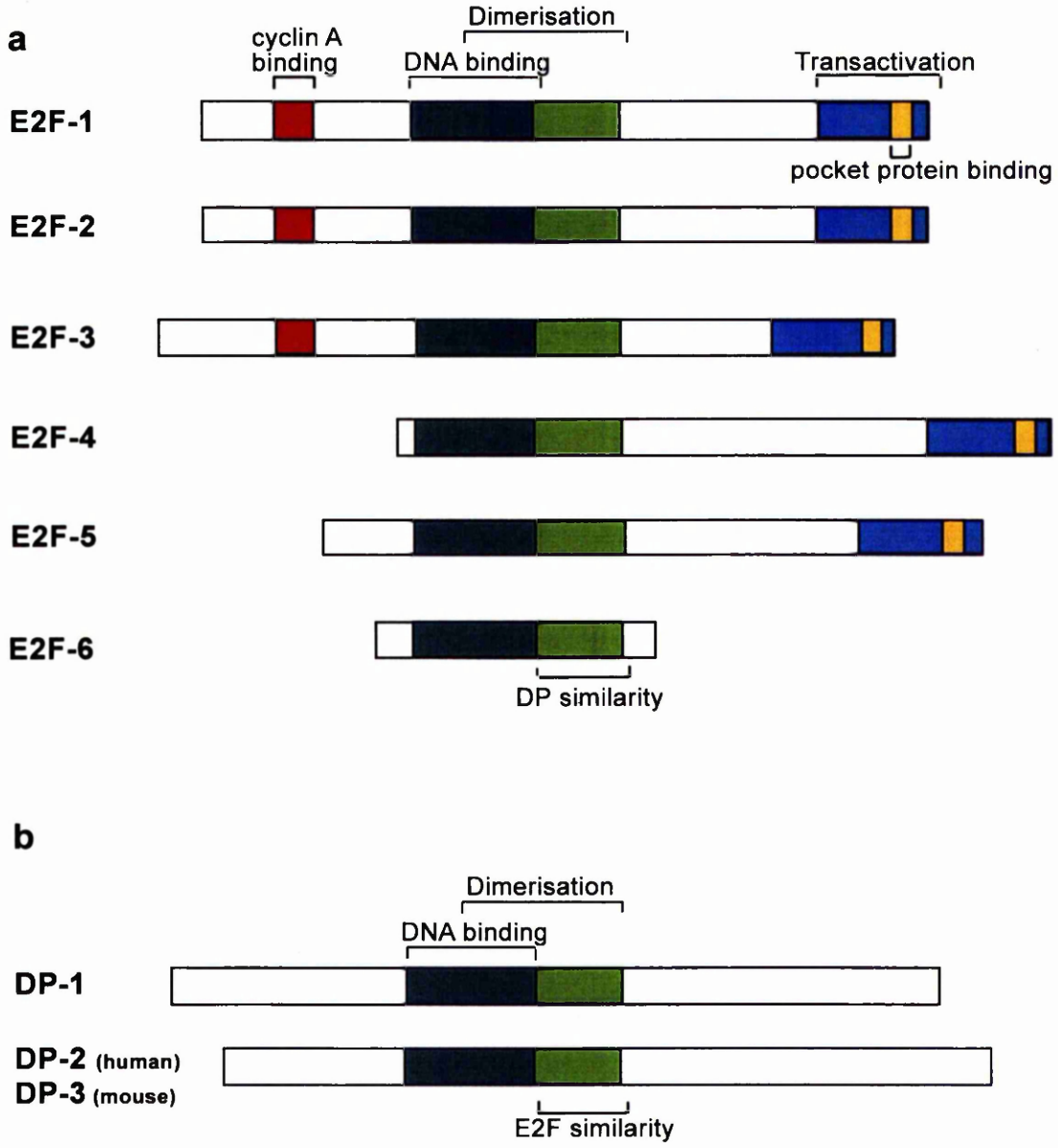


Figure 1.2 The organisation of the E2F and DP family members.

(a) Conserved domains within the E2F family.

(b) Conserved domains within the DP family, indicating the region with greatest sequence similarity to E2F family members.

cyclinA-CDK2 binding domain. Thus E2F-3b has a unique combination of characteristics shared with both the E2F-1 subfamily and the E2F-4 subfamily.

The first member of the DP family, DP-1 was isolated by purification from F9 embryonal carcinoma cells and was shown to be an E2F site specific DNA binding protein and a frequent component of E2F DNA binding activity (Girling *et al.*, 1993; Bandara *et al.,* 1994). A second DP, designated DP-2 was cloned in *Xenopus* (Girling *et al.*, 1994). Human DP-1 and DP-2 were cloned using a probe from mouse DP-1 (Wu *et al.*, 1995; Zhang and Chellapan, 1995; Rogers *et al.*, 1996). A third DP, murine DP-3 (also referred to as human DP-2) was shown to exist in multiple alternatively spliced forms termed α, β, γ and δ (Ormondroyd *et al.*, 1995). One consequence of this processing is the presence of a region, namely the E-region, in the α and δ forms which is necessary for localisation of DP-3 in the nucleus (see Section 1.3). The expression of human and *Xenopus* DP-1 and DP-2 is restricted to specific tissues (Wu *et al.*, 1995; Zhang and Chellappan, 1995; Girling *et al.*, 1994) and expression of the subtypes of DP-3 RNA also vary in different tissues (Ormondroyd *et al.*, 1995), suggesting the DP composition of E2F may reflect the proliferative requirements of a particular tissue. *Drosophila* homologues of E2F and DP have also been identified, designated dE2F and dDP (Hao *et al.*, 1995).

Bacterially expressed E2F-1 can bind DNA as a homodimer (Ivey-Hoyle *et al.*, 1993). However E2F and DP polypeptides synergistically bind DNA and activate transcription as a heterodimer (Bandara *et al.*, 1993; Krek *et al.*, 1993; Helin *et al.*, 1993b; Beijersbergen *et al.*, 1994; Buck *et al.*, 1995). DP-1 has a small region of sequence similarity to E2F-1 in the second helix of the E2F-1 DNA binding domain which enables heterodimerisation (Girling *et al.*, 1993). The crystal structure of E2F-

4/DP-2 has recently been determined and indicates that the residues of E2F that contact DNA are conserved among the E2F family members (Zheng *et al.*, 1999).

The existence of multiple forms of E2F opens up the possibility of particular E2F activities consisting of different E2F and DP combinations targeting specific promoters.

1.2.2 The pocket protein family

The activity of E2F is tightly regulated during the cell cycle by association with pRb and the related proteins, p107 and p130 (Dyson, 1998). The region of pRb that binds to E2F is called the 'pocket' and pRb, p107 and p130 are collectively referred to as the pocket proteins. The 'pocket' region also interacts with viral oncoproteins such as E1A, SV40 Large T antigen and E7 of human papilloma virus (La Thangue, 1994). Interaction of viral oncoproteins with pocket proteins disrupts their interaction with E2F, thereby preventing growth suppression. In p107 and p130 there is a discrete element in the pocket region, the spacer, which binds to cyclinA-CDK2 and cyclinE-CDK2 (Ewen *et al.*, 1992).

Like pRb, both p107 and p130 form a physical complex with E2F, but associate with E2F during different stages of the cell cycle. The pRb/E2F complex is detected predominantly in G1 cells and declines after the G1-S transition, correlating with the rise in activity of the cyclinD and cyclinE-CDK2 kinases (Shirodkar *et al.*, 1992). p107/E2F complexes are found in G1 phase and remain abundant throughout S phase (Lees *et al.*, 1992; Shirodkar *et al.*, 1992). In contrast, p130/E2F complexes are most prevalent in quiescent or differentiated cells and are rarely detected in cycling cells (Cobrinik *et al.*, 1993; Vairo *et al.*, 1995; Smith *et al.*, 1996). In late G1 both p130/E2F and p107/E2F complexes also contain cyclinE-CDK2 and during S phase p107/E2F

and cyclinA-CDK2 complexes have been detected (Lees *et al.*, 1992; Shirodkar *et al.*, 1992; Cobrinik *et al.*, 1993). The function of the cyclin-CDK associated with p107 and p130 and E2F is unknown. However it has been demonstrated that the cyclin-CDK2 binding domain in p107 and p130 can inhibit both cyclinA-CDK2 and cyclinE-CDK2 kinase activity and is related to and has comparable activity to the p21 CDI (Zhu *et al.*, 1995). Thus, p107 and p130 may have an additional role to pRb in growth suppression.

The pocket proteins may restrict repression mediated by a particular E2F to a certain stage of the cell cycle as they specifically associate with different subsets of E2F. E2F-1, E2F-2 and E2F-3 bind exclusively to pRb (Lees *et al.*, 1993), whereas E2F-4 and E2F-5 are regulated primarily by p107 and p130 (Beijersbergen *et al.*, 1994; Ginsberg *et al.*, 1994; Hijmans *et al.* 1995; Vairo *et al.*, 1995). However there are exceptions to this generalisation, for example E2F-4/pRb and E2F-4/p130 complexes have also been detected in cycling cells (Moberg *et al.*, 1996; Vairo *et al.*, 1995). In addition E2F-5 has been shown to bind preferentially to p130 in human CAMA breast carcinoma cells (Hijmans *et al.*, 1995). The presence of distinct E2F pocket protein combinations opens up the possibility of targeting repressive complexes to specific E2F regulated promoters at different times during the cell cycle.

1.2.3 E2F regulated genes

The consensus E2F binding site 'TTTCGCGC' is found in the adenovirus E2a promoter and similar sequences are found in the promoters of cellular E2F target genes. E2F target genes include genes that regulate the cell cycle (e.g. *cyclins* A, D1 and E, *cdc2*), genes that induce DNA synthesis (e.g. *DNA Polymerase α, dihydrofolate reductase* (*DHFR*)) and genes that encode S phase regulatory proteins (e.g. *cdc*6). Mutational analysis of E2F sites within the promoters of E2F target genes has been used to investigate the role of E2F in transcription and has revealed that E2F sites can have a stimulatory or inhibitory effect on transcription (Slansky and Farnham, 1998). Mutation of E2F-binding sites in the *DHFR* promoter resulted in loss of activation during cell cycle progression, suggesting that these binding sites mediate transcriptional activation during S phase entry (Slansky *et al.*, 1993; Fry *et al.*, 1997). In contrast, mutation of the E2F site in the *B-myb* promoter was sufficient to relieve transcriptional repression in G_0 and resulted in a promoter with constitutively high activity (Lam and Watson, 1993).The *cyclin A* promoter is also repressed during the G1 phase of the cell cycle (Henglein *et al.*, 1994). *In vivo* footprinting of the *cyclin A* promoter demonstrated that E2F sites were protected in cells arrested in G0/G1 by serum starvation and this protection was lost when cells entered S phase (Rech *et al.*, 1995).

The *cyclin E* promoter contains a combination of E2F sites that are involved in either activation or repression of transcription (Ohtani *et al.*, 1995; Botz *et al.*, 1996; Geng *et al*, 1996; Cam *et al.*, 1999). A variant E2F site in the *cyclin E* promoter can cooperate with an upstream AT-rich sequence to delay cyclin E expression until late G1 (Cam *et al.*, 1999). In extracts from Swiss 3T3 fibroblasts, a cyclin E repressor

complex (CERC) consisting of E2F-4, DP-1, p107 and additional unidentified proteins was shown to bind to this site but not to canonical E2F sites (Cam *et al.*, 1999).

E2F sites in the *E2F-1*, B-*myb* and *HsORC1* genes have been demonstrated to be important in repression in response to growth arrest by TGF-β (Li *et al.*, 1997). Figure 1.3 summarises the role of E2F sites in a variety of promoters.

Gene	Function of gene product	Effect of E2F sites
DNA Polymerase a	DNA synthesis	Not reported
DHFR	"	Activating
Thymidine kinase	"	Activating
ORC1	"	Repressing
CDC6	S phase regulation	Repressing
CDC2	Proliferation control	Repressing
Cyclin D	"	Activating
Cyclin E	"	Repressing and Activating
Cyclin A	"	Repressing
Rb	"	Not reported
p107	"	Activating
c-myc	"	Not reported
B-Myb	"	Repressing
E2F-1	"	Repressing
CDC25A	"	Repressing
CDC25C	"	Repressing
p19ARF	Apoptosis	Not reported

Figure 1.3 Role of E2F sites in a variety of E2F regulated promoters and function of their gene products. Adapted from: Helin, 1998 and data also from Cam *et al.*, 2000; Zwicker *et al* 1995; Zhu *et al.*, 1995; Rech *et al* 1995; Botz *et al.*, 1995; Neuman *et al.*, 1994; Johnson *et al.*, 1994; Li *et al.*, 1994; Slansky *et al.*, 1993; Lam and Watson, 1993.

1.3 Regulation of E2F activity

The activity of E2F is tightly regulated during the cell cycle. There are several mechanisms that influence E2F activity including: interaction of E2F with pocket proteins, regulation of E2F synthesis and degradation, phosphorylation of E2F and regulation of E2F subcellular localisation (reviewed in Müller and Helin, 2000). The regulation of E2F activity by phosphorylation of pRb and DP-1 as cells progress from G1 to S phase is summarised in figure 1.4.

1.3.1 Phosphorylation of pocket proteins

During G1 phase of the cell cycle pRb is phosphorylated by CDKs, a process which coincides with release of E2F from pRb and cell cycle progression (Figure 1.4; see section 1.1.5). Similarly the phosphorylation of the pRb related proteins, p107 and p130 are cell cycle regulated. Ectopically expressed cyclinD1-CDK4 but not cyclinE-CDK2 can phosphorylate p107, leading to its dissociation from E2F-4 (Beijersbergen *et al.*, 1995). Moreover the timing of endogenous p107 phosphorylation in cells correlates with cyclin D1 induction and occurs well before cyclin E induction, suggesting cyclin D1 is the most likely cyclin candidate to control p107 phosphorylation (Beijersbergen *et al.*, 1995; Xiao *et al.*, 1996).

The phosphorylation of p130 in mid-G1 correlates with the increase in cyclin D1 protein levels (Mayol *et al.*, 1995), resulting in its dissociation from E2F (Beijersbergen *et al.,* 1995), and also targeting it for destruction by the proteosome (Smith *et al.*, 1998). Although pocket proteins are phosphorylated by CDKs in G1, complexes of E2F with pocket proteins persist in S phase. The function of these is

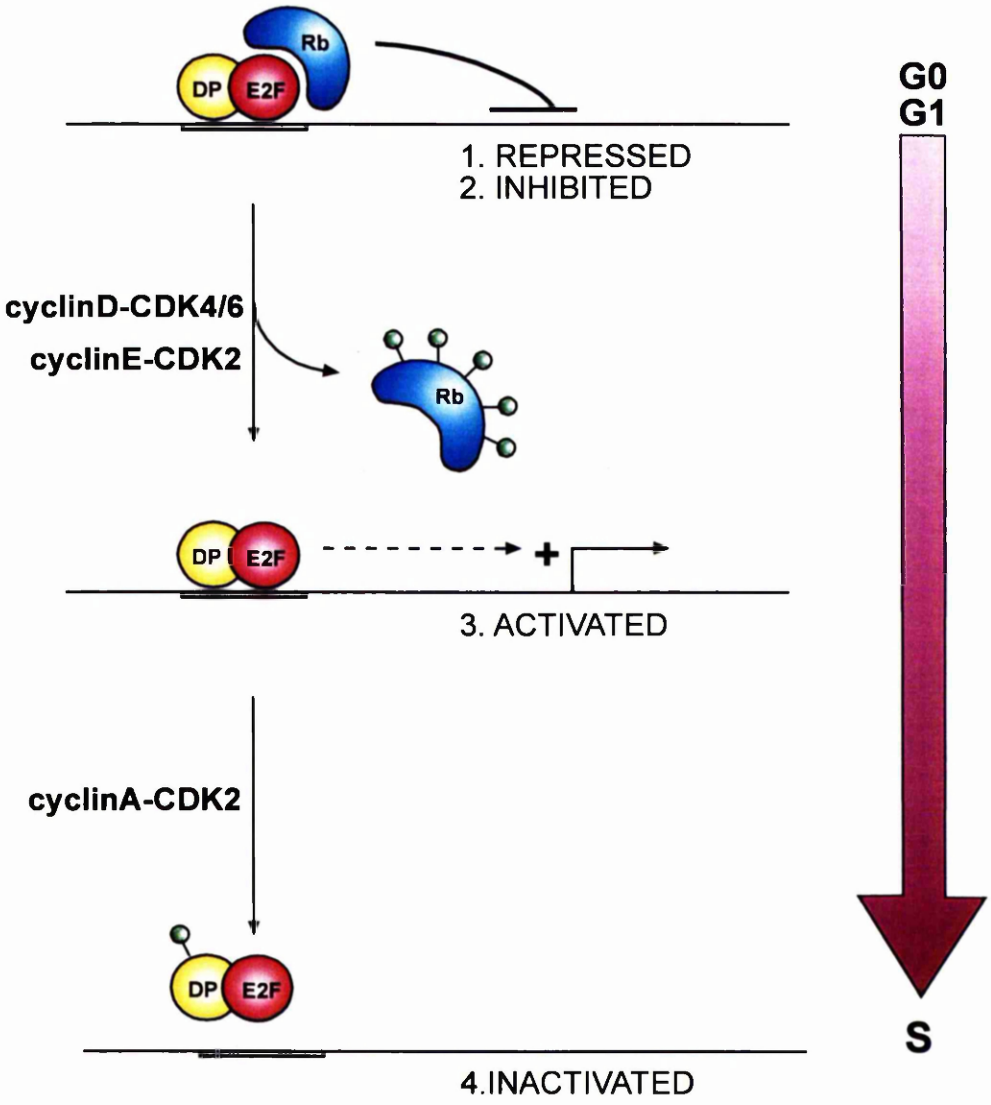


Figure 1.4 Sequential activation and inactivation of E2F by cyclin-CDKs
In G0 and G1 E2F regulated genes are (1) actively repressed by pRb, (2) pRb physically blocks the E2F activation domain. In late G1 pRb is phosphorylated by D-type cyclin-CDKs and cyclinE-CDK2, releasingit from E2F and allowing E2F to (3) activate transcription. In S phase the E2F heterodimer is released from DNA after phosphorylation of the DP subunit by cyclinA-CDK2. Green circles represent phosphate groups.

unknown. However some of these complexes have been detected in the cytoplasm so may not be associated with transcription (see Section 1.3.2).

1.3.2 Regulation of E2F subcellular localisation

The mechanism of nuclear uptake of E2F is an important consideration in the regulation of E2F activity as E2F must be present in the nucleus to regulate transcription. E2F-1, -2 and –3 contain a nuclear localisation signal (NLS) and ectopically expressed E2F-1, -2 and –3 are predominantly localised in the nucleus in transfected cells (Magae *et al.*, 1996; Allen *et al.*, 1997; Lindeman *et al.*, 1997). In contrast, ectopically expressed E2F-4 and E2F-5 are predominantly cytoplasmic, being devoid of an NLS. Two distinct mechanisms govern their nuclear localisation. Either pocket proteins or a nuclear DP heterodimeric partner can supply an NLS *in trans* to E2F-4 or E2F-5. DP-1 does not contain a NLS and ectopic DP-1 did not accumulate in the nucleus unless it was coexpressed with E2F-1, E2F-2, or E2F–3 (Magae *et al.*, 1996). However two alternatively spliced DP-3 isoforms, namely DP-3α and DP-3δ contain a region that functions as a NLS (de la Luna *et al.*, 1996), and both DP-3α and DP-3δ can transport E2F-4 and E2F-5 into the nucleus (Allen *et al.*, 1997).

Ectopically expressed E2F-4/5 and DP-1 are predominantly cytoplasmic and the heterodimer can be transported into the nucleus by co-expression of a pocket protein (Magae *et al.*, 1996; Allen *et al.*, 1997; Lindeman *et al.*, 1997). Moreover, the level of endogenous E2F-4 and E2F-5 in the cytoplasm increases progressively between early G1 and S phase (Allen *et al.*, 1997; Lindeman *et al.*, 1997), consistent with the idea that nuclear localisation of E2F-4 and E2F-5 in early G1 is mediated by pocket proteins. Thus, regulation of E2F nuclear localisation through different subunit composition

provides a means of controlling the presence of activating or repressive E2F complexes in the nucleus.

1.3.3 Phosphorylation of E2F

Several studies have shown that phosphorylation of E2F influences E2F activity. In serum stimulated 3T3 cells phosphorylation of DP-1 occurs at the G1-S transition correlating with the increase in E2F DNA binding activity observed at this stage of the cell cycle (Bandara *et al.*, 1994). E2F-1 and DP-1 are effectively phosphorylated *in vitro* by cyclinA-CDK2, but they are poor substrates for cyclinE-CDK2 (Dynlacht *et al.*, 1994; Kitagawa *et al.*, 1995). Furthermore in phosphopeptide mapping studies, tryptic phosphopeptides derived from E2F-1 phosphorylated *in vitro* by cyclinA-CDK2 overlap with phosphopeptides derived from E2F-1 phosphorylated *in vivo*, indicating that cyclinA-CDK2 may be responsible for the majority of E2F-1 phosphorylation *in vivo* (Xu *et al.*, 1994; Kitagawa *et al.*, 1995).

Phosphorylation of DP-1 *in vivo* is dependent on the presence of the cyclinA-CDK2 binding domain in E2F-1, as transfection of an E2F-1 mutant lacking the cyclinA-CDK2 binding domain abolishes DP-1 phosphorylation (Krek *et al.*, 1994; Dynlacht *et al.*, 1997). CyclinA-CDK2 phosphorylation of DP-1 coincided with a reduction in E2F DNA binding activity in S phase and may therefore contribute to the downregulation of E2F activity in late S phase (Krek *et al.*, 1994; Figure 1.4). In support of this idea *in vitro* phosphorylation of the E2F-1/DP-1 heterodimer by cyclinA-CDK2 reduced its ability to bind DNA (Dynlacht *et al.*, 1994; Xu *et al.*, 1994; Kitagawa *et al.*, 1995). Overexpression of E2F-1 and a phosphorylation deficient DP-1 in cells caused a delay in S phase followed by apoptosis, suggesting that E2F activity must be downregulated for normal progression through S phase (Krek *et al.*, 1995).

One of the sites in E2F-1 phosphorylated by cyclinA-CDK2 has been mapped to serine 375 and phosphorylation of this site *in vitro* has been correlated with increased binding to pRb (Peeper *et al.*, 1994). Conversely, phosphorylation of E2F-1 on serine 332 and serine 337 can inhibit the interaction of E2F-1 with pRb *in vivo*, although the kinase responsible is unknown (Fagan *et al.*, 1994). It has recently been demonstrated that phosphorylation of E2F-1 on serine 403 and threonine 433 can be mediated by a kinase associated with TFIIH (Vandel and Kouzarides, 1999).

E2F-4, E2F-5 and DP-2 have also been shown to be phosphoproteins but the functional significance of their phosphorylation is unknown (Ginsberg *et al.*, 1994; Hijmans *et al.*, 1995; Rogers *et al.*, 1996). E2F-1, E2F-2 and E2F-3 all bind to CDK3, a cyclin dependent kinase with an unknown cyclin partner, and this appears to be involved in activation of E2F in late G1 (Hofmann and Livingston, 1996).

1.3.4 Regulation of E2F abundance

The pool of E2F in a cell is determined by the level of transcription of the E2F genes, and the stability of the individual E2F proteins. The *E2F-1, E2F-2* and *E2F-3* genes have E2F sites in their promoters, which are repressed in G0 and early G1, likely mediated through an E2F-pocket protein complex (Neuman *et al.*, 1994; Johnson *et al.*, 1994; Adams *et al.*, 2000). As a result E2F-1, E2F-2 and E2F-3a mRNA and protein are undetectable in quiescent cells but induced in mid-late G1 (Kaelin *et al.*, 1992; Slansky *et al.*, 1993; Moberg *et al.*, 1996; Leone *et al.*, 1998).

E2F-4, E2F-5 and DP-1 mRNA levels are relatively constant throughout the cell cycle, although there is a marginal increase in E2F-5 and DP-1 mRNA levels in mid G1 (Ginsberg *et al.*, 1994; Sardet *et al.*, 1995; Moberg *et al.*, 1996). Thus E2F-4 and E2F-

5 may regulate E2F-dependent genes in G0 and early G1, prior to the synthesis of other E2Fs. The shorter E2F-3, namely E2F-3b, differs from E2F-3a in that its promoter is not regulated by E2F and consequently its expression during the cell cycle resembles that of E2F-4 and E2F-5 (Adams *et al.*, 2000). However E2F-3b is structurally similar to E2F-3 as both contain a NLS and associate with pRb. E2F-3b is the predominant E2F associated with pRb in quiescent cells and therefore may have a unique function at this stage of the cell cycle.

Many proteins involved in cell cycle regulation are degraded at defined points in the cell cycle, e.g. cyclins and CDIs. E2F-1 and E2F-4 are unstable proteins and their degradation is mediated by the ubiquitin-proteosome pathway (Hateboer *et al.*, 1996; Hofmann *et al.*, 1996; Campenero and Flemington, 1997). Ubiquitination of E2F-1 is dependent on a component of the SCF ubiquitin-protein ligase, $p45^{SKP2}$ and may be involved in the downregulation of E2F-1 activity in the S/G2 phase of the cell cycle (Marti *et al.*, 1999).

An epitope in the C-terminus of E2F-1 and E2F-4 is responsible for their instability and pocket protein binding stabilises both E2F-1 and E2F-4 (Hateboer *et al.*, 1996; Hofmann *et al.*, 1996; Campenero and Flemington, 1997). Adenovirus E1A and E1b also inhibit breakdown of free E2F, despite their interference with E2F/pocket protein interactions and may thus provide a mechanism to facilitate S phase entry in adenovirus infected cells (Hateboer *et al.*, 1996). Conversely the stabilisation of E2F by pocket proteins may have a role in differentiation or in the maintenance of quiescence. Consistent with this idea, during myogenic differentiation there is no change in E2F-1 protein levels, despite the fall in E2F-1 mRNA and the presence of a hypo-phosphorylated pRb (Martelli and Livingston, 1999).

1.4 Functional significance of the E2F pathway

The complexity in the regulation of E2F activity suggests that individual family members may have distinct roles at particular stages of the cell cycle. E2F can positively and negatively regulate the transcription of genes whose expression is required for entry into S phase of the cell cycle. There is strong evidence emphasising the importance of either E2F *trans*activation or E2F-mediated repression of genes in cell cycle progression but the overall contribution of each of these processes is unknown. Many studies are based on overexpression of E2F and pocket proteins, which may compensate for normal regulatory interactions. Furthermore cell type specific variations exist and the processes that regulate transformed cells can differ from those that regulate primary cells.

1.4.1 Importance of E2F in cellular proliferation

Overexpression of E2F-1 or dE2F in quiescent cells is sufficient to drive cells into S phase, an effect which is dependent on the *trans*activation domain of E2F-1, suggesting it is mediated by E2F dependent transcriptional activation (Johnson *et al.*, 1993; Qin *et al.*, 1994; Asano *et al.*, 1996). In support of this E2F and DP proteins are oncogenic. E2F-1, E2F-4, E2F-5 and DP-1 can cooperate with activated Ras to transform cells, and these cells produce tumours when implanted into nude mice (Johnson *et al.*, 1994b; Singh *et al.*, 1994; Jooss *et al.*, 1995; Polanowska *et al.*, 2000). In another study E2F-1, E2F-2 and E2F-3 alone were sufficient to transform NIH3T3 cells (Xu *et al.*, 1995).

The effect of different E2F proteins on transcription and cell cycle progression varies. Overexpression of the individual E2F proteins in cells induced the expression of a panel of E2F-target genes to different degrees (DeGregori *et al.*, 1997). E2F-1, E2F-2 and E2F-3 have been shown to induce S phase in serum starved Rat1 and REF52 cells, whilst E2F-4 and E2F-5 could only drive quiescent Rat1 cells into S phase when they were overexpressed together with DP-1 (Lukas *et al.*, 1996; DeGregori *et al.*, 1997). However E2F-1 and E2F-4 were demonstrated to have similar abilities to induce proliferation in transgenic mice (Wang *et al.*, 2000).

One approach to investigate E2F function has been to inactivate individual E2F proteins in cells. *E2F-1* knock-out mice develop normally, but exhibit defects in T-cell apoptosis and develop a broad spectrum of tumours (Field *et al.*, 1996; Yamasaki *et al.*, 1996), suggesting E2F-1 functions as a tumour suppressor. Consistent with these observations overexpression of E2F-1 in serum starved fibroblasts can trigger apoptosis (Shan and Lee, 1994). The ability to induce apoptosis may be a unique property of E2F-1 as expression of the other E2F proteins did not induce apoptosis in serum starved fibroblasts (DeGregori *et al.*, 1997). *E2F-5*$^{-/-}$ mice are viable but die after several weeks from hydrocephalus caused by an excessive secretion of cerebral spinal fluid in the choroid plexus, suggesting a specific role for E2F-5 in differentiation of the choroid plexus (Lindeman *et al.*, 1998). The absence of a more dramatic phenotype in *E2F-5* knockout mice may be explained by some degree of redundancy among the E2F proteins.

E2F-3$^{-/-}$ mice arise at one-quarter of the expected frequency demonstrating that E2F-3 is important for normal development (Humbert *et al.*, 2000). Furthermore, mouse embryonic fibroblasts (MEFs) derived from these animals have reduced expression of numerous E2F-responsive genes, including *B-myb*, *cyclin A* and *DHFR*

(Humbert *et al.*, 2000). *E2F -1*$^{-/-}$ MEFs exit G_0 between three and four hours later than wild-type cells indicating E2F-1 is essential for timely S phase entry from G_0 (Wang *et al.*, 1998). Microinjection of E2F-3 antibodies into fibroblasts synchronised in G2 caused a reduction in the number of cells entering S phase, whilst an E2F-1 antibody had no effect (Leone *et al.*, 1998). Moreover in synchronised cells E2F-3 DNA-binding activity accumulates at the G1/S transition of each cell cycle, whereas E2F-1 only accumulates during the initial G1 following a growth stimulus (Leone *et al.*, 1998). Taken together these results suggest that E2F-1 and E2F-3 have distinct roles in cell cycle progression. Whilst E2F-3 regulates expression of S phase genes in proliferating cells, E2F-1 is required for G_0 exit.

Many studies have investigated whether E2F activity is absolutely required for entry into S phase. Several observations have indicated that E2F activity is rate-limiting for entry into S phase. The proportion of cells in G1 is increased by the overexpression of a dominant-negative DP-1 (Wu *et al.*, 1996). In *Drosophila*, null mutations in dE2F and dDP cause lethality in late development (Duronio *et al.*, 1995; Royzmann *et al.*, 1997). *Drosophila* mutations of dDP or dE2F that reduce the activity of E2F diminished the rate of DNA replication suggesting a quantitative relationship between the levels of E2F/DP and the efficiency of DNA replication (Duronio *et al.*, 1998). Delays in S phase entry were only detected with severe reductions in E2F function. In contrast, partial loss of function of cyclin E resulted in a delay in S phase entry without detectable fall in the rate of DNA replication (Duronio *et al.*, 1998).

Cyclin E acts downstream of E2F as it is an E2F target gene (Botz *et al.*, 1996). However, cyclinE-CDK2 phosphorylates pRb resulting in E2F activation (Lundberg and Weinberg, 1998). Thus cyclin E and E2F cooperate in an autoregulatory feedback loop. The activity of cyclinE-CDK2 is necessary for entry into S phase in mammalian

cells (Ohtsubo *et al.*, 1995) and in *Drosophila* (Duronio *et al.*, 1996). Ectopic expression of cyclin E can override G1 arrest imposed by either p16 or a phosphorylation deficient mutant pRb and can also overcome a G1 block induced by expression of a dominant negative mutant DP-1 (Alevizopoulos *et al.*, 1997; Lukas *et al.*, 1997). Furthermore Myc and Ras can collaborate in the induction of cyclinE-CDK2 activity and stimulate cells to enter S phase in the presence of a hypophosphorylated pRb (Leone *et al.*, 1997). Taken together these results suggest the cyclin E can promote S phase independently of pRb phosphorylation and can compensate for E2F mediated *trans*activation. Therefore *cyclin E* is likely to be a key E2F-target gene and it is possible once a threshold of cyclin E is achieved cells can proceed into S phase and E2F activity is no longer required for DNA replication. Moreover cyclinE-CDK2 may target at least one other critical effector of cell cycle progression besides pRb. This idea is supported by the observation that, in growth-arrested G1 cells, expression of the E2F DNA-binding domain stimulates entry into S phase without significant phosphorylation of pRb, whereas complete cell-cycle progression requires the cyclinE-CDK2 complex (Zhang *et al.*, 1999).

Further understanding of E2F function *in vivo* has been acquired by analysing primary mouse cells deficient in specific pocket protein family members. $Rb^{-/-}$ embryos die *in utero* between day 13.5 and 15.5 of embryogenesis (Clarke *et al.*, 1992). In addition, ectopic cell cycle entry and increased levels of apoptosis are apparent in some tissues (Clarke *et al.*, 1992; Jacks *et al.*, 1992). $Rb^{-/-}$ mouse embryonic fibroblasts (MEFs) have a slightly shorter G1 phase and enter S phase earlier than wild type cells (Herrera *et al.*, 1996). Mice deficient in either p107 or p130 were viable, whereas double knockout ($p107^{-/-}$, $p130^{-/-}$) animals suffer lethal abnormalities, indicating p107 and p130 are functionally redundant (Cobrinik *et al.*, 1996). Deficiency of p107 also

enhances the developmental abnormalities of $Rb^{+/-}$ embryos indicating there is also some degree of functional overlap between p107 and pRb (Lee *et al.*, 1996). It was demonstrated that $Rb^{+/-}$, $p107^{-/-}$ pups have an increased mortality rate during the first three weeks after birth and those that survived developed multiple retinal dysplasia.

p107$^{-/-}$,*p130*$^{-/-}$ MEFs showed strongly derepressed expression of a broad range of genes, including *B-myb*, *cdc2* and *E2F-1* whereas there was no difference in expression of these genes in MEFS deficient in either of these proteins alone (Hurford *et al.*, 1997). Therefore, p107 and p130 share overlapping functions. Subtle derepression of the *cyclin E* and p107 genes have been detected in $Rb^{-/-}$ MEFs, whilst expression of the cyclin E gene was unchanged in *p107*$^{-/-}$, *p130*$^{-/-}$ MEFS (Hurford *et al.*, 1997). Furthermore cyclin E is prematurely activated in *Rb*$^{-/-}$ MEFS (Lukas *et al.*, 1997). These observations indicate the *cyclin E* gene is repressed in G0/G1 by E2F/pRb complexes.

In cells arrested by γ-irradiation E2F becomes a potent transcriptional repressor and sequestration of E2F in these cells allows the cells to bypass the cell cycle block and progress into S phase (He *et al.*, 2000). Moreover, overexpression of the E2F DNA binding domain in cells prevents G1 arrest by TGF–β, despite the accumulation of hypophosphorylated pRb (Zhang *et al.*, 1999). Taken together these observations suggest that G1 arrest requires active repression mediated by E2F and hypophosphorylated pRb. Mutational analysis of E2F sites has demonstrated that a broad range of E2F target genes primarily mediate repression (see Section 1.2.3). Thus direct repression of transcription mediated by E2F/pocket proteins complexes plays an important role in the timely expression of genes required for entry into S phase.

1.4.2 Mutation of E2F pathway in cancer

Cancer is a disease of cellular proliferation caused by genetic changes that lead to the activation of oncogenes or inhibition of tumour suppressor proteins. Given the importance of E2F in the regulation of proliferation any mutation that augments E2F transcriptional activity could provide a cell with a proliferative advantage and contribute to its tumourigenic potential. A strong correlation has been made between tumourigenesis and unregulated E2F activity.

The most widespread aberration in cancer is mutation of the tumour suppressor gene, p53 (Levine, 1997). p53 can promote cell cycle arrest or apoptosis via several mechanisms. Activation of the *p21(WAF1)* promoter by p53 causes cell cycle arrest due to inhibition of cyclin-CDK complexes and consequently pRb is hypo-phosphorylated. Apoptosis mediated by p53 can be either E2F-dependent or E2F-independent. E2F-dependent apoptosis may result from activation of $p19^{ARF}$, the promoter of which is directly regulated by E2F (Bates *et al.*, 1998). $p19^{ARF}$ stabilises p53 in response to DNA damage. Inactivation of $p19^{ARF}$ has been observed in a wide spectrum of human cancers (Ruas and Peters, 1998). Thus inactivation of p53 or $p19^{ARF}$ in tumour cells bypasses a G1 checkpoint, allowing cells to proliferate with damaged DNA.

There have been few reports of E2F genes being mutated in transformed cells. Chromosomal amplifications and overexpressions of the *E2F-5* gene have been detected in breast tumours (Polanowska *et al.*, 2000). Another study demonstrated amplification of the *E2F-1* locus in an erythroleukaemia cell line (Saito *et al.*, 1995).

Genetic aberrations that disrupt the pRb pathway, which involves pRb, cyclin D, CDK4 and p16 has been frequently detected in human cancer (Sherr, 1996).

Inheritance of a defective allele of *RB* provides a strong predisposition for developing the rare childhood cancer, retinoblastoma. Loss of the remaining functional allele of *RB* appears to be a rate-limiting event for tumour initiation (Whyte, 1995). The pRB gene *RB1* is mutated in approximately 30% of human cancers. All naturally occurring mutations occur within the pocket domain, which is necessary for binding to E2F. DNA tumour viruses such as simian virus 40 (SV40) and adenovirus can also transform cells, in part by interfering with the interaction of E2F and pocket proteins (Bandara and La Thangue, 1991; Li *et al.*, 1993; Zamanian and La Thangue 1992). The viral oncoproteins sequester pocket proteins, through regions in the viral proteins necessary for transformation, thereby releasing active E2F.

Unlike the frequent inactivation of Rb in human tumours, no mutations in the p107 gene and only a few mutations of the p130 gene have been demonstarted to occur in tumours (Helin *et al.*, 1997). This may be due to redundancy between p107 and p130, which has been highlighted in studies with mice carrying mutant alleles of p107 and p130 (see Section 1.4.1).

Many tumours that retain a wild-type Rb allele, have genetic alterations in other components of the pRb pathway. The *p16(INK4a)* gene is rearranged, deleted, or mutated in a majority of gliomas, leukaemias and melanomas (Kamb *et al.*, 1994). The *cyclin D1* locus is disrupted in a wide range of tumours, including lymphomas, squamous cell tumours and breast carcinomas (Pines, 1995). Hyperactivity of cyclin D can transform cells. The most recognised role of cyclin D is to initiate the phosphorylation of pRb, thereby releasing E2F and enabling activation of E2F target genes (see section 1.1.5). However it has been suggested that the transforming ability of cyclin D1 could also result from its ability to sequester CIP/KIP proteins leading to

the activation of cyclinE-CDK2 (see section1.1.4; reviewed in Sherr and Roberts, 1999).

In conclusion, mutation of *RB1* gene, overexpression of cyclin D1, and inactivation of p16 have all been detected in transformed cells. The consequence of each of these aberrations is to free E2F from pRb control thus overriding this critical G1 checkpoint and contributing to tumourgenesis.

1.5 Mechanism of transcriptional activation and repression by E2F

In eukaryotic cells genes are condensed and packaged into chromatin. To fully understand how transcription factors activate and repress genes in a physiological context the accessibility of genes in chromatin needs to be explored. The primary component of chromatin structure is the nucleosome. Nucleosomes organise chromosomal DNA into structures that resemble beads on a string. They are comprised of histones H2A, H2B, H3 and H4, which together form an octomeric core wrapped around approximately two turns of DNA. Nucleosome assembly is regulated by histone acetylation. Acetylation is generally associated with transcriptional activity of genes as it disrupts nucleosome structure, thereby unfolding DNA and allowing transcription factors to bind to DNA (Grunstein, 1997; Shikama *et al.*, 1997).

1.5.1 Mechanism of E2F activation

E2F proteins have a C-terminal *trans*activation domain which can activate transcription when attached to the DNA-binding domain of an heterologous protein (Kaelin *et al.*, 1992). The E2F-1 *trans*activation domain can interact with components of the basal transcription machinery, TBP and TFIIH, *in vitro* (Hagemeier *et al.*, 1993; Pearson and Greenblatt, 1997). Furthermore *trans*activation by E2F-1 has been correlated with the ability to bind TBP and TFIIH, suggesting that transcriptional activation is mediated by direct contact with components of the basal transcription machinery. Alternatively E2F-1 and TBP can interact with the p300/CBP family of co-activators resulting in the formation of a physical bridge between the E2F activation domain and components of the basal transcription machinery (see Section 1.6).

It has also been demonstrated that the E2F heterodimer can bend DNA, an effect which was abolished by the interaction of pRb with E2F (Cress and Nevins, 1996). The DNA bending contributed to transcriptional activation and thus may facilitate the ability of upstream activators to make appropriate contacts with the basal transcription machinery.

1.5.2 Mechanism of E2F repression

Pocket proteins can inhibit E2F transcriptional activation by masking the activation domain of E2F, which interferes with its interaction with the basal transcription machinery (Pearson and Greenblatt, 1997). There is also strong evidence that pocket proteins can act as active repressors of transcription. pRb can repress transcription when tethered to a promoter by a heterologous DNA binding domain (Bemner *et al.*, 1995; Sellers *et al.*, 1995). Furthermore there are numerous genes containing E2F sites which mediate repression, likely through an E2F/pocket protein complex (see section 1.2.3).

Two mechanisms of pocket protein repression have been described. One mechanism depends on the ability of pRb to interact with the *trans*activation domain of surrounding transcription factors on the promoter (Weintraub *et al.*, 1995). Sequence similarity between pRb, TBP and TFIIB suggests that pRb can mimic components of the basal transcription machinery, and consequently block the interaction between transcription factors and the basal transcription machinery (Hagemeier *et al.*, 1993; Weintraub *et al.*, 1995). This mechanism appears to be E2F dependent as E2F is required to tether pRb to the promoter (Weintraub *et al.*, 1995).

In the second mechanism, pRb is tethered to the promoter through E2F and recruits histone deacetylases (HDAC), which induces nucleosome assembly thereby blocking access of transcription factors to the promoter (Brehm *et al.*, 1998; Luo *et al.*, 1998; Magnaghi-Jaulin *et al.*, 1998). Several lines of evidence support a role for histone deacetylase in active repression of transcription mediated by pocket proteins. Trichostatin A, a specific and irreversible inhibitor of HDAC, can block Gal4-pRb mediated repression of a reporter construct consisting of the adenovirus major late promoter modified with upstream Gal4 sites (Luo *et al.*, 1998). Phosphorylation of pRb or interaction with viral oncoproteins disrupts the interaction of pRb with HDAC1 (Brehm *et al.*, 1998; Ferreira *et al.*, 1998). It has also been demonstrated that both p107 and p130 can interact with HDAC1 (Ferreira *et al.*, 1998). Furthermore p107 is able to interact with HDAC1 and E2F-4 simultaneously, suggesting it can recruit HDAC1 to E2F-4 containing heterodimers. pRb-mediated repression of certain transcription factors e.g. PU.1 is independent of histone deacetylase activity, supporting the idea that there is more than one mechanism of pRb-mediated repression (Luo *et al.*, 1998).

Sequential phosphorylation of pRb has been proposed to be important for its ability to inactivate E2F-1 and actively repress transcription in association with HDAC (Harbour *et al.*, 1999). First, phosphorylation of a set of CDK sites in pRb block its ability to bind to HDAC resulting in a loss of active repression mediated by pRb. Subsequently phosphorylation of another set of CDK sites disrupts the pRb pocket structure resulting in release of pRb from E2F, and a loss in inhibited complexes. Recently an additional level of control for the regulation of Rb-mediated repression has been described (Zhang *et al.*, 2000). The hSWI/SNF nucleosome remodelling complex can interact with pRb and repress transcription of the *cyclin E* and *cyclin A* genes. It was proposed that a trimeric complex between Rb, HDAC and hSWI/SNF is required

to repress the *cyclin E* gene and that after subsequent loss of HDAC from the complex during cell cycle progression, Rb-hSWI/SNF is sufficient to maintain repression of the *cyclin A* gene. Therfore different multiprotein complexes may be important in controlling the temporal gene expression as cell progress from G0/G1 into S phase.

1.6 The p300/CBP family of co-activators

1.6.1 Regulation of transcription by p300/CBP

The p300 protein and the related CREB binding protein (CBP) are highly homologous nuclear proteins that were originally identified by their ability to interact with adenovirus E1A and with the transcription factor CREB respectively (Chrivia *et al.*, 1993; Eckner *et al.*, 1994). Both possess co-activating properties as they do not contain a DNA binding domain but can stimulate transcription when tethered to a promoter by fusion with a foreign DNA-binding domain (reviewed in Shikama *et al.*, 1997). *Trans*activation domains (TADs) have been identified in both the N-terminus and C-terminus of CBP and deletion of the N-terminal TAD greatly reduced the ability of CBP to activate CREB-mediated transcription (Swope *et al.,* 1996). Several discrete functional domains have been identified in p300/CBP, including a central bromodomain and three cysteine/histidine rich domains (CH1, CH2 and CH3) (Shikama *et al.*, 1997; Figure 1.5). The modular organisation of p300/CBP allows the formation of multimeric transcription mediating complexes.

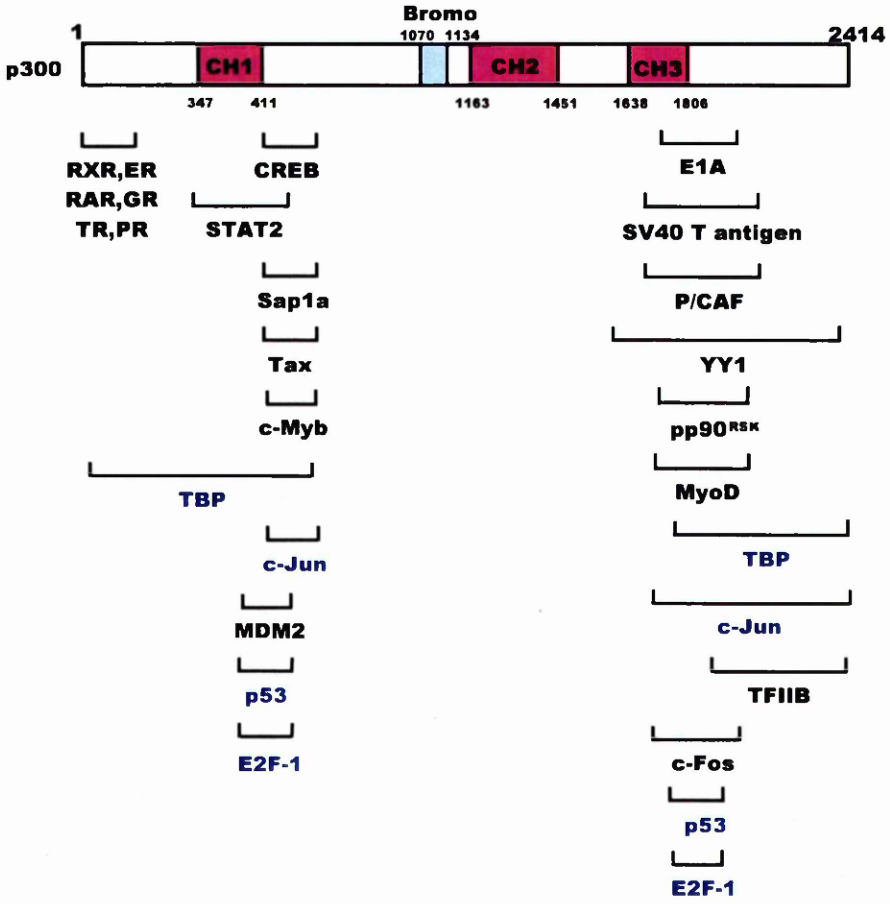


Figure 1.5
Schematic representation of p300/CBP. The cysteine/histidine-rich domains (CH1, CH2 and CH3) and the bromodomain are indicated. Note that the numbers indicate the amino acid residues in human p300. CBP has a similar organisation and contains 2440 (2441 in mouse) amino acid residues. The binding domains of various target proteins are indicated and blue text denotes a protein with more than one binding region. (Adapted from Shikama *et al.*, 1997).

A constantly increasing array of transcription factors are able to form stable physical complexes with and respond to the co-activating properties of p300/CBP (Shikama *et al.*, 1997; Figure 1.5). These include E2F-1, c-Jun, c-Fos, c-Myb, CREB, MyoD, Stat 2, YY1, p53 and nuclear hormone receptors. Some of these are the products of proto-oncogenes, for example c-Myb, whilst others are tumour supressors, for example p53, or are involved in differentiation, such as MyoD. p300/CBP can also physically interact with components of the basal transcription machinery and act as a bridge between sequence specific binding factors and the basal transcription machinery, thereby facilitating transcriptional activation (Shikama *et al.*, 1997).

Transcriptional activation mediated by p300/CBP and several transcriptional activators can be abolished directly and specifically by E1A binding to p300/CBP (Arany *et al.*, 1995; Yang *et al.*, 1996). The disruption of the MyoD-p300 interaction by E1A inhibits MyoD-dependent transcription (Yuan *et al.*, 1996). Thus, E1A may keep cells in the proliferative state by suppressing the p300/CBP-mediated activation of genes whose products are required differentiation. However, it is somewhat surprising that E1A counteracts the interaction between p300 and transcription factors implicated in enhancing proliferation, such as AP-1, a heterodimer composed of c-Fos and c-Jun (Arany *et al.*, 1995; Lundblad *et al.*, 1995). An understanding of the regulation of p300/CBP activity may shed light on how these different processes are controlled by p300/CBP.

In addition to their role as adapter proteins, p300/CBP also possesses intrinsic histone acetyltransferase (HAT) activity. Both p300 and CBP can acetylate all four core histones and thus may contribute directly to transcriptional regulation by remodelling chromatin (Bannister and Kouzarides, 1996; Ogryzko *et al.*, 1996). Moreover, p300/CBP may influence chromatin remodelling by recruiting other acetyltransferases

to the promoter (reviewed in Shikama *et al.*, 1997). One such protein is p/CAF (p300/CBP-associated factor). A possible scenario is that transcription factors need to recruit multiple HATs in order to overcome the repressive effects of deacetylation on promoter activity.

Recent studies have demonstrated that p300 and p/CAF can also directly acetylate several non-histone substrates, including p53, TFIIF, p53, MyoD and E2F-1 (Gu and Roeder, 1997; Imhof *et al.*, 1997; Sartorelli *et al.*, 1999; Martinez-Balbas *et al.*, 2000), opening up the possibility that acetylation of sequence specific DNA-binding proteins may play a role in the regulation of transcription.

Both p300 and CBP have been demonstrated to interact with E2F-1 and enhance E2F-1 dependent transcription (Trouche *et al.*, 1996; Trouche and Kouzarides, 1996; Lee *et al.*, 1998). Moreover, E2F-1 recruitment of CBP is critical for activation of the *DHFR* promoter (Fry *et al.*, 1999). A recent study has revealed that p/CAF and to a lesser extent, p300 can acetylate E2F-1 *in vitro* (Martinez-Balbas *et al.*, 2000). Acetylation of E2F-1 *in vivo* by p/CAF was shown to increase the half-life and DNA binding activity of E2F-1, thereby augmenting E2F transcriptional activity. Taken together these studies suggest that p300/CBP plays a critical role in mediating E2F-1 dependent transcription.

1.6.2 Regulation of differentiation, proliferation and apoptosis by p300/CBP

The importance of p300/CBP in differentiation and proliferation has been highlighted by studies of p300/CBP knockout mice and in the disruption of p300/CBP in human disease. An autosomal dominant inheritable disease called Rubinstein-Taybi

syndrome (RTS) is characterised by loss of one CBP allele (reviewed in Goodman and Smolik, 2000). Symptoms of RTS include mental retardation, physical abnormalities, such as bone malformation and an increased incidence of neoplasia. CBP heterozygous mutant mice have abnormal skeletal patterning (Tanaka *et al.*, 1997). Another study has shown that $CBP^{+/-}$ mice develop a high incidence of hematological malignancies as they age (Kung *et al.*, 2000). The phenotype of RTS patients is similar to that of mice deficient in one CBP allele. The inactivation of one p300 allele causes embryonic lethality in mice (Yao *et al.*, 1998). Moreover double heterozygosity for p300 and CBP was invariably associated with embryonic death. Thus, mouse development is sensitive to the overall gene dosage of p300 and CBP.

The studies from CBP and p300 heterozygotes have suggested that the levels of CBP and p300 are limited in the cell (Tanaka *et al.*, 1997; Yao *et al.*, 1998). Competition for a limiting amount of p300/CBP by different transcription factors may allow cross-talk between different signalling pathways. Consistent with this idea, nuclear hormone receptors can antagonise the AP-1 transcription factor, and expression of either CBP or p300 can abrogate this inhibition (Kamei *et al.*, 1996). Furthermore, in conditions where E2F-1 and p53 co-operate in apoptosis E2F-1 can effectively compete for p300, causing a reduction in p53-dependent transcription (Lee *et al.*, 1998). Thus, competition between the activation domains of p53 and E2F-1 for p300 may be instrumental in determining whether p53-dependent G1 arrest or E2F-1-dependent apoptosis ensues.

The effects of partial inactivation of CBP or p300 suggest p300/CBP is a tumour suppressor. In agreement with this role, various forms of leukaemia are associated with CBP-disrupting chromosomal translocations, for example the fusion of the gene encoding MOZ (monocytic leukaemia zinc finger protein) onto the amino

terminus of CBP (reviewed in Goodman and Smolik, 2000). The ability of p300/CBP to act as a tumour suppressor is epitomized by its interaction with p53. p300/CBP mediates transcriptional activation of the p53 responsive *p21WAF1, MDM-2* and *Bax* promoters, the gene products of which are important for G1 arrest and apoptosis (Avantaggiati *et al.*, 1997). The importance of p300 in mediating p53 apoptotic function has been highlighted by the recent identification of a novel p300-interacting protein called JMY, which augments p53-dependent apoptosis (Shikama *et al.*, 1999). Therefore, the interaction of p300 with additional proteins may be important in dictating a particular functional outcome.

The ability of adenovirus E1A to promote cell cycle progression by binding to pocket proteins was discussed in section 1.4.2. p300/CBP constitutes another major target of E1A and their interaction is sufficient to promote the entry of cells into S phase, suggesting that deregulation of p300/CBP activity has a role in cellular transformation (Moran, 1993). E1A mutants that lack the p300-binding site have lost the properties of E1A-driven cell cycle progression and its transforming potential. Moreover, p/CAF can compete with E1A for binding to CBP, thereby counteracting E1A-induced mitogenicity (Yang *et al.*, 1996). Thus, E1A may inhibit p300 by altering histone acetylation through competition with p/CAF. Alternatively, as E1A and TFIIB interact with a common domain in p300, (Felzien *et al.*, 1999), E1A may inhibit p300-dependent transcription by preventing p300 from interacting with the basal transcription machinery.

1.6.3 Regulation of p300/CBP activity

The ability of p300/CBP to regulate diverse cellular processes is a major paradox. Thus regulation of p300/CBP levels and activity is likely to be important in dictating when p300/CBP mediates the transcriptional activity of a particular target protein. The function of p300/CBP is modulated by a number of signalling pathways, including the cAMP activated protein kinase A (PKA) and nerve growth factor activation of the mitogen activated protein (MAP) kinases, p42/p44 (Chivria *et al.*, 1993; Liu *et al.*, 1998). Moreover, it is known that p300/CBP is phosphorylated in a cell cycle dependent manner (Yaciuk and Moran, 1991). Both cyclin/CDC2 and cyclin/CDK2 can phosphorylate p300 and binding of p300 to E1A can block these phosphorylation events (Banerjee *et al.*, 1994). It has been demonstrated that a region in the carboxy terminus of p300 binds to cyclinE-CDK2 (Perkins *et al.*, 1997). Furthermore, inhibition of p300 bound cyclinE-CDK2 by p21 correlated with enhanced *trans*activation by NF-κB.

Another study has demonstrated that the HAT activity of CBP is modulated in a cell cycle dependent manner, peaking around the G1-S transition (Ait-Si-Ali *et al.*, 1998). Moreover phosphorylation of CBP by cyclinE-CDK2 *in vitro*, was shown to enhance the HAT activity of CBP. The regulation of p300/CBP by cyclinE-CDK2 and p21 provide potential mechanisms for the coordination of p300/CBP-mediated transcription with cell cycle progression.

1.7 Regulation of transcription by phosphorylation

Phosphorylation is one of the major post-translational mechanisms by which the activity of transcription factors is regulated. To activate and repress transcription, transcription factors must localise in the nucleus, bind DNA and interact with the basal transcription machinery. Accordingly, phosphorylation may regulate transcription factor activity by affecting one or more of these processes (reviewed in Hunter and Karin, 1992). Several examples of transcription factors that are modulated by these mechanisms are described below and summarised in figure 1.6.

The Rel family of transcription factors, which includes NF-κB p50 and NF-κB p65, bind to their DNA recognition site as homodimers and heterodimers. In unstimulated cells, NF-κB is held in the cytoplasm in an inactive complex with the inhibitory protein, IκB (Inhibitor of NF-κB) (Karin, 1999). Upon treatment of cells with inducers such as tumour necrosis factor α, interleukin-1 and phorbal esters, IκB becomes phosphorylated, which acts as a signal for its destruction by the proteosome. Consequently NF-κB is released and translocates to the nucleus, where it activates transcription. This regulation ensures the rapid activation of NF-κB in response to a variety of signals.

More recently it has been demonstrated that the p65 subunit of NF-κB is phosphorylated by cAMP-dependent activation of protein kinase A (PKA) and this modification is required for efficient transcriptional activation (Zhong *et al.*, 1997). The mechanism of NF-κB activation by PKA was proposed to result from the unmasking of a domain within NF-κB which binds to the p300/CBP co-activator, thereby enabling p300/CBP to mediate NF-κB dependent transcription (Zhong *et al.*, 1998).

Transcription factor	Effect of phosphorylation	Kinase
CREB	Enhances its interaction with CBP	PKA
AP-1 (Jun)	Stabilises Jun by reducing ubiquitination	JNK
	Enhances interaction with CBP	JNK
	Inhibits DNA binding	CKII
AP-1 (ATF-2)	Increases HAT activity	SAPK/JNK
NF-κB	Increases interaction with p300/CBP	PKA
	Phosphorylation of I-κB releases NF-κB allowing it to enter the nucleus	IKK (I-κB kinase)
p53	Releases p53 from an inhibitory protein, MDM-2	Various *in vitro*: CKII Cdc2 PKC
	Stimulates DNA binding	DNA-PK
E2F	Phosphorylation of DP-1 subunit prevents DNA binding of the heterodimer	CyclinA-CDK2

Figure 1.6 Properties of transcription factors that are targets for kinases. Data from Kawasaki et al., 2000; Karin ,1999; Prives *et al.*, 1998; Zhong *et al.*, 1997; Shieh *et al.*, 1997; Musti *et al.*, 1997; Hill and Triesman, 1995; Bannister *et al.*, 1995; Chivria *et al.*,1993; Lin *et al.*, 1992; Pulverer *et al.*, 1991; Boyle *et al.*, 1991;

There are several examples of phosphorylation of transcription factors stimulating transcription through enhancement of their interaction with p300/CBP. A variety of genes induced by cAMP contain a cAMP-response element (CRE) in their promoters which binds to the CRE-binding protein, CREB. Phosphorylation of CREB by PKA at serine 133 stimulates the transcriptional activity of CRE-dependent genes in part by enabling the kinase-inducible domain (KID) in CREB to interact with CBP (Chrivia *et al.*, 1993).

Signal transduction cascades induced by extracellular mitogenic stimuli can ultimately lead to the activation of a group of kinases, the extracellular response kinases (ERK), a subfamily of the mitogen-activated protein (MAP) kinases. The ERKs are proline-directed serine/threonine kinases that recognize a proline at the +1 position of a potential substrate (Songyang *et al.*, 1996). Two other MAP kinase families have been found in vertebrates, Jun N-terminal kinase (JNK) and the p38 MAP kinases. MAP kinases have been shown to phosphorylate a variety of transcription factors that have been implicated in controlling the expression of genes required for cell proliferation (Hill and Treisman, 1995; Hunter, 1995).

Several kinases have been implicated in the phosphorylation of c-Jun, a subunit of the mitogen activated transcription factor AP-1 (Boyle *et al.*, 1991; Pulverer *et al.*, 1991). AP-1 consists of homo and heterodimers of Jun, Fos, ATF (activating transcription factor) and bZIP (basic region leucine zipper proteins) proteins (reviewed in Karin *et al.*, 1997). Phosphorylation of residues in the amino-terminal activation domain of c-Jun by JNK reduces the ubiquitination of c-Jun, thereby preventing its degradation (Musti *et al.*, 1997). The phosphorylation of c-Jun by JNK has also been demonstrated to enhance its interaction with CBP (Bannister *et al.*, 1995). Thus,

phosphorylation of specific sites in a transcription factor may regulate its activity by multiple mechanisms.

There is also evidence that phosphorylation of a set of sites in c-Jun inhibits its activity (Lin *et al.*, 1992). Phosphorylation of threonine 231 and serine 249 by casein kinase II (CKII) inhibits c-Jun DNA binding and their dephosphorylation in response to phorbol esters is associated with increased AP-1 activity (Boyle *et al.*, 1991). The transcription factor ATF-2 binds to the cyclic AMP response element as a homodimer or hereodimer with c-Jun (reviewed in Karin *et al.*, 1997). Recently it has been shown that ATF-2 has intrinsic HAT activity, which is essential for CRE-dependent transcription (Kawasaki *et al.*, 2000). Moreover, the HAT activity of ATF-2 was modulated by phosphorylation by JNK or signal-activated-protein kinases (SAPKs). This provides a link between signal activated protein kinase cascades and the regulation of transcription through the ability of a sequence specific DNA binding protein to regulate transcription by directly acetylating histones.

The phosphorylation of p53 is induced by DNA damage and correlates with enhanced transcription of p53 target genes (Prives, 1998). Serine 15 and serine 37 have been identified as sites of phosphorylation following DNA damage, in studies using phosphospecific antibodies (Shieh *et al.*, 1997; Siliciano *et al.*, 1997). Phosphorylation by several kinases, including DNA-PK, CKII, cdc2 and protein kinase C, *in vitro* enhanced the *in vitro* sequence-specific DNA-binding capability of p53 (Prives, 1998). However there is little information available about the kinases responsible for the phosphorylation of p53 *in vivo*.

The stability of p53 is regulated by MDM-2, which targets it for destruction by the proteosome. Phosphorylation of serine 15 inhibits binding of MDM-2 to p53 and blocks MDM-2 inhibition of p53-mediated *trans*activation (Shieh *et al.*, 1997).

However it has also been shown that mutation of various phosphorylation sites in p53, including serine 15, has no effect on the stabilisation of p53 following DNA damage (Ashcroft *et al.*, 1999). Thus the importance of individual phosphorylation sites in the regulation of p53 function requires further investigation.

1.8 Objectives

The regulation of E2F activity through phosphorylation of pRb by cyclin-CDKs provides a mechanism for co-ordinating cell cycle progression with the regulation of E2F-dependent transcription. However relatively little information is available on other levels of control that influence E2F activity. Phosphorylation of the DP-1 subunit of E2F by cyclinA-CDK2 is believed to be important for the downregulation of E2F activity in S phase. Furthermore, although E2F-1 can be phosphorylated *in vitro*, the physiological importance of E2F phosphorylation is unknown.

In this study the role of E2F-5 phosphorylation in cell cycle progression was investigated. Phosphorylation of E2F-5 at two sites in the *trans*activation domain was shown to be important in stimulating E2F-5-dependent transcription. E2F-5 contains a consensus CDK site positioned at threonine 251, which is absent in other E2F family members. A clue to the identity of the kinase responsible for the phosphorylaton of threonine 251 came from the analysis of E2F-5 during the Rat1 cell cycle. At the G1-S transition in Rat1 cells a slower migrating E2F-5 polypeptide was detected by SDS-PAGE analysis. This polypeptide was of an identical mobility to that detected by overexpressing E2F-5 and cyclinE-CDK2 in cells. *In vitro* and *in vivo* phosphorylation of E2F-5 with cyclinE-CDK2 confirmed that it was indeed the kinase responsible for phosphorylation of threonine 251.

The next part of this project aimed to understand the mechanisms by which phosphorylation of E2F-5 enhanced E2F-5 dependent transcription. To this end, mutant E2F-5 derivatives that could not be phosphorylated on threonine 251 or serine 307 were constructed. An analysis of these mutants revealed that phosphorylation at these sites did not effect the ability of E2F-5 to bind to DNA or interact with pocket proteins. However phosphorylation at threonine 251 by cyclinE-CDK2 enhanced the interaction of E2F-5 with p300, an important co-activator of E2F-5 dependent transcription. Thus a link between the cell cycle machinery and the activation of transcription was made.

An understanding of the role of phosphorylation in the regulation of E2F activity should ultimately shed light on the mechanisms whereby E2F causes the timely induction of target genes that promote cell cycle progression.

Chapter 2

Materials and Methods

2.1 Plasmids

The following plasmids have been described previously: pG4-DP-1 (Bandara *et al.*, 1993), pCMV-βgal (Zamanian and La Thangue, 1992), pCMV-HA-E2F-5, pCMV-HA-E2F-5$^{198-335}$ (Allen *et al.*, 1997), pGDP-3δ (de la Luna *et al.*, 1996), pCMV-p107 (Beijersbergen *et al.*, 1994), pCMV-HAp130 (Vairo *et al.*, 1995), pCE-luciferase (Botz *et al.*, 1996), pG5E1b-luciferase (Lee *et al.*, 1998), pVP16-TAD (Lee *et al.*, 1998), pSG5 (Green *et al.*, 1988), pX-cdk2, pX-cdk4, pX-cycE and pX-cycD1 (Zerfass-Thome *et al.*, 1997), p300$^{1572-1903}$ and his-E2F-5$^{1195-1673}$ (Yang *et al.*, 1996). Flag-tagged p300$^{1135-2414}$ was a gift from Ho Man Chan (Shikama *et al.*, 2000), Baculovirus vectors for his-cycE, his-cycA and his-cdk2 were gratefully received from David Morgan (Desai *et al.*, 1995).

To construct Gal4-E2F5$^{198-335}$, pCMV-HAE2F-5$^{198-335}$ was digested with Xho1 and the resulting insert cloned into pG4polyII (Bandara *et al.*, 1993). His-E2F5 and His-T251A were constructed by inserting into pET28c (Novagen) an EcoR1 fragment from pCMV-HA-E2F-5/HA-T251A/HA-S307A (Morris *et al.*, 2000). GST-E2F-5, GST-T251A and GST-S307A were generated from EcoR1 fragments of pCMV-HAE2F-5 (or the relevant mutant construct) cloned into pGEX2T (Pharmacia).

For the mammalian two-hybrid assay, pVP16-E2F-5, pVP16-T251A, pVP16-S307A, were constructed by digesting pCMV-HAE2F-5/T251A/S307A with EcoR1 and EcoRV and cloning the insert into pVP16-TAD (Lee *et al.*, 1998). The resulting constructs contained an N-terminal VP16 TAD and residues 90-335 of E2F-5.

2.2 Site-directed mutagenesis

pCMV-HAE2F-5 or HAE2F-5$^{198-335}$ was used as a template to make T251A, T251D, S307A and S307D using the Sculptor *in vitro* mutagenesis system (Amersham). Single stranded DNA was prepared from pCMV-HAE2F-5 or pCMV-HAE2F-5$^{198-335}$, using the M13K07 derivative of bacteriophage M13 (Sambrook *et al.*, 1989). The following oligonucleotides were annealed to the single stranded template DNA to introduce single site mutations into E2F-5 (bases which differ from wild-type E2F-5 are indicated in bold):

T251A: 5′ T CTG TGG AG**C** CAC TGA AGT TG 3′
T251D: 5′ T CTG TGG A**TC** CAC TGA AGT TG 3′
S307A: 5′ G GGT AGG AG**C** AAG CCG TAA AAG 3′
S307D: 5′ G GGT AGG A**TC** AAG CCG TAA AAG 3′

A summary of the reactions and enzymes used is shown in figure 2.1.

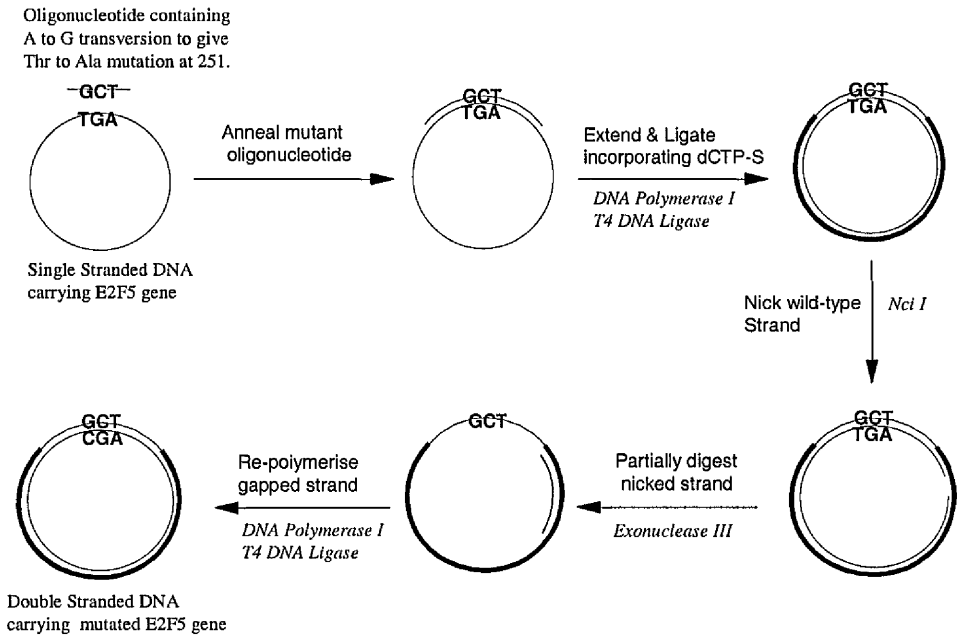


Figure 2.1 Summary of site-directed mutagenesis procedure.

The DNA from the mutagenesis reactions was transformed into TG-1 bacteria and DNA minipreps were prepared. DNA sequencing was carried out either manually for both strands (Sequenase, Amersham) or with an ABI dye terminator cycle sequencing-ready reaction kit (Perkin Elmer) and an automated DNA sequence analyser.

2.3 Cell Culture and Transfection

Mammalian cells were grown in Dulbecco's modified eagles medium (DMEM) supplemented with 10% foetal calf serum (GIBCO) and antibiotics, 10μg/ml streptomycin and 100U/ml penicillin (GIBCO), at 37°C in a 5% CO_2/ H_2O-saturated atmosphere. Rat1 cells were serum starved by transfering cells at 50% confluency into DMEM supplemented with 0.2% foetal calf serum for 72 hours. *Sf9* cells were grown in TC100 (GIBCO) supplemented with 5% FCS.

Cells for transient transfection were plated out 24 hours before transfection at approximately 1.5 x 10^6 cells per 100mm dish (for immunoprecipitation and flow cytometric analyses), 5 x 10^5 cells per 60mm dish (for luciferase and β-galactosidase assays, extracts for immunoblotting and gel retardation) or 2 x 10^5 cells per 35mm dish (on coverslips for immunofluorescence). Two hours before the transfection, the medium from the cells was removed and replaced with fresh growth medium.

Transient transfection of U2OS and SAOS2 cells was performed by the calcium phosphate precipitation method. The relevant plasmid DNA was mixed with a final concentration of 250mM $CaCl_2$ and the resulting solution (100μl per 35mm dish, 250μl per 60mm dish and 500μl per 100mm dish) was added drop wise to an equal volume of 2xHBS solution (50mM HEPES pH7.1, 280mM NaCl, and 1.5mM Na_2HPO_4) and

added to the media on cells. COS7 cells were transfected using Lipofectin Reagent (GIBCO). For every 1μg of DNA transfected, 2μl of Lipofectin was used.

To maintain a constant amount of plasmid DNA in each sample, either pcDNA3 (Invitrogen) or pSG5 was transfected as appropriate. Cells were washed three times with PBS at 14-16 hours (U2OS and COS7) or 8-10 hours (SAOS2) post transfection. U2OS and SAOS2 cells were harvested 40-46 hours post transfection and COS7 cells were harvested 64-70 hours post transfection.

U2OS cell-lines stably transfected with doxycyclin inducible HA-tagged E2F-5 (refered to as WT1) and S307A (refered to as S5) were prepared by Liz Allen (Allen and La Thangue, personal communication).

2.4 Cell extract preparation and phosphatase treatment

Cells were lysed in ice-cold buffer (20mM HEPES pH 7.6, 400mM KCl, 1mM EDTA, 25% glycerol, 1mM DTT, 0.1% NP40, 5mM NaF, 1mM Na_3VO_4, 1mM PMSF) for 30 minutes and centrifuged for 10 minutes to remove insoluble material. The protein content of extracts from Rat1 and F9EC cells was estimated by the method of Bradford (1976).

Extracts to be used for phosphatase treatment were lysed in the absence of NaF and Na_3VO_4. Phosphatase treatment of F9EC and Rat1 cell extract (50μg) was carried out in a 50μl reaction containing 200U of λ-protein phosphatase (NE Biolabs), 50mM Tris pH 7.8, 5mM DTT, 2mM $MnCl_2$, 100 μg/ml BSA. The reactions were incubated at 30°C for 1 hour and terminated by addition of SDS loading buffer.

2.5 Immunoblotting

Typically, 50μg of extract was blotted but where transfected extracts were analysed one third of the cells harvested from a 60mm dish was used. Samples were separated by 10% SDS-PAGE, except for p300 detection where 6% SDS-PAGE was used. The gels containing the resolved proteins were transferred to a nitrocellulose membrane (Inverclyde Biochemicals Ltd), blocked in PBS-10% non-fat milk for 30 minutes, then incubated with primary antibody for at least one hour. After washing in PBS-0.1%Tween-20 the blot was incubated with secondary antibody, either anti-rabbit or anti-mouse alkaline phosphatase conjugated antibody (Promega; 1:7,500). Alternatively, horseradish peroxidase-conjugated goat anti-rabbit or -mouse IgG (1:4,000) was used for ECL detection (Amersham).

2.6 Immunoprecipitation and binding assays

For all immunoprecipitations the antibodies were prebound to Protein A beads (Roche) in 50mM Tris (pH8), 5mM EDTA, 120mM NaCl (TNE). For immunoprecipation of kinases to be used for *in vitro* phosphorylation reactions Rat1 cell extract (200μg) was diluted with 2 volumes of 50mM Tris (pH8), 5mM EDTA and immunoprecipitated with anti-cdk2, anti-cycE M20 or anti-Gal4 (all from Santa Cruz). The beads were washed twice with TNE and twice with kinase buffer (see section 2.18) before being used in a kinase assay.

For immunoprecipitation of proteins from Rat1, RAGI or F9EC extract, between 0.5 and 1mg of extract (as indicated in the results section) was diluted with 2 volumes of 50mM Tris (pH8), 5mM EDTA to give a final salt concentration of

130mM. The diluted extracts were precleared by incubating with Protein A beads for 1 hour at 4°C and thereafter combined with the relevant antibody bound to Protein A beads and agitated on a Vibrax machine for three hours at 4°C. The bead pellet was washed (3x 10 minutes) in TNE containing 0.1% NP-40 and after the final wash solution was removed the pellet was resuspended in 20-40µl of SDS-loading buffer (250mM Tris-HCl, pH6.8, 20% glycerol (v/v), 4% SDS (w/v), 0.1% bromophenol blue (w/v), 200mM DTT and 5% β-mercaptoethanol). The samples were then analysed by SDS-PAGE and immunoblotting.

Purified flag-p300 was incubated with *in vitro* phosphorylated His-E2F-5 or His-T251A for 2 hours at 4°C and washed 4 times in 50mM Tris (pH8), 12.5mM $MgCl_2$, 120mM NaCl, 1mM PMSF, 1mM Na_3VO_4. The bound proteins were subjected to 10% SDS-PAGE and detected by immunoblotting with anti-E2F5 (see section 2.20). Pulldowns were quantified with a BIORAD imaging densitometer GS-670 using Molecular Analyst software.

2.7 Immunofluoresence

Cells were transfected in 35mm dishes containing three coverslips (see section 2.3). The cells were fixed to the coverslips in 4% paraformaldehyde at room temperature for 15 minutes, washed in PBS, then permeabilised in PBS containing 0.2% Triton X-100 for 10 minutes. Fixed cells were then washed three times with PBS-1% FCS and incubated in primary antibody diluted in PBS-5% FCS for 30 minutes. Subsequently the coverslips were washed in PBS-1% FCS, cells were incubated in secondary antibody; goat anti-mouse or anti-rabbit IgG conjugated to

fluorescein isothiocyanate (FITC) or tetramethyl rhodamine isothiocyanate (TRITC) (Southern Biotechnology Associates Inc.) diluted in PBS-10% FCS for 30 minutes at room temperature. After three final washes in PBS, the coverslips were mounted onto slides using citifluor (Citifluor Ltd), and viewed using a fluorescence microscope (Olympus).

2.8 Gel retardation analysis

The E2F recognition site from the adenovirus E2a promoter (or a mutant site) was used in all gel retardation analysis (Girling *et al.*, 1993):

Wild-type 5'-GATCTAGTTTTCGCGCTTAAATTTGA-3' and
3'-ATCAAAAGCGCGAATTTAAACTCTAG-5'

Mutant 5'-GATCTAGTTTTCG**ATA**TTAAATTTGA-3' and
3'-ATCAAAAGC**TAT**AATTTAAACTCTAG-5'

Typically, 10μg of cell extract was used per gel retardation sample. When transfected cell extract was analysed, 3-5% of cells harvested from a subconfluent 60mm tissue culture dish for used for one sample. The cell extract was combined with binding buffer (10mM HEPES pH7.6, 0.1 M KCl, 1mM EDTA, 4% Ficoll, 0.5mM DTT), 2μg of sheared salmon sperm DNA and 200ng of mutant promoter oligo to reduce the non-specific DNA binding activity. Antibodies for supershifts were added and complexes were allowed to form at room temperature. After 15 minutes, 1ng of a ^{32}P-labelled E2F site was added for a further 20 minutes. Complexes were resolved on a 4% polyacrylamide gel in 0.5x Tris-borate EDTA (TBE) at 4°C for 2 hours (200V).

2.9 Heparin-Sepharose column chromatography

A 3ml heparin-Sepharose column was poured using a slurry of heparin-Sepharose CL-6B (Pharmacia) suspended in NEP buffer (20mM, HEPES pH7.9, 100mM KCl, 12.5mM $MgCl_2$, 10mM EDTA, 0.5mM PMSF, 0.5mM DTT). The plunger was inserted to exclude all air bubbles in the column. The column was washed at a rate of two column volumes per hour for two hours. F9EC extract (6mls) was thawed rapidly and loaded onto the column at a rate of 1 column volume per hour. The column was washed for a further hour and proteins were eluted in a linear KCl gradient (between 0.1M and 1.0M). The gradient was set up using an Econo system controller (Bio-Rad), and NEP buffer containing either 0.1 or 1.0M KCl. Fractions (200µl) were collected using a Bio-Rad Model 2110 Fraction Collector and dialysed against NEP buffer overnight. 10% glycerol was added before storage at –80°C.

2.10 Affinity matrix preparation

The E2F recognition site from the adenovirus E2a promoter (see Section 2.8) was covalently coupled to Sepharose to prepare an affinity matrix, based on the method by Kadonaga and Tijan (1986). Briefly, annealed, 5'-phosphorylated and ligated oligos (100µg of the wild-type and mutant E2F site) were prepared. Approximately 0.6g of cyanogen bromide-activated Sepharose CL4B (Pharmacia) was combined with 1mM HCl and washed for 15 minutes in 1mM HCl using a sintered glass funnel. To activate the coupling groups the matrix was then washed with 100mls of 10mM KPO_4 buffer.

The activated resin was divided between two 50ml falcon tubes, each containing 4mls of 10mM potassium phosphate buffer pH 8.35 and either the wild-type

or mutant pre-ligated DNA, and rotated for 16 hours at 4°C. Each slurry was then washed with dH_2O (50ml), followed by 1M ethanolamine pH 8.5 and thereafter rotated for 4 hours in ethanolamine at room temperature. Finally, the beads were washed sequentially, using the following buffers: 50ml 10mM KPO_4 buffer pH 8.35, 50ml 1M KPO_4 buffer pH 8.35, 50ml 1M KCl, 50ml dH_2O, 50ml (10mM Tris pH 7.6, 0.3M NaCl, 1mM EDTA, 0.02% NaN_3). The matrix was stored in the final wash buffer at 4°C.

2.11 Metabolic labelling

At 36 hours post-transfection U2OS cells (100mm dishes) were washed twice with phosphate-free medium and labelled with [^{32}P]orthophosphate (0.2mCi/ml) in phosphate-free medium for 12 hours. The cells were lysed in 50mM Tris pH8.0, 150mM NaCl, 1mM EDTA, 1% NP40, pre-cleared for 30 minutes with protein G beads and immunoprecipitated for 2 hours with anti-HA11 monoclonal antibody (Babco). Immunoprecipitates were washed three times in RIPA buffer (150mM NaCl, 1.0% NP-40/Igepal (v/v), 0.5% DOC (w/v), 0.1% SDS (w/v), 50mM Tris-HCl (pH8.0), once in 50mM Tris pH8.0, 400mM NaCl, 1mM EDTA, 1% NP40 followed by a final wash in 50mM Tris pH8.0, 150mM NaCl before resuspending in SDS sample buffer and resolving on a 10% gel. The immunoprecipitated proteins were transferred to nitrocellulose and visualised by autoradiography.

2.12 CnBr phosphopeptide mapping

Cyanogen Bromide (CnBr) cleavage was performed according to the method by Luo *et al.*, (1990). The phosphorylated E2F-5 polypeptides were excised from nitrocellulose and cleaved with CnBr (100mg/ml CnBr in 50% formic acid) for 2 hours at room temperature. Phosphopeptides were resolved on a 24% acrylamide gel and visualised after a two week exposure on a phosphoimager. CnBr cleaves at methionine residues and the predicted sizes of the E2F-5 peptides are 153, 62, 43, 39 and 38 residues; the 62-residue peptide contains threonine 251 and was clearly resolved from the other cleavage products.

2.13 Flow Cytometry

Cells were harvested in PBS and fixed in 50%(v/v) ethanol/PBS. Cells were treated with RNase (125U/ml) for 30min, harvested by centrifugation and suspended in propidium iodide (20μg/ml) in PBS at 4°C for 1hour. Cells were analysed in a Becton Dickinson FACScan.

Cells that had been labelled by BrdU were fixed in 70% ethanol containing 2mM glycine (pH 2.0) for 20 minutes at -20°C and labelled with anti-BrdU (see section 2.14), prior to propidium iodide treatment.

2.14 BrdU incorporation

Following transfection and overnight incubation, U2OS cells were incubated with bromodeoxyuridine BrdU (Boehringer Mannheim) for 10 minutes. The cells were fixed in 70% ethanol containing 2mM glycine (pH2.0) for 20 minutes at -20°C. BrdU incorporation was detected by incubating for 30 minutes with a primary mouse anti-BrdU, followed by three washes with PBS and a further incubation with an anti-mouse IgG conjugated to fluorescein isothiocyanate (FITC) (all supplied in a kit from Boehringer Mannheim).

Transfected cells were identified by immunostaining with an anti-DP-1 antiserum and anti-rabbit tetramethyl rhodamine isothiocyanate (TRITC) (Southern Biotechnology Associates Inc.) and the level of BrdU incorporation expressed relative to the total number of transfected cells. The same procedure was followed with the stable cell lines (WT1 and S5), although thereafter these were analysed by flow cytometry (see section 2.13).

2.15 Protein purification from Baculovirus infected *Sf9* cells

Sf9 insect cells were co-infected with the appropriate recombinant baculoviruses and whole cell extracts were prepared 48 hours post-infection. Briefly cells were lysed in buffer A (25mM HEPES pH7.6, 400mM KCl, 12.5mM $MgCl_2$, 0.1mM EDTA, 30% glycerol, 0.1% NP40), centrifuged to removed unsoluble material and the resulting lysate was diluted dropwise with 2 volumes of buffer B (25mM HEPES pH7.6, 100mM KCl, 12.5mM $MgCl_2$, 0.1mM EDTA, 10%glycerol, 0.1% NP40), then incubated with either anti-FlagM2-beads (Sigma) or Ni^{2+}-beads (Qiagen), as appropriate for 1 hour at

4°C. The beads were washed three times with buffer B containing 200mM KCl (and 20mM imidazole for His-tagged proteins). His-tagged proteins were eluted with 250mM imidazole in buffer B and dialysed against the same buffer without the imidazole. Flag-tagged p300 was eluted with 1mg/ml of Flag peptide (Sigma) in buffer B.

2.16 GST and His-tagged recombinant protein-purification

Transformed BL21 (DE3) bacteria (Invitrogen) were grown to mid-logarithmic stage in 500ml of LB-broth containing the appropriate antibiotic at 37°C. Thereafter, protein expression was induced by the addition of 0.5 mM (final concentration) isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 hours at 30°C.

For GST purification, bacterial pellets were resuspended in 10ml PBS/1% Triton X-100 and 0.5mM PMSF on ice, and then sonicated briefly (3 x 10sec) on ice. Bacterial debris was pelletted by centrifugation and re-centrifugation at 10,000rpm for 15min (at 4°C) each. A 400µl suspension of glutathione-Sepharose beads (50% v/v; Pharmacia), which had been prewashed in PBS, were added to the supernatant and mixed with constant rotation at 4°C for 30 minutes. The suspension was washed three times with 50ml PBS by spinning in a bench-top centrifuge at 5,000rpm for 5 minutes (at 4°C). The GST proteins were eluted from the beads by incubating the bead pellet with an equal volume of 50mM Tris pH8, containing 10mM of glutathione.

For his-tagged purification the bacterial pellets were resuspended in 10ml of denaturing buffer (4M Urea, 100mM KCl, 20mM Tris pH8, 10% Glycerol, 0.1% NP-40) and gently stirred for 12 hours at room temperature. $MgCl_2$ was added to a final concentration of 5mM, and cellular debris was cleared by repeated centrifugation at

4°C. A 500µl suspension of nickel-agarose (50% v/v, Qiagen) and 10ml of wash buffer (100mM KCl, 20mM Tris-HCl pH7.9, 10% Glycerol, 0.1% NP-40, 10mM imidazole pH 8) were added to the supernatant, which was then rotated for 1 hour at room temperature. The urea in the supernatant was diluted by the addition of a further 20ml of wash buffer and the tube was rotated at 4 °C for two hours, to allow the proteins bound to the nickel-agarose to refold. The supernatant was removed and the beads were washed twice and rotated for a further 2-4 hours at 4 °C. Protein was eluted by incubating the resin with an equal volume of imidazole buffer (200mM Imidazole, 100mM NaCl and 20mM Tris-HCl pH7.9). Imidazole was removed from the eluted protein by dialysis with 100mM NaCl and 20mM Tris-HCl (pH7.9) buffer, for 6 to 8 hours.

Protein expression was measured by Coomassie after SDS-PAGE.

2.17 In vitro protein expression

In vitro transcription and translation was carried out using a TNT T7 coupled reticulocyte lysate system (Promega) as recommended by the manufacturer, in the presence of ^{35}S methionine. To check expression 1µl was subjected to SDS-PAGE and autoradiographed.

2.18 *In vitro* kinase assays

Phosphorylation reactions (40µl) were performed in 20mM HEPES (pH7.6), 10mM $MgCl_2$, 1mM EGTA, 2mM $MnCl_2$, 10% glycerol, 1mM DTT, 50µM ATP and 5µCi [γ-^{32}P]ATP and incubated with 1µg of His-E2F-5, His-T251A or histone H1. Reactions to be used in subsequent p300 binding assays were performed cold with 1mM ATP without [γ-^{32}P]ATP. The kinase activity was supplied to the reaction by either purified baculovirus cyclinE-CDK2 or immunoprecipitated cyclinE or CDK2 from Rat1 cells. After 30 minutes at 30°C the reactions were terminated with SDS loading buffer and analysed by 10% SDS-PAGE.

2.19 *In vitro* acetylation assays

In vitro acetylation reactions (30µl) were performed in 50mM Tris pH 8, 5% Glycerol, 0.1% EDTA, 50mM KCl, 1mM DTT, 1 mM PMSF, 10mM sodium butyrate and C^{14}-Acetyl CoA (Amersham) and incubated with 1-2µg of his-E2F-5 or GST-E2F-5, together with 100ng of flag-p300. A histone octomer preparation (prepared by L Smith) was used as a positive control. After 30 minutes at 30°C the reactions were terminated with SDS loading buffer and analysed by 10% SDS-PAGE and autoradiography.

2.20 Antibodies

The following antibodies were used:

E2F-5 Polyclonal anti-E2F-5 antiserum (directed against a C-terminal peptide: residues 320-335 **(C)NEGVCDLFDVQILNY**) and pre-immune serum (Buck *et al.*, 1995)

HA monoclonal antibody HA11 (BabCO).

p300 Anti-p300 N15 rabbit polyclonal antiserum (Santa Cruz).

DP-1 Affinity purified polyclonal anti-DP-1 (anti-D) directed against peptide D (Sørensen *et al.*, 1996):

Peptide D (residues 385-400) (C)RVETPVSYVGEDDDDD

Affinity purified polyclonal anti-DP-1 (anti-A) directed against peptide A (Sørensen *et al.*, 1996):

Peptide A (residues 3-15) (C)KDASLIEANGELK

Monoclonal anti-DP-1 (32.3), directed against peptide D (Sørensen *et al.*, 1996).

p107 mouse monoclonal SD9 (SantaCruz)

βGal mouse monoclonal (Roche)

cdk2 anti-CDK2 M2 rabbit polyclonal (Santa Cruz)

cyclinE rabbit polyclonal anti-cyclinE M20 (Santa Cruz)

Gal4 anti-Gal4 DBD (Santa Cruz).

Chapter 3

Characterisation of E2F-5 in cells

3.1 Introduction

E2F is a heterodimeric transcription factor consisting of an E2F family member (E2F-1 to –6) and a DP family member (DP-1 to –3). It is becoming apparent that different E2F heterodimers have distinct roles in cell cycle control (DeGregori *et al.*, 1997). This chapter describes the study of the endogenous E2F-5 protein and its interaction with other proteins, in an attempt to gain insight into the specific function of this E2F family member.

E2F-5 was isolated on the basis of its interaction with DP-1 in a yeast two-hybrid screen (Buck *et al.*, 1995). The protein sequence, molecular organisation and certain functional properties of E2F-5 are more closely related to E2F-4 than other members of the E2F family (Buck *et al.*, 1995; Hijmans *et al.*, 1995; Sardet *et al.*, 1995). Based on these similarities E2F-4 and E2F-5 make up a subfamily of E2F. Both E2F-4 and E2F-5 are physiological components of E2F DNA binding activity in a variety of cell lines. The level of their mRNA remains relatively constant throughout the cell cycle (Ginsberg *et al.*, 1994; Sardet *et al.*, 1995). E2F-5 mRNA levels are particularly high in leukaemic cell lines, for example RAGI and DAUDI and in F9 embryonal carcinoma (EC) cells (Buck *et al.*, 1995). In contrast E2F-1 transcription is cell cycle regulated and the levels of E2F-1 mRNA rise dramatically towards the end of G1 phase. Moreover E2F-1 expression is relatively low in a range of cell lines that highly express E2F-4 or E2F-5 (Buck *et al.*, 1995). An observation perhaps related to the ability of E2F-1, but not E2F-4 or E2F-5 to cause apoptosis.

Much of endogenous E2F-5 is found as a heterodimer with DP-1, some of which is associated with the pocket protein p130 (Hijmans *et al.*, 1995). DP-1 is a frequent component of E2F DNA binding activity throughout the cell cycle in several cell lines (Bandara *et al.*, 1994), whilst its E2F partner varies and has been shown to be cell type and cell phase specific (reviewed in Dyson, 1998).

3.2 E2F-5 is an abundant component of E2F DNA binding activity

E2F-5 is an abundant component of E2F DNA binding activity in a variety of cell types, including F9EC cells and DAUDI cells (Buck *et al.*, 1995; Fig 3.1a and 3.1b). E2F DNA binding activity was examined by gel retardation analysis with an E2F-site probe containing the E2F binding sequence from the adenovirus (Ad5) E2a promoter. This site was chosen as it is a high affinity E2F binding site and has previously been used to define E2F in F9EC cells (La Thangue *et al.*, 1990). Several complexes were resolved in gel retardation analysis using extracts from F9EC and DAUDI cells. All three of the complexes detected in F9EC cell extract were specific for E2F site recognition since binding to the labelled probe was competed by addition of excess unlabelled probe (Fig 3.1b, compare lanes 1 and 2). However in DAUDI cell extract there were two specific complexes and one non-specific complex (Fig 3.1a, compare lanes 1 and 4). The identities of the complexes were assessed using a panel of antibodies. A polyclonal anti-E2F5 antiserum directed against a peptide in the C terminus of E2F-5 (anti-E2F-5) supershifted the fastest migrating complex in F9EC cells and DAUDI cells (Fig 3.1a, lanes 2 and 3; Fig 3.1b, compare lanes 3 and 4). The pre-immune antiserum did not shift any complex indicating the supershift generated by

Figure 3.1

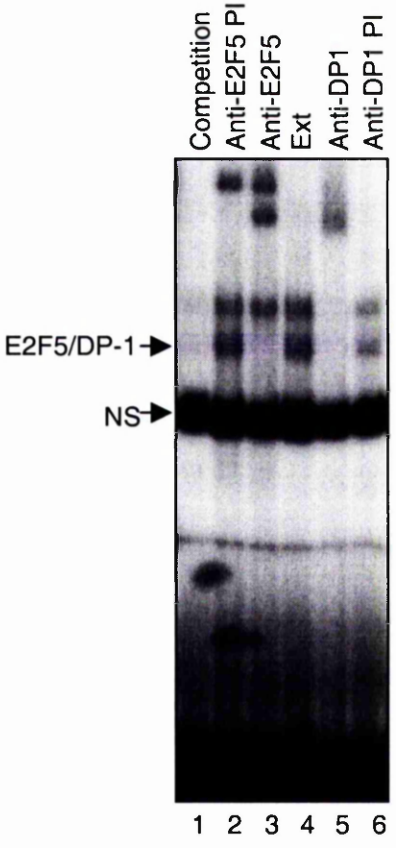
E2F-5 is an abundant component of DNA binding activity in DAUDI and F9EC cells.

Gel retardation analysis of E2F complexes in DAUDI and F9EC extracts using the sequence of the E2F site taken from the adenovirus E2A promoter.

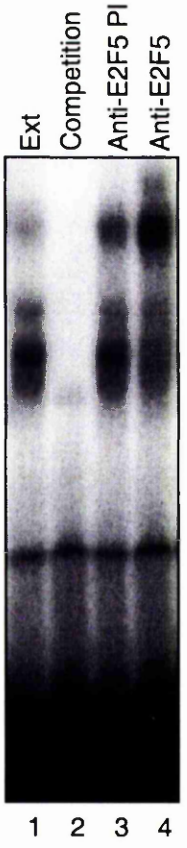
(a) The DNA binding activity of DAUDI extract is shown in lane 4. Lane 1 included the addition of a 100-fold unlabelled E2F site as a competitor to the labelled E2F probe. Extracts in lane 2,3,5 and 6 were supershifted with the indicated antisera. PI: rabbit pre-immune serum.

(b) The DNA binding activity of F9EC extract is shown in lane 1. Lane 2 included the addition of a 100-fold unlabelled E2F site as a competitor to the labelled E2F probe. Extracts in lane 3 and 4 were supershifted with either anti-E2F-5 or the pre-immune (PI) serum as indicated.

a


Competition
Anti-E2F5 PI
Anti-E2F5
Ext
Anti-DP1
Anti-DP1 PI
E2F5/DP-1
NS
1 2 3 4 5 6

b


Ext
Competition
Anti-E2F5 PI
Anti-E2F5
1 2 3 4

anti-E2F-5 was specific. An anti-DP-1 (αD) antiserum supershifted all of the complexes in DAUDI extract (Fig 3.1a, compare lanes 5 and 6). Thus the fastest migrating complex in DAUDI cells consisted of an E2F-5/DP-1 heterodimer.

The E2F-5 protein was detected as a heterogenous set of polypeptides by western blot analysis with anti-E2F-5 (Fig 3.2a). In COS7 cells, transiently transfected with an E2F-5 expression vector, exogenous E2F-5 was resolved as three closely migrating polypeptides, two of which had an identical mobility to the endogenous E2F-5 polypeptides detected in F9EC cells (Fig 3.2a, compare lane 2 and 3). By including the homologous peptide in the primary antiserum incubation the recognition of these three bands was abolished, demonstrating that the polypeptides were specifically recognised by the anti-E2F-5 antiserum (Fig 3.2a, lanes 1 and 4).

The E2F-5 polypeptides were defined as, Lower (E2F-5L), Middle (E2F-5M) and Upper (E2F-5U), in order of decreasing mobility on SDS-PAGE (Fig 3.2a). The distribution of these polypeptides varied in F9EC cells and Rat1 cells (Fig 3.2b). In F9EC cells the L and M forms were most abundant, whereas in Rat1 fibroblasts forms M and U were most readily detected (Fig 3.2b).

3.3 E2F-5 may be modified by phosphorylation

One study has suggested that E2F-5 is phosphorylated *in vivo* (Hijmans *et al.* 1995). The authors characterised E2F-5 from cells metabolically labelled with [^{32}P]-orthophosphate, from which they immunoprecipitated a range of polypeptides with two different E2F-5 antibodies that likely represented differentially phosphorylated forms of E2F-5.

To investigate whether phosphorylation was responsible for the presence of the multiple forms of E2F-5 in F9EC and Rat1 cells, extracts from these cells were treated with λ-phosphatase. Western blot analysis of the treated extracts revealed a shift in mobility of the E2F-5U and E2F-5M bands to the position of E2F-L, suggesting that the bands reflected different phosphorylated forms of E2F-5 (Fig 3.2c).

In vitro translation of E2F-5 using a rabbit reticulocyte lysate produced several E2F-5 polypeptides, possibly the result of differential phosphorylation of E2F-5 by kinases in the lysate (Fig 3.2d, lane 1). A similar pattern of polypeptides was observed when a C-terminal fragment of E2F-5 (198-335) was *in vitro* translated (Fig 3.2d, lane 2), implying phosphoacceptor sites may be concentrated between amino acids 198 and 335 of the E2F-5 sequence.

3.4 Analysis of the DNA binding properties of the E2F-5/DP-1 heterodimer

It is well known that phosphorylation regulates the DNA binding activity of numerous diverse transcription factors (Hunter and Karin, 1992). Phosphorylation of the tumour suppressor protein, p53 dramatically stimulates its ability to bind to target sites in the *p21WAF1* promoter (Wang and Prives, 1995). The DNA binding activity of

Figure 3.2

Cell extracts contain multiple E2F-5 polypeptides with different electrophoretic mobilities and the slowest mobility polypeptide becomes more abundant after phosphatase treatment.

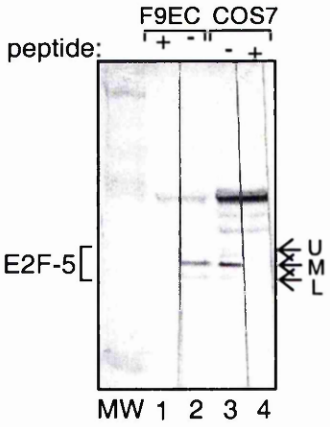
(a) Immunoblot of E2F-5 in F9EC cell extract and in an extract from COS7 cells transfected with an E2F-5 expression vector. Gel lanes were cut in half and probed with either anti-E2F-5 (lane 2 and 3) or anti-E2F-5 in the presence of the homologous peptide from which the antibody was generated (lane 1 and 4).

(b) Immunoblot of E2F-5 in F9EC and Rat1 cell extract. The presence of the different forms (U, M and L) is shown.

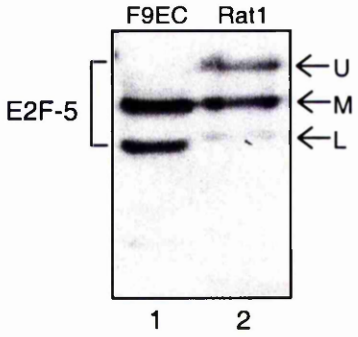
(c) Immunoblot of E2F-5 after treatment of an F9EC and Rat1 cell extract with λ-phosphatase (lane 2 and 4) and control treatments performed in the absence of λ-phosphatase (lane 1 and 3).

(d) Expression vectors for full length E2F-5 and E2F-$5^{198-335}$ were *in vitro* transcribed and translated in the presence of ^{35}S-Methionine (see Materials and Methods) and subjected to SDS-PAGE and autoradiography.

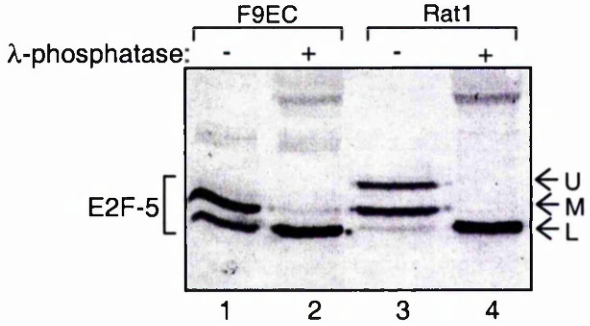
a


F9EC COS7
peptide: + - - +
E2F-5
U
M
L
MW 1 2 3 4

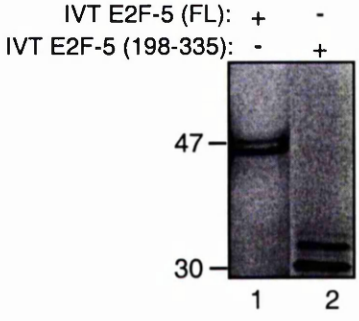
b


F9EC Rat1
E2F-5
U
M
L
1 2

c


F9EC Rat1
λ-phosphatase: - + - +
E2F-5
U
M
L
1 2 3 4

d


IVT E2F-5 (FL): + -
IVT E2F-5 (198-335): - +
47
30
1 2

E2F itself is regulated during the cell cycle by cyclinA-CDK2 dependent phosphorylation (see Introduction: section 1.3.3). One study has shown that two immunochemically distinct forms of DP-1, which the authors interpreted as differentially phosphorylated DP-1, differed in their ability to bind to the E2F consensus site (Bandara *et al.*, 1994). To further explore the regulation of E2F DNA binding activity it was decided to investigate whether E2F-5L, E2F-5M or E2F-5U differed in their ability to bind DNA.

Heparin-Sepharose chromatography was used to purify E2F DNA binding activity from F9EC cells. Proteins were eluted in a linear KCl gradient (0 to 1M) and the fractions assessed for DNA binding activity by gel retardation. DNA binding activity peaked in fractions 32 to 38, corresponding to a KCl concentration ranging from 300 to 400mM (Fig 3.3a, lanes 6-12). Immunoblotting of these fractions with anti-E2F-5 revealed they were enriched with E2F-5 and two forms of E2F-5 (L and M) were detected in these 'peak' fractions in a similar ratio as detected in crude extract (Fig 3.3b, compare lane 5 and lane 9). Similarly E2F-5L and E2F-5M were detected in column flow through-fractions, which were non-DNA binding (Fig 3.3b, lanes 2-4).

Two DP-1 antibodies, anti-D and anti-A, were used to investigate the presence of immunochemically distinct forms of DP-1 in these fractions. Anti-D is a polyclonal antibody directed against a peptide in the extreme C-terminus of DP-1 that recognises a single DP-1 polypeptide at 55kDa, designated p55L (Bandara *et al.*, 1994: see Materials and Methods). This polypeptide is also recognised, albeit much more weakly, by anti-A (directed against an N-terminal peptide) and anti-A also recognises an additional DP-1 polypeptide designated p55U (Bandara *et al.*, 1994). DNA binding fractions were enriched with p55L (Fig 3.3c, compare lane 5 and lane 1), whilst the

Figure 3.3

Separation of E2F DNA binding activity by binding to heparin-Sepharose.

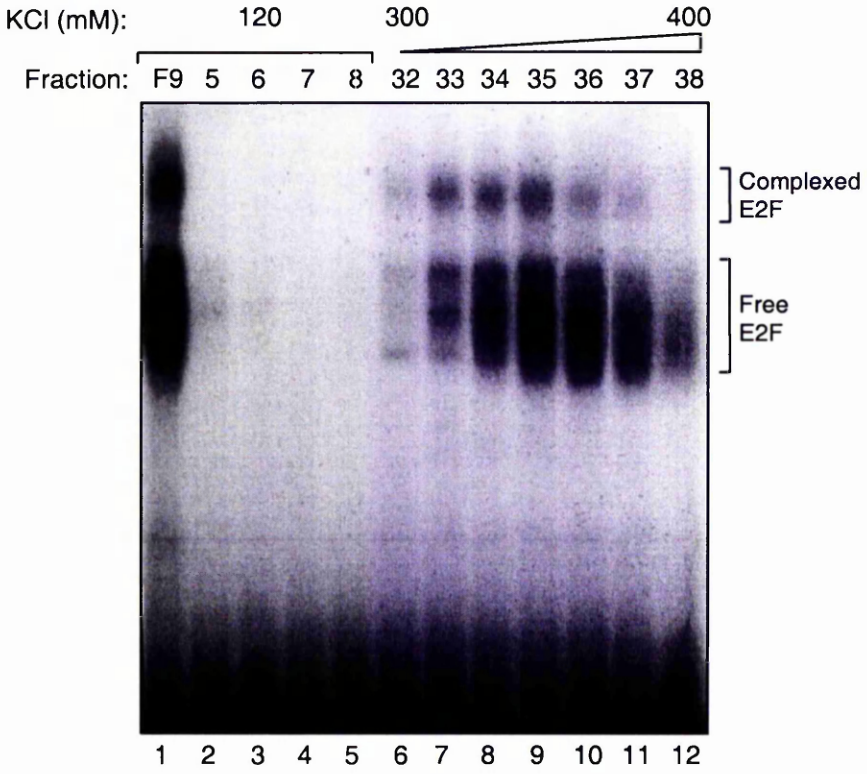
(a) Gel retardation analysis of F9EC extract fractionated over a heparin-Sepharose column, using the E2F site taken from the adenovirus E2A promoter. Lane 1 shows the DNA binding activity of crude F9EC extract. Lanes 2-12 show the DNA binding activity of the indicated fractions. Fractions were eluted from the column at the indicated KCl concentrations.

(b) Immunoblot of E2F-5 in the indicated heparin-Sepharose fractions described in (a). Lane 1 and lane 5 show the reactivity of the E2F-5 antibody with crude F9EC extract.

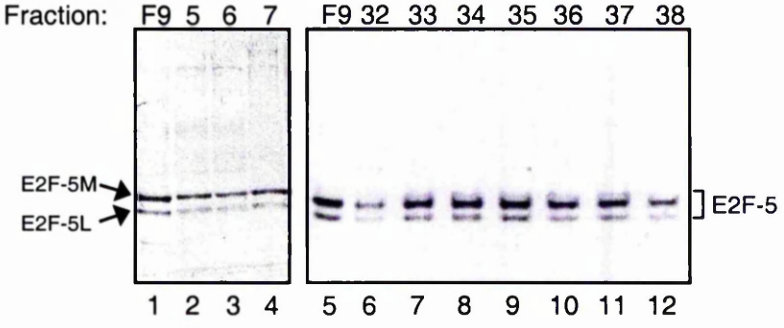
(c) Immunoblot of DNA binding fractions from (a) with two different DP-1 antibodies. The gel lanes were cut in half and probed with either anti-A or anti-D as indicated (see Materials and Methods). Lane 1 shows the crude F9EC extract. The fraction number is indicated above each lane (lanes 2-8).

(d) Immunoblot of DP-1 to compare DP-1 in crude extract (lane 1) with DP-1 in heparin-Sepharose fraction 5 and 6 (non-DNA binding). The gel lanes were cut in half and probed with either anti-A or anti-D as indicated. The asterisk indicates the position of the p55U form of DP-1 in fractions 5 and 6. MW is a lane showing the sizes of the molecular weight size markers.

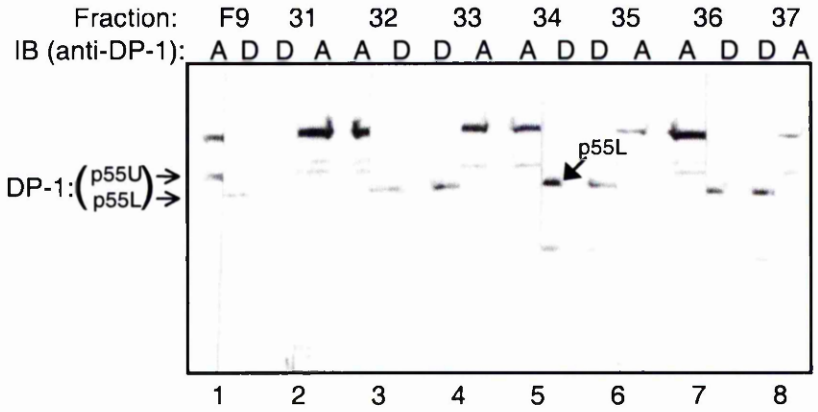
a


KCl (mM):
120
300
400
Fraction:
F9 5 6 7 8 32 33 34 35 36 37 38
Complexed E2F
Free E2F
1 2 3 4 5 6 7 8 9 10 11 12

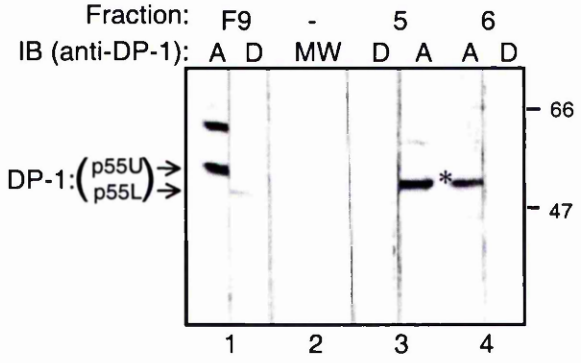
b


Fraction:
F9 5 6 7
F9 32 33 34 35 36 37 38
E2F-5M
E2F-5L
E2F-5
1 2 3 4 5 6 7 8 9 10 11 12

c


Fraction: F9 31 32 33 34 35 36 37
IB (anti-DP-1): A D D A A D D A A D D A A D D A
DP-1: p55U p55L
p55L
1 2 3 4 5 6 7 8

d


Fraction: F9 - 5 6
IB (anti-DP-1): A D MW D A A D
DP-1: p55U p55L
*
66
47
1 2 3 4

'flow-through' from the column contained only p55U (Fig 3.3d, lanes 3 and 4). Thus two immunochemically distinct forms of DP-1 were separated in the 'peak' and 'flow-through' fractions. These results are in agreement with a previous study that showed an immunochemically distinct form of DP-1, namely p55U, could exist in a non-DNA binding state (Sørensen *et al.,* 1996). In conclusion, heparin-Sepharose chromatography had fractionated two forms of DP-1 with distinct electrophoretic mobilities, into a DNA-binding an non DNA-binding fraction. By contrast, E2F-5L and E2F-5M were present in both of these fractions.

A more direct way of determining the ability of different forms of E2F-5 to interact with the E2F site is to apply cell extracts to an affinity matrix containing the E2F DNA-binding site. The DNA binding site was taken from the adenovirus E2a promoter, identical to the sequence used in the previous gel retardation experiments. E2F-5 from F9EC cell extracts bound to the wild-type E2F binding site but not to a mutant site (Fig 3.4a, compare lanes 1 and 5). Co-incubation of the wild-type site affinity matrix with a 100-fold excess of free wild-type DNA binding site disrupted this interaction, indicating the binding was specific for the E2F site (Fig 3.4a, compare lanes 1 and 3). All forms of E2F-5 in the F9EC extract bound to the E2F site (Fig 3.4a, lane1), whereas there was no detectable binding to the mutant E2F site (Fig 3.4a, lane 5). In Rat1 extracts considerably more E2F-5 was detected bound to the wild-type site than was bound to the mutant site (Fig 3.4b, compare lanes 1 and 3). Moreover both E2F-5M and E2F-5U bound to the E2F site. When the mutant E2F-site affinity matrix was co-incubated with Rat1 extract the majority of E2F-5 remained in the non-binding fraction (Fig 3.4b, lane 4). In conclusion all three forms of E2F-5 are capable of binding to DNA and there appeared to be no detectable difference in their affinity for the E2F site.

Figure 3.4

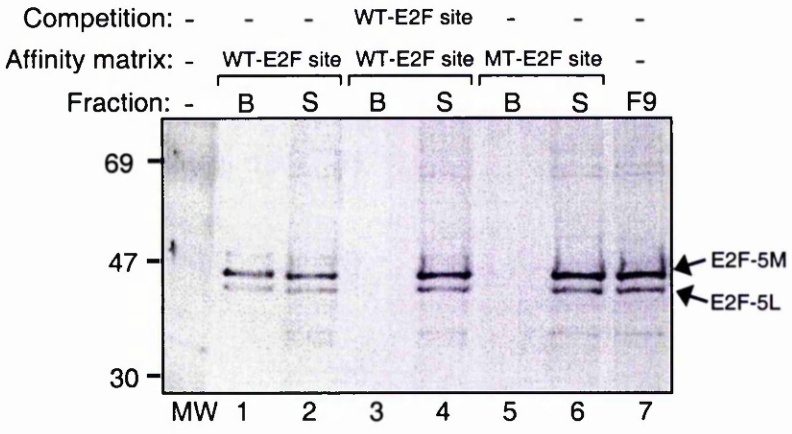
E2F-5L, E2F-5M and E2F-5U can all interact with the consensus E2F binding site from the adenovirus E2A promoter.

Cell extracts were incubated with an affinity matrix consisting of the E2F site from the adenovirus E2A promoter or a mutant E2F site (see Materials and Methods). Lanes labelled with 'B' indicate fractions of extract that remained bound to the matrix after three washes, whilst 'S' (Supernatant) indicates the fraction of extract that did not bind to the affinity matrix.

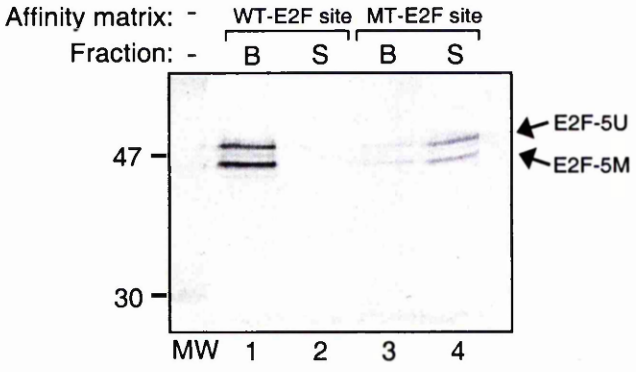
(a) F9EC extract (400μg) was incubated with the wild-type E2F site affinity matrix (lanes 1-4) or mutant E2F-site affinity matrix (lane 5 and 6). 100-fold excess of 'free' wild-type E2F site was added as a competitor to the wild-type E2F site matrix in lane 3 and 4. Thereafter, the bound proteins were released and immunoblotted with anti-E2F-5, alongside 20% of the unbound proteins remaining in the supernatant. Track 7 shows the reactivity of 10% of the amount of crude F9EC extract used in the affinity matrix incubations.

(b) Rat1 extract (400μg) was incubated with either the wild-type E2F site affinity matrix or mutant E2F-site affinity matrix, as indicated. Thereafter, the bound proteins were released and immunoblotted with anti-E2F-5 (lane 1 and 3), alongside 25% of the unbound proteins remaining in the supernatant (lane 2 and 4).

a


Competition: - - - WT-E2F site - - -
Affinity matrix: - WT-E2F site WT-E2F site MT-E2F site -
Fraction: - B S B S B S F9
69
47
30
E2F-5M
E2F-5L
MW 1 2 3 4 5 6 7

b


Affinity matrix: - WT-E2F site MT-E2F site
Fraction: - B S B S
47
30
E2F-5U
E2F-5M
MW 1 2 3 4

3.5 E2F-5 associates with DP-1 and p107 *in vivo*

Previously gel retardation analysis demonstrated that E2F-5 exists as a heterodimer with DP-1 on the consensus E2F binding site (see Fig 3.1). However gel retardation cannot distinguish whether a particular form of E2F-5, either U, M or L, preferentially binds to DP-1. Therefore co-immunoprecipitation experiments were performed to investigate the ability of these different forms of E2F-5 to associate with DP-1. Extracts from F9EC cells were subjected to immunoprecipitation with a monoclonal anti-DP1 directed against a peptide in the C-terminus of DP-1 (see Materials and Methods) in the presence of either a homologous or control peptide. Subsequent immunoblotting with anti-E2F-5 revealed that two forms of endogenous E2F-5 were detected in F9EC extract and both of these bound specifically to DP-1 (Fig 3.5a, compare lane 2 and 3). Similarly extracts from COS7 cells, transfected with either E2F-5 alone or E2F-5 and DP-1 together, were subjected to immunoprecipitation with the anti-DP1 monoclonal antibody. Three forms of exogenous E2F-5 were detected in DP-1 immunoprecipitates from cells co-transfected with E2F-5 and DP-1, whilst E2F-5 was absent in DP-1 immunoprecipitates from cells transfected with DP-1 alone (Fig 3.5b, compare lane 1 and 2). These results indicate that all three forms of E2F-5 (L, M and U) are capable of interacting with DP-1 *in vivo*.

One way of gaining further insight into processes that regulate E2F-5 is to identify proteins that interact with E2F-5 *in vivo*. It has been reported that E2F-5 preferentially binds to p130, which is most abundant in quiescent cells (Hijmans *et al.*, 1994; Cobrinik *et al.*, 1993). However, in asynchronous cultures of F9EC cells and many other cells during S phase the major pocket protein complexing with E2F is p107 (Lees *et al.*, 1992; Shirodkar *et al.*, 1992). Several studies have shown that these

Figure 3.5

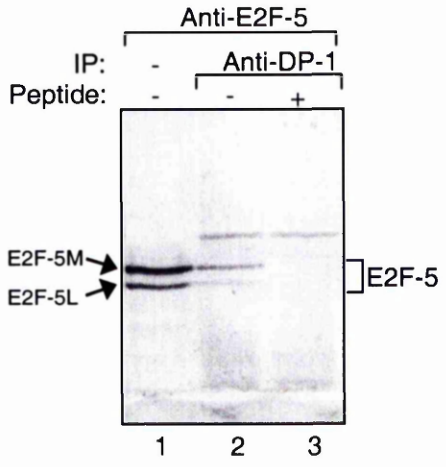
E2F-5 associates with DP-1 and p107 *in vivo*.

(a) Extract prepared from F9EC cells (400μg) was subjected to immunoprecipitation (IP) with an anti-DP-1 monoclonal antibody (see Materials and Methods), in either the presence (lane 3) or absence (lane 2) of the homologous peptide from which the antibody is directed. The immunoprecipitates were resolved by SDS-PAGE and then immunoblotted with anti-E2F-5. The reactivity of 40μg of F9EC extract with anti-E2F-5 is shown in lane 1.

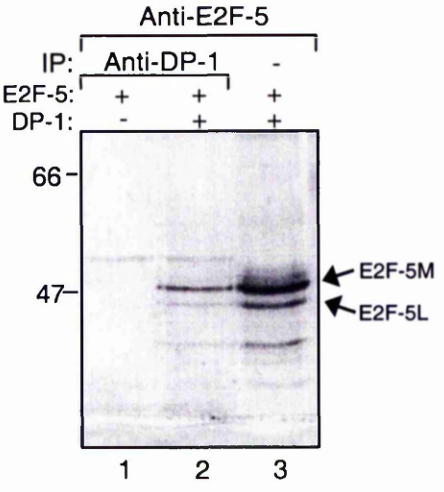
(b) Expression vectors for E2F-5 (5μg) and DP-1 (5μg) were introduced into a 10cm plate of COS7 cells as indicated and extracts were prepared and subjected to immunoprecipitation (IP) with an anti-DP-1 monoclonal antibody (see Materials and Methods). The immunoprecipitates were resolved by SDS-PAGE and then immunoblotted with anti-E2F-5, alongside 10% of the quantity of crude extract used in the immunoprecipitation (lane 3).

(c) RAGI extract (1mg) was immunoprecipitated with an anti-p107 monoclonal antibody (SD9, Santa Cruz) or a control antibody (IG4), as indicated. The immunoprecipitates were resolved by SDS-PAGE and then immunoblotted with anti-E2F-5. The reactivity of 100μg of RAGI extract with anti-E2F-5 is shown in lane 3.

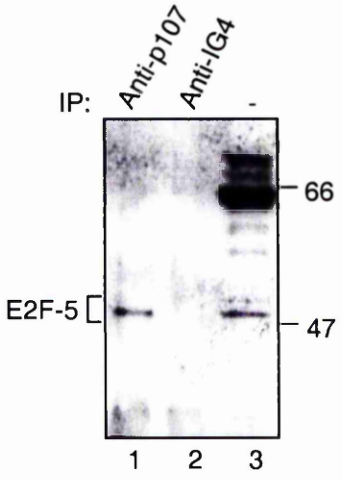
a


Anti-E2F-5
IP:
-
Anti-DP-1
Peptide:
-
-
+
E2F-5M
E2F-5L
E2F-5
1
2
3

b


Anti-E2F-5
IP:
Anti-DP-1
-
E2F-5:
+
+
+
DP-1:
-
+
+
66
47
E2F-5M
E2F-5L
1
2
3

c


IP:
Anti-p107
Anti-IG4
-
66
E2F-5
47
1
2
3

complexes specifically contain E2F-4 (Shirodkar *et al.*, 1992; Moberg *et al.*, 1996). To investigate whether E2F-5 and p107 were also associated *in vivo* an immunoprecipitation assay was employed. E2F-5 was found associated with p107 in immunoprecipitates from RAGI cell extract using an anti-p107 monoclonal antibody (SD9), but not when an irrelevant antibody (IG4) was used (Fig 3.5b). Therefore E2F-5 interacts with p107 in this cell line.

3.6 E2F-5 co-operates with DP-1 and DP-3 in transcriptional activation

The E2F family of proteins is known to activate transcription synergistically with DP proteins through the formation of heterodimers (Bandara *et al.*, 1993). The ability of E2F-5 to activate transcription in association with a DP partner was studied by transient transfection using a luciferase reporter gene driven by the murine *cyclin E* promoter, a well-studied E2F regulated promoter (Botz *et al.*, 1996). Neither DP-1 nor DP-3δ significantly activated the *cyclin E* promoter when expressed without E2F (Fig 3.6a). Moreover a titration of the E2F-5 expression vector resulted in at best 2-fold activation of the *cyclin E* promoter. A synergistic effect in *trans*activation was seen when the levels of E2F-5 were titrated together with a constant amount of DP-1 or DP-3δ (Fig 3.6a). DP-3δ was more effective than DP-1 in cooperative activation of *cyclin E* promoter.

E2F-1 and DP-1 cooperate in transcriptional activation of the *DHFR* promoter (Lee *et al.*, 1998). However E2F-5 did not cooperate with DP-1 in transcriptional activation of the *DHFR* promoter (Fig 3.6b). Therefore the response of promoters to overexpression of different heterodimers may be variable. Based on the results shown

Figure 3.6

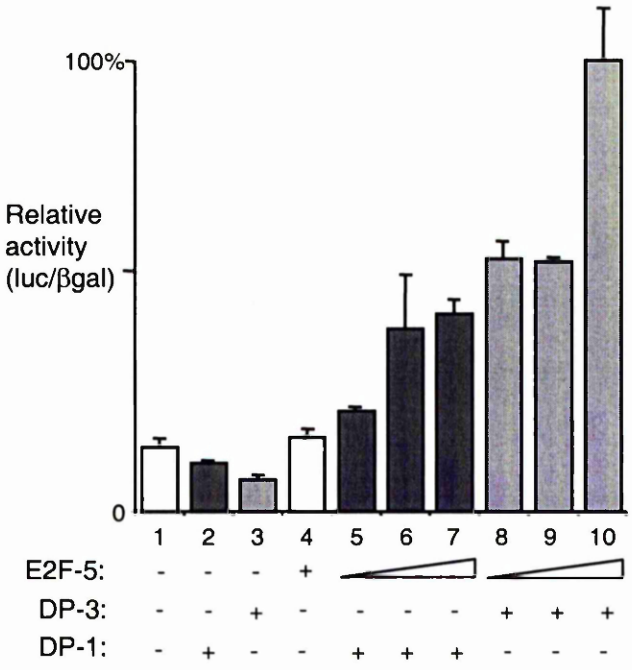
Synergistic activation of the *cyclin E* promoter by E2F-5 and either DP-1 or DP-3δ.

(a) U2OS cells were transfected with the cyclin E-luciferase reporter (1μg), together with expression vectors for HA-E2F-5 (0.5, 0.75, 1.0μg), DP-1 (0.75μg) or DP-3δ (0.75μg) as indicated. The '+' in lane 4 indicates that 1.0μg of HA-E2F-5 was transfected.

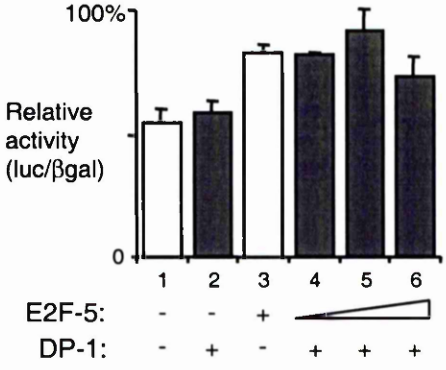
(b) U2OS cells were transfected with the DHFR-luciferase reporter (1μg), together with expression vectors for HA-E2F-5 (0.5, 0.75, 1.0μg) and DP-1 (0.75μg) as indicated. The '+' in lane 3 indicates that 1.0μg of HA-E2F-5 was transfected.

In both (a) and (b) pCMV-βGal (1μg) was transfected throughout as an internal control. Values shown are the means of two separate readings and represent the ratio of luciferase to β-galactosidase activity.

a


100%
Relative activity (luc/βgal)
0
1 2 3 4 5 6 7 8 9 10
E2F-5: - - - + + + + + + +
DP-3: - - + - - - - + + +
DP-1: - + - - + + + - - -

b


100%
Relative activity (luc/βgal)
0
1 2 3 4 5 6
E2F-5: - - + + + +
DP-1: - + - + + +

earlier, the murine *cyclin E* promoter was chosen as a model system to study E2F-5 *trans*activation in subsequent experiments.

3.7 Regulation of E2F-5 during the cell cycle

Cell cycle control of E2F-1 occurs at the transcriptional level, E2F-1 mRNA levels peaking at the G1-S transition at the time when E2F activity is at its highest (Kaelin *et al.*, 1992; Slansky *et al.*, 1993; Johnson *et al.*, 1994). However the mRNA which encodes either E2F-4 or E2F-5 is relatively constant throughout the cell cycle and both genes are expressed in quiescent cells (Beijersbergen *et al.*, 1994; Ginsberg *et al.*, 1994; Sardet *et al.*, 1995). Post-translational mechanisms of control, such as regulation of nuclear localisation, may provide one way of regulating these E2F proteins.

To determine whether E2F-5 was subject to cell cycle regulation an analysis of the E2F-5 protein during the Rat1 cell cycle was performed. Rat1 cells are particularly amenable to cell cycle analysis, being easily arrested in G0/G1 by serum deprivation. Rat1 cells were arrested by serum starvation for 70 hours and stimulated to enter the cell cycle by the addition of 20% serum. At the indicated time intervals, flow cytometry was performed to monitor the proportion of cells in different phases of the cell cycle (Fig 3.7a). For example, in serum starved cultures 67% of the cells were in G1, 13% were in S, and 20% were in G2/M (Fig 3.7a, panel ii). In western blot analysis of extracts from the arrested cells E2F-5 was resolved as a single polypeptide species (form M). At 16 hours post serum stimulation, coincident with cells entering S phase, a slower migrating polypeptide (form U) was detected (Fig 3.7a, panel v; Fig 7.3b). The

Figure 3.7

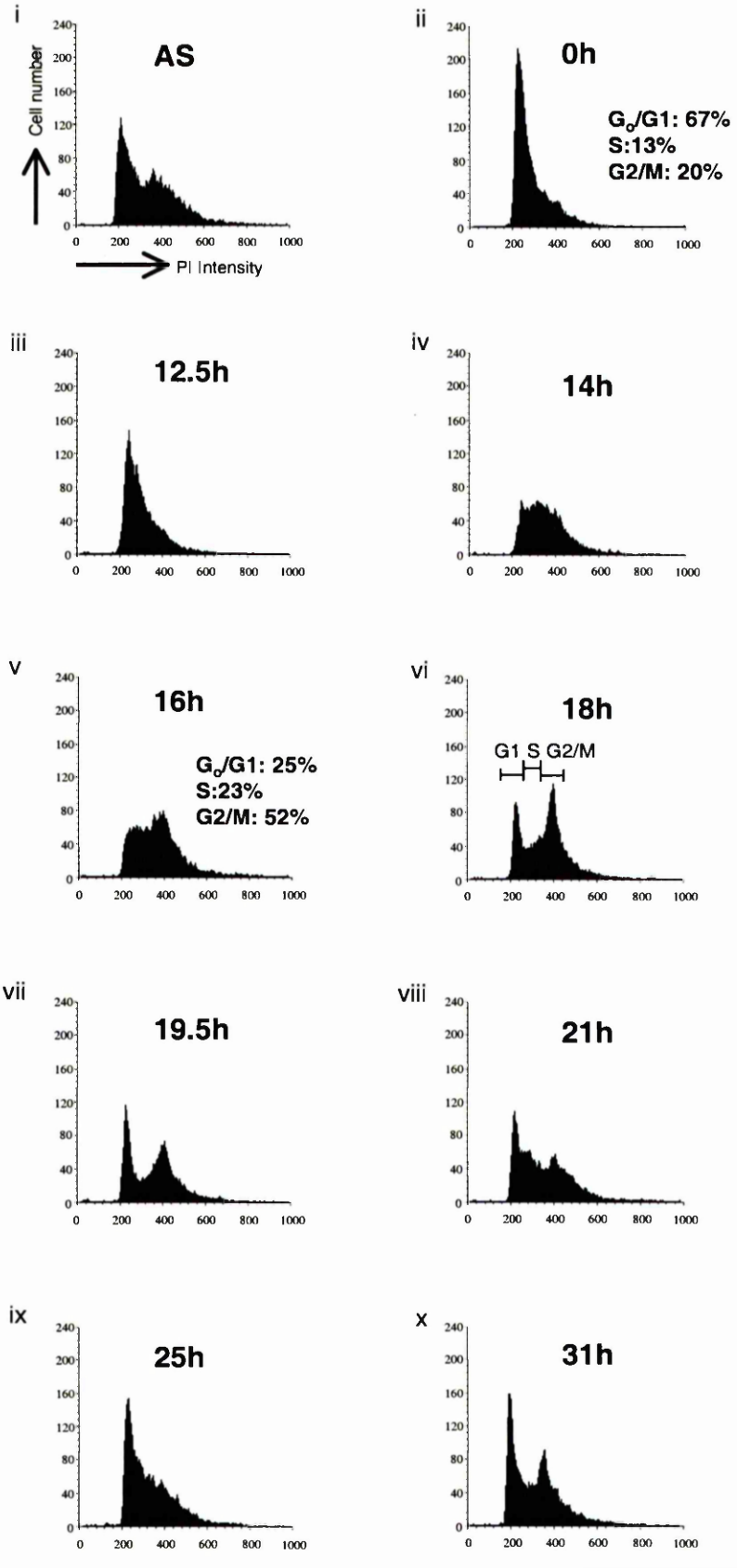
E2F-5U is a cell cycle regulated form of E2F-5.

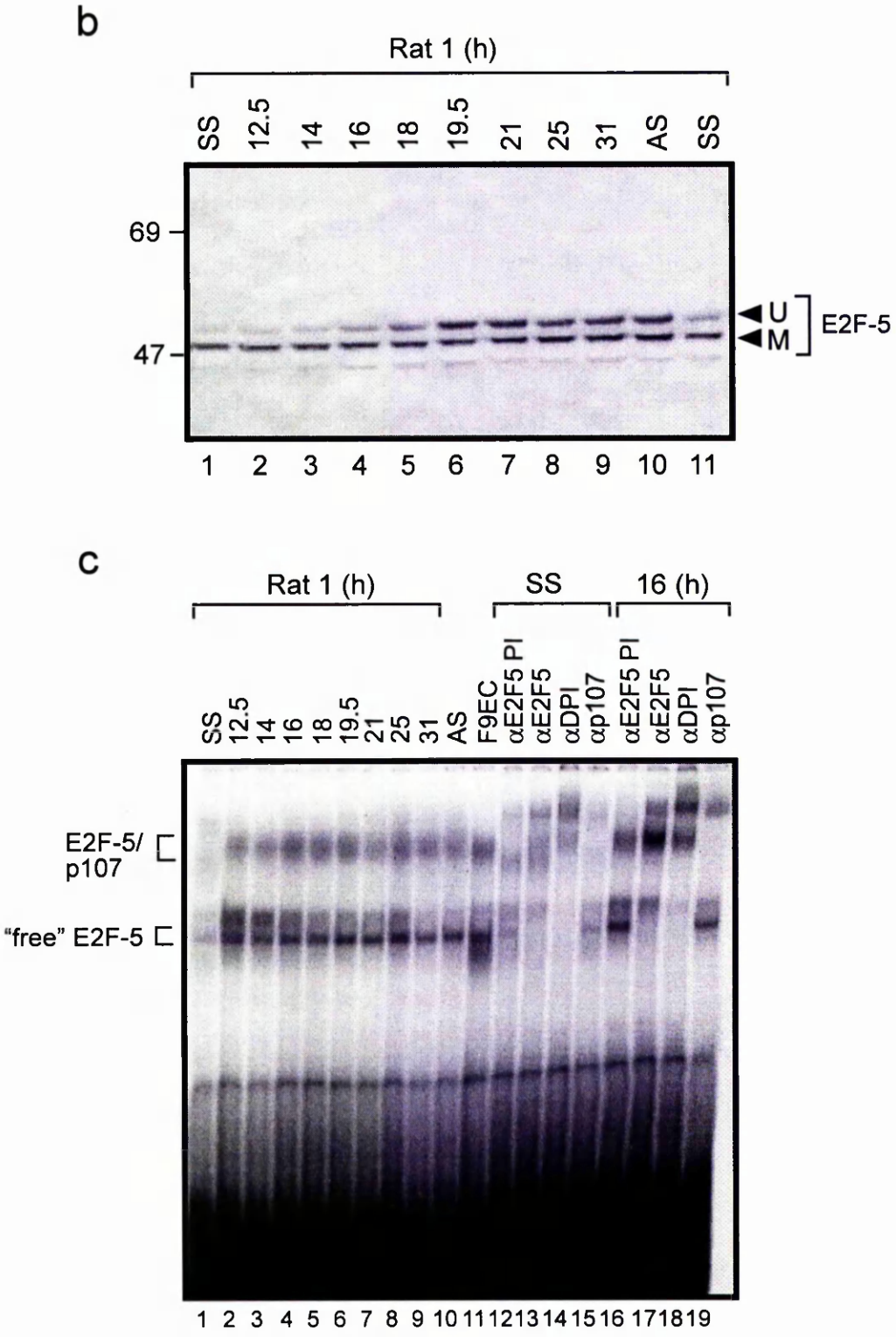
(a) Cell cycle distribution of Rat1 cells after serum starvation (panel ii), and at the indicated time points (in hours) after addition of serum (panels iii to x). Panel i shows the cell cycle distribution of the asynchronous population. The histograms show the cell number along the y-axis and the DNA content (as measured by PI intensity) along the x-axis. The number of cells in the different cell cycle phases in the serum starved population was G1: 67%, S: 13%, G2: 20% (panel ii) and at 16 hours post serum stimulation was G1: 25%, S: 23%, G2: 52% (panel v). The markers used for the estimation of G1:S:G2/M are shown in panel vi.

(b) Extracts from Rat1 cells treated identically to those in (a) were prepared; AS indicates asynchronous cultures, SS indicates the serum starved cultures. The time in hours after serum addition is indicated at the top of each lane. 50μg of extract from each cell cycle stage were immunoblotted using an anti-E2F-5 specific antiserum. The presence of the E2F-5M and E2F-5U are indicated.

(c) Gel retardation analysis of E2F complexes in Rat1 extracts at the indicated time-points on a consensus E2F site taken from the adenovirus E2A promoter. Extracts from serum starved cells and cells 16h post serum stimulation were supershifted with the indicated antisera, and an F9 embryonal carcinoma (EC) cell extract is shown for comparison (lane 11); PI indicates the rabbit pre-immune serum. The predominant species of 'free' E2F is an E2F-5/DP-1 heterodimer during S phase and at least some of the E2F/p107 complex contains E2F-5.

a


i
AS
Cell number
PI Intensity
ii
0h
G0/G1: 67%
S:13%
G2/M: 20%
iii
12.5h
iv
14h
v
16h
G0/G1: 25%
S:23%
G2/M: 52%
vi
18h
G1 S G2/M
vii
19.5h
viii
21h
ix
25h
x
31h


b
Rat 1 (h)
SS
12.5
14
16
18
19.5
21
25
31
AS
SS
69
47
U
M
E2F-5
1 2 3 4 5 6 7 8 9 10 11
c
Rat 1 (h)
SS
16 (h)
SS
12.5
14
16
18
19.5
21
25
31
AS
F9EC
αE2F5 PI
αE2F5
αDPI
αp107
αE2F5 PI
αE2F5
αDPI
αp107
E2F-5/
p107
"free" E2F-5
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

previous phosphatase experiment suggested that E2F-5U is a hyper-phosphorylated E2F-5 (see Fig 3.2c). Therefore the timing of the appearance of E2F-5U during cell cycle progression suggests it is the result of phosphorylation by a kinase whose activity is cell cycle regulated.

To assess the significance of the observed regulation of E2F-5 an investigation of the DNA binding properties of E2F during the Rat1 cell cycle was performed by gel retardation analysis. Rat1 cell extracts contain a number of discrete DNA binding complexes, which migrated in equivalent positions to those previously characterised in F9EC extract (Fig 3.7c, lanes 10 and 11). Relevant antisera revealed the presence of a p107 containing complex and 'most' of the free E2F consisted of an E2F-5/DP-1 heterodimer (Fig 3.7c, lanes 16-19). When the synchronised extracts were assessed for their ability to bind to the E2F site, there was an overall increase in DNA binding activity as cells entered the cell cycle. This was most striking at 12 hours post serum stimulation when an increase in 'free' E2F and the p107 complex were detected (Fig 3.7c, compare lane 1 and 2). At 14 to 16 hours after serum addition some of the 'free' E2F started to decline. This 'free' E2F DNA binding activity was not supershifted by the anti-E2F-5 antibody so it is likely to consist of another E2F family member (Fig 3.7c, compare lane 16 and 17). In contrast there was no change in the level of E2F-5/DP-1 heterodimer as cells progressed through the cell cycle (Fig 3.7c, compare lanes 2 to 10). Therefore the regulation of E2F-5U did not correlate with any changes in the level of E2F-5 DNA binding activity, suggesting that the presence of E2F-5U may reflect another aspect of E2F-5 activity.

3.8 Conclusion

Data presented in this chapter demonstrated that E2F-5 is an abundant component of E2F DNA binding activity in a range of different cell types, such as fibroblasts and cancer cell lines. Thus it may make a significant contribution to the control of the G1 to S phase transition in these cells. At the protein level E2F-5 was detected as a heterogeneous set of polypeptides that likely result from differential phosphorylation of E2F-5. These were designated E2F-5U, E2F-5M and E2F-5L, in order of increasing mobility on SDS-PAGE. Interestingly one of these polypeptides, namely E2F-5U, was absent in Rat1 cells arrested in G0/G1, but appeared as the cells entered the cell cycle. This observation opens up the possibility that E2F-5 may be phosphorylated in a cell cycle dependent manner. The appearance of E2F-5U during the Rat1 cell cycle did not correlate with any change in the level of E2F-5 DNA-binding activity. Furthermore in DNA affinity purification of Rat1 cell extract both E2F-5U and E2F-5M bound to DNA with equal efficiency. Therefore the regulation of E2F-5 which results in the appearance of E2F-5U during the Rat1 cell cycle may be controlling some other aspect of E2F activity.

Chapter 4

Analysis of E2F-5 phosphorylation by site-directed mutagenesis

4.1 Introduction

The activity of E2F is regulated through the phosphorylation of pocket proteins. Direct phosphorylation of E2F also occurs, but the functional significance of this is not fully understood. The best studied mechanism of E2F phosphorylation is the phosphorylation of E2F-1 and DP-1 by cyclinA-CDK2 kinase. CyclinA-CDK2 binds to a domain in the N-terminal region of E2F-1, which is conserved in E2F-2 and E2F-3 but not in E2F-4 and E2F-5. The cyclinA-CDK2 binding domain is required for *in vitro* phosphorylation of E2F-1 by cyclinA-CDK2 (Krek *et al.,* 1994). CyclinA-CDK2 can phosphorylate DP-1 (Krek *et al.*, 1995) and this phosphorylation has been correlated with down-regulation of E2F DNA binding (Krek *et al.,* 1994), thereby reducing the activity of the E2F heterodimer as cells progress through S phase.

Phosphorylation sites have been identified in E2F-1 by site-directed mutagenesis (Fagan *et al.*, 1994, Peeper *et al.*, 1995). Phosphorylation of serine 332 and serine 337 in the C-terminal of E2F-1 prevented E2F-1 from binding pRb (Fagan *et al.*, 1994). In contrast, phosphorylation of E2F-1 on serine 375 greatly enhanced its affinity for pRb *in vitro* (Peeper *et al.*, 1994). However the *in vivo* significance of phosphorylation at these sites has yet to be determined.

It has also been suggested that E2F-4 and E2F-5 are phosphoproteins (Beijersbergen *et al.,* 1994, Hijmans *et al.*, 1995). The objectives of this chapter were to define phosphorylation sites in E2F-5 and investigate their role in the regulation of E2F-5 activity.

4.2 Site directed mutagenesis of E2F-5

Data presented in the previous chapter suggested that E2F-5 was phosphorylated in cells in a cell cycle-dependent manner, and therefore may be a target for cyclin-dependent kinases (CDKs). To investigate whether E2F-5 is regulated by phosphorylation a mutational analysis of E2F-5 was performed at potential CDK phosphorylation sites. The consensus amino acid sequence for phosphorylation by CDKs is S/T-P-X-K/R. There are several CDK consensus phosphorylation sites in E2F-5. One site, positioned at threonine 251, fits the consensus for CDK phosphorylation perfectly. In addition there are several threonines and serines preceding proline residues that are potential targets for phosphorylation by either CDKs or other proline directed kinases, for example MAP kinases.

In the previous chapter *in vitro* translation of a C-terminal fragment of E2F-5 generated several polypeptides, which were suggested to result from differential phosphorylation (see Fig 3.2d). In agreement with this idea, the majority of putative CDK phosphorylation sites are in the C-terminus of E2F-5. A sequence and structure-based prediction of phosphorylation sites has been made using an artificial neural network method (Blom *et al.*, 1999; Net Phos@cbs.dtu.dk). The polypeptide sequence of the protein of interest is input and the prediction results are output as a score between 0 and 1. True phosphorylation sites should obtain a score close to 1, whereas non-phosphorylation sites should obtain a score close to 0. In E2F-5, serine 307 is one of the highest scoring residues at 0.995 and threonine 251 scored at 0.220. Therefore, two individual point mutations were made in the C-terminal region of E2F-5 at threonine 251 and serine 307 (Fig 4.1a). The mutants were designated T251A and S307A, to indicate that the selected residues were substituted with alanine (single

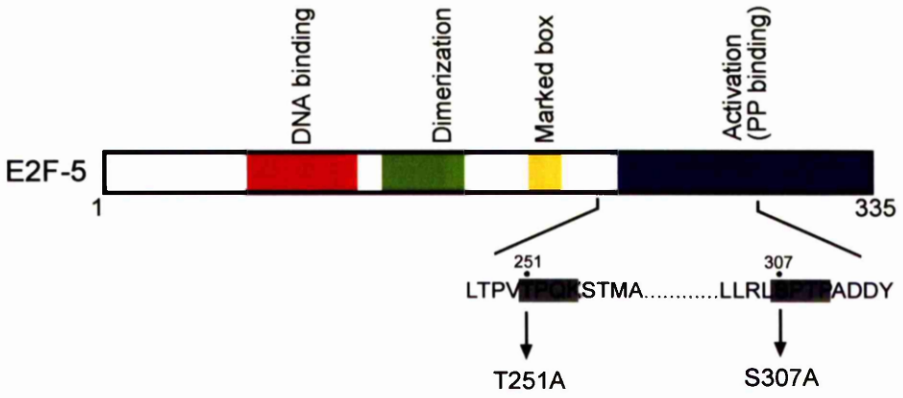
Figure 4.1

Mutant derivatives of E2F-5.

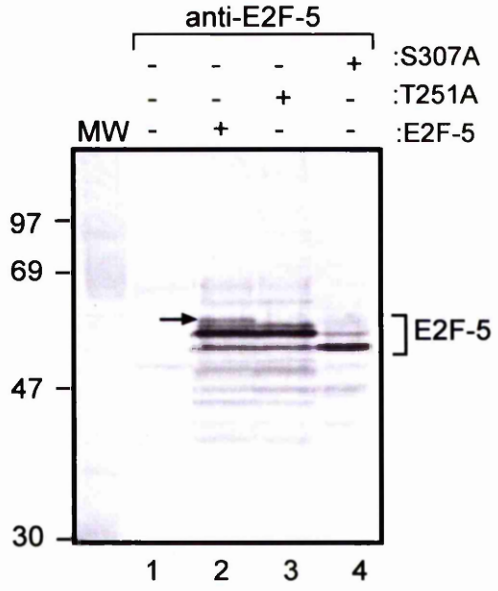
(a) Diagram of E2F-5 indicating the domains conserved with other family members and the threonine residue (T251) and serine residue (S307) which were mutated to alanine (A), the mutant derivatives being referred to as T251A and S307A respectively. The sequences around T251 and S307 are shown, and PP indicates the pocket protein-binding domain.

(b) COS7 cells were co-transfected with expression vectors for DP1 (1μg) and either HA-tagged E2F-5, HA-tagged T251A or HA-tagged S307A (1μg) as indicated. Extracts were prepared from transfected cells and subjected to immunoblotting using an anti-HA monoclonal antibody. The arrow indicates an E2F-5 polypeptide species which is absent from T251A expressing cells.

a


DNA binding
Dimerization
Marked box
Activation (PP binding)
E2F-5
1
335
251
307
LTPV
STMA.....LLRL
ADDY
T251A
S307A

b


anti-E2F-5
- - - + :S307A
- - + - :T251A
MW - + - - :E2F-5
97
69
47
30
E2F-5
1 2 3 4

amino acid code). Alanine substitution results in the replacement of the OH group in serine/threonine with a CH_3 side chain, thereby preventing phosphorylation. Furthermore being a small, uncharged residue it is less likely than other amino acids to cause conformational changes to a protein. The serine at 307 is conserved in E2F-1, E2F-2, E2F-4 and E2F-5, whereas threonine 251 is unique to E2F-5.

Synthetic peptides have been used as substrates to show specificity between CDK sites preferentially phosphorylated by cyclin D1-CDK4 and cyclin A/E-CDK2 (Kitagawa *et al.*, 1996). The authors showed that S/T-P-X-K/R, such as the sequence mutated at T251, is preferentially phosphorylated by cyclinA/E-CDK2. The sequence surrounding serine 307 (306-LSPTP-310) more closely resembles the site of phosphorylation of other proline directed kinases, such as MAP kinases or cyclin D1-CDK4, rather than a cyclin A/E-CDK2 site.

4.3 Analysis of mutants derivatives of E2F-5

When wild-type E2F-5 is over-expressed in COS-7 cells, four E2F-5 polypeptides were detected by western analysis (Fig 4.1a, lane 2). Three of these polypeptides were equivalent in mobility to E2F-5U, E2F-5M and E2F-5L, defined in the previous chapter (see Fig 3.2a), and the fourth form was resolved between E2F-5M and E2F-5U. In cells transfected with the T251A mutant derivative, the slowest mobility E2F-5 polypeptide, E2F-5U, was absent implying that phosphorylation on threonine 251 was unique to this form of E2F-5 (Figure 4.1b, compare lanes 2 and 3). In cells transfected with S307A only two E2F-5 polypeptides were detected in western blot analysis (Figure 4.1b). One of these polypeptides, E2F-5L, was more abundant than it was in cells transfected with wild-type E2F-5 (Figure 4.1b, compare lanes 2 and

4). However, in cells transfected with S307A mutant derivative, E2F-5U was undetectable and the level of E2F-5M was significantly lower than that detected in cells transfected with wild-type E2F-5 (Figure 4.1b, compare lanes 2 and 4). There are two possible explanations for this pattern. Firstly, E2F-5M may consist of E2F-5 polypeptides that are phosphorylated at serine 307. Secondly, phosphorylation of serine 307 in E2F-5 may be required prior to phosphorylation of E2F-5 at other sites, which results in the appearance of the heterogenous set of wild-type E2F-5 polypeptides.

4.4 The DNA binding activity of S307A or T251A is not compromised

To determine the functional consequences of E2F-5 phosphorylation at serine 307 or threonine 251 the mutants derivatives were assessed for their ability to bind to DNA in association with a DP partner. U20S cells were transfected with E2F-5, T251A or S307A, alone and in combination with DP-1. Gel retardation analysis with an E2F site probe (see Section 3.1) revealed that extracts from cells transfected with DP-1, E2F-5, T251A or S307A had a similar level of DNA binding activity to extracts from untransfected cells (Fig 4.2b). However when E2F-5 or either of the mutants were co-transfected with DP-1 a distinct complex of E2F-5/DP-1 heterodimer was detected, that was of a similar intensity for wild-type E2F-5 and both of the mutants (Fig 4.2b, lanes 6-8). Western blotting of the same extracts showed that the level of E2F-5 was equal and thus directly proportional to the DNA binding activity (Fig 4.2b). Figure 4.2 is a representative example of three experiments. The results therefore suggest that phosphorylation of threonine 251 and serine 307 is unlikely to influence the ability of E2F-5 to heterodimerise with DP-1 and bind DNA.

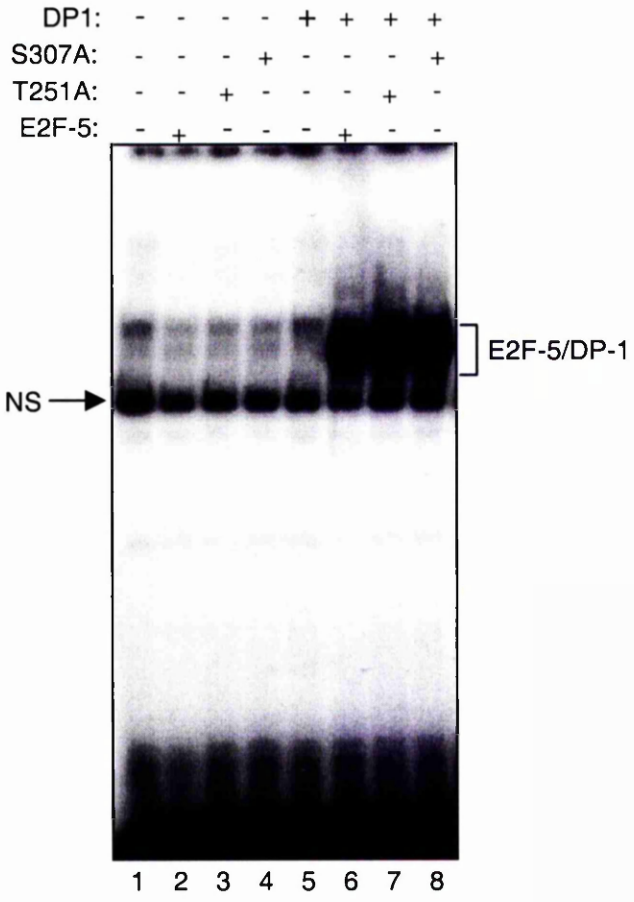
Figure 4.2

The DNA binding activity of T251A and S307A is not compromised.

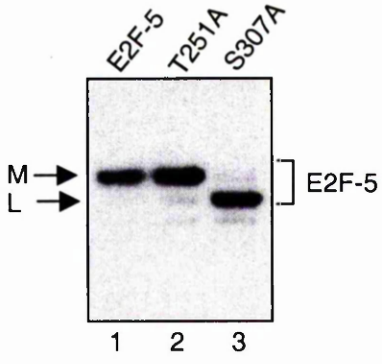
(a) Gel retardation analysis of extracts from U2OS cells transfected with expression vectors for HA-tagged E2F-5, HA-tagged T251A or HA-tagged S307A (4μg), DP-1 (4μg), as indicated. The position of the E2F-5/DP-1 heterodimer and a non-specific (NS) band are indicated.

(b) Immunoblot using the HA monoclonal antibody of extracts from lanes 6,7,8 in (a), containing HA-tagged E2F-5, HA-tagged T251A or HA-tagged S307A, together with DP-1.

a


DP1: - - - - + + + +
S307A: - - - + - - - +
T251A: - - + - - - + -
E2F-5: - + - - - + - -
E2F-5/DP-1
NS
1 2 3 4 5 6 7 8

b


E2F-5
T251A
S307A
M
L
E2F-5
1 2 3

It is noteworthy that exogenous E2F-5 was resolved as a single polypeptide in a western blot of the U2OS extracts used in the above gel retardation analysis (Fig 4.2b). This is clearly different from the cluster of E2F-5 polypeptides detected in COS7 cells (Fig 4.1b). In other U2OS transfections exogenous E2F-5 was resolved as multiple polypeptides and therefore the expression of individual E2F-5 polypeptides may vary with transfection efficiency. In conclusion wild-type E2F5, T251A and S307A do not differ in their DNA binding activity when over-expressed with DP-1 in U2OS cells. This is consistent with the data in chapter 3, which showed that both E2F5L and E2F-5M bound to DNA (see Fig 3.4a).

4.5 T251A and S307A retain the ability to bind to pocket proteins

In order to test whether phosphorylation of threonine 251 or serine 307 in E2F-5 influenced binding to pocket proteins, expression vectors for E2F-5 and the mutant derivatives were transfected into U2OS cells together with DP-1 and either p130 or p107. Gel retardation analysis of extracts containing exogenous wild-type E2F-5, DP-1 and p130 resolved a distinct p130 complex (Fig 4.3a, lane 7). This complex was dependent on the transfection of all three plasmids as it was not detected in control extracts containing the individual plasmids were transfected alone (Fig 4.3a, lanes 2,3 and 6). An identical complex was detected when extracts containing T251A or S307A, DP-1 and p130 were subject to gel retardation analysis. In addition to the p130 complex some of the E2F-5 in these extracts resolved as a 'free' E2F-5/DP-1 heterodimer, suggesting the level of p130 was limiting. Furthermore the ratio of 'free' E2F-5/DP-1 to complexed E2F-5/DP-1/p130 was identical in all three transfections, suggesting the mutant derivatives did not differ from E2F-5 in their affinity for p130. Western blotting

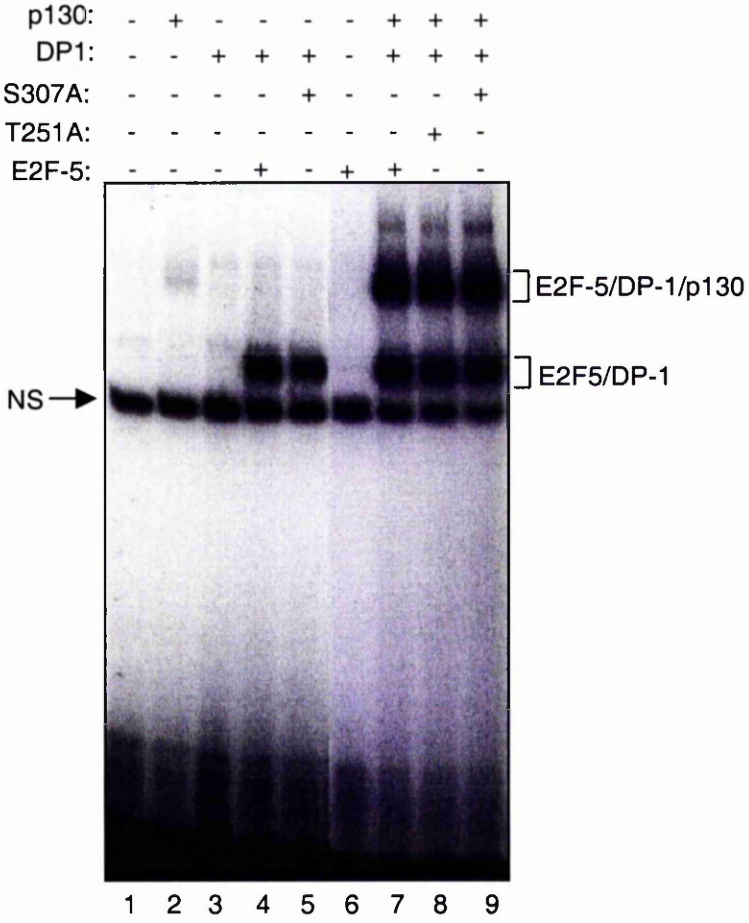
Figure 4.3

T251A and S307A retain the ability to bind to p130.

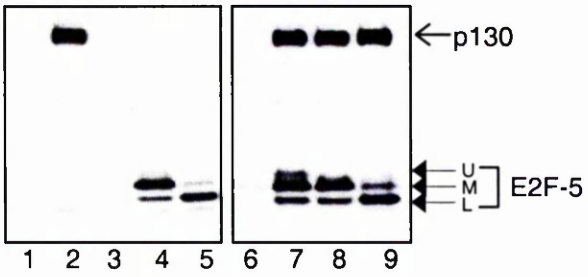
(a) Gel retardation analysis of extracts from U2OS cells transfected with expression vectors for HA-tagged E2F-5, HA-tagged T251A or HA-tagged S307A (4μg), DP-1 (4μg) and HA-tagged p130 (4μg), as indicated. The position of the 'free' E2F-5/DP-1 heterodimer and E2F-5/DP-1/p130 complex are indicated. NS is a non-specific band.

(b) Immunoblot using the HA monoclonal antibody of extracts (a). The lane numbers in (a) and (b) correspond to the same extract. The position of the HA-tagged p130 and HA-tagged E2F-5 and the mutant derivatives are indicated.

a


p130: - + - - - - + + +
DP1: - - + + + - + + +
S307A: - - - - + - - - +
T251A: - - - - - - - + -
E2F-5: - - - + - + + - -
E2F-5/DP-1/p130
E2F5/DP-1
NS
1 2 3 4 5 6 7 8 9

b


p130
U
M
L
E2F-5
1 2 3 4 5 6 7 8 9

confirmed that p130 expression was equal in each of these treatments (Fig 4.3b). An increase in E2F-5 protein occurred when E2F-5 or the mutant derivatives were co-expressed with DP-1 and p130 (Fig 4.3b, compare lanes 4-5 with lanes 7-9). This was not due to increased transfection efficiency, as the β-galactosidase activity in the extracts, resulting from the β-galactosidase plasmid transfected as an internal control, remained constant. A potential explanation for this observation is suggested by recent evidence that E2F-1 and E2F-4 are stabilised by binding to pocket proteins (Hateboer *et al.*, 1996; Hofmann *et al.*, 1996). An analogous situation may occur when E2F-5 and p130 interact.

Figure 4.3b also shows a clear difference in the polypeptide profile of the wild-type and mutant derivatives of E2F-5, with E2F-5U appearing only when wild-type E2F-5 but not when T251A is transfected (Fig 4.3b, compare lane 7 and 8). In addition the S307A mutant is seen to clearly consist of a combination of two polypeptides, E2F-5L being the most abundant and E2F-5M is also present at reduced levels (Fig 4.3b, lane 9). The presence of E2F-5M is possibly a result of phosphorylation of S307A on threonine 251 which remains intact in this protein, although there may be several other sites phosphorylated. Phosphopeptide mapping is required to confirm this.

The above gel retardation analysis was repeated using extracts transfected with p107 instead of p130 but a p107 complex was not detected (data not shown). This observation may reflect a reduced affinity of E2F-5 for p107 compared to p130, as E2F-5 has been shown to preferentially interact with p130 (Hijmans *et al.*, 1995). The interaction of p107 with each of the mutants is investigated further in section 4.6 and chapters 5 and 6.

4.6 Cellular Localisation of E2F-5

During these studies work was published to show that the majority of E2F-5 and DP-1 is cytoplasmic when over-expressed in U2OS cells, as both proteins lack an inherent nuclear localisation signal (NLS) (Allen *et al.*, 1997). Moreover p107 can supply a NLS to the E2F heterodimer, transporting it into the nucleus. It was possible therefore that the phosphorylation status of E2F-5 affected the ability of the E2F-5/DP-1 heterodimer to bind and co-localise in the nucleus with p107. To explore this idea the cellular localisation of the E2F-5 mutant derivatives and p107 was determined.

HA-tagged E2F-5, T251A, S307A and DP-1 were expressed in U2OS cells by transient transfection and their cellular distribution determined by indirect immunofluorescence. Two different secondary fluorescent-tagged antibodies were used, rhodamine-conjugated anti-mouse antibody (for HA or p107) or fluorescein-conjugated anti-rabbit antibody (for DP-1). U2OS cells were either immuno-stained for HAE2F-5 and DP-1 or p107 and DP-1. The majority of E2F-5 and the mutant derivatives were cytoplasmic when over-expressed with DP-1 (Fig 4.4a, panel i, iii, v). Similarly DP-1 was predominantly cytoplasmic in these cells (Fig 4.4a, panel ii, iv, vi). However, E2F-5 co-localised with DP-1 in the nucleus when they were expressed with p107, suggesting that the heterodimer binds to p107 and utilises the p107 NLS to enter the nucleus (Fig 4.4c, panel i and ii). Consistent with this, in all cells transfected with E2F-5, DP-1 and p107, a nuclear staining of p107 was observed, whereas DP-1 was only nuclear when expressed in the same cell as p107 (Fig 4.4b, compare i and ii).

The mutant derivatives of E2F-5 did not differ from E2F-5 in their sub-cellular localisation in either the absence (Fig 4.4a, iii to vi) or presence of co-transfected p107 (Fig 4.4c, iii to vi). Therefore, phosphorylation of E2F-5 at threonine 251 or serine 307

Figure 4.4

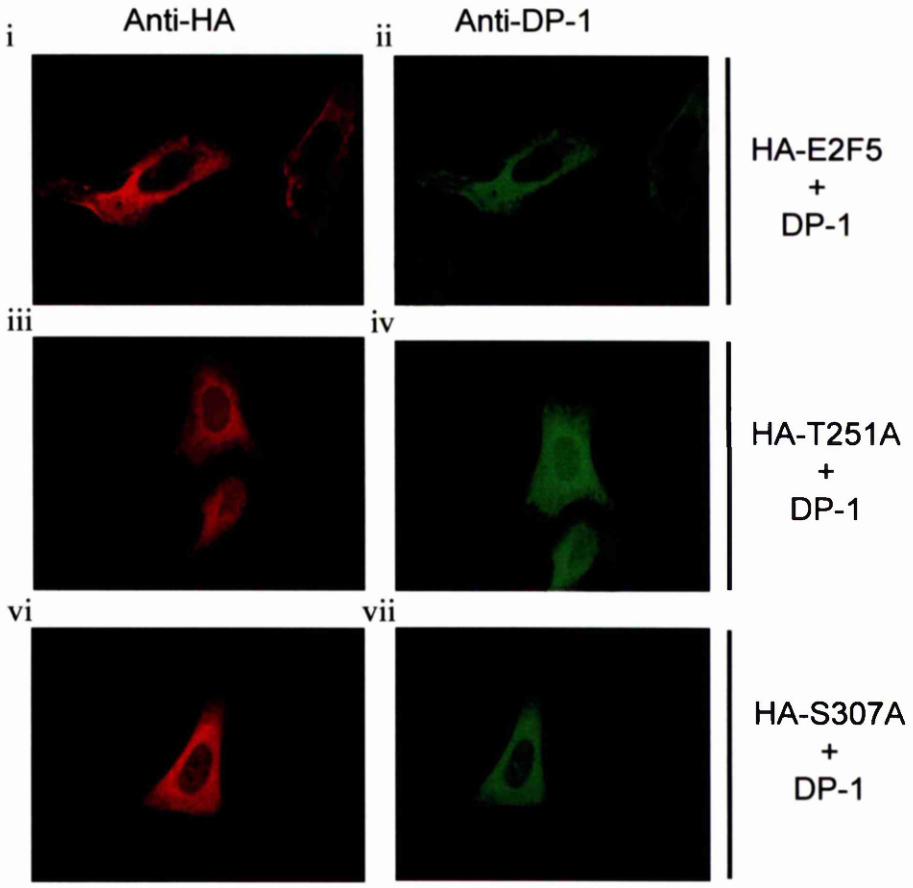
E2F-5, T251A and S307A are cytoplasmic and their nuclear accumulation is mediated by p107.

(a) U2OS cells were transfected with either 1μg of HA-tagged E2F-5 (i and ii), 1μg of HA-tagged T251A (iii and iv), or 1μg of HA-tagged S307A (vi and vii), together with DP-1 (1μg). The intracellular distribution of the DP-1 subunit and the HA-tagged E2F heterodimeric partner was assessed by immunofluorescence with a rabbit polyclonal anti-DP-1 antibody (ii, iv, vii) and an anti-HA monoclonal antibody (i, ii, and vi). The majority of exogenous E2F-5 (i), T251A (iii), S307A (vi) and DP-1 (ii, iv, vii) is cytoplasmic.

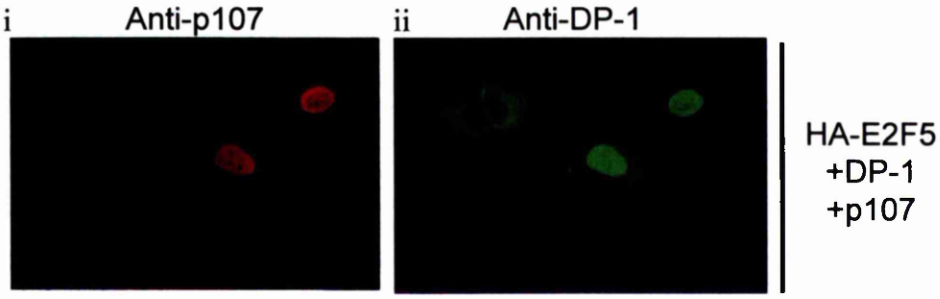
(b) U2OS cells were transfected with 1μg of HA-tagged E2F-5, 1μg of DP-1 and 1μg of p107. The intracellular distribution of DP-1 and p107 was determined by immunofluorescence with a rabbit polyclonal anti-DP-1 antibody (ii) and an anti-p107 (SD9) monoclonal antibody (i). A nuclear staining pattern was observed for p107 (i). DP-1 was nuclear in cells co-expressing p107, but cytoplasmic in cells where p107 expression was undetectable (compare i and ii).

(c) U2OS cells were transfected with either HA-tagged E2F-5, HA-tagged T251A or HA-tagged S307A (1μg), together with 1μg of DP-1 and 1μg of p107. The intracellular distribution of exogenous DP-1 and HA-tagged E2F-5 and the mutant derivatives was determined by immunofluorescence with a rabbit polyclonal anti-DP-1 antibody (ii, iv, vii) and an anti-HA monoclonal antibody. A nuclear staining pattern was observed for exogenous E2F-5, T251A and S307A (i, iii and vi, respectively) and DP-1 (ii, iv, vii).

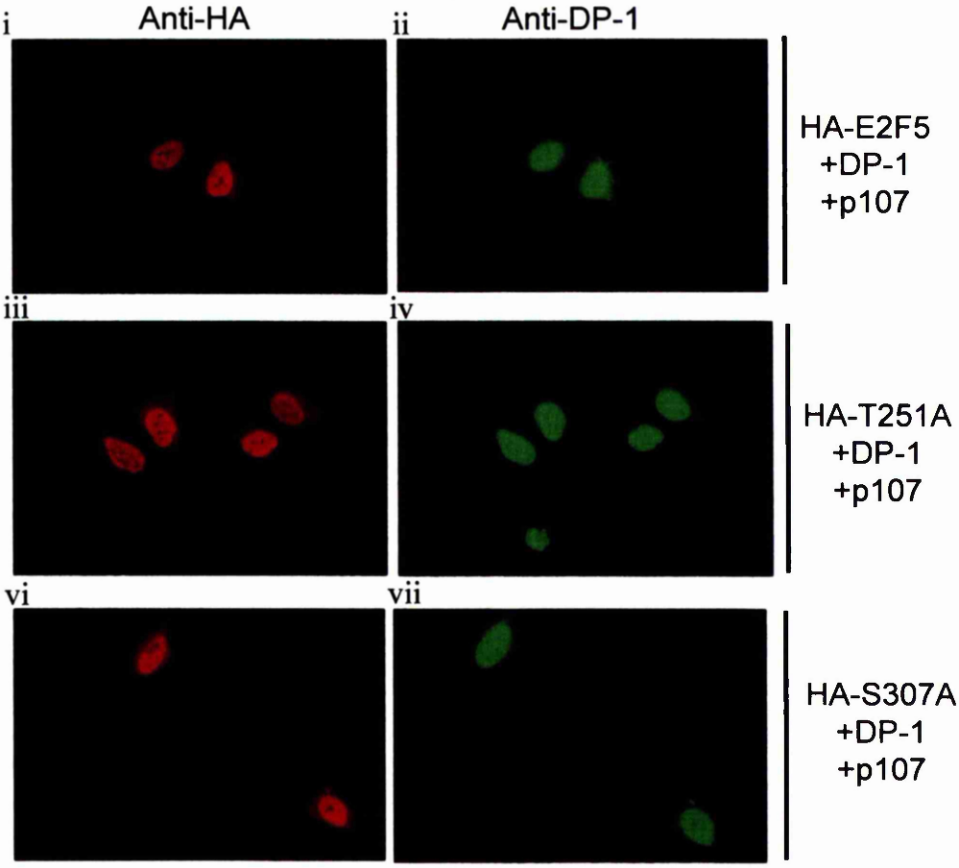
a


i
Anti-HA
ii
Anti-DP-1
HA-E2F5
+
DP-1
iii
iv
HA-T251A
+
DP-1
vi
vii
HA-S307A
+
DP-1

b


i
Anti-p107
ii
Anti-DP-1
HA-E2F5
+DP-1
+p107

c


i
Anti-HA
ii
Anti-DP-1
HA-E2F5
+DP-1
+p107
iii
iv
HA-T251A
+DP-1
+p107
vi
vii
HA-S307A
+DP-1
+p107

does not effect its ability to bind to p107 and enter the nucleus via the p107-directed nuclear import mechanism.

4.7 Conclusion

There are several consensus phosphorylation sites in E2F-5. Two of these sites, positioned at serine 307 and threonine 251 were mutated to alanine to produce mutant derivatives of E2F-5, designated S307A and T251A respectively. It was demonstrated that when E2F-5 was over-expressed in COS-7 cells a heterogenous cluster of polypeptides was detected on SDS-PAGE (Fig 4.1b). However, the number and distribution of these polypeptides was different in cells expressing mutant derivatives of E2F-5, incapable of phosphorylation at either serine 307 or threonine 251. Specifically, T251A expressing cells lacked one slowly migrating polypeptide relative to the cluster produced by wild-type E2F-5. Moreover, two E2F-5 polypeptides were absent in extracts of cells expressing S307A. These observations imply that phosphorylation at serine 307 and threonine 251 is important for the appearance of specific slower mobility E2F-5 polypeptides.

Several properties of E2F-5 were unaffected in the mutant derivatives, namely DNA binding activity, interaction with p107 and sub-cellular localisation. These observations establish that mutagenesis of E2F-5 did not alter the integrity of the E2F-5 protein. Furthermore, they rule out possible mechanisms for the functional consequence of phosphorylation of E2F-5 at serine 307 and threonine 251. The E2F-5 mutant derivatives are employed in the following chapters to explore the functional consequences of phosphorylation at these sites on E2F activity.

Chapter 5

Regulation of E2F activity by phosphorylation

5.1 Introduction

Many of the genes that are believed to play an important role in regulating early cell cycle progression contain E2F binding sites in their promoters. Among these are genes that encode regulators of the cell cycle (e.g. *cdc-2*, *cyclins A*, *D* and *E*) and genes encoding enzymes required for DNA synthesis (e.g. *DHFR*, *thymidine kinase* and *DNA polymerase* α). Physiological E2F consists of an E2F and DP component which together bind synergistically to the E2F site and activate transcription through the *trans*activation domain of the E2F subunit (Dyson, 1998). The *trans*activation domain also contains the interface recognised by pRb, which upon binding E2F is believed to hinder the interaction between the activation domain and the transcription apparatus (Helin, 1998). However, some E2F binding sites can also mediate transcriptional repression (Weintraub *et al.*, 1992). The *cyclin E* promoter contains several E2F sites which have been shown to be involved in both activation and repression of transcription (Butz *et al.*, 1996; Geng *et al.*, 1996; Cam *et al.*, 1999)

Data presented in the previous chapters raised the possibility that phosphorylation of E2F-5 may be important for some other aspect of E2F function besides DNA binding. In this chapter the S307A mutant derivative of E2F-5 was studied to explore the role of serine 307 phosphorylation in E2F-5-dependent transcriptional activation and repression.

5.2 S307A has a compromised transcriptional activity

In order to investigate the role of E2F-5 phosphorylation in E2F-dependent transcription the mutant derivative of E2F-5, S307A, was assessed for its ability to activate the *cyclin E* promoter. Transient transfection of U2OS cells with cyclinE-luciferase and expression vectors for either E2F-5 or S307A yielded an insignificant stimulation of reporter activity (Fig 5.1a). However when E2F-5 or S307A were co-transfected with DP-1 a significant induction in reporter activity was observed. Titration of E2F-5 levels in the presence of a constant amount of DP-1 induced reporter activity in a dose-dependent manner, to a maximum of 14-fold above that produced by DP-1 alone (Fig 5.1a, lanes 4-8, lane 14). The maximal trancriptional activation achieved by S307A and DP-1 was 8-fold (Fig5.1a, lanes 9-13). Furthermore, the rise in reporter activity in response to increasing amounts of S307A was not as steep as that detected when equivalent amounts of wild-type E2F-5 were transfected. These observations imply that S307A has a compromised transcriptional activity.

To determine whether the effect of S307A on E2F dependent transcription was specific for heterodimers containing DP-1, the reporter assay was repeated replacing DP-1 with DP-3δ. E2F-5 and DP-3δ synergistically activated the *cyclin E* promoter by 9-fold, whereas the maximum activation caused by S307A and DP-3δ was 5-fold (Fig 5.1b, compare lane 7 and 11). Thus the *trans*activation ability of S307A was compromised by approximately 45%, irrespective of its heterodimerisation partner.

Figure 5.1

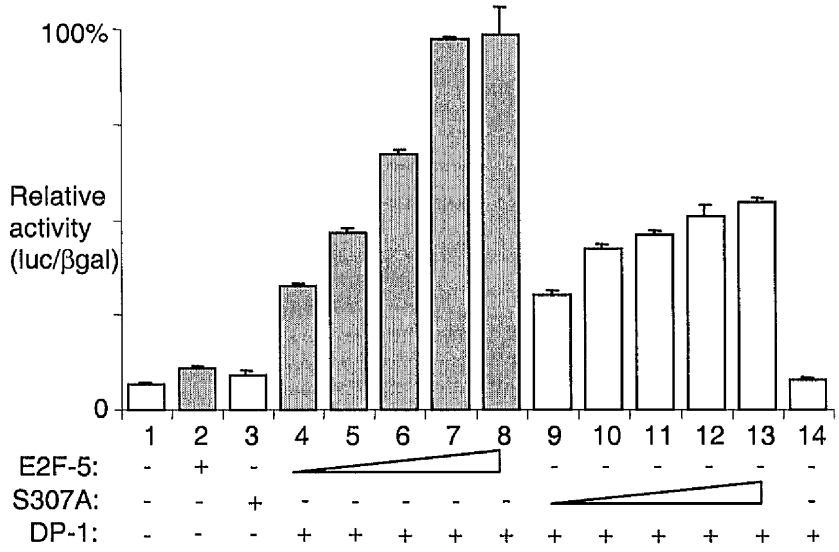
S307A has a compromised transcriptional activity.

(a) The S307A/DP-1 heterodimer is less efficient at activating the *cyclin E* promoter than the E2F-5/DP-1 heterodimer. U2OS cells were transfected with the cyclin E-luciferase reporter (1μg), together with expression vectors for HA-E2F-5 or HA-S307A (0.25, 0.5, 0.75, 1.0, 1.5μg), DP-1 (1μg), as indicated. The'+' below lane 2 and 3 indicates that 1.5μg of HA-E2F-5 or HA-S307A was transfected, respectively.

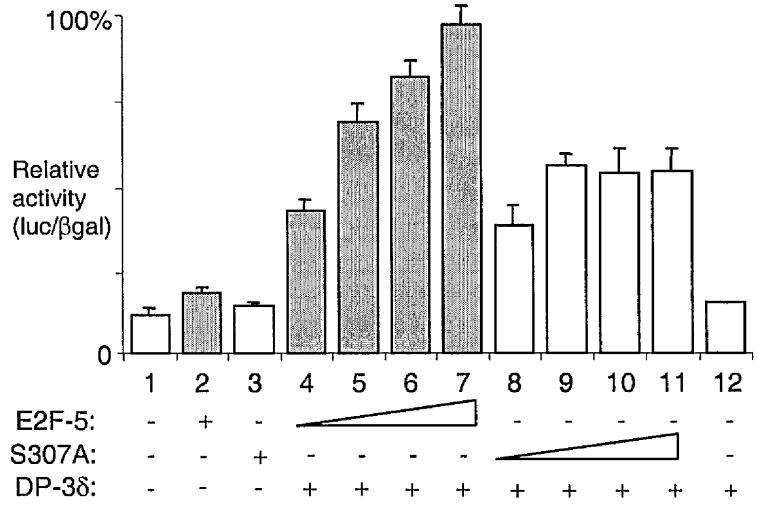
(b) The S307A/DP-3δ heterodimer is less efficient at activating the *cyclin E* promoter than the E2F-5/DP-3δ heterodimer. U2OS cells were transfected with the cyclin E-luciferase reporter (1μg), together with expression vectors for HA-E2F-5 or HA-S307A (0.25, 0.5, 0.75, 1.0), DP-3δ (0.75μg), as indicated. The'+' below lane 2 and 3 indicates that 1.0μg of HA-E2F-5 or HA-S307A was transfected, respectively.

In both (a) and (b) pCMV-βGal (1μg) was transfected throughout as an internal control. Values shown are the means of two separate readings and represent the ratio of luciferase to β-galactosidase activity.

a


100%
Relative activity (luc/βgal)
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14
E2F-5: - + - - - - - - - - - - - -
S307A: - - + - - - - - - - - - - -
DP-1: - - - + + + + + + + + + + +

b


100%
Relative activity (luc/βgal)
0
1 2 3 4 5 6 7 8 9 10 11 12
E2F-5: - + - - - - - - - - - -
S307A: - - + - - - - - - - - -
DP-3δ: - - - + + + + + + + + +

5.3 Mutation of E2F-5 to S307A does not alter its ability to bind p107

The pocket protein p107 can physically associate with the E2F-5/DP-1 heterodimer resulting in accumulation of the heterodimer in the nucleus (Allen *et al.* 1997). In the previous chapter it was shown that this aspect of E2F-5 regulation was not altered in S307A. Although these data showed that E2F-5 or S307A and p107 could co-localise in the nucleus, they did not give an indication of the binding efficiency of the two proteins for each other. Therefore, an immunoprecipitation assay was performed to determine whether E2F-5 and S307A have different affinities for p107.

Co-expression of E2F-5 with p107 resulted in an overall increase in E2F-5 protein levels, with a proportionally greater increase in the levels of E2F-5L (Fig 5.2a, compare lane 1 and 4). A possible explanation for this is that binding of E2F-5 to p107 stabilises E2F-5. Thus it is conceivable that p107 binds with greater affinity to E2F-5L or S307A. To test this hypothesis U2OS cells were transfected with either HA-tagged E2F-5 or S307A, in combination with DP-1 and p107. The levels of p107 were equal in extracts from these cells as confirmed by western blotting (Fig 5.2b). The extracts were immunoprecipitated with an anti-p107 monoclonal antibody (SD9), followed by immunoblotting with anti-HA to assess whether E2F-5 and p107 formed a complex (Fig 5.2c). Both E2F-5 and S307A were co-immunoprecipitated with p107 (Fig 5.2c, lane 4 and 6). To quantify the relative binding efficiency of E2F-5 and S307A for p107, the ratio of input E2F-5/S307 in the crude extract to the level of E2F-5 in the p107 immunoprecipitate was calculated. The level of E2F-5 and S307A binding to p107 was 11% of that in crude extract, indicating that S307A had the same affinity for p107 as wild-type E2F-5 in these extracts.

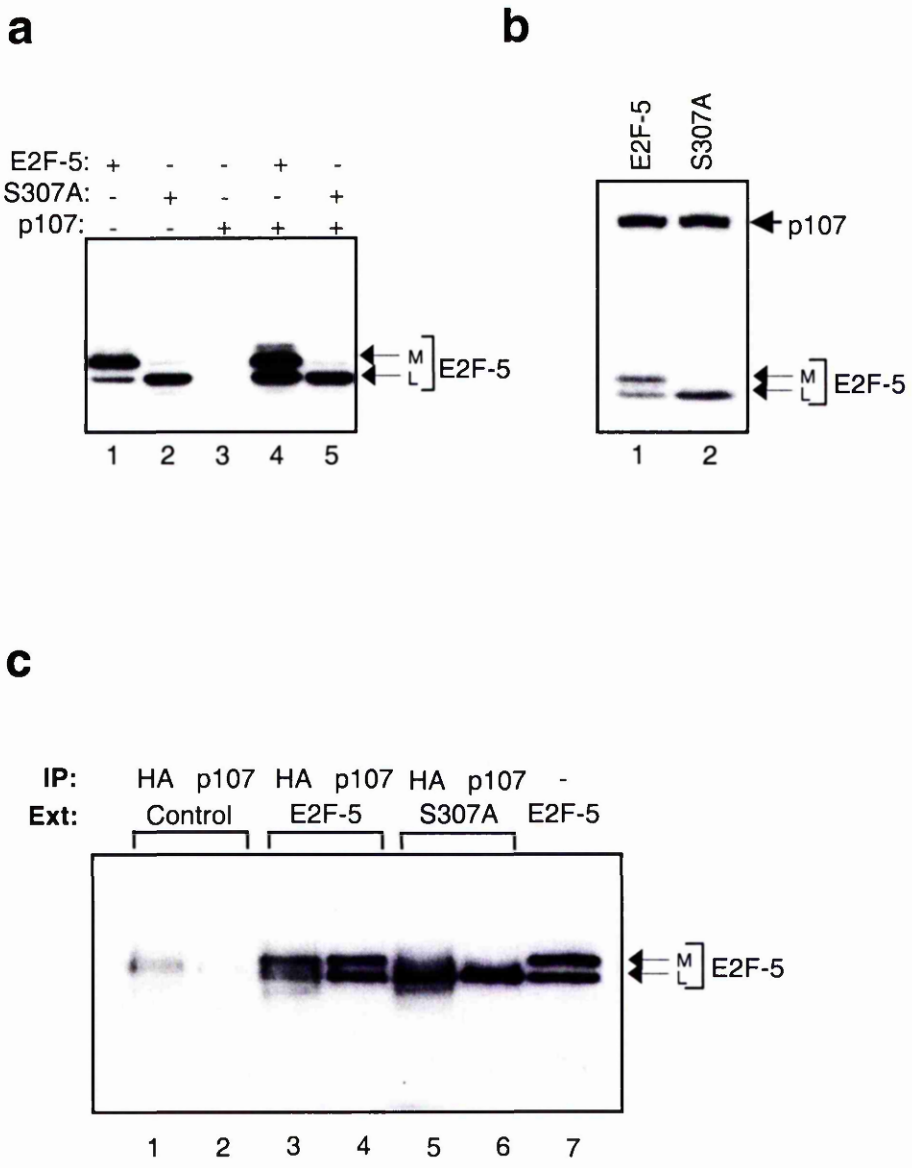
Figure 5.2

E2F-5 and S307A bind to p107 with similar efficiencies.

(a) Immunoblot with anti-HA monoclonal antibody of extracts from U2OS cells, transfected with HA-tagged E2F-5 (4μg), HA-tagged-S307A (4μg) and p107 (4μg), as indicated.

(b) Immunoblot with anti-HA monoclonal antibody and anti-p107 monoclonal antibody (SD9) of extracts from U2OS cells, transfected with expression vectors for p107 (10μg) and either 10μg of HA-tagged E2F-5 (lane 1), or 10μg of HA-tagged-S307A (lane 2).

(c) Extracts described in (b) and a control extract from U2OS cells, transfected with the relevant empty vector DNA, were immunoprecipitated (IP) with an anti-p107 monoclonal antibody (SD9) or an anti-HA monoclonal antibody, as indicated. The immunoprecipitates were resolved by SDS-PAGE and then immunoblotted with anti-HA. Lane 7 shows the reactivity of 10% of the input extract from lane 3 and 4.


a
E2F-5: + - - + -
S307A: - + - - +
p107: - - + + +
M
L
E2F-5
1 2 3 4 5
b
E2F-5
S307A
p107
M
L
E2F-5
1 2
c
IP: HA p107 HA p107 HA p107 -
Ext: Control E2F-5 S307A E2F-5
M
L
E2F-5
1 2 3 4 5 6 7

5.4 Transcriptional activation and repression mediated by Gal4-E2F5

To study the effects of E2F-5 phosphorylation on the E2F-5 mediated transcriptional activitation domain, a C-terminal region of E2F-5 (amino acids 198-335) was fused to a heterologous DNA binding domain (DBD). The resulting construct consisted of Gal4 DBD and the *trans*activation domain, but not the DNA binding or dimerisation domain of E2F-5. Transient transfection of the Gal4E1B-luciferase reporter and increasing amounts of the Gal4-E2F5 expression vector stimulated reporter activity between 100 and 700-fold in a dose-dependant manner (Fig 5.4a, lanes 2-4). However a maximum of 400-fold induction of reporter activity was achieved by Gal4-S307A (Fig 5.4a, lanes 5-7). A similar result was observed in C33A cells, where the ability of Gal4-S307A to activate transcription was reduced by approximately 50% compared to Gal4-E2F5 (Fig 5.4b). Thus the phenotype of full length S307A can be transferred to Gal4-S307 (198-335), suggesting that sequences in this C-terminal region of E2F-5 were responsible for the compromised ability of full length S307A to activate transcription.

The *trans*activation and pocket protein-binding domain remain intact in Gal4-E2F-5 so it was reasoned that proteins that interact with these domains might be important in mediating the effects of phosphorylation of serine 307 in E2F-5. Previously, by immunoprecipitation, it was shown that the efficiency of binding between S307A and p107 was indistinguishable from that of wild-type E2F-5 (see Section 5.3). To further investigate whether phosphorylation of serine 307 has a role in modulating the response of E2F-5 to pocket proteins, Gal4-E2F-5 and Gal4-S307A were assessed for their ability to be repressed by pocket proteins.

Figure 5.3

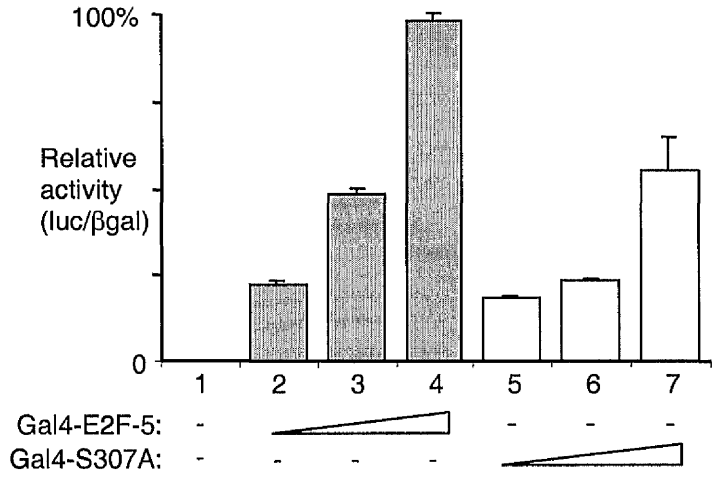
A Gal4-S307A fusion protein has the same effect on transcriptional activation as full length S307A.

(a) U2OS cells were transfected with the Gal4E1B-luciferase reporter (1μg), together with expression vectors for Gal4-E2F-5 or Gal4-S307A (50, 100, 150ng), as indicated.

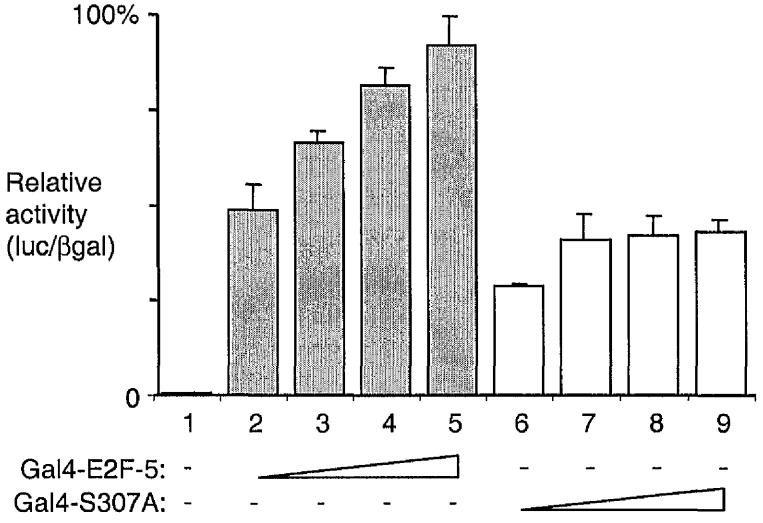
(b) C33A cells were transfected with the Gal4E1B-luciferase reporter (1μg), together with expression vectors for Gal4-E2F-5 or Gal4-S307A (50, 75, 100, 125ng), as indicated.

In both (a) and (b) pCMV-βGal (1μg) was transfected throughout as an internal control. Values shown are the means of two separate readings and represent the ratio of luciferase to β-galactosidase activity.

a


100%
Relative activity (luc/βgal)
0
1 2 3 4 5 6 7
Gal4-E2F-5: - - - -
Gal4-S307A: - - - -

b


100%
Relative activity (luc/βgal)
0
1 2 3 4 5 6 7 8 9
Gal4-E2F-5: - - - - -
Gal4-S307A: - - - - -

SAOS-2 cells were transfected with Gal4E1B-luciferase and either expression vectors for Gal4-E2F5 or Gal4-S307, together with increasing amounts of a p130 expression vector. The level of *trans*activation mediated by either Gal4-E2F-5 or Gal4-S307A was inhibited in a p130 concentration-dependent fashion, with maximal repression ocurring at 200ng of p130 (Fig 5.4c). A similar response was observed when the transfection was repeated substituting p130 with p107, with maximal repression requiring 600ng of transfected p107 (Fig 5.4d). Therefore maximum repression of both the wild-type and mutant E2F-5 required the same quantity of p130 or p107, suggesting that both E2F-5 and S307A bind to either pocket protein with the same efficiency. However, the overall percentage of p107 induced repression was greater with wild-type E2F-5 than S307A. This effect is unlikely to reflect a preferential interaction between wild-type E2F-5 and p107, rather wild-type E2F-5 is intrinsically more active than S307A and therefore when wild-type E2F-5 interacts with p107 the consequence is a more marked reduction in *trans*activation.

Figure 5.4

p107 and p130 both repress Gal4-E2F-5 and Gal4-S307A to a similar level.

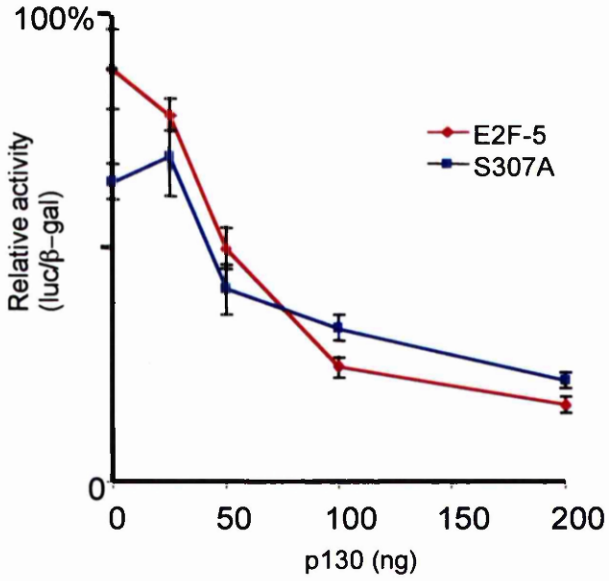
U2OS cells were transfected with the Gal4E1B-luciferase reporter (1μg), together with expression vectors for Gal4-E2F-5 or Gal4-S307A (100ng) and:

(a) the indicated amounts of a p130 expression vector;

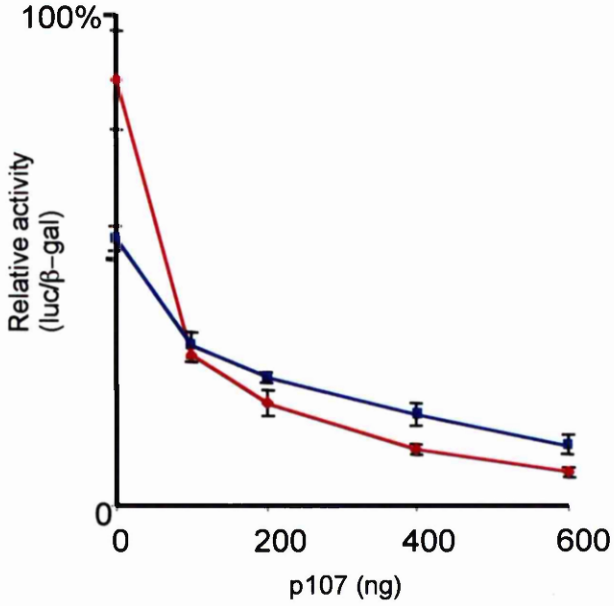
(b) the indicated amounts of a p107 expression vector.

The red line represents wild-type Gal4-E2F-5 and the blue line represents Gal4-S307A. In both (a) and (b) pCMV-βGal (1μg) was transfected throughout as an internal control. Values shown are the means of two separate readings and represent the ratio of luciferase to β-galactosidase activity.

a


100%
0
Relative activity
(luc/β-gal)
E2F-5
S307A
0
50
100
150
200
p130 (ng)

b


100%
0
Relative activity
(luc/β-gal)
0
200
400
600
p107 (ng)

5.5 The transcriptional activity of E2F-5 and S307D are equal

Phosphorylation confers a negative charge to a protein which can be mimicked by substituting the phosphorylated residue with aspartate. A mutant E2F-5 derivative was created with an aspartate at position 307, refered to as S307D. U2OS cells were transiently transfected with expression vectors for either E2F-5, S307A or S307D and cell extracts were analysed by western blotting (Fig 5.5a). S307D resolved as a single polypeptide of a slower mobility than S307A and of approximately the same mobility as the major wild-type E2F-5 polypeptide (E2F-5M). Thus, introducing a negative charge at serine 307 in E2F-5 resulted in a mobility shift of the faster migrating E2F-5L to a more slowly migrating polypeptide, E2F-5M. To explore the reasons for these mobility differences cell extracts, transfected with expression vectors for E2F-5 or S307D together with DP-1, were treated with lambda-phosphatase. Phosphatase treatment induced a mobility shift of E2F-5 from the M to the L form, but did not effect the mobility of S307D (Fig 5.5b). This was the expected result if phosphorylation on serine 307 is the only reason for the mobility difference between E2F-5L and E2F-5M, although the possibility that there are other differences in post translational modification of these polypepetides cannot be excluded.

The transcriptional activity of S307D was found to be indistinguishable from E2F-5 when they were assessed for their ability to activate cyclinE-luciferase in combination with a DP-1 partner (Fig 5.5c). Thus S307D had greater *trans*activation ability than S307A. Taken together these results suggest that when E2F-5 is over-expressed by transient transfection it is phosphorylated on serine 307 by an endogenous kinase. This modification enhances the transcriptional activity of E2F-5 and is mimicked in S307D, but is lost in S307A.

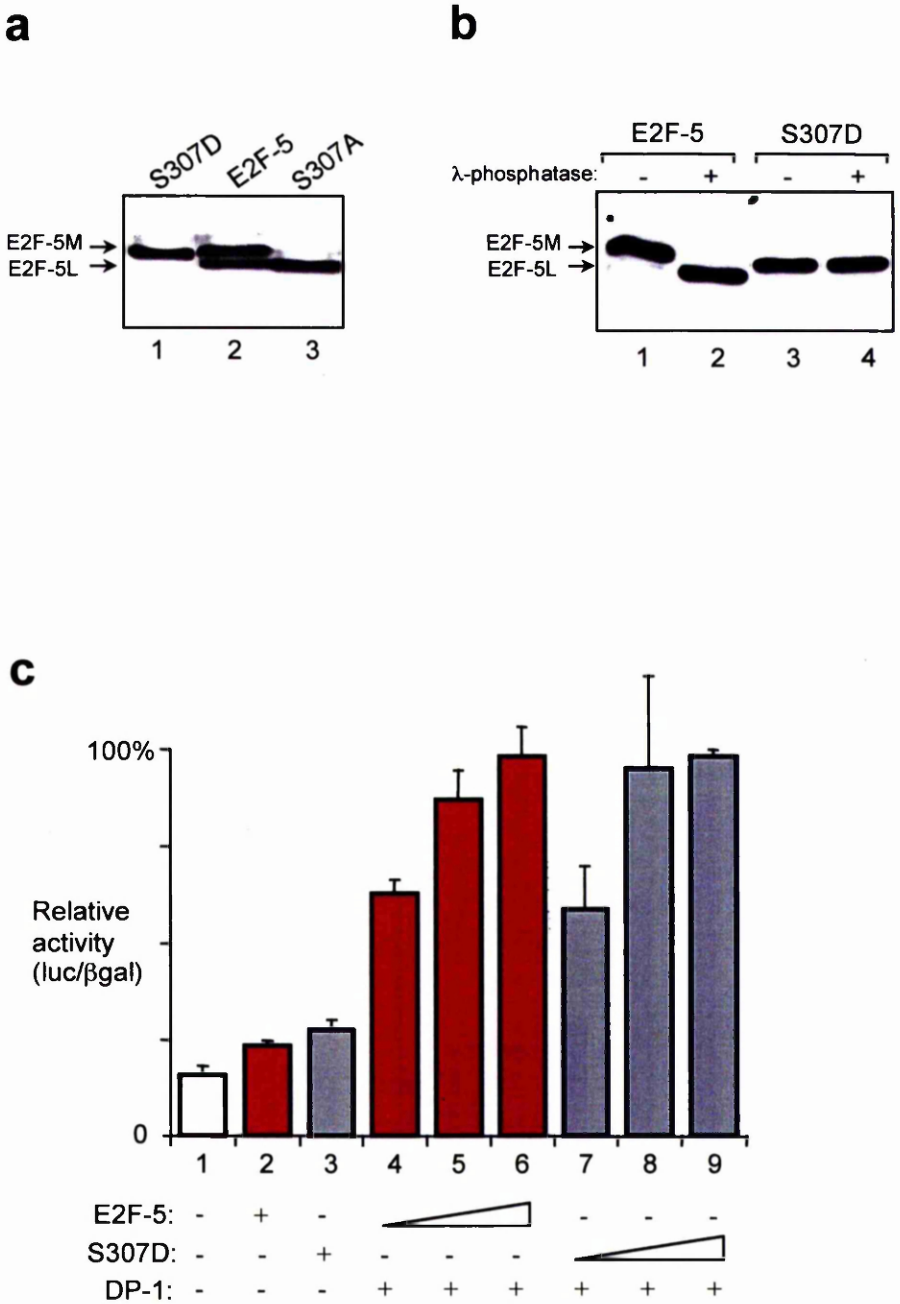
Figure 5.5

Mutation of serine 307 in E2F-5 to aspartate alters E2F-5 electrophoretic mobility but does not affect E2F-5 dependent transcription.

(a) Immunoblot with anti-HA of extracts from U2OS cells transfected with 5μg of either HA-tagged E2F-5, HA-tagged S307A or HA-tagged S307D, as indicated.

(b) U2OS cells were transfected with 5μg of HA-tagged E2F-5 or HA-tagged S307D and extracts were treated with λ-phosphatase (lane 2 and 4). Control reactions were treated in the same way except λ-phosphatase was omitted from the reaction mixture (lane 1 and 3). E2F-5 was detected by immunoblotting with anti-HA monoclonal antibody.

(c) U2OS cells were transfected with the cyclin E-luciferase reporter (1μg), together with expression vectors for HA-E2F-5 or HA-S307D (0.5, 0.75, 1.0), DP-1 (1μg), as indicated. The'+' below lane 2 and 3 indicates that 1.0μg of HA-E2F-5 or HA-S307D was transfected, respectively. Values were calculated as for figure 5.1.


a
S307D
E2F-5
S307A
E2F-5M
E2F-5L
1
2
3
b
E2F-5
S307D
λ-phosphatase:
-
+
-
+
E2F-5M
E2F-5L
1
2
3
4
c
100%
Relative activity (luc/βgal)
0
1
2
3
4
5
6
7
8
9
E2F-5: - + - - - - - - -
S307D: - - + - - - - - -
DP-1: - - - + + + + + +

5.7 Effect of S307A on the Cell Cycle

U2OS cells were stably transfected with doxycyclin inducible HA-tagged E2F-5, HA-tagged S307A or empty expression plasmid (Allen and La Thangue, personal communication). These cells were used to investigate the effect of the serine to alanine mutation in S307A on cell cycle progression. One cell-line expressing wild-type E2F-5 (designated WT1) and another expressing S307A (designated S5) expressed high levels of E2F-5 when compared to control cells stably transfected with empty expression plasmid (Fig 5.6a). However when these cells were tested for inducibility in the absence and presence of doxycyclin the levels of E2F-5 and S307A remained similar (Allen and La Thangue, personal communication).

There were only minor differences in the cell cycle profiles of cells expressing wild-type E2F-5 and cells expressing S307A (Fig 5.6b). BrdU labelling was employed to measure the number of cells in S phase and the BrdU labelled cells were counted by FACS. In addition propidium iodide staining was used to assess the DNA content of the cells so the number of cells in G1 and G2 phases could also be measured. The number of S phase cells in both the WT1 and S5 cell lines was 60-62% greater than the number in the control cell line (Fig 5.6b). To accommodate this increase in S phase there was a fall in the G1 and G2/M population of both WT1 and S5 cells, relative to the control. However the fall in G1 phase was greater in WT1 cells and the fall in G2/M was greater in S5 cells.

The stable cell lines were serum starved to investigate whether overexpression of E2F-5 or S307A influenced the ability of the cells to undergo a cell cycle arrest. Serum starvation increased the G1 population of control cells and cells expressing S307A, but had a much smaller effect on the cells expressing wild-type E2F-5,

indicating cells that over-expressed S307A could be more readily arrested than cells that over-expressed wild-type E2F-5 (Fig 5.6c).

5.8 Conclusion

The data presented in this chapter suggests that E2F-5 transcriptional activity is regulated by direct phosphorylation of E2F-5 at serine 307. Evidence supporting this conclusion came from the analysis of a mutant derivative of E2F-5 incapable of phosphorylation at serine 307 (S307A). S307A has a reduced transcriptional activity, both as a heterodimer with DP-1 or DP-3, and as a Gal4-hybrid protein. Data presented in this and the previous chapter suggest that phosphorylation of E2F-5 at serine 307 does not regulate its DNA binding activity or ability to heterodimerise with a DP partner. Furthermore there was no difference in the ability of E2F-5 and S307A to interact with pocket proteins. Therefore phosphorylation of E2F-5 at serine 307 is unlikely to mediate its effect on transcription through any of these mechanisms.

The ability of E2F to promote cell cycle progression is believed to involve the activation of key target genes (Dyson, 1998). Both E2F-5 and S307A were capable of stimulating cell cycle progression when stably expressed in U2OS cells. This was demonstrated by an increase in BrdU incorporation, relative to the control cell line. Cells stably expressing S307A could be arrested by serum starvation. However cells expressing wild-type E2F-5 were incapable of arrest by serum starvation. Therefore phosphorylation of E2F-5 on serine 307 may play a role in cell cycle re-entry from G0/G1 into S phase.

Fig 5.6

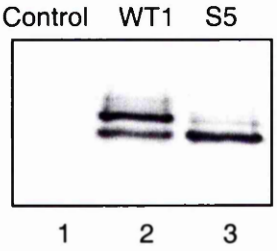
Differences in the cell cycle profile between cells expressing E2F-5 and S307A.

(a) Immunoblot with anti-HA monoclonal antibody of extracts from cells stably transfected with HA-tagged E2F-5 (WT1), HA-tagged S307A (S5), or empty expression vector (Control).

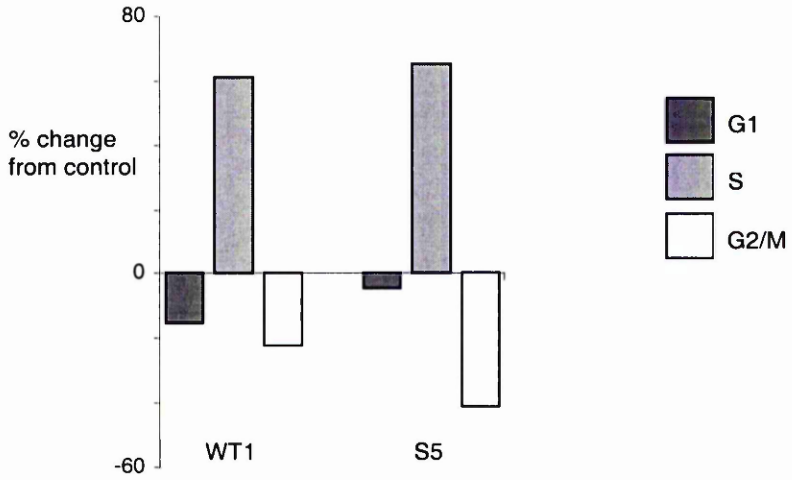
(b) The stable cell lines, WT1, S5 and the control cell line, were analysed by flow cytometry after labeling with BrdU and staining with propridium iodide. The data are presented as the percentage change of cells in each phase of the cell cycle, compared to the control cell line.

(c) WT1, S5 and control cells were serum starved for 72 hours. The serum starved cells and asynchronous populations of WT1, S5 and control cells were analysed by flow cytometry. The data are presented as the percentage change in the number of G1 cells in the serum starved populations, relative to the number of G1 cells in the asynchronous population.

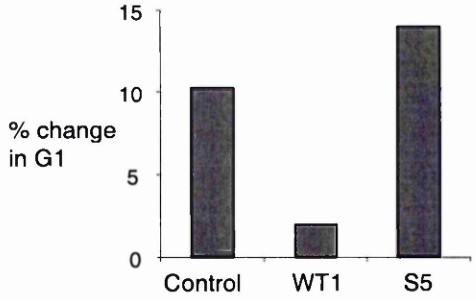
a


Control
WT1
S5
1
2
3

b


80
% change
from control
0
-60
WT1
S5
G1
S
G2/M

c


15
10
% change
in G1
5
0
Control
WT1
S5

Chapter 6

Regulation of E2F-5 by CyclinE-CDK2

6.1 Introduction

The G1 cyclin dependent kinases (CDK) control entry into S phase by phosphorylating key substrates (Sherr, 1993). Members of the cyclin D family and their catalytic partners, CDK4 and CDK6, are most recognised for their role in phosphorylating pRb, which plays an important role in the regulation of the G1/S transition. Phosphorylation of pRb releases it from E2F enabling the activation of E2F target genes, such as *cyclin E*. In most cells, the accumulation of cyclin D-dependent kinase activity is followed by the activation of cyclinE-CDK2 kinase, which is believed to be involved in completing the process of pRb phosphorylation at additional sites (Lundberg and Weinberg, 1998). However, in cells arrested through the expression of a phosphorylation-deficient mutant pRb, the introduction of cyclinE-CDK2 allows cells to progress into S phase (Alevizopoulos *et al.*, 1997; Lukas *et al.*, 1997), suggesting that cyclinE-CDK2 targets at least one other critical effector of cell cycle progression. Although it is known that cyclinE-CDK2 activity is necessary for entry into S phase few *in vivo* substrates for cyclinE-CDK2 have been identified (Ohtsubo *et al.*, 1995). In this chapter the phosphorylation of E2F-5 by cyclinE-CDK2 and the functional consequence of phosphorylation of the threonine 251 site in E2F-5 are explored.

6.2 E2F-5 is phosphorylated *in vitro* by cyclinE-CDK2

In chapter 4 it was shown that in COS7 cells transfected with E2F-5, a number of E2F-5 polypeptides with different electrophoretic mobility were detected by western blot analysis, with an anti-E2F-5 antibody. One of these polypeptides was absent in cells transfected with the T251A mutant derivative of E2F-5 (see Fig 4.1b). The polypeptide lost in the T251A mutant form of E2F-5 is of an equivalent mobility to the endogenous E2F-5 polypeptide (E2F-5U) in Rat1 cells which occurs at the G1-S phase transition, suggesting it is the result of phosphorylation of E2F-5 by a G1 cyclin/CDK complex (see Fig 3.7b).

To explore the idea that E2F-5 is phosphorylated by cyclinE-CDK2, *in vitro* phosphorylation reactions were performed with recombinant E2F-5. Initially E2F-5 and the mutant derivative, T251A, were expressed as GST fusion proteins in bacteria. When the purified preparations were subjected to SDS-PAGE and Coomassie Blue staining a 73kDa band, the estimated size of the GST fusion protein, was detected (Fig 6.1a). In addition a ladder of smaller bands was detected, probably as a result of protein degradation or bacterial contamination in the GST-E2F-5 preparation. The identity of full length E2F-5 was confirmed by western blotting with anti-E2F-5 and an abundant band at 73kDa and four smaller polypeptides were detected (Fig 6.1a, lane 3). The expression and purity of the 73kDa full-length E2F-5 fusion protein was poor. It corresponded to approximately 1% of the overall GST-E2F-5 preparation.

In vitro kinase assays were performed using GST-E2F-5 and either cyclinE-CDK2 or cyclinA-CDK2 purified from baculovirus infected *Sf9* cells. Histone H1 was used as a positive control to determine the relative activity of the kinases, cyclinA-CDK2 being the most efficient at phosphorylating Histone H1 in this assay (Fig 6.1b,

Figure 6.1

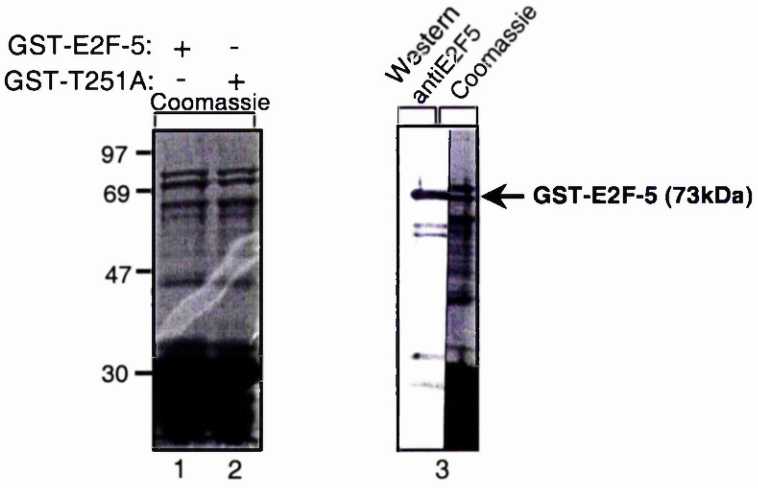
GST-E2F-5 but not GST-T251A is phosphorylated by cyclinE-CDK2 *in vitro*.

(a) Coomassie stained SDS-PAGE of GST-E2F-5 and GST-T251A.

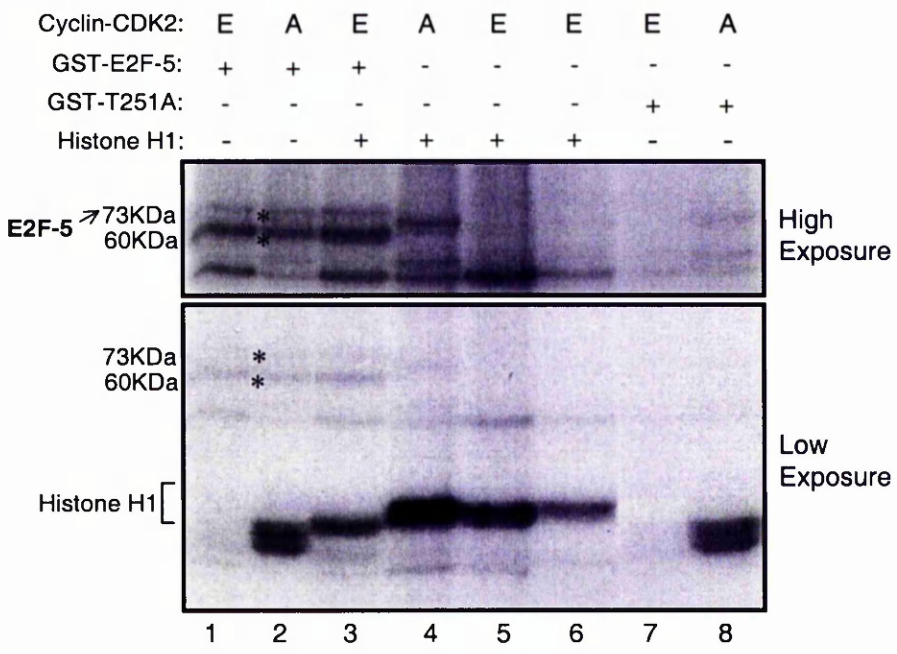
Purified GST-E2F-5 was resolved on SDS-PAGE and transferred to nitrocellulose. The nitrocellulose strip was cut in half and one half lane was probed with anti-E2F-5 antiserum (see Materials and Methods) and the other half was stained with Coomassie.

(c) Purified GST-E2F-5 and GST-T251A (approximately 0.5μg of each full-length protein) were used as phosphorylation substrates for cyclinE-CDK2 and cyclinA-CDK2 (50ng), purified from baculovirus-infected *Sf9* cells. Phosphorylation of histone H1 served as a positive control (lanes 3, 4 and 5 contain 1μg, lane 6 contains 0.5μg). The asterisks indicate the position of full-length E2F-5 (73kDa) and a smaller specific E2F-5 polypeptide (60kDa). A higher exposure of the upper part of the gel is shown so the low intensity bands corresponding to E2F-5 phosphorylation can be detected. A: cyclin A; E: cyclin E.

a


GST-E2F-5: + -
GST-T251A: - +
Coomassie
97
69
47
30
1 2
Western
antiE2F5
Coomassie
GST-E2F-5 (73kDa)
3

b


Cyclin-CDK2: E A E A E E E A
GST-E2F-5: + + + - - - - -
GST-T251A: - - - - - - + +
Histone H1: - - + + + + - -
E2F-5
73KDa
60KDa
High Exposure
73KDa
60KDa
Histone H1
Low Exposure
1 2 3 4 5 6 7 8

compare lanes 4 and 5). CyclinA-CDK2 and cyclinE-CDK2 both phosphorylated polypeptides of 73kDa and 60kDa in the GST-E2F-5 preparation but, relative to Histone H1, these two polypeptides were more efficiently phosphorylated by cyclinE-CDK2 (Fig 6.1b, lanes 1 and 2). A 73kDa and 60kDa polypeptide were also detected by anti-E2F-5 and were therefore deemed to be E2F-5 polypeptides (Fig 6.1a, lane 3). However cyclinA-CDK2 or cyclinE-CDK2 phosphorylated neither the 73kDa nor the 60kDa polypeptides in the GST-T251A preparation (Fig 6.1b, lanes 7 and 8). Polypeptides of approximately 30kDa, present in both the GST-E2F-5 and GST-T251A preparations, were phosphorylated by cyclinA-CDK2 to the same extent. These are likely breakdown products consisting of GST and N-terminal fragments of E2F-5, which judging by their size would not contain the C-terminal T251 site and therefore served as an internal control for phosphorylation. However the 60kDa polypeptide is large enough to be a C-terminally degraded E2F-5 polypeptide which still contains the T251 site (Fig 6.1b, lanes 2 and 8). As the only difference between the two preparations is a single amino acid at position 251 in T251A then the absence of phosphorylation in GST-T251A must be due to the loss of this important threonine residue. This implies either that threonine 251 is phosphorylated in E2F-5 or the absence of threonine 251 in T251A has prevented GST-T251A from becoming phosphorylated on other sites.

One drawback of using bacterially expressed GST-E2F-5 was that it was not sufficiently pure to prove cyclinE-CDK2 phosphorylation of E2F-5 was direct. In an attempt to improve purification, His-tagged bacterial expression vectors of E2F-5 were constructed. Purification of His-tagged E2F-5 expressed in bacteria proved the most successful system for preparing greater levels of pure E2F-5 (Fig 6.2a, lane 1).

Figure 6.2

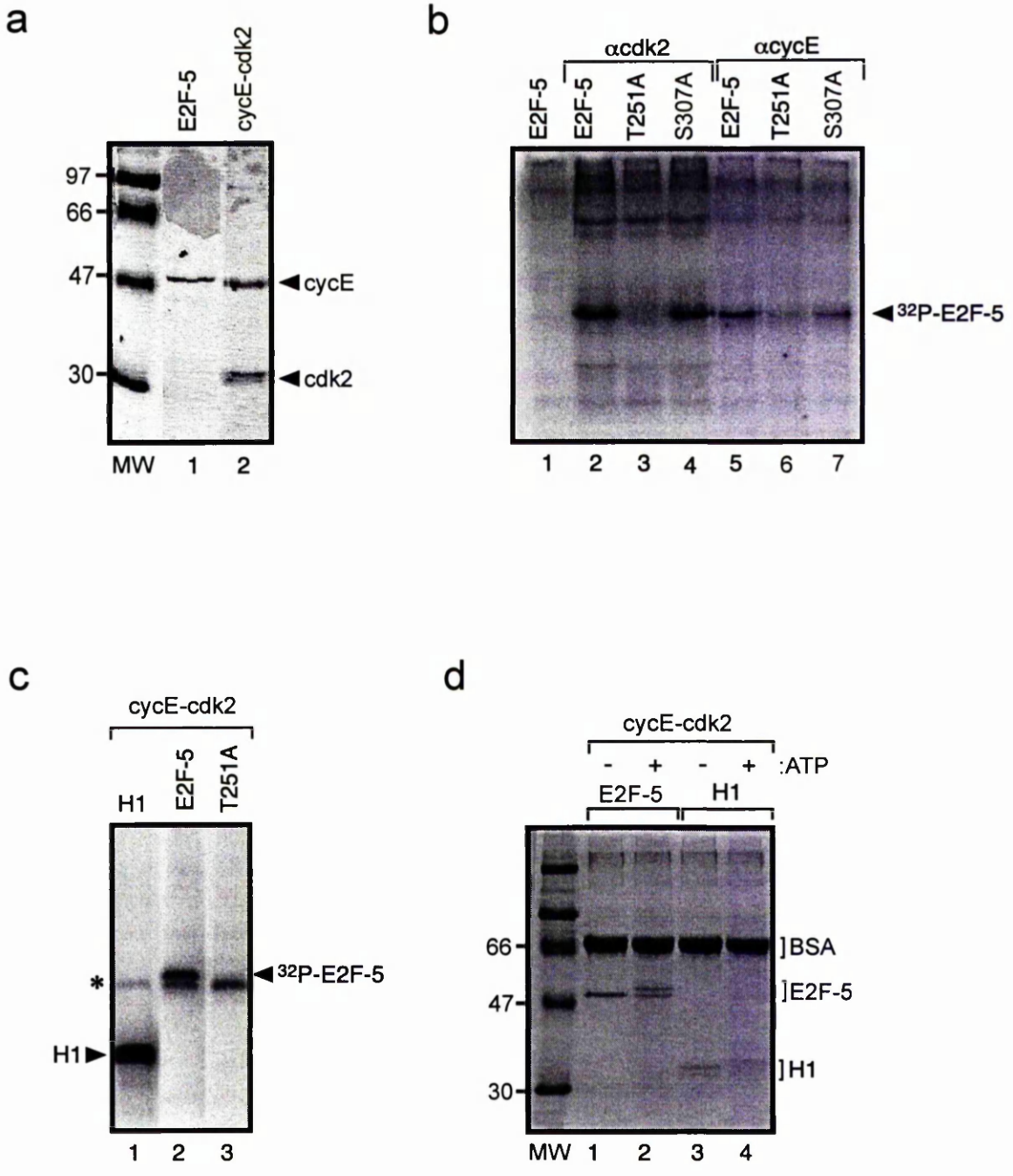
Mutation of threonine 251 in E2F-5 to alanine prevents phosphorylation of E2F-5 by cyclinE-CDK2 *in vitro*.

(a) Coomassie stained SDS-PAGE of bacterially expressed purified his-E2F-5 and cyclinE-CDK2 purified from baculovirus infected *Sf9* cells.

(b) Purified His-E2F-5 (1μg), His-T251A (1μg) and His-S307A (1μg) were used as substrates for phosphorylation by protein complexes immunoprecipitated from Rat1 cell extracts with control anti-Gal4 (lane 1), anti-CDK2 (lanes 2, 3 and 4) or anti-cyclinE (lanes 5, 6 and 7). The arrow indicates phosphorylated E2F-5.

(c) The cyclinE-CDK2 complex (50ng) purified from baculovirus infected *Sf9* cells was used to phosphorylate his-E2F-5 (1μg: lane2), his-T251A (1μg; lane 3); the arrow indicates phosphorylated E2F-5. Phosphorylation of histone H1 served as a positive control (lane 1). The asterisk indicates a non-specific polypeptide, which migrates in an equivalent position to purified cyclin E, therefore may be autophosphorylation of cyclin E.

(d) His-E2F-5 (1μg) and histone H1 (1μg) were *in vitro* phosphorylated by cyclinE-CDK2 (50ng) purified from baculovirus infected *Sf9* cells, in the presence of non-radioactive ATP. Reactions were also performed in the absence of ATP (lane 1 and 3). Thereafter the reactions were subjected to SDS-PAGE and Coomassie staining.


a
E2F-5
cycE-cdk2
97
66
47
30
cycE
cdk2
MW 1 2
b
αcdk2
αcycE
E2F-5
E2F-5
T251A
S307A
E2F-5
T251A
S307A
32P-E2F-5
1 2 3 4 5 6 7
c
cycE-cdk2
H1
E2F-5
T251A
*
H1
32P-E2F-5
1 2 3
d
cycE-cdk2
- + - + :ATP
E2F-5
H1
66
47
30
]BSA
]E2F-5
]H1
MW 1 2 3 4

Initially kinase reactions were performed with His-E2F-5/S307A/T251A using kinase complexes immunoprecipitated from Rat1 cells. Both E2F-5 and S307A were phosphorylated by anti-CDK2 and anti-cyclinE immunoprecipitates, but T251A was not phosphorylated by either complex (Fig 6.2b). Moreover, E2F-5 was not phosphorylated by immunoprecipitates using an irrelevant antibody (Gal4), indicating that complexes specifically immunoprecipitated by cyclin E and CDK2 antibodies were responsible for phosphorylating E2F-5 (Fig 6.2b, lane 1). To gain evidence that cyclinE-CDK2 directly phosphorylated E2F-5, His tagged E2F-5 and T251A were used as substrates for purified cyclinE-CDK2 from baculovirus-infected *Sf9* cells. CyclinE-CDK2 phosphorylated wild-type E2F-5 but did not phosphorylate the T251A mutant derivative (Fig 6.2c). Moreover the phosphorylation of E2F-5 led to the appearance of a slower migrating band in Coomassie blue stained gels (Fig 6.2d), an expected result if E2F-5U represents a form of E2F-5 that has been phosphorylated by cyclinE-CDK2 in cells.

6.3 E2F-5 is phosphorylated *in vivo* by cyclinE-CDK2

It does not always follow that a phosphorylation event that occurs readily *in vitro* will also occur physiologically. Thus to explore further the possibility that a G1 kinase phosphorylates E2F-5, expression vectors for either cyclinE-CDK2 or cyclinD1-CDK4 were co-transfected into U2OS cells with either E2F-5 or T251A. The appearance of the slower mobility E2F-5U was observed in transfections with cyclinE-CDK2 and E2F-5 but not when cyclinE-CDK2 was transfected with T251A, consistent with phosphorylation on the threonine 251 being responsible for the observed mobility shift (Fig 6.3a).

Figure 6.3

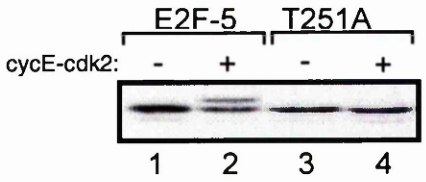
A threonine residue at position 251 in E2F-5 is phosphorylated by cyclinE-CDK2 *in vivo*.

(a) U2OS cells were transfected with either HA-E2F-5 (2μg; lanes 1,2), HA-T251A (2μg; lanes 3,4), in combination with expression vectors (3μg of each vector) for Cyclin E and CDK2, as indicated. Transfected cells were harvested and immunoblotted with anti-HA antisera. Note that phosphorylated E2F-5 only appears when wild-type E2F-5 is co-transfected with cyclinE-CDK2 (lane 2).

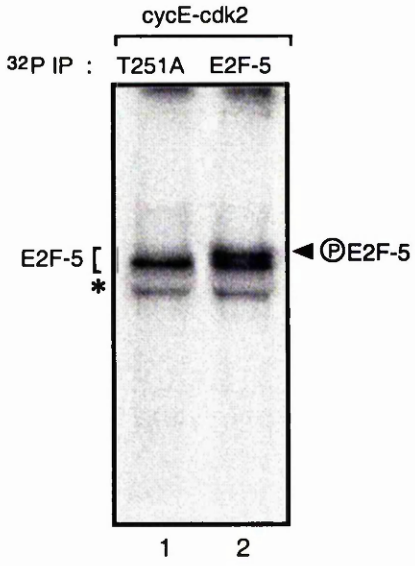
(b) U2OS cells were transfected with HA-T251A (2μg; lane 1) or HA-E2F-5 (2μg; lane 2) together with expression vectors for cyclinE-CDK2 (2μg of each) and metabolically labelled with [^{32}P] orthophosphate (see Materials and Methods). Cells were harvested and immunoprecipitated with the anti-HA monoclonal antibody. The E2F-5 polypeptides are indicated. Note that the upper polypeptide (track 2 indicated by **P**-E2F-5) is absent from the T251A immunoprecipitate (track 1), and * represents a non-specific band.

(c) CnBr cleavage of the *in vivo* ^{32}P labelled HA-T251A (track 2) and HA-E2F-5 (track 3). For comparison, recombinant His-E2F-5 protein was phosphorylated *in vitro* with ^{32}P-ATP by cyclinE-CDK2 kinase (track 1) and similarly treated with CnBr. The expected size of the E2F-5 cleavage product containing phosphorylated T251 and the positions of the size markers (KD) are indicated.

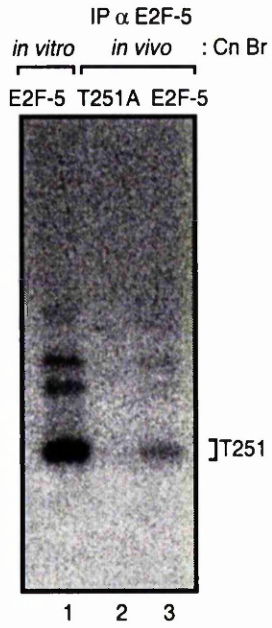
a


E2F-5
T251A
cycE-cdk2:
-
+
-
+
1
2
3
4

b


cycE-cdk2
32P IP :
T251A
E2F-5
E2F-5 [
*
◀ ⓟE2F-5
1
2

c


IP α E2F-5
in vitro
in vivo
: Cn Br
E2F-5
T251A
E2F-5
]T251
1
2
3

To investigate whether the threonine residue at position 251 in E2F-5 is phosphorylated *in vivo*, U2OS cells were transfected with either HA-tagged E2F-5 or T251A together with cyclin E and CDK2 expression vectors and the cells were metabolically labeled with [^{32}P]-orthophosphate. Thereafter the cell extracts were immunoprecipitated with an anti-HA monoclonal antibody. The wild-type E2F-5 immunoprecipitate appeared as two closely migrating polypeptides, which had identical mobility to E2F-5U and E2F-5M defined by immunoblotting E2F-5 in cell extracts (Figure 6.3b, lane 2). In contrast, the T251A immunoprecipitate appeared as a single polypeptide with mobility equivalent to E2F-5M (Figure 6.3b, lane 1). These results support the earlier conclusion that phosphorylation in E2F-5U is dependent upon the integrity of the CDK site at T251. Furthermore, they argue that in addition to T251, at least one other phosphorylation site exists in E2F-5.

To define the position of the phosphorylated residue(s) in E2F-5, peptide mapping was performed by cyanogen bromide (CnBr) cleavage of the *in vivo* ^{32}Pi-phosphorylated E2F-5 immunoprecipitate. CnBr cleavage mapped the major phosphorylation site within the C-terminally derived peptide (residue 194 to 256), in which the only CDK site is located at T251 (Fig 6.3c). Most importantly, both *in vitro* and *in vivo* phosphorylated E2F-5 yielded the same size phosphopeptide which, as expected, was absent in the T251A mutant derivative (Fig 6.3c). Overall, these results support the idea that threonine 251 is phosphorylated *in vivo* by cyclinE-CDK2.

6.4 Functional relevance of cyclinE-CDK2 phosphorylation of E2F-5

Having established that E2F-5 can be phosphorylated by cyclinE-CDK2 *in vitro* and *in vivo* the physiological consequence of this phosphorylation was investigated. The effect of cyclinE-CDK2 on E2F-5 dependent trancriptional activation of the *cyclin E* promoter was measured. Co-expression of cyclin E and CDK2 expression vectors into U2OS cells resulted in a dose dependent rise in E2F-5 transcriptional activity (Fig 6.4a). This stimulation was markedly reduced when E2F-5 was replaced by T251A in the transfection. The same effect was observed when the transfection was repeated in SAOS2 cells (Fig 6.4b, compare lanes 5,6 with 10,11). In extracts from these cells the appearance of the slower mobility form of E2F-5, E2F-5U, correlated with the enhanced trancriptional activity (Fig 6.4b, lanes 5 and 6).

CyclinD1-CDK4 also stimulated the transcriptional activity of wild-type E2F-5, but consistently to a lesser degree than cyclinE-CDK2 (Fig 6.4b, lanes 3 and 4). However cyclinD1-CDK4 and cyclinE-CDK2 had very similar effects on T251A dependent transcriptional activation (Fig 6.4b, lanes 8-11). To determine whether there was any change in the mobility of E2F-5 upon co-expression of cyclinD1-CDK4, extracts from this reporter assay were also immunoblotted with anti-E2F-5. CyclinD1-CDK4 did not induce a shift in mobility of E2F-5 when cotransfected with E2F-5 (Fig 6.4b, lanes 3 and 4), although cyclinD1-CDK4 was able to reverse p107 induced repression, confirming that an active kinase was expressed (see Fig 6.5). This suggests that the G1 kinases have a general effect stimulating E2F activity, for example through the phosphorylation of endogenous pocket proteins. In addition cyclinE-CDK2 has a specific effect which depends on the integrity of the T251 site in E2F-5.

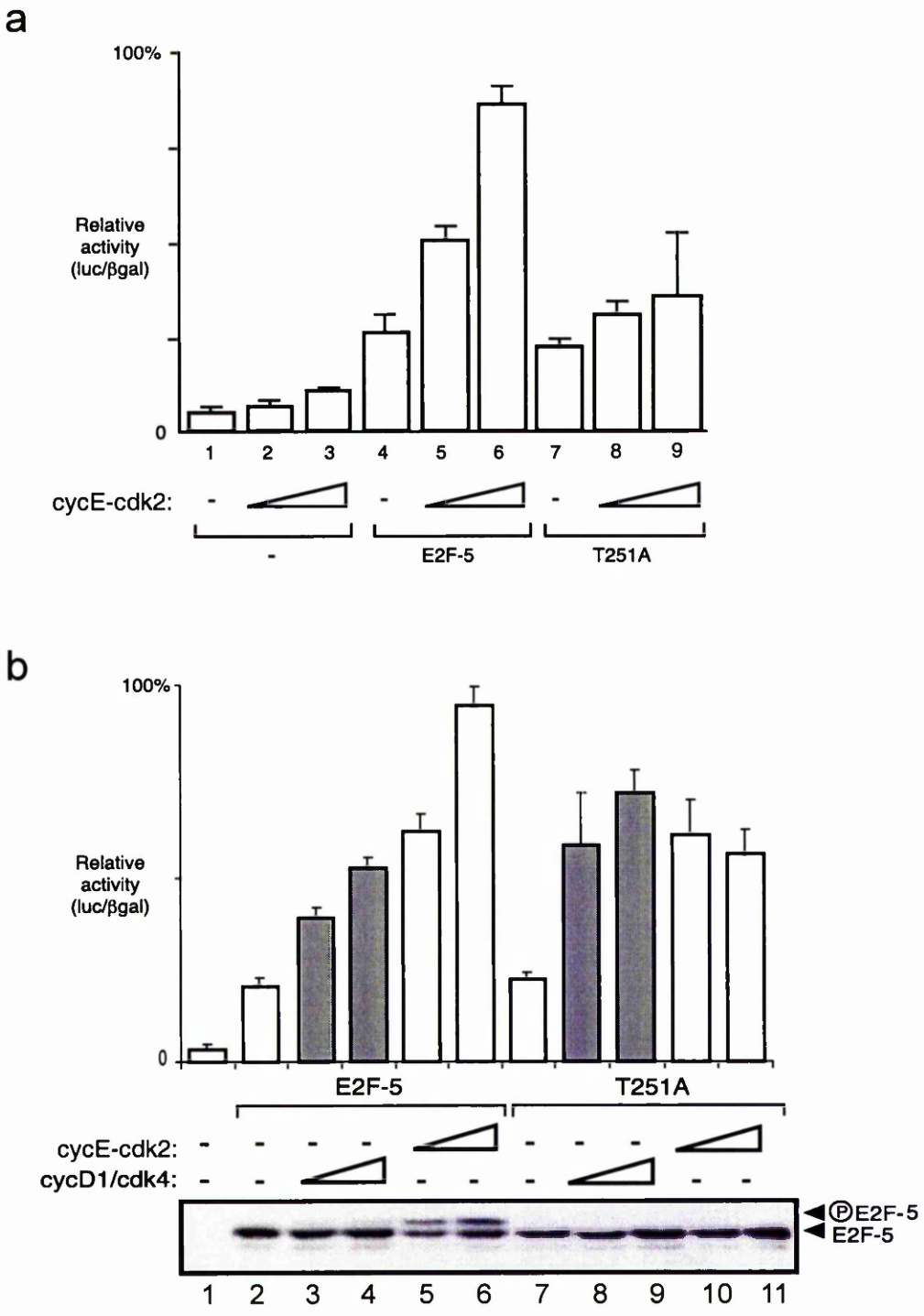
Figure 6.4

CyclinE-CDK2 stimulates E2F-5-dependent transcription.

(a) U2OS cells were transfected with the cyclinE-luciferase reporter (2μg) together with expression vectors for HA-E2F-5 or HA-T251A (2μg) in the presence of increasing levels of cyclinE-CDK2 (1 or 2μg of each vector) as indicated; an expression vector for wild-type DP-1 (2μg) was transfected throughout.

(b) U2OS cells were transfected with the cyclinE-luciferase reporter (2μg) together with expression vectors for HA-E2F-5 or HA-T251A (2μg), as indicated. Expression vectors for cyclinD1-CDK4 (1 or 2μg of each vector) and cyclinE-CDK2 (1 or 2μg of each vector) were also transfected as indicated; an expression vector for wild-type DP-1 (2μg) was transfected throughout. Transfected cells were harvested and immunoblotted with anti-HA antisera. The levels of E2F-5 are shown in the lower panel.

In both (a) and (b) pCMV-β gal (1μg) was transfected as an internal control, and the values shown are the means of three separate readings (+ or - s.d.), and represent the ratio of luciferase to β-galactosidase activity.


a
100%
Relative activity (luc/βgal)
0
1 2 3 4 5 6 7 8 9
cycE-cdk2: - - - - - -
-
E2F-5
T251A
b
100%
Relative activity (luc/βgal)
0
E2F-5
T251A
cycE-cdk2: - - - - - - - - -
cycD1/cdk4: - - - - - - - - -
ⓅE2F-5
E2F-5
1 2 3 4 5 6 7 8 9 10 11

6.5 CyclinD1-CDK4 but not cyclinE-CDK2 reverses p107 mediated repression of Gal4-E2F5

The data presented above suggests that phosphorylation of E2F-5 by cyclinE-CDK2 facilitates E2F-5 dependent *trans*activation. A possible mechanism to explain this effect is that phosphorylation of E2F-5 contributes to the release of endogenous pocket proteins, analogous to the phosphorylation of pocket proteins leading to their dissociation from E2F. To investigate this idea, cyclinE-CDK2 and cyclinD1-CDK4 were assessed for their ability to overcome p107-mediated repression of E2F-5. Transfection of SAOS2 cells with Gal4E1B-luciferase and the Gal4-E2F-5 expression vector resulted in approximately 200-fold stimulation of reporter activity, which was repressed by 90% upon co-expression of p107 (Fig 6.5a/b, lane 2 and 6). This repression was relieved by cyclinD1-CDK4 but not by cyclinE-CDK2, although both kinases resulted in activation of Gal4-E2F-5 (Fig 6.5a/b, compare lanes 3-6 with lanes 7-9). This suggests cyclinD1-CDK4 is regulating E2F-5 indirectly, possibly through release of endogenous pocket proteins, whereas cyclinE-CDK2 activates E2F-5 through an alternative mechanism. As cyclinE-CDK2 augments transactivation of wild-type E2F-5 and not T251A, one mechanism may involve direct phosphorylation of threonine 251 in E2F-5 by cyclinE-CDK2.

6.6 CyclinE-CDK2 phosphorylation of E2F-5 does not alter its affinity for DNA or pocket proteins

Previously it was shown that endogenous E2F-5 polypeptides, E2F-5U and E2F-5M, did not differ in their DNA binding ability. Although exogenous E2F-5 resulting from overexpression of E2F-5 and cyclinE-CDK2 corresponds in its mobility

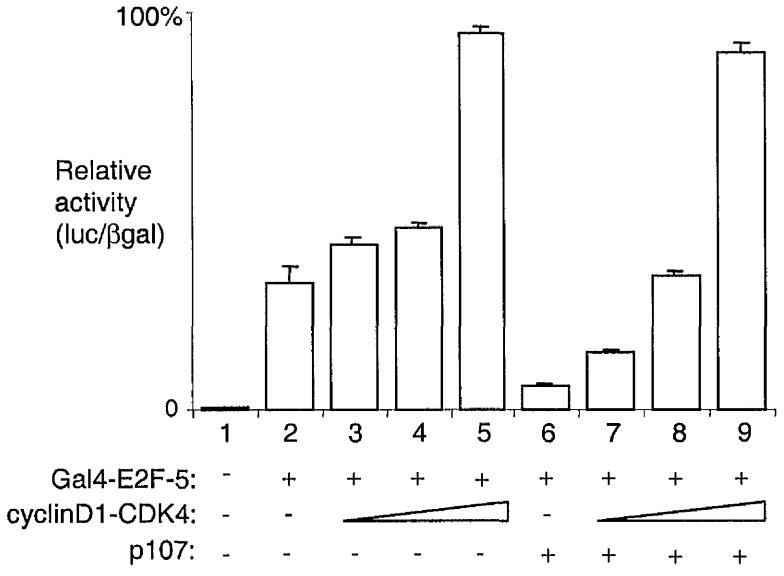
Figure 6.5

CyclinD1-CDK4 but not cyclinE-CDK2 reverses p107 mediated repression of Gal4-E2F-5.

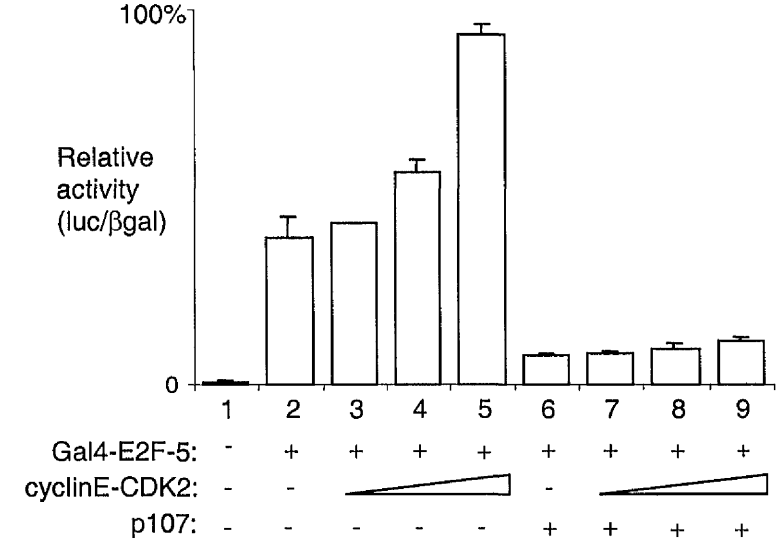
U2OS cells were transfected with the Gal4E1B-luciferase reporter (1μg), together with expression vectors for Gal4-E2F-5 (100ng), p107 (200ng) and either:
(a) CyclinD1-CDK4 (0.2, 0.4, 0.8μg of each expression vector).
(b) CyclinE-CDK2 (0.2, 0.4, 0.8μg of each expression vector).

In both (a) and (b) pCMV-βGal (1μg) was transfected throughout as an internal control. Values shown are the means of two separate readings and represent the ratio of luciferase to β-galactosidase activity.

a


100%
Relative activity (luc/βgal)
0
1 2 3 4 5 6 7 8 9
Gal4-E2F-5: - + + + + + + + +
cyclinD1-CDK4: - - - -
p107: - - - - - + + + +

b


100%
Relative activity (luc/βgal)
0
1 2 3 4 5 6 7 8 9
Gal4-E2F-5: - + + + + + + + +
cyclinE-CDK2: - - - -
p107: - - - - - + + + +

to E2F-5U and E2F-5M it may not be post-translationally modified in exactly the same way. Thus U2OS cell extracts, transfected with expression vectors for E2F-5 and cyclinE-CDK2, were subjected to gel retardation analysis to determine whether the effect of cyclinE-CDK2 on E2F-5-dependent *trans*activation correlated with the level of E2F-5 bound to DNA. There was no change in DNA binding activity of either wild-type E2F-5 or T251A upon co-expression of cyclinE-CDK2 (Fig 6.6a, lanes 1,2 and 5,6). Thus phosphorylation of E2F-5 by cyclinE-CDK2 does not increase the affinity of E2F-5 for DNA.

Gel retardation assays were carried out to determine whether the hyper-phosphorylated E2F-5 present in cells transfected with E2F-5 and cyclinE-CDK2 interacted with pocket proteins. A discrete p130 complex was resolved in extracts transfected with expression vectors for E2F5, DP-1 and p130, which disappeared upon co-expression of cyclinE-CDK2 (Fig 6.6a, lanes 3 and 4). CyclinE-CDK2 had a similar effect when transfected with T251A, DP-1 and p130 and thus the effect of cyclinE-CDK2 on p130 did not depend on the integrity of the threonine 251 site. Furthermore there was an increase in E2F-5/T251A levels when p130 was co-transfected which dropped upon co-transfection of cyclinE-CDK2 (Fig 6.6b, lane 3 and 4). Recent studies have suggested that E2F is stabilised by binding to pocket proteins (Hateboer *et al.*, 1996; Hofmann *et al.*, 1996). The results presented here are consistent with p130 stabilising E2F-5 and cyclinE-CDK2 dissociating the E2F-5/p130 complex as a consequence of p130 phosphorylation, detected as a reduction in E2F-5 levels and the absence of a E2F-5/p130 complex in the bandshift analysis. The transfection efficiency was similar throughout because the levels of a coexpressed B-galactosidase plasmid, the internal control, were unchanged (Fig 6.6b).

Figure 6.6

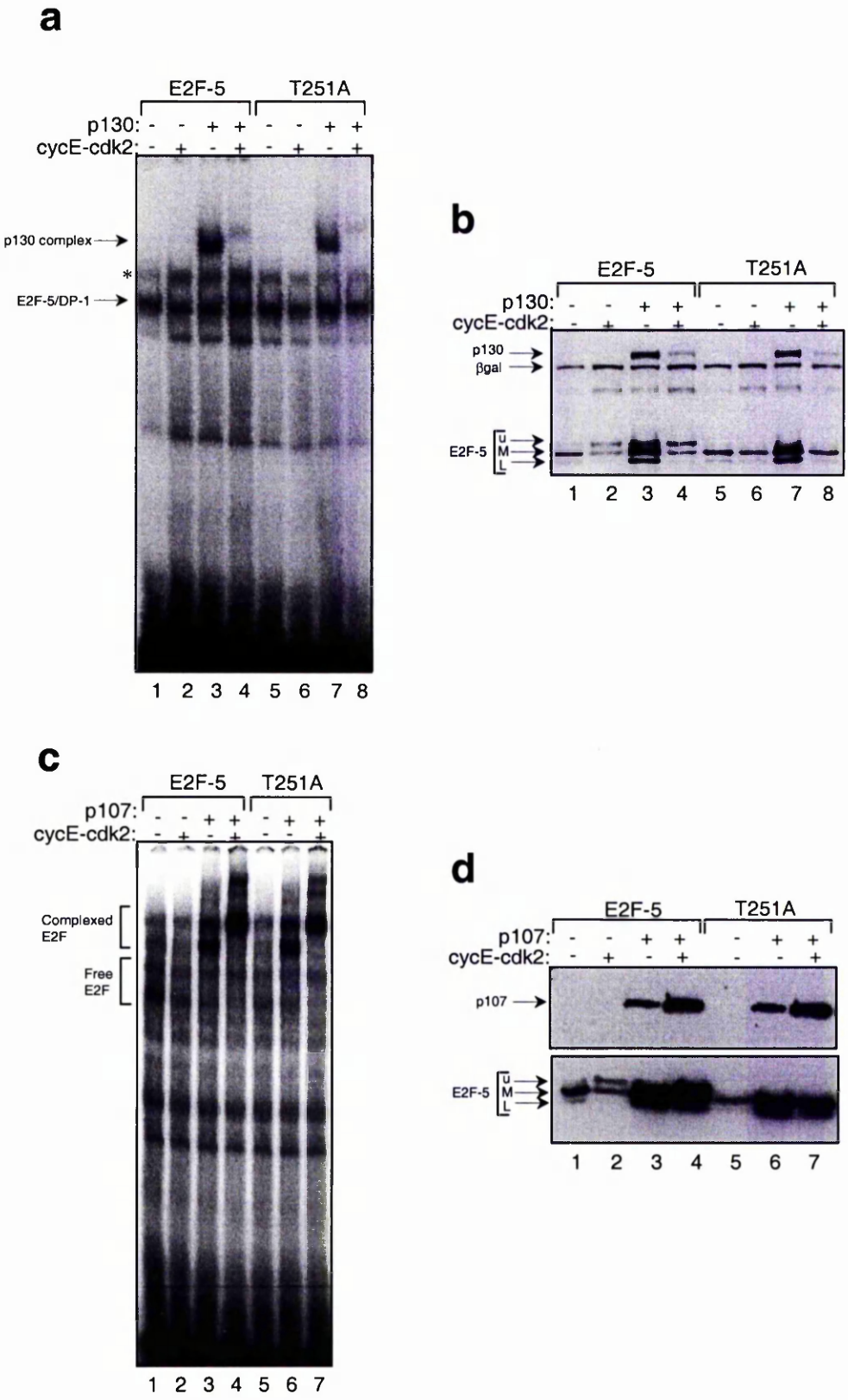
CyclinE-CDK2 phosphorylation of E2F-5 does not affect the ability of E2F-5 to bind to DNA or pocket proteins.

(a) U2OS cells were transfected with expression vectors for HA-tagged E2F-5 (4μg), HA-tagged T251A (4μg), HA-tagged p130 (4μg) and cyclinE-CDK2 (4μg of each vector). Expression vectors for DP-1 (4μg) and β-galactosidase (2μg) were transfected throughout. Extracts were prepared and analysed by gel retardation using the E2F site from the adenovirus E2a promoter (see Materials and Methods). The positions of the E2F-5/DP-1 heterodimer and the E2F/p130 complex, resulting from overexpression, are indicated. The asterisk indicates a complex that is present in untransfected U2OS cells and therefore may be an endogenous E2F complex.

(b) Cell extracts from (a) were immunoblotted with an anti-HA monoclonal antibody and anti-βgalactosidase (see Materials and Methods). The position of βgalactosidase, HA-tagged E2F-5 and HA-tagged p130 are indicated.

(c) U2OS cells were transfected with expression vectors for HA-tagged E2F-5 (4μg), HA-tagged T251A (4μg), p107 (4μg) and cyclinE-CDK2 (4μg of each vector). Expression vectors for DP-1 (4μg) and β-galactosidase (2μg) were transfected throughout. Extracts were prepared and analysed by gel retardation using the E2F site from the adenovirus E2a promoter (see Materials and Methods). The positions of 'free' E2F and E2F complexed to pocket proteins are indicated. Note the increase in intensity of the bands corresponding to complexed E2F, upon expression of p107 and the fall in intensity of free E2F (compare lanes 1,2 with lanes 3,4). Due to the low stringency of the buffer, several non-specific complexes were also detected.

(d) Cell extracts from (c) were immunoblotted with an anti-HA monoclonal antibody and anti-p107 (SD9) (see Materials and Methods). Note that the level of p107 (lanes 3,4,6,7) corresponds to the increase in intensity of complexed E2F in (c) (lanes 3,4,6,7).


a
E2F-5
T251A
p130:
cycE-cdk2:
p130 complex
*
E2F-5/DP-1
1 2 3 4 5 6 7 8
b
E2F-5
T251A
p130:
cycE-cdk2:
p130
βgal
E2F-5
U
M
L
1 2 3 4 5 6 7 8
c
E2F-5
T251A
p107:
cycE-cdk2:
Complexed E2F
Free E2F
1 2 3 4 5 6 7
d
E2F-5
T251A
p107:
cycE-cdk2:
p107
E2F-5
U
M
L
1 2 3 4 5 6 7

When the gel retardation analysis was repeated with p107 in place of p130 the outcome was different. Previous attempts to detect E2F-5/p107 complexes by gel retardation had not been successful. Therefore the KCl levels in the gel retardation buffer were reduced from 100mM to 75mM to reduce the stringency of the assay. In these conditions a higher order complex was detected when p107 was expressed together with E2F-5 and DP-1 and this was retarded further upon co-expression of cycE/CDK2 (Fig 6.6c, lanes 3 and 4). Complexes containing E2F-4/p107 and cyclinE-CDK2 have been detected in endogenous cell extracts during S phase in several studies (Lees *et al.*, 1992; Shirodkar *et al.*, 1992; Cobrinik *et al.*, 1993). Thus it is likely that overexpression of these proteins had reconstituted this S phase complex, containing E2F-5 in place of E2F-4, as both of these E2F's are capable of binding p107. There was no difference in the abundance of these complexes in cells transfected with E2F-5 or T251A. Furthermore the levels of E2F-5/T251A and p107 in western blot analysis of these extracts correlated with the level of E2F-5/T251A complexed to p107 (Fig 6.6d). To conclude, there appeared to be no apparent difference in the ability of wild-type E2F-5 or T251A to bind pocket proteins in the presence of cyclinE-CDK2.

6.7 T251D mimics phosphorylation of E2F-5 by cyclinE-CDK2

To investigate whether introducing a negative charge on threonine 251 could mimic phosphorylation at this site, an aspartate derivative of E2F-5, designated T251D, was constructed. T251D had a slower mobility than E2F-5 on SDS-PAGE and underwent no further mobility shift upon co-transfection of cyclinE-CDK2 (Fig 6.7a). T251D was resolved as two polypeptides on SDS-PAGE, which were almost equivalent in their mobility to the two forms resolved when E2F-5 and cyclinE-CDK2

Figure 6.7

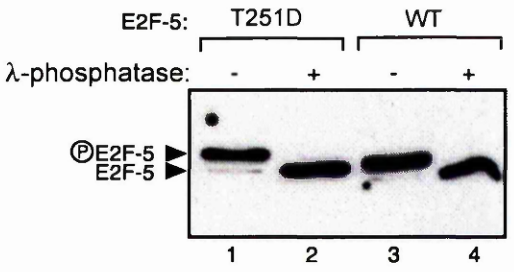
T251D mimics phosphorylation of E2F-5 by cyclinE-CDK2.

(a) U2OS cells were transfected with 5μg of DP-1 and either HA-tagged E2F-5 (5μg) or HA-tagged T251D (5μg) and extracts were prepared and treated with λ-phosphatase (lane 2 and 4). Control reactions were treated in the same way except λ-phosphatase was omitted from the reaction mixture (lane 1 and 3). E2F-5 was detected by immunoblotting with anti-HA monoclonal antibody.

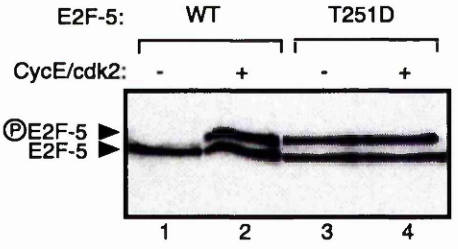
(b) U2OS cells were transfected with expression vectors for HA-tagged E2F-5 (2μg) or HA-tagged T251D (2μg) together with DP-1 (2μg) and cyclinE-CDK2 (2μg of each vector) as indicated. Extracts were prepared from transfected cells and subjected to immunoblotting with an anti-HA monoclonal antibody.

(c) U2OS cells were transfected with the cyclinE-luciferase reporter (1μg) together with expression vectors for wild-type HA-E2F-5 or HA-T251D as indicated, and DP-1 (750ng), with pCMV-β-gal (1μg) transfected as an internal control. The values shown are the average of three separate readings, and represent the ratio of luciferase to β-galactosidase.

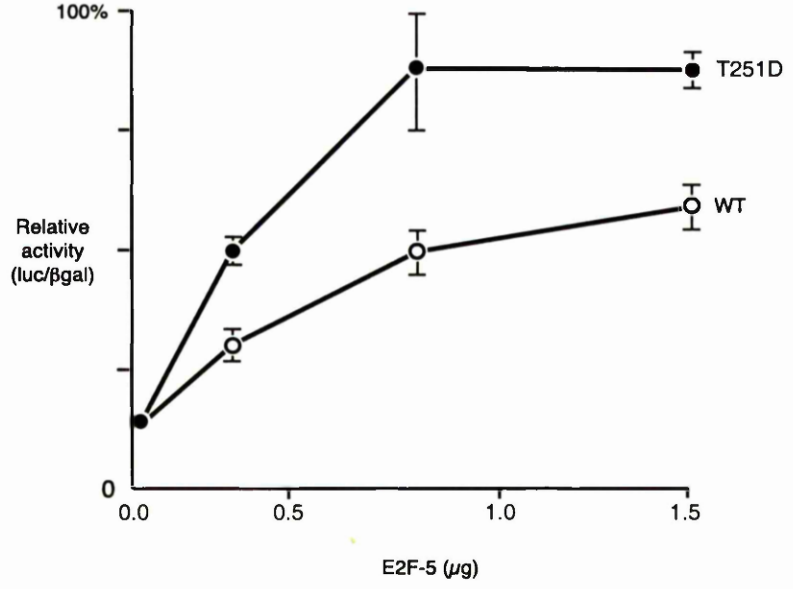
a


E2F-5:
T251D
WT
λ-phosphatase:
-
+
-
+
ⓅE2F-5
E2F-5
1
2
3
4

b


E2F-5:
WT
T251D
CycE/cdk2:
-
+
-
+
ⓅE2F-5
E2F-5
1
2
3
4

c


100%
0
Relative activity (luc/βgal)
T251D
WT
0.0
0.5
1.0
1.5
E2F-5 (μg)

were over-expressed, suggesting phosphorylation on threonine 251 occurs in both of these forms. Furthermore T251D can still undergo a mobility shift upon treatment with λ-phosphatase, indicating the form corresponding to E2F-5U is phosphorylated on at least one additional site, apart from threonine 251. Results presented in the previous chapter, using the S307D mutant, suggest one of the other phosphorylation sites is likely to be serine 307. The mobility of S307D in a SDS gel was not effected by λ-phosphatase treatment. Thus, as the S307 phosphoacceptor site remains intact in wild-type E2F-5 and T251D, it can be inferred that dephosphorylation of serine 307 may be responsible for the response of E2F-5 and T251D to phosphatase.

T251D is more active than E2F-5 in co-operation assays with DP-1, although the affect of the aspartate mutation is not as striking as the induction by cyclinE-CDK2 on wild-type E2F-5 (Fig 6.7c). This suggests that the negative charge on threonine 251 as a result of phosphorylation is at least partly responsible for the effect of cyclinE-CDK2 on E2F-5 transcriptional activity.

6.8 Phosphorylation of E2F-5 promotes cell cycle progression

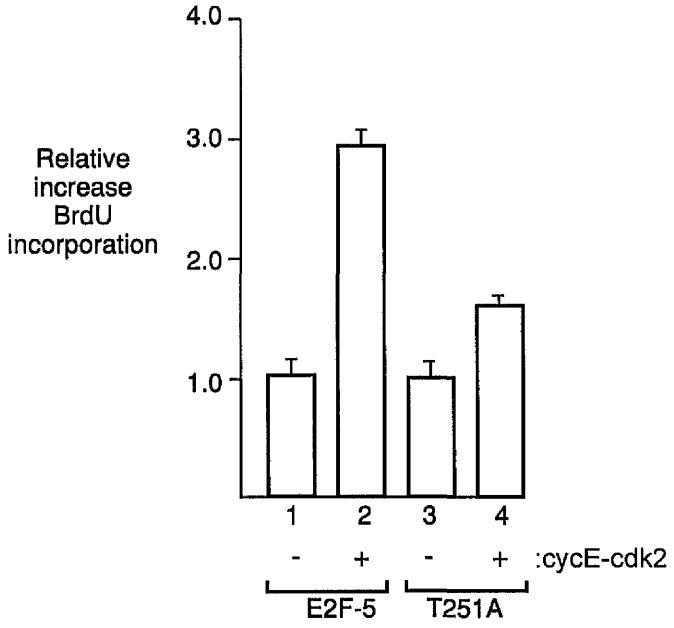
The incorporation of BrdU into cells was used to assess the ability of E2F-5 and cyclinE-CDK2 to stimulate entry into S phase. Co-expressing cyclinE-CDK2 with E2F-5 in U2OS cells caused a 3-fold induction of BrdU incorporation relative to E2F-5 alone, whereas the increase in S phase cells was far less marked when T251A was co-expressed with cyclinE-CDK2 (Figure 6.8). This result suggests that the phosphorylation of the threonine 251 in E2F-5 by cyclinE-CDK2, promotes cell cycle progression.

Figure 6.8

Phosphorylation of E2F-5 by cyclinE-CDK2 promotes cell cycle progression.

(a) U2OS cells were transfected with HA-E2F-5 or HA-T251A (2μg) together with DP-1 (2μg) in the presence or absence of cyclinE-CDK2 (2μg of each vector) as indicated. BrdU incorporation was measured as described, and immunostaining with the anti DP-1 antibody was performed in parallel to determine the proportion of double positive cells. In the absence of cyclinE-CDK2, the level of BrdU incorporation was similar for E2F-5 and T251A (about 20% of the transfected cell population). The data shown are a representative example of four different experiments.

a


Relative increase BrdU incorporation
4.0
3.0
2.0
1.0
1
2
3
4
-
+
-
+
:cycE-cdk2
E2F-5
T251A

6.9 Conclusion

In this chapter E2F-5 but not T251A was phosphorylated *in vitro* by cyclinE-CDK2. This suggests that threonine 251 is either the only target site for cyclinE-CDK2 in E2F5 or that phosphorylation on this site is required prior to phosphorylation of other CDK sites within E2F5 by cyclinE-CDK2. E2F-5 can also be phosphorylated *in vivo*, as detected by incorporation of ^{32}P-orthophosphosphate and an E2F-5 mobility shift induced in extracts of cells transfected with cyclinE-CDK2 and E2F-5. Furthermore CnBr cleavage mapped the site of phosphorylation in E2F-5 *in vivo* to a peptide where the only CDK site is positioned at threonine 251. Evidence that cyclinE-CDK2 phosphorylation of endogenous E2F-5 occurs in a regulated fashion, and is thus not dependent on manipulating the levels of E2F-5 and cyclinE-CDK2, was shown by cell cycle analysis of Rat1 cells presented in chapter 3. The appearance of E2F-5U during the Rat1 cell cycle corresponded to the time when cyclinE-CDK2 became active and is consistent with the phosphorylation of E2F-5 on threonine 251. Thus E2F-5 is likely to be a physiological target for cyclinE-CDK2.

The transcriptional activity of E2F-5 is enhanced by cyclinE-CDK2 when assayed on a cyclinE-luciferase reporter, a well-studied E2F-regulated gene. These observations enable an attractive model to be proposed, where a positive feedback loop exists between E2F-5 and cyclin E. E2F-5 transcriptionally activates the *cyclin E* promoter, causing a surge in cyclin E protein levels which can, on complexing with CDK2, further feedback on E2F-5 transcriptional activity and thereby enhance its own levels, to promote irreversible entry into S phase (see Chaper 8 for discussion and Fig 8.1). The mechanism of transcriptional activation by cyclinE-CDK2 phosphorylated E2F-5 is explored in the next chapter.

Chapter 7

Regulation of E2F-5 by p300/CBP co-activators

7.1 Introduction

The p300/CBP family of co-activators are transcriptional adapters that interact with diverse transcription factors and facilitate *trans*activation (Shikama *et al*, 1997). They are part of multi-protein complexes that connect the activation domains of transcription factors with the transcription apparartus. Both p300 and CBP possess intrinsic acetyl transferase activity, which can acetylate histones and a number of non-histone proteins, such as p53 and TFIIE (Gu and Roeder, 1997; Imhof *et al.*, 1997). Acetylation of histones modifies chromatin increasing the accessibility of DNA for transcription factors (Grunstein, 1997), whilst acetylation of p53 is believed to facilitate transcription by increasing the DNA binding activity of p53 (Gu and Roeder, 1997).

Given that the p300/CBP family can bind to E2F-1 and that CBP is an important co-activator for E2F-1 regulation of the *DHFR* promoter (Trouche and Kouzarides, 1996; Lee *et al.*, 1998; Fry *et al.*, 1999), it is possible that E2F-5 dependent transcription also utilises p300/CBP. Therefore this chapter explores the importance of p300/CBP in E2F-5-dependent transcription.

Evidence presented in the previous two chapters implied that phosphorylation of E2F-5 on serine 307 and threonine 251 enhanced E2F-5-dependent transcription. To elucidate the mechanism responsible for these observations the possibility that phosphorylation of E2F-5 may regulate the interaction of the activation domain with p300/CBP was considered.

7.2 E2F-5 interacts with p300

A variety of approaches were taken to assess the role of p300 in E2F-5-dependent transcription. A mammalian two-hybrid assay was employed to determine whether E2F-5 interacted with p300. E2F-5, T251A and S307A (residues 90-335) were fused to VP16 *trans*activation domain to construct VP16-E2F-5, VP16-T251A and VP16-S307A. A construct consisting of full-length p300 fused to the Gal4 DNA-binding domain (Gal4-p300) was used as 'bait'. Expression of Gal4-p300 had an insignificant effect on the *trans*activation ability of the Gal4-E1b-luciferase reporter in U2OS cells (Fig 7.1a, compare lane 1 and 2). However, co-expression of VP16-E2F-5 with Gal4-p300 caused a 10-fold induction in reporter activity (Fig 7.1a, compare lane 1 with lanes 6-8). This induction was not due to an interaction between Gal4-p300 and the VP16 *trans*activation domain, as these two proteins did not significantly activate the reporter (Fig 7.1a, compare lane 2 and 15). VP16-T251A and VP16-S307A also interacted with the Gal4-p300 bait although the induction in reporter activity was marginally less than that of wild-type E2F-5 (Fig 7.1a, compare lanes 6-14). These data indicate that E2F-5 and the mutant derivatives interact with p300, although it does not establish whether the interaction is direct.

To determine whether endogenous E2F-5 and p300 were physically associated in cells Rat1 cell extract, prepared from asynchronous cells, was immunoprecipitated with anti-E2F-5. Western blot analysis and subsequent immunoblotting with an anti-p300 N15 rabbit polyclonal antiserum (Santa Cruz) revealed the presence of p300 in the E2F-5 immunocomplex (Fig 7.1b). Moreover the interaction between E2F-5 and p300 was specific as it was prevented by pre-incubation of the anti-E2F-5 antibody with the homologous peptide (Fig 7.1b, compare lane 2 and 3).

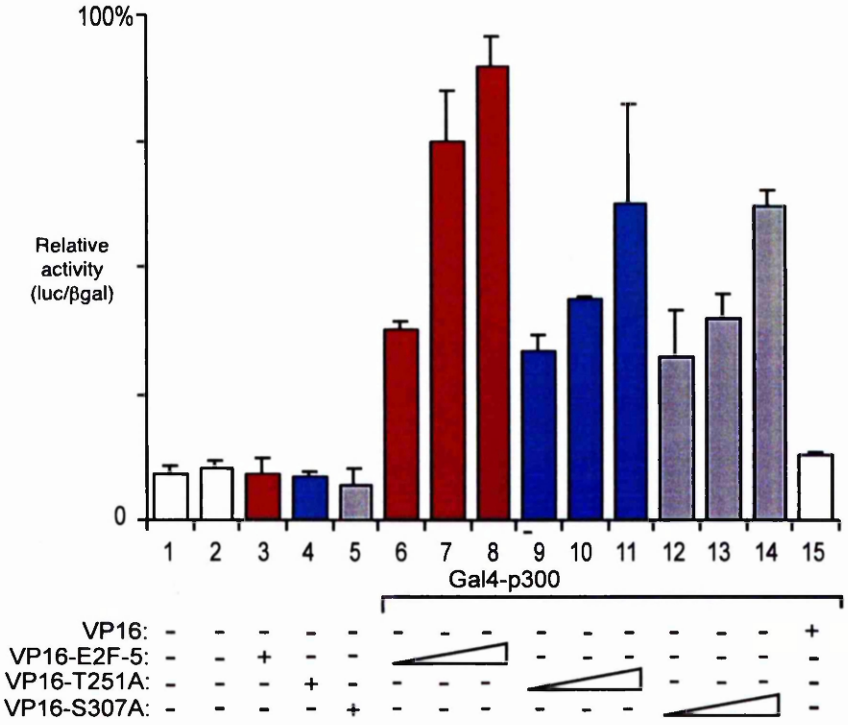
Figure 7.1

p300 interacts with E2F-5 *in vivo.*

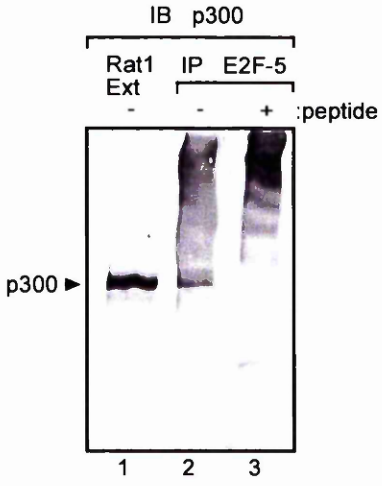
(a) Mammalian two-hybrid interaction assay between between p300 fused to Gal4 DNA binding domain and E2F-5, T251A, or S307A (residues 90-335) fused to the *trans*activation domain of VP16 (see Materials and Methods). The quantities of expression plasmids used were: Gal4-p300 (0.5μg) and VP16-E2F-5/ VP16-T251A/ VP16-S307A (0.25, 0.5, 1.0 μg), with 1.0 μg of the indicated VP16 construct in lanes 3, 4 and 5. The Gal4-p300 bait was co-expressed with VP16TAD alone (1.0 μg) in lane 15. The Gal4-E1B-luciferase reporter (1μg) and pCMV-βGal (1μg) were transfected throughout and the results are presented as a ratio of luciferase activity to β–Galactosidase activity and are means of three separate readings (+/-s.d.).

(b) Extracts prepared from asynchronously growing Rat1 cells were immunoprecipitated with anti-E2F-5 peptide antiserum in the presence (+) and absence (-) of competing E2F-5 peptide. The immunoprecipitate was resolved by SDS-PAGE and subsequently immunoblotted with the anti-p300 antibody (see Materials and Methods). Track 1 shows the input (5%) reactivity of the anti-p300 with the crude Rat1 extract.

a


100%
0
Relative activity (luc/βgal)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Gal4-p300
VP16:
VP16-E2F-5:
VP16-T251A:
VP16-S307A:

b


IB p300
Rat1 Ext
IP E2F-5
:peptide
p300
1 2 3

7.3 Role of p300 in E2F-5 dependent transcriptional activation

To determine whether endogenous p300/CBP proteins are functionally important for E2F-5 activity *in vitro* the effects of a truncated p300 molecule, namely $p300^{1572\text{-}1903}$ were studied. As residues 1572 to 1903 of p300 harbour the E2F interaction domain it was anticipated that $p300^{1572\text{-}1903}$ would act in a dominant-negative fashion by competing with endogenous p300 for E2F-5 activation domain. Expressing $p300^{1572\text{-}1903}$ caused a marked reduction in the E2F-5-dependent activation of the *cyclinE* promoter without affecting the level of E2F-5 protein (Figure 7.2a). In a similar type of experiment, expression of $p300^{1572\text{-}1903}$ also compromised the transcriptional activity of the Gal4-E2F-5 hybrid protein (Figure 7.2b). These data establish that p300/CBP proteins are important effectors of E2F-5-dependent transcription *in vitro*.

7.4 Phosphorylation of E2F-5 on threonine 251 augments its interaction with p300

Data presented in the previous two chapters revealed that phosphorylation of E2F-5 on threonine 251 and serine 307 augment E2F-5 transcriptional activity. Given that p300/CBP proteins are required for E2F-5-dependent transcription, the phosphorylation of threonine 251 or serine 307 may enhance the interaction of p300 with the E2F-5 activation domain. The mammalian two-hybrid assay showed that the mutants, T251A and S307A can still interact with p300 , albeit more weakly. The individual phosphorylation status of each of these mutant derivatives is not known when expressed as VP16 fusion proteins *in vivo*. Therefore a biochemical-binding

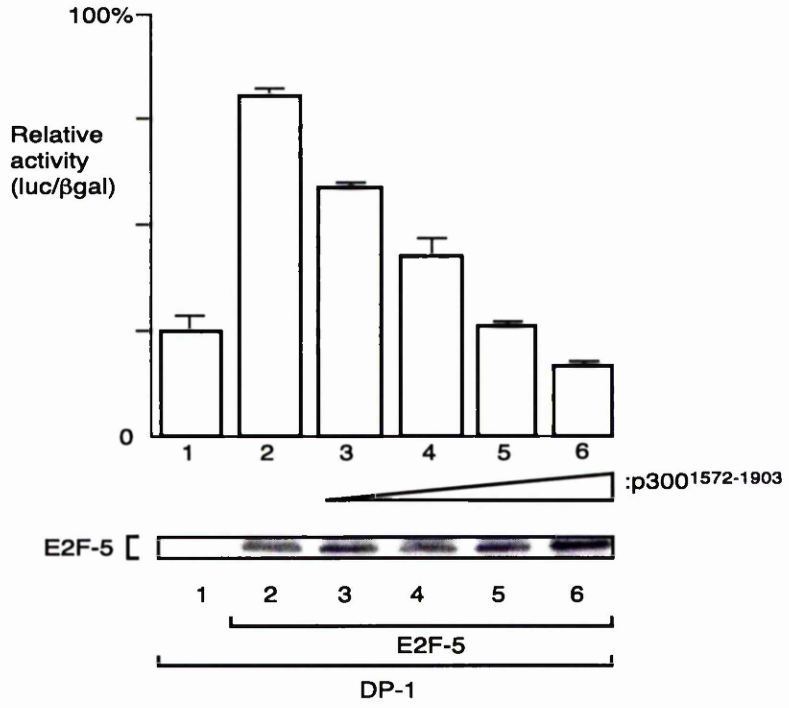
Figure 7.2

p300 is important for E2F-5 dependent transcription.

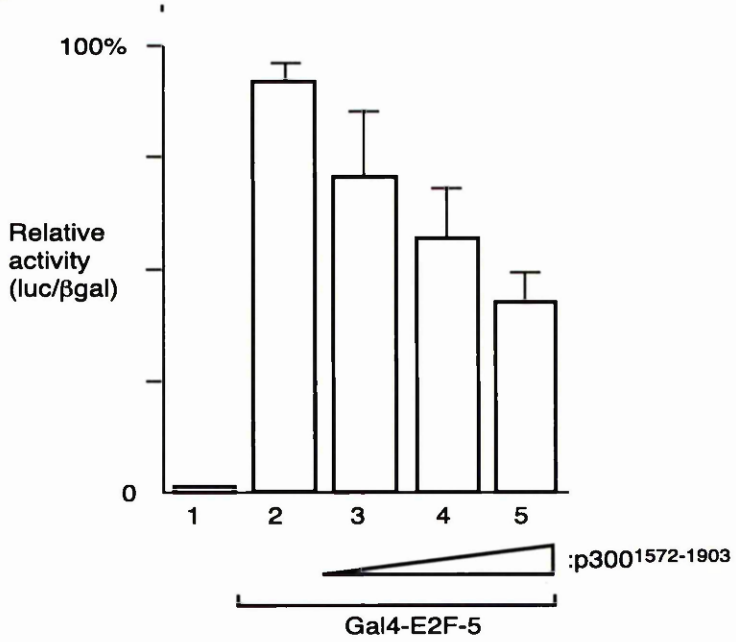
(a) U2OS cells were transfected with the cyclinE-luciferase reporter (2μg) together with expression vectors for wild-type HA-E2F-5 (2μg) and DP-1 (2μg) in the presence of increasing $p300^{1572-1903}$ (2, 4, 6 and 8μg), as indicated. pCMV-β-gal (1μg) was transfected as an internal control, and the values calculated as a ratio of luciferase to β-galactosidase activity and presented as the means of three separate readings (+/- s.d.). Transfected cells were harvested and immunoblotted with anti-HA antiserum (shown underneath) to monitor the levels of E2F-5.

(b) U2OS cells were transfected with the pGal4-E1B-luc (2μg), together with expression vectors for Gal4-E2F-5 (150ng) and an increasing level of $p300^{1572-1903}$ (1, 2, and 4μg) as indicated. pCMV-β gal (1μg) was transfected as an internal control, and the values calculated from triplicate readings as described in (a).

a


100%
Relative activity (luc/βgal)
0
1
2
3
4
5
6
:p300 1572-1903
E2F-5
1
2
3
4
5
6
E2F-5
DP-1

b


100%
Relative activity (luc/βgal)
0
1
2
3
4
5
:p300 1572-1903
Gal4-E2F-5

assay, using purified $p300^{1135-2414}$ and *in vitro* phosphorylated recombinant E2F-5, was employed to explore the effect of E2F-5 phosphorylation on its interaction with p300.

An interaction between purified His-tagged E2F-5 and Flag-tagged $p300^{1135-2414}$ was detected, as the amount of E2F-5 bound to Flag-tagged $p300^{1135-2414}$ was greater than that bound to the Flag antibody control (Figure 7.3a, compare lane 5 and 6). In the previous chapter it was shown that cyclinE-CDK2 phosphorylates threonine 251, but the kinase responsible for phosphorylation of serine 307 is unknown. Thus it was decided to investigate whether phosphorylation of E2F-5 by cyclinE-CDK2 had an effect on the interaction between E2F-5 and p300. A shift in mobility of recombinant E2F-5 as a result of *in vitro* phosphorylation was readily detected by western blot analysis with anti-E2F-5 (Fig 7.3a, lane 1 and 2). This mobility shift did not occur when ATP was omitted from the *in vitro* phosphorylation reaction. This observation is in agreement with the data from the previous chapter concluding that cyclinE-CDK2 phosphorylation of E2F-5 causes a shift in its mobility in SDS-PAGE. To determine the p300 binding affinity of E2F-5 the ratio of the level of E2F-5 bound to Flag-p300 to level of E2F-5 bound to the Flag-antibody was calculated by measuring the intensity of the bands with a densitometer, in each of the different treatments (Fig 7.3b). Phosphorylation of E2F-5 by cyclinE-CDK2 enhanced the interaction between E2F-5 and p300 by between three to five-fold (Figure 7.4a, lanes 3 and 4). Furthermore, the interaction between E2F-5 and p300 required the integrity of the T251 CDK site, as in a similar binding assay, a specific interaction between the T251A mutant and p300 was not seen (Figure 7.3a and 7.3b). These data strongly suggest that phosphorylation of E2F-5 at threonine 251 by cyclinE-CDK2 augments transcription by enhancing the interaction of E2F-5 with p300/CBP co-activator proteins.

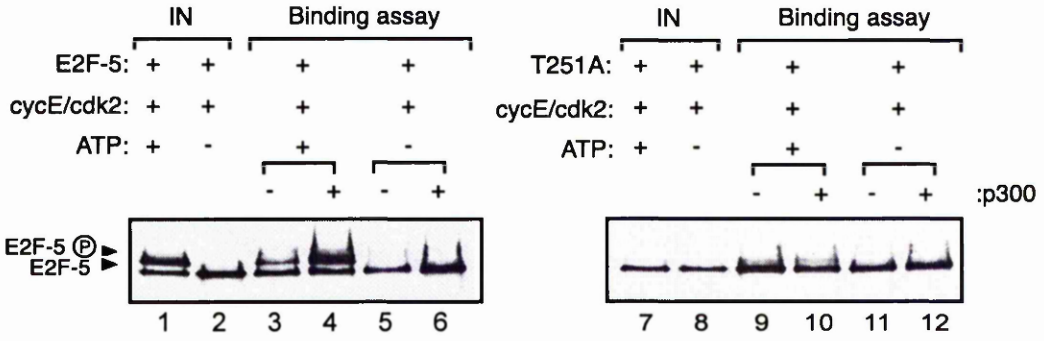
Figure 7.3

Phosphorylation of E2F-5 by cyclinE-CDK2 facilitates its interaction with p300.

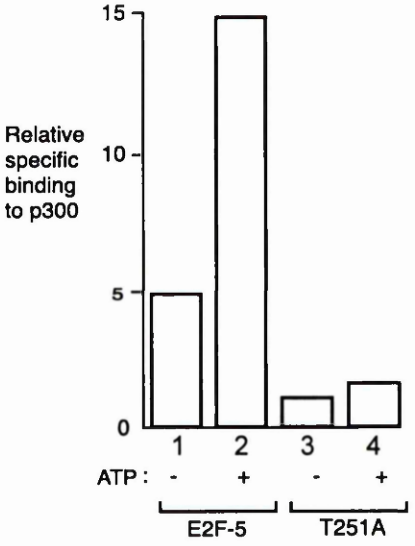
(a) The indicated input His-tagged E2F-5 or T251A (IN; tracks 1 and 2, 7 and 8) were treated as shown and thereafter immunoblotted with anti-E2F-5. Binding to flag-tagged-p300$^{1135\text{-}2414}$ (1μg) or flag-control beads was assessed for the pretreated input E2F-5 and T251A as indicated (lanes 3-6 and 9-12). The level of E2F-5 and T251A bound to flag-tagged-p300$^{1135\text{-}2414}$ or flag-control beads was determined by immunoblotting with anti-E2F-5. 1μg of E2F-5 or T251A was used in the binding assay; 100ng of E2F-5 and T251A were immunoblotted in lanes 1,2,7 and 8. Phosphorylated E2F-5, which is absent in the T251A mutant, is indicated.

(b) Quantification of the data shown in (a) where the level of pre-and post-phosphorylated E2F-5 and T251A bound to p300 is indicated. The relative specific binding represents the difference between the amount of E2F-5 or T251A bound to flag-tagged p300 compared to the flag-control beads. A value of 1.0 would indicate that there was no evidence of specific binding to p300.

a


IN
Binding assay
E2F-5:
cycE/cdk2:
ATP:
E2F-5 Ⓟ
E2F-5
1 2 3 4 5 6
IN
Binding assay
T251A:
cycE/cdk2:
ATP:
:p300
7 8 9 10 11 12

b


Relative specific binding to p300
15
10
5
0
1 2 3 4
ATP : - + - +
E2F-5
T251A

7.5 p300 acetylates E2F-5 *in vitro*

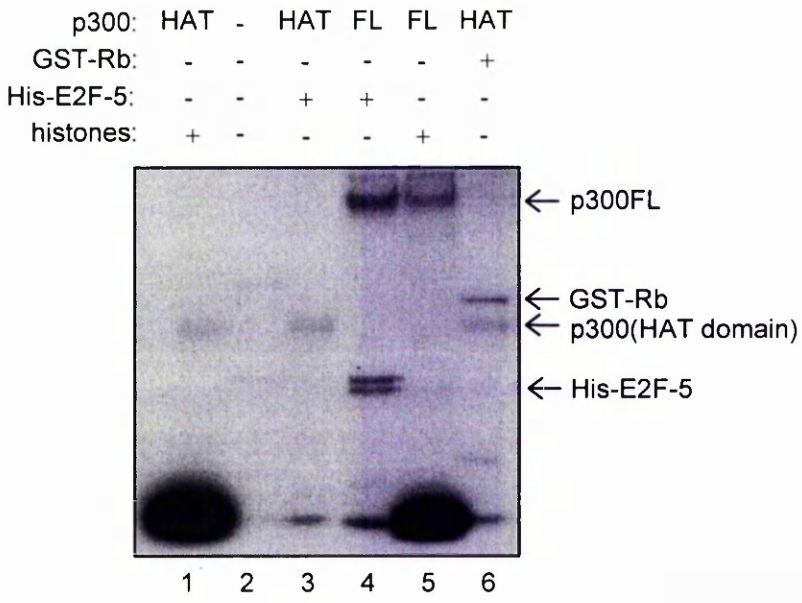
A recent study has reported that p/CAF (p300/CBP associated factor) and to a lesser extent p300 can acetylate E2F-1 (Martinez-Balbas *et al.*, 2000). Moreover acetylation of E2F-1 was shown to be involved in stimulating gene transcription. Thus E2F-5 acetylation may be important in the ability of E2F-5 to activate transcription. To determine whether E2F-5 could be acetylated, *in vitro* acetylation reactions were performed using purified flag-tagged full length p300 and his-p300 HAT domain (residues 1195-1673). Full-length p300 efficiently acetylated histones and to a lesser extent also acetylated E2F-5 (Fig 7.4a, lane 4 and 5). However the p300 HAT domain, whilst acetylating both histones and pRb (Fig 7.4a, lane 1 and 6), was unable to acetylate E2F-5 (lane 3), suggesting regions outside the HAT domain were necessary for p300 to acetylate E2F-5.

Figure 7.4

E2F-5 is acetylated by p300 *in vitro*.

In vitro acetylation was performed using full-length flag-p300 (100ng), GST-p300 (residues 1195-1673, encompassing the HAT domain) (100ng), GST-Rb (1.5μg), His-E2F-5 (1.5μg) and histones, as indicated (see Materials and Methods). The samples were resolved on SDS-PAGE and subjected to autoradiography for one week. FL: refers to Full-Length p300. HAT: refers to the HAT domain. Track 2 is a damaged lane and does not contain any relevant sample.

a


p300: HAT - HAT FL FL HAT
GST-Rb: - - - - - +
His-E2F-5: - - + + - -
histones: + - - - + -
p300FL
GST-Rb
p300(HAT domain)
His-E2F-5
1 2 3 4 5 6

7.6 Conclusion

Data presented in this chapter has demonstrated that p300 can interact directly with E2F-5. Moreover phosphorylation of E2F-5 on threonine 251 by cyclinE-CDK2 enhanced the interaction between E2F-5 and p300. A region of p300 that interacts with E2F-5, namely $p300^{1572\text{-}1903}$, blocked E2F-5 transcriptional activity implying E2F-5 dependent transcription utilises endogenous p300. Thus p300 is a critical effector protein involved in E2F-5 transcriptional regulation. Taken together these results suggest that phosphorylation of E2F-5 by cyclinE-CDK2 may augment transcription by stabilising the interaction between E2F-5 and p300.

E2F-5 was also shown to be a substrate for p300 acetyl transferase activity *in vitro*. It is possible that the acetylation of E2F-5 by p300 may be important in the mechanism of E2F-5 activation. Thus it will be interesting to determine whether the ability of E2F-5 to bind to p300 or the phosphorylation status of E2F-5 influences the level of E2F-5 acetylation.

Chapter 8

Discussion

The important role that E2F plays in the control of early cell cycle progression is mediated principally through regulating the activity of banks of genes necessary for entry into and through S phase. The activity of E2F must be tightly regulated in order to ensure the timely activation and repression of E2F target genes during cell cycle progression. In this study, the role of phosphorylation of the E2F-5 family member in the regulation of E2F activity has been investigated.

8.1 E2F-5 is an abundant component of E2F DNA binding activity

E2F-5 is an abundant component of 'free' E2F DNA binding activity in Rat1 fibroblasts, F9EC cells and leukaemic cell lines, for example DAUDI and RAGI cells (Chapter 3). Consequently, E2F-5 may make a significant contribution to the control of cell cycle progression and DNA synthesis in these cell lines. Previous studies have reported the selective induction of E2F-5 in various differentiated cells and the association of E2F-5 with p130 in G0 cells (Moberg *et al.*, 1996; Dagnino *et al.*, 1997). E2F-5 is structurally and functionally related to E2F-4. Given that E2F-4 is often associated with p107 *in vivo*, it is noteworthy that E2F-5 can be detected in p107 immunoprecipitates in RAGI cell extract (Fig 3.5b). However, E2F-5 was not a major component of DNA bound E2F/p107 complexes in Rat1 cell extracts (Fig 3.7c). The majority of E2F-5 in Rat1 extract bound to DNA as a 'free' heterodimer with DP-1. The distinct E2F/p107 complex observed in Rat1 cell extract must therefore be composed of another E2F family member, a likely candidate being E2F-4, as E2F-4 is the most abundant E2F found in a variety of cells types and is the only E2F that has

been reported to bind in significant amounts to p107 (Beijersbergen *et al.*, 1994; Ginsberg *et al.*, 1994; Moberg *et al.*, 1996). If this is the case, then there is likely to be a major difference in the regulation of E2F-4 and E2F-5 in Rat1 cells. However, it is possible that E2F-5/p107 complexes are present in Rat1 cell extracts but not in a form that is capable of binding to DNA in the conditions used in this assay.

The presence of a high level of 'free' E2F-5 DNA binding activity relative to E2F-5 complexed to pocket protein suggests that E2F-5 may make a significant contribution to the activation of E2F-target genes in Rat1 cells. Accordingly, the level of E2F-5/DP-1 was high at the G1-S transition and therefore would be expected to coincide with the induction of E2F target genes. However, it has been suggested that E2F-5, and the related E2F-4, are primarily involved in the repression of E2F-dependent genes in G0 and G1, through the recruitment of pocket proteins. Several studies support this idea, including the demonstration that E2F-5 is relatively abundant in quiescent and differentiated cells (Sardet *et al.,* 1995; Moberg *et al.*, 1996; Dagnino *et al.*, 1997). Furthermore, E2F-4 and E2F-5 do not contain a nuclear localisation signal (NLS), but can enter the nucleus when associated with p107 and p130 (Magae *et al.*, 1996; Buck *et al.*, 1997; Lindeman *et al.*, 1997). Although E2F-5 mRNA is prevalent in G0 and G1 cells, a notable increase in E2F-5 mRNA occurs towards the end of G1 phase in HaCat cells (Sardet *et al.,* 1995). A role for E2F-5 strictly as a repressor of genes in G0/G1 does not explain the function of the increase in E2F-5 expression towards the end of G1 phase. Moreover the oncogenic effect of E2F-5 and the increase in E2F-5 RNA in certain breast tumour cell lines (Polanowska *et al.,* 2000) are supportive of a role for E2F-5 as an activator of proliferation, presumably be *trans*activating E2F target genes required for cell cycle progression.

The results presented here demonstrate that 'free' E2F-5 DNA binding activity is present in late G1 and during S phase of the Rat1 cell cycle and therefore, once released from p130, E2F-5 may contribute to the activation of E2F target genes (Fig 3.7c). In this respect E2F-5 may differ from the closely related E2F-4, as E2F-4 may be largely present in a complex with p107 and in this form would not be capable of activating transcription (Shirodkar *et al.*, 1992; Moberg *et al.*, 1996). It is noteworthy that, although the level of E2F-5/DP-1 DNA binding activity remained relatively constant throughout the Rat1 cell cycle, an overall fall in the level of 'free' E2F DNA binding activity as cells entered S phase was observed (Fig 3.7c). The prevalence of E2F-5/DP-1 DNA binding activity during S phase in Rat1 cells may reflect a requirement of E2F-5/DP-1 for the regulation of a specific subset of E2F target genes that are transcribed in S phase, at a stage when other free E2F DNA binding activity has been down-regulated. Alternatively, it could be argued that inefficient cell synchronisation may account for the detection of E2F-5/DP-1 DNA binding activity in the later stages of S phase.

8.2 E2F-5 is a phosphorylated protein

Several lines of evidence indicate that E2F-5 is a phosphorylated protein. Firstly, multiple forms of E2F-5 with different electrophoretic mobilities exist in extracts from Rat1 cells and F9EC cells, and phosphatase treatment of these extracts causes a shift in these polypeptides to a faster mobility (Chapter 3). Specifically, three E2F-5 polypeptides, defined according to mobility as U, M and L, were detected. Secondly, mutant derivatives of E2F-5 that contain alanine substitutions at potential phosphorylation sites, namely S307A and T251A, exhibited different electrophoretic

mobilities in SDS-PAGE (Chapter 4). Finally, E2F-5 can be phosphorylated *in vitro* by cyclinE-CDK2 and metabolically labelled with ^{32}P-orthophosphate *in vivo* (Chapter 5).

The presence of exogenous E2F-5U, M and L in extracts from U2OS cells transfected with an E2F-5 expression vector was variable, suggesting that the expression of individual differentially phosphorylated E2F-5 polypeptides may be related to transfection efficiency. For example, good transfection efficiency results in high expression of exogenous E2F-5 and therefore it is possible that the availability of the endogenous kinase, responsible for the phosphorylation dependent mobility shift of E2F-5, becomes limiting. Despite unavoidable variations between transfections, E2F-5M was consistently observed as the most abundant exogenous E2F-5 polypeptide detected when wild-type E2F-5 and DP-1 were co-transfected (see Fig 4.1b, Fig 4.2b as examples). E2F-5U was always absent in cells transfected with T251A, whilst E2F-5L was the predominant polypeptide detected in cells transfected with S307A. This is consistent with E2F-5U being phosphorylated on threonine 251 and E2F-5M being phosphorylated on serine 307.

The identity of the sites phosphorylated in E2F-5U, M and L can be postulated by comparison of the mobility of T251D and S307D, both in the absence and presence of phosphatase. T251D resolved as a mixture of E2F-5U and E2F-5M, and upon treatment with phosphatase E2F-5U disappeared and E2F-5M increased. S307D resolved as E2F-5M and did not undergo a mobility shift upon treatment with phosphatase. Therefore, E2F-5M is most likely a mixture of polypeptides, either phosphorylated on threonine 251 or on serine 307, whilst E2F-5U polypeptides may be phosphorylated on both serine 307 and threonine 251 (Fig 8.1). Moreover, E2F-5L represents a hypophosphorylated E2F-5, as it increases in abundance on treatment of wild-type E2F-5 with phosphatase. This conclusion is by no means exhaustive as the

E2F-5 polypeptides may contain other post-translational modifications which this study has not addressed.

The relatively high level of E2F-5M compared to E2F-5L detected is cells transfected with wild-type E2F-5, suggests that the kinase responsible for the appearance of E2F-5M is not limiting. Thus, a kinase that is constitutively active in the various cell lines tested, rather than a cell cycle regulated kinase may be responsible for phosphorylation of serine 307.

The distribution of endogenous E2F-5L, M and U also varied in different cell lines. Rat1 cells contain predominantly E2F-5U and M, whereas in F9EC cells E2F-5U was barely detectable and forms M and L were most abundant. Thus, E2F-5 may be regulated by different kinases and phosphorylated on different sites in these two cell lines.

E2F-5	**Relative mobility on SDS-PAGE**	**Predicted phosphorylation status**
E2F-5U	Slowest	Phosphorylated on T251 and S307
E2F-5M	Intermediate	Mixture of polypeptides either phosphorylated on T251 or S307
E2F-5L	Fastest	Hypophosphorylated (neither phosphorylated on T251 or S307)

Figure 8.1 Prediction of the phosphorylation status of E2F-5 polypeptides detected on SDS-PAGE. The prediction is based on the relative mobilities of mutant derivatives of E2F-5, in which serine 307 or threonine 251 were substituted with alanine or aspartate residues.

The data presented here implies that E2F-5 is subject to cell-cycle dependent phosphorylation in Rat1 cells. The appearance of E2F-5U occurs as Rat1 cells approach and enter S phase, suggesting that E2F-5 may be phosphorylated by a kinase that is active at this stage of the cell cycle (Fig 3.7b). This timing correlates well with the induction of cyclinE-CDK2 kinase, which has been shown to peak in activity as cells progress towards S phase (Sherr *et al.,* 1993, 1996). Consistent with this idea the results presented here indicate that E2F-5 phosphorylation is mediated by cyclinE-CDK2, rather than the mitogen-responsive cyclinD-dependent kinase. Phosphorylation of His-tagged E2F-5 *in vitro* with purified cyclinE-CDK2 from baculovirus infected cells results in a mobility shift of E2F-5 in SDS-PAGE (see Fig 6.2d). An equivalent mobility shift was observed in extracts from cells co-transfected with E2F-5 and cyclinE-CDK2. Taken together these results infer that the mobility shift seen in transfected extracts is the result of direct phosphorylation of E2F-5 by cyclinE-CDK2.

Cyanogen bromide mapping of wild-type E2F-5, which had been *in vitro* phosphorylated by cyclinE-CDK2 identified a phosphopeptide in which the only CDK site is positioned at threonine 251. Moreover a peptide of identical size was derived from cyanogen bromide cleavage of *in vivo* phosphorylated wild-type E2F-5, but not T251A. These findings suggest that cyclinE-CDK2 phosphorylates E2F-5 on threonine 251 *in vivo*. That phosphorylation of this site is likely to occur in a physiological context was implied by the appearance of E2F-5U towards the end of G1 phase in the Rat1 cell cycle.

A comparison of polypeptide sequences of the E2F family members reveals that the threonine at 251 is unique to E2F-5, therefore E2F-5 may be the only E2F family member that is phosphorylated by cyclinE-CDK2. Consistent with this idea it has previously been shown that E2F-1 was efficiently phosphorylated by cyclinA-CDK2 *in*

vitro but was not phosphorylated by an excessive amount of cyclinE-CDK2 (Dynlacht *et al.*, 1994). However, to control for differences in the assay conditions, the specificity of cyclinE-CDK2 for the different E2F family members should be compared within the same assay. Thus the possibility that different sites are phosphorylated by cyclinE-CDK2 in the other E2F family members cannot be excluded.

8.3 Phosphorylation of serine 307 may regulate E2F-5 activity

Phosphorylation can affect the activity of a transcription factor by modulating its DNA binding activity, subcellular localisation and its interaction with co-activators and components of the basal transcription machinery (reviewed in Hill and Triesman, 1995; see Section 1.7).

The mutant derivatives of E2F-5, T251A and S307A were competent in their ability to bind to the E2F-recognition site as a heterodimer with DP-1 and as a complex with p130 and DP-1. Moreover, the subcellular localisation of T251A and S307A was similar to that of wild type E2F-5. These findings imply that E2F-5 DNA binding, heterodimerision, subcellular localisation and interaction with p130, are not dependent upon phosphorylation of E2F-5 at either of these two sites. In addition, they demonstrate that the integrity of E2F-5 was not altered by mutagenesis.

The analysis of S307A and S307D presented in chapter 5, suggests that phosphorylation of serine 307 may regulate E2F-5 activity. S307A has a reduced transcriptional activity, both as a heterodimer with DP-1 or DP-3δ when assessed for its ability to activate the cyclinE-luciferase reporter (Fig 5.1). Furthermore, the rise in reporter activity in response to increasing amounts of S307A was not as steep as that detected when equivalent amounts of wild-type E2F-5 were transfected. There are a

number of possible explanations for this observation. One possibility is that as increasing levels of S307A are transfected an endogenous protein involved in transcriptional activation becomes limiting. This may be because S307A has a lower affinity for this protein so cannot efficiently recruit it to the promoter. Many proteins have been shown to have a role in E2F-dependent gene regulation, such as the p300/CBP co-activator, TBP and Sp1 (Hagemeier *et al.*, 1993; Lin *et al.*, 1996; Trouche and Kouzarides 1996; Lee *et al.*, 1998). It is possible that E2F-5 phosphorylation on serine 307 has a role in recruiting one of these proteins to the promoter. Alternatively the DP-1 component of the heterodimer itself may become limiting, since the levels of exogenous DP-1 were kept constant in the transfection. Another possibility is that as the level of S307A expression plasmid was increased in cells, the resulting rise in S307A expression was offset by an accompanying increase in the degradation of S307A.

The phenotype of full-length S307A can be transferred to a Gal4 fusion protein, consisting of the Gal4 DNA binding domain and the *trans*activation domain of E2F-5. This observation allows a number of important conclusions to be made. In agreement with the previous gel retardation results, the compromised transcriptional activity of S307A cannot be explained by a change in its ability to bind DNA or heterodimerise with a DP subunit. Moreover, the phenotype of S307A in the Gal4- fusion protein gives a direct indication of the activity of the S307A *trans*activation domain. Whereas the results from overexpression of the full-length E2F-5 and S307A proteins, whilst useful for mimicking the physiological proteins, may be indirect. For example the full-length proteins may modulate the transcription of genes encoding endogenous E2F proteins, which in turn regulate the *cyclin E* reporter. The phenotype of Gal4-S307A

demonstrates that the *trans*activation domain of S307A is intrinsically less active than that of wild-type E2F-5.

Serine 307 is within the pocket protein-binding region of E2F-5. Given the importance of pocket protein binding in the regulation of E2F activity, it was therefore possible that phosphorylation of E2F-5 itself modified the interaction between E2F-5 and pocket proteins. Several experiments indicated that this was not the case. S307A was demonstrated to interact with p107 by immunoprecipitation and with p130 as a DNA-binding complex to a similar extent as wild-type E2F-5. Moreover both pocket proteins inhibited *trans*activation mediated by Gal4-E2F-5 or Gal4-S307A to a similar level.

Serine 307 is conserved in E2F-1, E2F-2, E2F-4 and E2F-5. Thus it may be a physiologically relevant site of phosphorylation in several E2F proteins. The homologous serine in E2F-1 is positioned at 403. Interestingly, a recent study demonstrated that mutation of serine 403 in E2F-1 to alanine increased the stability of E2F-1 (Vandel and Kouzarides, 1999). In the results presented here, the steady state levels of S307A did not differ from wild-type E2F-5 (see Fig 4.2, 4.3; data not shown). However the rate of degradation of S307A relative to wild-type E2F-5 was not determined. Thus the possibility that the stability of S307A differs from wild-type E2F-5 cannot be ruled out. However if, by analogy to E2F-1 (S403A), the stability of S307A were greater than wild-type E2F-5 then this would create a paradox whereby an increased level of S307A was accompanied by a reduction in the ability of S307A to activate transcription. Therefore further investigation is required to determine whether phosphorylation of serine 307 in E2F-5 and the homologous serines in the other E2F family members have similar functional consequences.

Expression of E2F-5 or S307A in cells can cause an increase in the number of cells in S phase. This was demonstrated by labelling cells expressing either wild-type E2F-5 or S307A, with BrdU. BrdU labelling measures DNA synthesis in cells during a short time period. However, quantifying the number of cells that incorporate BrdU does not establish whether cells are being forced into S phase at an increased rate or whether there is an accumulation of cells in S phase. It is possible that cells expressing S307A proceed more slowly through S phase due to a reduced capacity to activate the appropriate genes, and therefore they accumulate in S phase. To investigate this hypothesis cells could be treated with Nocodazole, a drug that causes cells to accumulate in G2/M. If cells are accumulating in S phase then they should not proceed into M phase, and therefore they will not be responsive to a Nocodazole block.

Studies of the function of individual E2F proteins has revealed that E2F-5 is considerably weaker than E2F-1, -2, and –3 in promoting cell cycle progression and a significant induction in S phase required the co-expression of E2F-5 and DP-1 (Lukas *et al.*, 1996). It has also been shown that E2F-5/DP-3δ promotes cell cycle progression (Allen *et al.*, 1997). Therefore the ability of E2F-5 or S307A to promote cell cycle progression may be more marked if co-transfected in cells together with DP-1 or DP-3δ.

The results presented here also showed that cells stably expressing S307A could be arrested by serum starvation, whilst cells expressing wild-type E2F-5 were incapable of arrest by serum starvation. This is compatible with phosphorylation of E2F-5 on serine 307 playing a role in cell cycle re-entry from G0/G1 into S phase. However some caution should be taken in the interpretation of these results. It is possible that differences exist, between the stable cell-lines expressing wild-type E2F-5 and S307A,

besides the alanine substitution at position 307. Therefore an inducible system of E2F-5 and S307A expression would provide a more reliable control, as it would enable a comparison of cell cycle profile of cells before and after E2F-5 induction in the same cell line.

Several important questions remain to be resolved concerning the phosphorylation of serine 307 in E2F-5. Firstly *in vivo* mapping would determine whether serine 307 is a physiological site of phosphorylation. Secondly the identity of the kinase responsible for phosphorylation of serine 307 may enable a link to be made between the signals that trigger the phosphorylation of E2F-5 and the regulation of E2F-5 dependent transcription. In this respect, the possibility that MAP kinases, for example the ERKs, phosphorylate serine 307 warrants further investigation. ERKs can be triggered in response to mitogenic signals and promote proliferation by modulating the activity of a variety of transcription factors, for example Elk-1, a transcriptional activator of the *c-fos* promoter (reviewed in Hill and Triesman, 1995). Given that MAP kinase activation is often linked with proliferation and that phosphorylation of serine 307 increases the activity of E2F and may be required for progression of cells into S phase, the MAP kinases are potential candidates for the phosphorylation of serine 307.

8.4 Regulation of E2F-5 activity by cyclinE-CDK2

The results presented here demonstrate that cyclinE-CDK2 stimulates E2F-5 dependent transcription in a dose-dependent fashion, and this correlates with the appearance of the slower migrating phosphorylated E2F-5U polypeptide. Although cyclinD1-CDK4 caused a marginal increase in E2F-5 dependent transcription, it did not cause a phosphorylation dependent mobility shift of E2F-5. Moreover cyclinD1-CDK4 but not cyclinE-CDK2 can reverse p107-mediated repression of Gal4-E2F-5, suggesting the effect of cyclinD1-CDK4 on E2F-5 dependent *trans*activation may be caused by disruption of the interaction between E2F-5 and endogenous pocket proteins (Fig 6.5).

The evidence that threonine 251 in E2F-5 is phosphorylated by cyclinE-CDK2 was discussed above (see Section 8.2). The E2F-5 mutant incapable of phosphorylation on threonine 251, namely T251A, displayed a markedly reduced response to cyclinE-CDK2 in *trans*activation, the expected outcome if phosphorylation of this site augments E2F-5-dependent *trans*activation. In support of this a mutant containing an aspartate residue at position 251, T251D, had a greater transcriptional activity than wild-type E2F-5. In addition, cyclinD1-CDK4 and cyclinE-CDK2 had similar effects on the activity of T251A, but cyclinE-CDK2 had a considerably greater effect on the activity of wild-type E2F-5. Therefore the regulation of E2F-5 activity by the phosphorylation of threonine 251 appears to be a specific property of cyclinE-CDK2.

It was demonstrated that cells co-transfected with T251A and cyclinE-CDK2 exhibited less BrdU labelling than cells transfected with wild-type E2F-5 and cyclinE-CDK2. This suggests that threonine 251 is important for facilitating the entry of cells

into S phase. Therefore phosphorylation of E2F-5 by cyclinE-CDK2 may contribute to the necessary induction of target genes required for cell cycle progression.

Although cyclinE-CDK2 is essential in allowing cells to move from G1 into S phase (Ohtsubo *et al.*, 1995), physiological protein substrates other than pRb that are subject to cyclinE-CDK2 phosphorylation, and which are necessary for cell cycle progression, have not yet been defined. Of relevance to this point is that cells arrested by a constitutively hypophosphorylated mutant pRb protein can enter S phase upon the expression of exogenous cyclinE-CDK2 (Lukas *et al.*, 1997), arguing that cyclinE-CDK2 has at least one other target in addition to pRb that is necessary for cell cycle progression. Considering the role that E2F has in activating genes required for cell cycle progression, the ability of cyclinE-CDK2 to directly phosphorylate E2F-5 and to increase E2F-5 activity opens up the possibility that E2F may be an important target required for cyclinE-CDK2 to exert its effects on cell cycle progression.

The regulation of E2F-5 dependent transcription by cyclinE-CDK2 presented here, along with the well-established role of pRb in regulating E2F, allows a model to be postulated whereby E2F and cyclinE-CDK2 cooperate in the induction of S phase. As cells leave quiescence the mitogen-dependent induction of cyclinD-CDK4 kinase plays a primary role in the phosphorylation control of pRb family proteins, which is followed by the induction of cyclinE-CDK2 and the completion of pRb phosphorylation. The subsequent release of E2F results in the activation of E2F target genes including *cyclinE*, causing a surge in cyclinE-CDK2 activity which can then further feedback and increase the activity of E2F-5 through phosphorylation. This positive feedback loop may cause the necessary increase in E2F activity to drive the expression of E2F target genes to a level that allows progress into S phase (Figure 8.2). As DNA replication approaches completion several mechanism are believed to be

involved in E2F inactivation. These include phosphorylation control of the DP subunit by cyclinA-CDK2 kinase (Krek *et al.*, 1995), the regulated ubiquitin-dependent degradation of E2F (Marti *et al.*, 1999) combined with the down-regulation of cyclinE-CDK2 (Koff *et al.*, 1992) and the relocation of E2F-4 and E2F-5 to the cytoplasm (Buck *et al.*, 1997; Lindeman *et al.*, 1997). Therefore through the sequential action of the cyclin-CDKs, E2F activity is increased and decreased in a temporal pattern as cells progress from G0/G1 into S phase.

Interestingly, a similar model of positive feedback control has been proposed to occur during the yeast cell cycle. In budding yeast, the G1 transcription factor complexes SWI4/SWI6 and MBP/SWI6 regulate a similar set of genes to E2F in mammalian cells (Johnston *et al.*, 1992). Their activity is thought to be in part regulated by the G1 cyclin-CDK complexes, the genes for which are themselves under the direct control of SWI4/SWI6 and MBP/SWI6. As in the proposed model of E2F activation by cyclinE-CDK2, it has been suggested that this positive feedback loop facilitates the rapid and timely induction of yeast genes necessary for entry into S phase.

The proposed positive feedback model may also incorporate other cyclinE-CDK2 substrates, which upon phosphorylation augment cyclinE-CDK2 activity. For example the CDK inhibitor, p27 inhibits the kinase activity of cyclinE-CDK2 and is also phosphorylated by cyclinE-CDK2, resulting in its elimination from the cell (Sheaff *et al.*, 1997). It was suggested that the binding of ATP to CDK2 governs which state predominates. Thus it is conceivable that several cyclinE-CDK2 substrates may contribute to the ability of cyclinE-CDK2 to promote irreversible entry into S phase, by positive feedback.

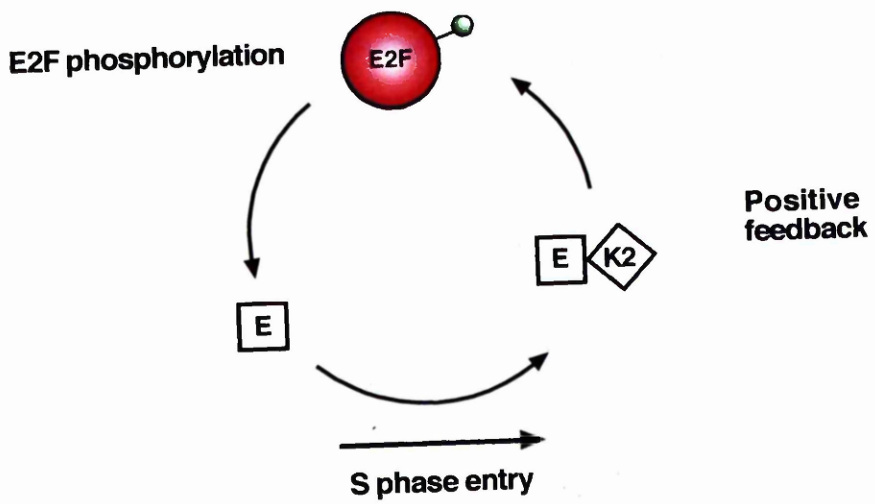


Figure 8.2 Regulation of E2F-5 by Cyclin E In this model the induction of the E2F-responsive cyclin E gene allows the formation of the cyclinE-CDK2 complex, which acts through an autoregulatory feedback loop to phosphorylate E2F-5 and thereby augment E2F-dependent transcription. E: cyclin E; K2:CDK2; red circle represents E2F-5, green circle represents a phosphate group.

8.5 Phosphorylation of E2F facilitates p300 co-activator recruitment

The p300/CBP family of co-activators has been the subject of intense investigation over recent years. Of particular interest is how p300/CBP regulate diverse cellular processes through their interaction with different transcription factors. Previously the interaction between E2F-1 and p300/CBP has been demonstrated to regulate E2F-1 dependent transcription (Trouche and Kouzarides, 1996; Lee *et al.*, 1999).

The results presented here demonstrate that cyclinE-CDK2 phosphorylates a site in the activation domain of E2F-5 that stimulates binding of E2F-5 to the p300 co-activator. The phosphorylation-dependent interaction of p300/CBP co-activators occurs with other transcription factors, such as CREB and p53 (Chivria *et al.*, 1993; Lambert *et al.*, 1998). However this may be the first example of a CDK-dependent phosphorylation event which stimulates the binding of p300/CBP to a transcription factor. Several lines of evidence support the importance of this interaction for the proper regulation of E2F target genes and orderly progression of the cell cycle. Firstly, the isolated E2F-interaction domain in p300 has a dominant negative effect on E2F-5-dependent transcription. This implies that p300/CBP is an important co-activator of E2F-5. Secondly, the reduced transcriptional activity of the T251A mutant in the presence of cyclinE-CDK2 suggests that cyclinE-CDK2 phosphorylation of this site increases E2F-5-dependent transcription. Finally, the T251 CDK site was demonstrated to facilitate entry into S phase.

It has become clear that p300/CBP proteins exist in cells bound to a variety of other proteins in a co-activator complex (reviewed in Shikama *et al.,* 1997). The histone acetyltransferase (HAT) activity that is associated with the co-activator

complex is believed to be important in regulating chromatin and thus the accessibility of genes to the transcription machinery (Bannister and Kouzarides, 1996; Ogryzko *et al.*, 1996). Recently it has been shown that the HAT activity of p300/CBP and p300/CBP-associated factor (p/CAF) can acetylate a number of non-histone proteins, for example p53, E2F-1 and TFIIE (Gu and Roeder *et al.,* 1997; Imhof *et al.,* 1997; Martinez-Balbas *et al.,* 2000). The consequence of E2F-1 acetylation was to potentiate E2F-1 activity by increasing its DNA-binding ability, activation potential and protein half-life (Martinez-Balbas *et al.,* 2000). As the results presented here demonstrate that E2F-5 can also be acetylated *in vitro* by p300 it would be interesting to determine whether the mechanism of transcriptional activation of E2F-5 by p300/CBP involves the acetylation of E2F-5.

Both p300 and CBP are believed to be limiting in the cell relative to the levels of transcription factors they bind to and regulate. Therefore a role for a cyclin-dependent kinase in the recruitment of p300/CBP to E2F, a transcription factor important for the control of genes necessary for cell cycle progression, provides a mechanism whereby p300/CBP can be directed away from processes such as differentiation and engaged in the regulation of proliferation. Perhaps of significance to these results is the discovery that p300/CBP can bind to cyclinE-CDK2 (Perkins *et al.,* 1997). CyclinE-CDK2 may regulate p300/CBP function, since inhibition of p300 associated cyclinE-CDK2 by p21 increases NF-κB *trans*activation. Moreover cyclinE-CDK2 can increase CBP HAT activity (Ait-Si-Ali *et al.*, 1998). Therefore, it is possible to speculate that the regulation of p300/CBP by cyclinE-CDK2 may enhance the activity of transcription factors that require CBP HAT activity and are involved in proliferation and compromise the activity of transcription factors involved in other cellular outcomes, such as growth arrest. Since p300/CBP interacts with cyclinE-

CDK2, it is conceivable that it recruits cyclinE-CDK2 to E2F-5, thereby augmenting its phosphorylation. Thus a weak interaction occurring between unphosphorylated E2F-5 and p300/CBP, could be dramatically increased at the G1-S transition through the phosphorylation of E2F-5 by p300/CBP bound cyclinE-CDK2, stimulating E2F-5 *trans*activation and thereby contributing to the surge of gene expression necessary for entry into S phase. Moreover the increase in HAT activity that occurs at this stage of the cell cycle (Ait-Si-Ali *et al.*, 1998), may be mechanistically important for enhancing transcription through acetylating histones, the basal transcription machinery or E2F-5 itself. Thus it will be important to establish whether E2F-5 acetylation occurs *in vivo* and whether acetylation of E2F-5 regulates E2F-5 activity. This line of inquiry will surely create further questions such as whether acetylation and phosphorylation have synergistic or antagonistic effects on E2F-5 activity.

The results presented here provide a plausible pathway through which G1 CDK complexes can directly act to increase E2F activity and stimulate the transcription of E2F target genes by facilitating the recruitment of the p300/CBP co-activator complex. As such, this mechanism is likely to be of central importance in regulating and co-ordinating gene expression during early cell cycle progression.

8.5 General Discussion

The data presented in these studies focused on the regulation of E2F-5 activity by phosphorylation. Prior to this work it was known that E2F DNA binding activity was negatively regulated by phosphorylation of the DP-1 subunit by cyclinA-CDK2. The results presented here now reveal that E2F activity is also regulated by cyclinE-CDK2. Specifically cyclinE-CDK2 stimulates E2F-5 dependent transcription by phosphorylation of a single site positioned at threonine 251 in the activation domain of E2F-5, which increases its ability to interact with p300/CBP. At least one other site is phosphorylated in E2F-5, a likely candidate being serine 307. Evidence that E2F-5 may be phosphorylated on other sites besides threonine 251 was provided by the observation that T251A mutant was still phosphorylated in cells, as measured by metabolic labelling with ^{32}P-orthophosphate. Moreover, the S307A and S307D mutants have different electrophoretic mobilities in SDS-PAGE. S307A, unlike T251A, was phosphorylated by cyclinE-CDK2 *in vitro* to a similar extent as wild-type E2F-5; therefore another kinase may be responsible for phosphorylation of serine 307.

Considering that the phosphorylation of either serine 307 or threonine 251 appears to have similar functional consequences, it would be interesting to determine whether phosphorylation of both of these sites has a synergistic effect on E2F activity. Alternatively the possibility that each of these sites are involved in different mechanisms of transcriptional activation would be interesting to explore. It could be speculated that phosphorylation of threonine 251 regulates a subset of E2F target genes that have a requirement for the co-activator, p300/CBP, whilst phosphorylation of serine 307 activates another set of E2F target genes through the recruitment of a different co-activator (Fig 8.3).

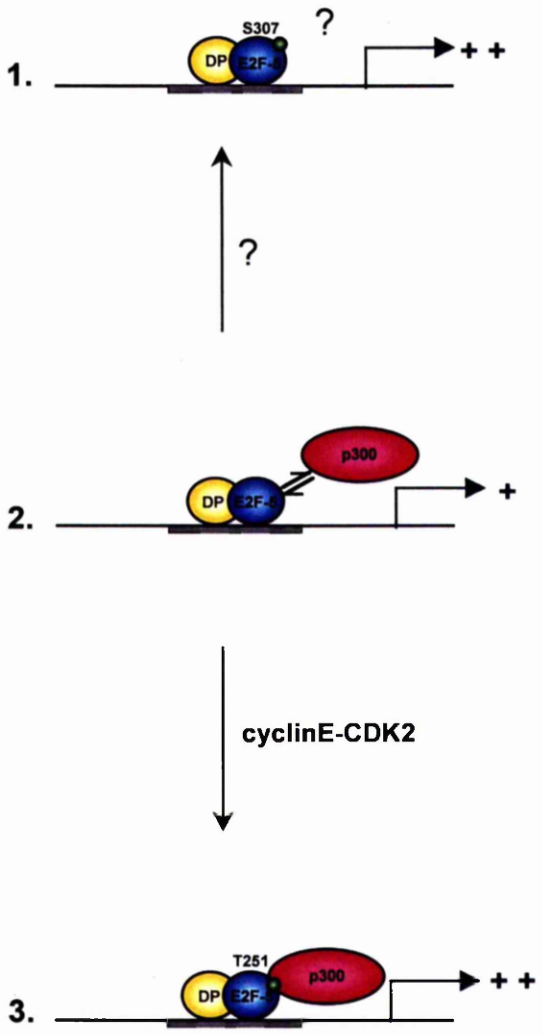


Figure 8.3 Phosphorylation of E2F-5 activates E2F-5 dependent transcription. (1) Phosphorylation of E2F-5 on serine 307 may enhance transcription by stimulating an interaction between the E2F-5 *trans*activation domain and a protein involved in transcriptional activation. (2) Unphosphorylated E2F-5 may activate transcription by binding weakly to p300/CBP. (3) Phosphorylation of E2F-5 on threonine 251 by cyclinE-CDK2 facilitates the interaction with p300/CBP. '+' indicates the relative *trans*activation. '?' represents an unknown protein

Antibodies that specifically recognise phosphorylated serine 307 or phosphorylated threonine 251 in E2F-5 would be useful to confirm whether phosphorylation of these sites occurs physiologically. Moreover they could also be employed to determine whether specific proteins interact with E2F-5 when it is phosphorylated on either of these sites. It is likely that phosphorylation on threonine 251 and serine 307 occurs to different degrees in different cell lines, as was suggested by the different electrophoretic mobilities of E2F-5 in F9EC and Rat1 cells. As E2F-5 activity was found to be up-regulated in certain breast tumour cell lines (Polanowska *et al.*, 2000), it will be interesting to determine the phosphorylation status of E2F-5 in these cell lines to establish whether phosphorylation of a particular site in E2F-5 is linked to aberrant growth.

Analysis of the cellular processes that enhance the activity of E2F should provide further understanding of the mechanisms controlling the growth of normal and cancer cells. Ultimately this should yield important targets for the production of specific therapies to treat cancer.

References

Adams, M. R., Sears, R., Nuckolls, F., Leone, G., Nevins, J. R. 2000. Complex Transcriptional Regulatory Mechanisms Control Expression of the E2F3 Locus. *Mol. Cell. Biol.* **20:** 3633-3639.

Ait-Si-Ali, S., S. Ramirez, F.-X. Barre, F. Dkhissi, L. Magnaghi-Jaulin, J.A.Girault, P. Robin, M. Knibiehler, I.L. Pritchard, B.Ducommun, D. Trouche, and A. Harel-Bellan, 1998. Histone acetyltransferase activity of CBP is controlled by cyclin-dependent kinases and oncoprotein E1A. *Nature* **396**:184-186.

Alevizopoulos, K., J. Vlach, S. Hennecke, and B. Amati. 1997. Cyclin E and c-Myc promote cell proliferation in the presence of p16^{INK4a} and of hyphophosphorylated retinoblastoma family proteins. *EMBO J.* **16**:5322-5333.

Allen, K.E., S. de la Luna, R.M. Kerkhoven, R. Bernards, and N.B. La Thangue. 1997. Distinct mechanisms of nuclear accumulation regulate the functional consequence of E2F transcription factors. *J. Cell Sci.* **110**:2819-2831.

Arany, Z., D. Newsome, E. Oldread, D.M. Livingston, and R. Eckner. 1995. A family of transcriptional adaptor proteins targeted by the E1A oncoprotein. *Nature* **374:**81-84.

Asano, M., J.R. Nevins, and R.P. Wharton. 1996. Ectopic E2F expression induces S phase and apoptosis in *Drosophila* imaginal discs. *Genes Dev.* **10**:1422-1432.

Ashcroft, M., M.H. Kubbutat, and K.H. Vousden. 1999. Regulation of p53 function and stability by phosphorylation. *Mol Cell Biol* **19:** 1751-8

Avantaggiati, M.L., Carbone, C., Graessmann, A., Nakatani, Y., Howard, B. and Levine, A.S. 1996. The SV40 large T antigen and adenovirus E1A oncoproteins interact with distinct isoforms of the transcriptional co-activator p300. *EMBO J.* **15**:2236-2248.

Baldin, V., J. Lukas, M.J. Marcote, M. Pagano, and G. Draetta. 1993. Cyclin D1 is a nuclear protein required for cell cycle progression in G_1. *Genes Dev.* **7:** 812-821

Bandara, L.R., V.M. Buck, M. Zamanian, L.H. Johnston, and N.B. La Thangue. 1993. Functional synergy between DP-1 and E2F-1 in the cell cycle-regulating transcription factor DRTF1/E2F. *EMBO J.* **12:**4317-4324.

Bandara, L.R. and N.B. La Thangue. 1991. Adenovirus E1A prevents the retinoblastoma gene product from complexing with a cellular transcription factor. *Nature* **351:**494-497.

Bandara, L.R., V.M. Buck, M. Zamanian, L.H. Johnston, and N.B. La Thangue. 1993. Functional synergy between DP-1 and E2F-1 in the cell cycle-regulating transcription factor DRTF1/E2F. *EMBO J.* **12:**4317-4324.

Bandara, L.R., E.W.-F. Lam, T.S. Sorensen, M. Zamanian, R. Girling, and N.B. La Thangue. 1994. DP-1; a cell cycle-regulated and phosphorylated component of transcription factor DRTF/E2F which is functionally important for recognition by pRb and the adenovirus E4 orf 6/7 protein. *EMBO J.* **13:**3104-3114.

Banerjee, A.C., Recupero, A.J., Mal, A., Piotrkowski, A.M., Wang, D.M., and Harter, M.L. 1994. The adenovirus E1A 289R and 243R protein inhibits the phosphorylation of p300. *Oncogene* **9:** 1733-1737.

Bannister A.J., T. Oehler, D. Wilhelm D, P. Angel and T. Kouzarides. 1995. Stimulation of c-Jun activity by CBP: c-Jun residues Ser63/73 are required for CBP induced stimulation in vivo and CBP binding in vitro. *Oncogene* **11:**2509-2514

Bannister, A.J. and Kouzarides, T. 1996. The CBP co-activator is a histone acetyltransferase. *Nature* **384**:641-643.

Bates, S., A.C. Phillips, P.A. Clark, F. Stott, G. Peters, R.L. Ludwig, and K.H. Vousden.1998. $p14^{ARF}$ links the tumour suppressors RB and p53. *Nature*. **395**:124-5.

Beijersbergen, R.L., R.M. Kerkhoven, L. Zhu, L. Carlee, P.M. Voorhoeve, and R. Bernards. 1994. E2F-4, a new member of the E2F gene family, has oncogenic activity and associates with p107 *in vivo*. *Genes Dev.* **8:**2680-2690.

Beijersbergen, R.L., L. Carlee, R.M. Kerkhoven, and R. Bernards. 1995. Regulation of the retinoblastoma protein-related p107 by G_1 cyclin complexes. *Genes Dev.* **9:** 1340-1353.

Blom, N. S. Gammeltoft, and S.Brunak. 1999. Sequence and Structure-based Prediction of Eukaryotic Protein Phosphorylation sites. *J. Mol. Biol.* **294:** 1351-1362.

Botz, J., K. Zerfass-Thome, D. Spitkovsky, H. Delius, B. Vogt, M. Eilers, A. Hatzigeorgiou, and P. Jansen-Dürr. 1996. Cell cycle regulation of the murine cyclin E gene depends on an E2F binding site in the promoter. *Mol. Cell. Biol.* **16:**3401-3409.

Boyle, W.J., Smeal, T., Defize, L.H.K., Angel, P., Woodgett, J.R., Karin, M. and Hunter, T. 1991. Activation of protein kinase C decreases phosphorylation of c-Jun at sites that negatively regulate its DNA-binding activity. *Cell*, **64:** 573-584.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. *Anal. Biochem.*, **72,** 248-254.

Brehm, A., Miska, E.A., McCance, D.J., Reid, J.L., Bannister, A.J., and Kouzarides, T. 1998. Retinoblastoma protein recruits histone deacetylase to repress transcription. *Nature* **391:** 597-601.

Bremner, R., B.L. Cohen, M. Sopta, P.A. Hamel, C.J. Ingles, B.L. Gallie, and R.A. Phillips. 1995. Direct transcriptional repression by pRB and its reversal by specific cyclins. *Mol. Cell. Biol.* **15:** 3256-3265.

Buck, V., K.E. Allen, T.S. Sørensen, A. Bybee, E.M. Hijmans, P.M. Voorhoeve, R. Bernards, and N.B. La Thangue. 1995. Molecular and functional characterisation of E2F-5, a new member of the E2F family. *Oncogene* **11:**31-38.

Cam, L., Polanowska, J., Fabbrizio, E., Olivier, M., Philips, A., Eaton, E. N., Classon, M., Geng, Y., Sardet, C. 1999. Timing of cyclin E gene expression depends on the regulated association of a bipartite repressor element with a novel E2F complex. *EMBO J.* 18: 1878-1890

Campenero, M.R. and E.K. Flemington. 1997. Regulation of E2F through ubiquitin-proteasome-dependent degradation: Stabilisation by the pRB tumor suppressor protein *Proc. Natl. Acad. Sci. USA* **94**: 2221-2226.

Cartwright, P., H. Muller, C. Wagener, K. Holm, and K. Helin. 1998. E2F-6: A novel member of the E2F family is an inhibitor of E2F-dependent transcription. *Oncogene* **17**:611-623.

Cheng, M., P. Olivier, J.A. Diehl, M. Fero, M.F. Roussel, J.M. Roberts, and C.J. Sherr. 1999. The $p21^{Cip1}$ and $p27^{Kip1}$ CDK `inhibitors' are essential activators of cyclin D-dependent kinases in murine fibroblasts. *EMBO J.* **18:** 1571-1583

Chew YP; Ellis M; Wilkie S; Mittnacht S. 1998. pRB phosphorylation mutants reveal role of pRB in regulating S phase completion by a mechanism independent of E2F. *Oncogene* **17:** 2177-2186

Chrivia, J.C., R.P. Kwok, N. Lamb, M. Hagiwara, M.R. Montminy, and R.H. Goodman. 1993. Phosphorylated CREB binds specifically to the nuclear protein CBP. *Nature* **365**:855-859.

Clarke, A.R., E.R. Mazndag, M. Van Roon, N.M.T. Van der Lugt, M. Van der Valk, M.J. Hooper, A. Berns, and H. Te Riele. 1992. Requirement for a functional *Rb-1* gene in murine development. *Nature* **359:**328-330.

Cobrinik, D., P. Whyte, D.S. Peeper, T. Jacks, and R.A. Weinberg. 1993. Cell cycle-specific association of E2F with the p130 E1A-binding protein. *Genes Dev.* **7**:2392-2404.

Cobrinik, D., M.H. Lee, G. Hannon, G. Mulligan, R.T. Bronson, N. Dyson, E. Harlow, D. Beach, R.A. Weinberg, and T. Jacks. 1996. Shared role of the pRb-related p130 and p107 proteins in limb development. *Genes Dev.* **10:**1633-1644.

Dagnino, L., C. Fry, S. Bartley, P. Farnha, B. Gallie, and R.A. Phillips. 1997. Expression patterns of the E2F family of transcription factors during murine epithelial development. *Cell Growth and Differ.* **8**:553-563.

de la Luna, S., J. Morwenna, C.-W. Lee, and N.B. La Thangue. 1996. Nuclear accumulation of the E2F heterodimer regulated by subunit composition and alternative splicing of a nuclear localisation signal. *J. Cell Sci.* **109**:2443-2452.

DeGregori, J., G. Leone, A. Miron, L. Jakoi, and J.R. Nevins. 1997. Distinct roles for E2F proteins in cell growth control and apoptosis. *Proc. Natl. Acad. Sci. USA* **94**:7245-7250.

Deng, C., P. Zhang, J.W. Harper, S. Elledge, and P. Leder. 1995. Mice lacking $p21^{Cip1/Waf1}$ undergo normal development, but are defective in G1 checkpoint control. *Cell* **82:**675-684.

Desai, D., H.C. Wessling, R.P. Fisher, and D.O. Morgan. Effects of phosphorylation by CAK on cyclin binding by cdc2 and cdk2. *Mol Cell Biol* **15:** 345-350.

Duronio, R.J., P.H. O'Farrell, J.-E. Xie, A. Brook, and N. Dyson. 1995. The transcription factor E2F is required for S phase during *Drosophila* embryogenesis. *Genes Dev.* **9:** 1445-1455

Duronio, R.J., A. Brook, N. Dyson, and P.H. O'Farrell. 1996. E2F-induced S phase requires *cyclin E. Genes Dev.* **10:** 2505-2513.

Duronio, R.J., P.C. Bonnette, and P.H. O'Farrell. 1998. Mutations of the *Drosophila dDP, dE2F,* and *cyclin E* genes reveal distinct roles for the E2F-DP transcription factor and cyclin E during the S-phase transition. *Mol. Cell. Biol.* **18:** 141-151

Dynlacht, B.D., O. Flores, J.A. Lees, and E. Harlow. 1994. Differential regulation of E2F transcription by cyclin/cdk complexes. *Genes Dev.* **8**:1772-1786.

Dynlacht, B.D., K. Moberg, J.A. Lees, J.A, E. Harlow, and L. Zhu. 1997. Specific regulation of E2F family members by cyclin-dependent kinases. *Mol. Cell. Biol.* **17:** 3867-3875.

Dyson, N. 1998. The regulation of E2F by pRb-family proteins. *Genes Dev.* **12:** 2245-2262

Eckner, R., M.E. Ewen, D. Newsome, Gerdes, M., DeCaprio, J.A., Lawrence, J. B., and Livingston, D.M. 1994. Molecular cloning and functional analysis of the adenovirus E1A-associated 300-KD protein (p300) reveals a protein with properties of a transcriptional adaptor. *Genes Dev.* **8:** 869-884.

Ewen, M.E., B. Faha, E. Harlow, D.M. Livingston. 1992. Interaction of p107 with cyclin A independent of complex formation with viral oncoproteins. *Science* **255:** 85-87

Fagan R., K.J. Flint K, and N. Jones. 1994. Phosphorylation of E2F-1 Modulates Its Interaction with the Retinoblastoma Gene Product and the Adenoviral E4 19 KDa Protein. *Cell* **78:** 799-811.

Felzien, L.K., S. Farrell, J.C.Betts, R. Mosavin, and G. Nabel. 1999. Specificity of Cyclin E-Cdk2, TFIIB, and E1A interactions with a common domain of the p300 co-activator. *Mol. Cell. Biol.* **19**: 4241-4246.

Ferreira, R., L. Magnaghi-Jaulin, R. Phillipe, A. Harel-Bellan, and D. Trouche. 1998. The three members of the pocket proteins family share the ability to repress E2F activity through recruitment of a histone deacetylase. *Proc. Natl. Acad. Sci. USA.* **95**:10493-10498.

Field, S.J., F.-Y. Tsai, F. Kuo, A.M. Zubiaga, W.G. Kaelin Jr., D.M. Livingston, S.H. Orkin, and M.E. Greenberg. 1996. E2F-1 functions in mice to promote apoptosis and suppress proliferation. *Cell* **85:**549-561.

Fisher, R.P. & Morgan, D.O. 1994. A novel cyclin associates with MO15/CDK7 to form the CDK-activating kinase. *Cell*, **78,** 713-724.

Fry C.J. , J.E. Slansky, and P.J. Farnham. 1997. Position-dependent transcriptional regulation of the murine dihydrofolate reductase promoter by the E2F transactivation domain *Mol. Cell. Biol.* **17:**1966-1976.

Fry, C. J., Pearson, A., Malinowski, E., Bartley, S. M., Greenblatt, J., Farnham, P. J. 1999. Activation of the Murine Dihydrofolate Reductase Promoter by E2F1. A REQUIREMENT FOR CBP RECRUITMENT. *J. Biol. Chem.* **274:** 15883-15891

Geng, Y., E.N. Eaton, M.Picon, J.M. Roberts, A.S. Lundberg, A. Gifford, C. Sardet, and R.A Weinberg. Regulation of cyclin E transcription by E2Fs and retinoblastoma protein. *Oncogene.* **12**:1173-1180.

Ginsberg, D., G. Vairo, T. Chittendon, Z.-X. Xiao, G. Xu, K.K. Wydner, J.A. DeCaprio, J.B. Lawrence, and D.M. Livingston. 1994. E2F-4, a new E2F transcription factor family member, interacts with p107 and has transforming potential. *Genes Dev.* **8:**2939-2952.

Girling, R., J.F. Partridge, L.R. Bandara, N. Burden, N.F. Totty, J.J. Hsuan, and N.B. La Thangue. 1993. A new component of the transcription factor DRTF1/E2F. *Nature* **362:**83-87.

Girling, R., L.R. Bandara, E. Ormondroyd, E.W.-F. Lam, S. Kotecha, T. Mohun, and N.B. La Thangue. 1994. Molecular characterisation of *Xenopus laevis* DP proteins. *Mol. Biol. Cell* **5**:1081-1092.

Goodman, R.H., and Smolik, S. 2000. CBP/p300 in cell growth, transformation, and development. *Genes Dev.* **14**: 1553-1577.

Grunstein, M. 1997. Histone acetylation in chromatin structure and transcription. *Nature* **389**: 349-352.

Gu, W. and R.G. Roeder, 1997. Activation of p53 sequence-specific DNA binding by acetylation of the p53 C-terminal domain. *Cell* **90**:595-606.

Hagemeier, C., Bannister, A.J., Cook, A. & Kouzarides, T. 1993. The Activation Domain of Transcription Factor-Pu.1 Binds the Retinoblastoma (RB) Protein and the Transcription Factor-TFIID *in vitro* - RB Shows Sequence Similarity to TFIID and TFIIB. *Proc. Natl Acad. Sci. U.S.A.*, **90:** 1580-1584.

Hall, M., S. Bates, and G. Peters. 1995. Evidence for different modes of action of cyclin-dependent kinase inhibitors: p15 and p16 bind to kinases, p21 and p27 bind to cyclins. *Oncogene* **11:** 1581-1588.

Hao, X. F., L. Alphey, L.R. Bandara, E. W.-F. Lam, D. Glover, and N. B. La Thangue, 1995. Functional conservation of the cell cycle-regulating transcription factor DRTF1/E2F and its pathway of control in Drosophila melanogaster. *J. Cell Sci.* **108:** 2945-2954

Harbour, J.W., R.X. Luo, A. Dei Santi, A.A. Postigo, and D.C. Dean. 1999. Cdk phosphorylation triggers sequential intramolecular interactions that progressively block Rb functions as cells move through G1. *Cell* **98**:859-869.

Hateboer, G., R.M. Kerkhoven, A. Shvarts, R. Bernards, and R.L. Beijerbergen. 1996. Deregulation of E2F by the ubiquitin-proteasome pathway: Regulation by retinoblastoma family proteins and adenovirus transforming proteins. *Genes Dev.* **10**:2960-2970.

Hatakeyama, M., Brill, J.A., Fink, G.R. & Weinberg, R.A. 1994. Collaboration of G1 cyclins in the functional inactivation of the retinoblastoma protein. *Genes Dev.* **8:** 1759-1771.

He, S., Cook, B. L., Deverman, B. E., Weihe, U., Zhang, F., Prachand, V., Zheng, J., Weintraub, S. J. 2000. E2F Is Required To Prevent Inappropriate S-Phase Entry of Mammalian Cells. *Mol. Cell. Biol.* 20: 363-371

Helin, K., J. Lees, M. Vidal, N. Dyson, E. Harlow, and A.R. Fattaey. 1992. A cDNA encoding a pRb-binding protein with properties of the transcription factor E2F. *Cell* **70**: 337-350.

Helin, K., E. Harlow, and A.R. Fattaey. 1993a. Inhibition of E2F-1 trans-activation by direct binding of the retinoblastoma protein. *Mol. Cell. Biol.* **13:**6501-6508.

Helin, K. and E. Harlow. 1993b. The retinoblastoma protein as a transcriptional repressor. *Trends. Cell Biol.* **12**: 43-46.

Helin, K., K. Holm, A. Nielbuhr, H. Eiberg, N. Tommerup, S. Hougaar, H.S. Poulsen, M. Spang-Thomsen, and P. Norgaard. 1997. Loss of the retinoblastoma protein-related p130 protein in small cell lung carcinoma. *Proc. Natl. Acad. Sci USA.* **9**:6933-6938.

Helin, K. 1998. Regulation of cell proliferation by the E2F transcription factors. *Curr. Opin. Genet. Dev.* **8**:28-35.

Henglein, B., X. Chenivesse, J. Wang, D. Eick, C. Brıchot 1994. Structure and cell cycle-regulated transcription of the human cyclin A gene. *Proc Natl Acad Sci USA USA* **91:** 5490-4

Herrera, R.E., V.P. Sah, B.O. Williams, T.P, Makela, R.A. Weinberg, and T.Jacks. 1996. Altered cell cycle kinetics, gene expression, and G1 restriction point regulation in Rb-deficient fibroblasts. *Mol. Cell. Biol.* **16**:2402-2407.

Hijmans, E.M., P.M. Voorhoeve, R.L. Beijersbergen, L.J. van't Veer, and R. Bernards. 1995. E2F-5, a new E2F family member that interacts with p130 *in vivo*. *Mol. Cell. Biol.* **15:**3082-3089.

Hill C.R., and R. Treisman 1995. Transcriptional regulation by extracellular signals: mechanisms and specificity. *Cell* **80:** 199-211

Hinds, P.W., S. Mittnacht, V. Dulic, A. Arnold, S.I. Reed, and R.A. Weinberg. 1992. Regulation of retinoblastoma protein functions by ectopic expression of human cyclins. *Cell* **70:**993-1006.

Hofmann, F., F. martelli, D.M. Livingston, and Z. Wang. 1996. The retinoblastoma gene product protects E2F-1 from degradation by the ubiquitin-proteasome pathway. *Genes Dev.* **10**:2949-2959.

Hofmann F., and D.M. Livingston. 1996. Differential effects of cdk2 and cdk3 on the control of pRb and E2F function during G1 exit. *Genes Dev* **10:** 851-61

Hsieh, J.-K., S. Fredersdorf, T. Kouzarides, K. Martin, and X. Lu. 1997. E2F1-induced apoptosis requires DNA binding but not transactivation and is inhibited by the retinoblastoma protein through direct interaction. *Genes Dev.* **11**:1840-1852.

Humbert P.O, R. Verona, J.M. Trimarchi, C. Rogers, S. Dandapani, J.A. Lees. 2000 E2f3 is critical for normal cellular proliferation. *Genes Dev* **14:** 690-703

Hunter, T. and M. Karin. 1992. The regulation of transcription by phosphorylation. *Cell* **70**:375-387.

Hurford, R.K., D. Cobrinik, M.H. Lee, and N. Dyson. 1997. pRB and p107/p130 are required for the regulated expression of different sets of E2F responsive genes. *Genes Dev.* **11:**1447-1463.

Imhof, A., X.J. Yang, V.V. Ogryzko, Y. Nakatani, A.P. Wolffe, and H. Ge. 1997. Acetylation of general transcription factors by histone acetyltransferase. *Curr. Biol.* **7**:689-692.

Ivey-Hoyle,M., R. Conroy, H. Huber, P. Goodhart, A. Oliff, and D.C Heinbrook. 1993. Cloning and characterisation of E2F-2, a novel protein with the biochemical properties of transcription factor E2F. *Mol Cell Biol.* 13: 7802-7812.

Jacks, T., A. Fazeli, E.M. Schmitt, R.T. Bronson, M.A. Goodell, and R.A. Weinberg. 1992. Effects of a Rb mutation in the mouse. *Nature* **359**:295-300.

Jeffrey, P.D., A.A. Russo, K. Polyak, E. Gibbs, J. Hurwitz, J. Massague and Pavletich, N.P. 1995. Crystal structure of a cyclin A-cdk2 complex at 2.3Å: Mechanism of CDK activation by cyclins. *Nature* **376:** 313-317.

Jiang, H., H.S. Chou, and L. Zhu. 1998. Requirement of cyclin E-cdk2 inhibition in $p16^{INK4a}$-mediated growth suppression. *Mol. Cell. Biol.* **18:** 5284-5290

Johnson, D.G., J.K. Schwartz, W.D. Cress, and J.R. Nevins. 1993. Expression of transcription factor E2F1 induces quiescent cells to enter S phase. *Nature* **365:**349-351.

Johnson, D.G., K. Ohtani, and J.R. Nevins. 1994. Autoregulatory control of E2F-1 expression in response to positive and negative regulators of cell cycle progression. *Genes Dev.* **8:**1514-1525.

Johnson, D.G., W.Douglas Cress, L. Jakoi, and J.R. Nevins. 1994b. Oncogenic capacity of the E2F1 gene. *Proc. Natl. Acad. Sci.* **91**:12823-12827.

Johnston, L.H. and N.F. Lowndes. 1992. Cell cycle control of DNA synthesis in budding yeast. *Nucl. Acids Res.* **20:** 2403-2410

Jooss, K., E.W.-F. Lam, A. Bybee, R. Girling, R. Muller, and N.B. La Thangue. 1995. Proto-oncogenic properties of the DP family proteins. *Oncogene* **10:**1529-1536.

Kaelin Jr., W.G., W. Krek, W.R. Sellers, J.A. DeCaprio, F. Ajchenbaum, C.S. Fuchs, T. Chittenden, Y. Li, P.J. Farnham, M.A. Blanar, D.M. Livingston, and E.K. Flemington. 1992. Expression cloning of a cDNA encoding a retinoblastoma-binding protein with E2F-like properties. *Cell* **70:**351-364.

Kawasaki H; Schiltz L; Chiu R; Itakura K; Taira K; Nakatani Y; Yokoyama. 2000. ATF-2 has intrinsic histone acetyltransferase activity which is modulated by phosphorylation. *Nature* **405:** 195-200

Kadonaga, J.T. and Tijan, R. 1986. Affinity purification of sequence-specific DNA binding proteins. *Proc. Natl. Acad. Sci. USA.* **83:** 5889-5893.

Kamb, A., N.A. Gruis, J. Weaver-Feldhaus, Q. Liu, K. Harshman, S.V. Tavitian, E. Stockert, R. Day, B.E. Johnson, and M.H. Skolnik. 1994. A cell cycle regulator potentially involved in genesis of many tumor types. *Science* **264**:436-440.

Kamei, Y., L. Xu, T. Heinzel, J. Torchia, R. Kurokawa, B. Gloss, S.C. Lin, R.A. Heyman, D.W. Rose, and C.K. Glass. 1996. A CBP integrator complex mediates transcriptional activation and AP1 inhibition by nuclear receptors. *Cell* **85**:403-414.

Karin. M. 1999. The Beginning of the End: IκB Kinase (IKK) and NF-κB activation. *J Biol Chem* **274:** 27339-27342.

Kitagawa, M., H. Higashi, I. Suzuki-Takahashi, K. Segawa, S.K. Hanks, Y. Taya, S. Nishimura, and A. Okuyama. 1995. Phosphorylation of E2F-1 by cyclinA-cdk2. *Oncogene* **10:** 229-236.

Kitagawa, M., H. Higashi, H.K. Jung, I. Suzuki-Takahashi, M. Ikeda, K. Tamai, J. Kato, K. Segawa, E. Yoshida, S. Nishimura, and Y. Taya. 1996. The consensus motif for phosphorylation by cyclin D1-Cdk4 is different from that for phosphorylation by cyclin A/E-Cdk2. *EMBO J.* **15:** 7060-7069

Ko, L.J. and C. Prives. 1996. p53: puzzle and paradigm. *Genes Dev.* **10:**1054-1072.

Koepp, D.M., J.W. Harper, S.J. Elledge. 1999. How the cyclin became a cyclin: regulated proteolysis in the cell cycle. *Cell* **97:** 431-4

Koff, A., Giordano, A., Desai, D., Yamashita, K., Harper, J.W., Elledge, S., Nishimoto, T., Morgan, D.O., Franza, B.R. & Roberts, J.M. 1992. Formation and activation of a cyclin E-CDK2 complex during the G1 phase of the human cell cycle. *Science* **257:** 1689-1694

Krek, W., D. M. Livingston, and S. Shirodkar. 1993. Binding to DNA and the retinoblastoma gene product promoted by complex formation of different E2F family members. *Science.* **262**:1557-1560

Krek W., M.E. Ewen, S. Shirodkar, Z. Arany, W.G. Kaelin, and D.M. Livingston. 1994. Negative regulation of the growth-promoting transcription factor E2F-1 by a stably bound cyclinA-dependent protein kinase. *Cell* **78**:161-172.

Krek W., G. Xu, and D. M. Livingston. 1995. Cyclin A-kinase Regulation of E2F-1 DNA Binding Function Underlies Suppression of an S Phase Checkpoint. *Cell* 1995 **83:** 1149-1158.

Kung, A.L., Rebel, V.I., Bronson, R.T., Ch'ng, L.-E., Sieff, C.A., Livingston, D.M., and Yao, T.-P. 2000. Gene dose-dependent control of hematopoiesis and hematologic tumor suppression by CBP. *Genes Dev.* **14:** 272-277

LaBaer, J., M.D. Garrett, L.F. Stevenson, J.M. Slingerland, C. Sandhu, H.S. Chou, A. Fattaey, and E. Harlow. 1997. New functional activities for the p21 family of CDK inhibitors. *Genes Dev.* **11:** 847-862

La Thangue, N.B. 1994. DRTF1/E2F: an expending family of heterodimeric transcription factors implicated in cell-cycle control. *Trends Biochem. Sci.* **19:**108-114.

Lam, E.W.-F. and N.B. La Thangue. 1994. DP and E2F proteins: co-ordinating transcription with cell cycle progression. *Curr. Op. Cell. Biol.* **6:**859-866.

Lam, E.W., and R.J. Watson. 1993. An E2F-binding site mediates cell cycle regulated expression of mouse B-myb transcription. *EMBO J.* **12**:2705-2713.

Lambert, P.F., Kashanchi, F., Radonovich, M.F., Shiekhattar, R., and Brady, J.N. 1998. Phosphorylation of p53 serine 15 increases interaction with CBP. *J Biol. Chem.* **273**: 33048-33053

Lee, M.-H., B.O. Williams, G. Mulligan, S. Mukai, R.T. Bronson, N. Dyson, E. Harlow, and T. Jacks. 1996. Targeted disruption of p107: functional overlap between p107 and Rb. *Gen & Dev.* **10**:1621-1632.
Lee, C.-W., T.S. Sørensen, N. Shikama, and N.B. La Thangue. 1998. Functional interplay between p53 and E2F through co-activator p300. *Oncogene* **16**:2695-2710.

Lees, J.A., M. Saito, M. Vidal, M. Valentine, T. Look, E. Harlow, N. Dyson, and K. Helin. 1993. The retinoblastoma protein binds to a family of E2F transcription factors. *Mol Cell Biol.* **13**:7813-7825.

Lees, E. 1995. Cyclin dependent kinase regulation. *Curr. Op. Cell. Biol.* **7:**773-780.

Leone, G., J. DeGregori, R. Sears, L. Jakoi, and J.R. Nevins. 1997. Myc and Ras collaborate in inducing accumulation of active cyclin E/Cdk2 and E2F. *Nature* **387**:422-426.

Leone, G., J. DeGregori, Z. Yan, L. Jakoi, S. Ishida, R.S. Williams, and J.R. Nevins. 1998. E2F-3 activity is regulated during the cell cycle and is required for the induction of S phase. *Genes Dev.* **12**:2120-2130.

Leone, G., Nuckolls, F., Ishida, S., Adams, M., Sears, R., Jakoi, L., Miron, A., Nevins, J. R. 2000. Identification of a Novel E2F3 Product Suggests a Mechanism for Determining Specificity of Repression by Rb Proteins. *Mol. Cell. Biol.* 20: 3626-3632.

Levine, A.J. 1997. p53, the cellular gatekeeper for growth and division. *Cell* **88**:323-331.

Li, Y., C. Graham, S. Lacy, A.M.V. Duncan, and P. Whyte. 1993. The adenovirus E1A-associated 130-kD protein is encoded by a member of the retinoblastoma gene family and physically interacts with cyclins A and E. *Genes Dev.* **7:**2366-2377.

Li J.M, P.P.-C Hu, X. Shen, Y. Yu, and X,-F, Wang. 1997. E2F4-RB and E2F4-p107 complexes suppress gene expression by transforming growth factor through E2F binding sites. *Proc. Natl. Acad. Sci. USA.* **94:**4948-4953.

Lin, A., Frost, J., Deng, T., Al-Alawi, N., Smeal, T., Kikkawa, U., Hunter, T., Brenner, D. and Karin, M. 1992. Casein kinase II is a negative regulator of c-Jun DNA binding and AP-1activity. *Cell* **70:** 777-789.

Lindeman G.J., L. Dagnino, S. Gaubatz, Y. Xu, R.T. Bronson, H.B. Warren, and D.M. Livingston. 1998. A specific, nonproliferative role for E2F-5 in choroid plexus function revealed by gene targeting. *Genes Dev.* **12:** 1092-1098.

Lindeman G.J., S. Gaubatz, D.M. Livingston, and D. Ginsberg. 1997. The subcellular localisation of E2F-4 is cell-cycle dependent *Proc. Natl. Acad. Sci.* **94:** 5095-5100.

Liu, Y.Z., Chrivia, J.C., and Latchman, D.S. 1998. Nerve growth factor up-regulates the transcriptional activity of CBP through activation of the p42/p44 (MAPK) cascade. *J. Biol. Chem.* **273:** 32400-32407

Lundblad, J.R., R.P.S. Kwok, M.E. Laurance, M.L. Harter, and R.H.Goodman. 1995. Adenoviral E1A-associated protein p300 as a functional homologue of the transcriptional co-activator CBP. *Nature* **374**:85-88.

Lundberg A.S., and R.A. Weinberg . 1998. Functional Inactivation of the Retinoblastoma Protein Requires Sequential Modification by at Least Two Distinct Cyclin-cdk Complexes. *Mol. Cell. Biol.* **18:** 753-761.

Lukas, J., D. Parry, L. Aagaard, D.J. Mann, J. Bartkova, M. Strauss, G. Peters, and J. Bartek. 1995. Retinoblastoma-protein-dependent cell-cycle inhibition by the tumour suppressor p16^{INK4a}. *Nature* **375:**503-506.

Lukas, J., B.O. Petersen, K. Holm, J. Bartek, and K. Helin. 1996. Deregulated expression of E2F family members induces S-phase entry and overcomes p16^{INK4a}-mediated growth suppression. *Mol. Cell. Biol.* **16:**1047-1057.

Lukas, J., T. Herzinger, K. Hansen, M.C. Moroni, D. Resnitsky, K. Helin, S.I. Reed, and J. Bartek. 1997. Cyclin E-induced S phase without activation of the Rb/E2F pathway. *Genes Dev.* **11:** 1479-1492

Luo, K. and B.M. Sefton. 1990. Transfer of proteins to membranes facilitates both cyanogen bromide cleavage and two dimensional proteolytic mapping. *Oncogene* **5:** 921-923.

Luo, R.X., A.A. Postigo, and D.C. Dean. 1998. Rb interacts with histone deacetylase to repress transcription. *Cell* **92**:463-473.

Magae, J., C.-L. Wu, S. Illenye, E. Harlow, and N.H. Heintz. 1996. Nuclear localisation of DP and E2F transcription factors by heterodimeric partners and retinoblastoma protein family members. *J. Cell Sci.* **109**:1717-1726.

Magnaghi-Jaulin, L., R. Groisman, I. Naguibneva, P. Robin, S. Lorain, J.P. Le Villain, F. Troalen, D. Trouche, and A. Harel-Bellan. 1998. Retinoblastoma protein represses transcription by recruiting a histone deacetylase. *Nature* **391:** 601-604

Mann, D.J., and N.C. Jones. 1996. E2F-1 but not E2F-4 can overcome p16-induced G1 cell cycle arrest. *Curr. Biol.* **6**:474-483.

Martelli, F., and D.M. Livingston. 1999. Regulation of endogenous E2F1 stability by the retinoblastoma family proteins. *Proc. Natl. Acad. Sci.* **96**:2858-63.

Marti, A., C. Wirbelauer, M. Scheffner and W. Krek. 1999. Interaction between ubiquitin-protein ligase SCF^{SKP2} and E2F-1 underlies the regulation of E2F-1 degradation. *Nature Cell Biol.* **1:** 14-19.

Martinez-Balbas M.A., Bannister A.J., Martin K., Haus-Seuffert P., Meisterernst, M., and Kouzarides, T. 1998. The acetyltransferase activity of CBP stimulates transcription. *EMBO J.* **17**:2886-2893.

Martinez-Balbás, M.A., Bauer, U-M., Nielsen, S.J., Brehm, A., and Kouzarides, T. 2000. Regulation of E2F-1 activity by acetylation. *EMBO J.* **19**: 662-271.

Mayol X., J.Garriga , X. Grapa. 1995. Cell cycle-dependent phosphorylation of the retinoblastoma-related protein p130. *Oncogene* **11:** 801-8

Mittnacht, S. 1998. Control of pRB phosphorylation. *Curr. Opin. Genet. Dev.* **8**: 21-27.

Moberg, K., M.A. Starz, and J.A. Lees. 1996. E2F-4 switches from p130 to p107 and pRB in response to cell cycle re-entry. *Mol. Cell. Biol.* **16**:1436-1449.

Montagnoli A, F. Fiore, E. Eytan, A.C. Carrano, G.F. Draetta, A. Hershko, M. Pagano 1999. Ubiquitination of p27 is regulated by Cdk-dependent phosphorylation and trimeric complex formation. *Genes Dev* **13:** 1181-9

Moran, E. 1993. DNA tumor virus transforming proteins and the cell cycle. *Curr. Opin. Genet. Dev.* **3:**63-70.

Morkel, M., J. Wenkel, A.J. Bannister, T. Kouzarides, and C. Hagemeier. 1997. An E2F-like repressor of transcription. *Nature* **390**: 567-568.

Morris, L, K.E. Allen, and N.B. La Thangue. 2000. Regulation of E2F transcription by cyclin E-Cdk2 kinase mediated through p300/CBP co-activators. *Nat. Cell Biol.* **2**:232-239.

Müller H., and K. Helin 2000. The E2F transcription factors: key regulators of cell proliferation. Biochim Biophys Acta **1470:** M1-12.

Musti A.M., M. Treier and D.Bohmann. 1997. Reduced ubiquitin-dependent degradation of c-Jun after phosphorylation by MAP kinases. *Science* **275:**400-402

Nakanishi, M., R.S. Robetorge, G.R. Adami, O.M. Pereira-Smith, and J.R. Smith. 1995. Identification of the active region of the DNA synthesis inhibitory gene $p21^{Sdi1/CIP1/WAF1}$. *EMBO J.* **14:** 555-563

Neuman E., E.K. Flemington, W.R. Sellers, and W.G. Kaelin, Jr. 1994. Transcription of the E2F-1 gene is rendered cell cycle dependent by E2F DNA-binding sites within its promoter. *Mol. Cell. Biol.* **14**:6607-6615.

Nigg, E.A. 1993. Cellular substrates of p34 cdc2 and its companion cyclin-dependent kinases. *Trends Cell Biol.* **3:** 296-301

Ogryzko, V.V., R.L. Schitz, V. Russanova, B.H. Howard, and Y. Nakatani. 1996. The transcriptional co-activators p300 and CBP are histone acetyltransferases. *Cell* **87**:953-959.

Ohtsubo, M., Theodoras, A.M., Schumacher, J., Roberts, J.M. and Pagano, M. 1995. Human cyclin E, a nuclear protein essential for the G1 to S phase transition. *Mol. Cell Biol.* **15:** 2612-2624.

Ormondroyd, E., S. de la Luna, and N.B La Thangue. 1995. A new member of the DP family, DP-3, with distinct protein products suggests a regulatory role for alternative splicing in the cell cycle transcription factor DRTF1/E2F. *Oncogene* **11**: 1437-1446.

Peeper, D.S., P. Keblusek, K. Helin, M. Toebes, A.J. Vandereb and A. Zantema. 1994. Phosphorylation of a Specific cdk Site in E2F-1 Affects its Electrophoretic Mobility and Promotes pRB-Binding *in vitro*. *Oncogene* **10**: 39-48.

Pearson, A., and J. Greenblatt 1997. Modular organisation of the E2F1 activation domain and its interaction with general transcription factors TBP and TFIIH. Oncogene 15: 2643-2658

Perkins, N.D., L.K. Felzien, J.C. Betts, K. Leung, D.H. Beach, and G.J. Nabel. 1997. Regulation of NF-*K*B by Cyclin-dependent kinases associated with the p300 co-activator. *Science* **275**: 523-527.

Perez R.I., S.H. Kim, B. Griffiths, A. Sewing A, H. Land. 1999. Cyclins D1 and D2 mediate myc-induced proliferation via sequestration of p27(Kip1) and p21(Cip1). *EMBO J* **18:** 5310-20

Pines, J. 1995. Cyclins, CDKs and cancer. *Seminars in Cancer Biology* **6**: 63-72.

Polanowska, J., L. Le Cam, B. Orsetti, H. Valles, E. Fabbrizio, L. Fajas, S. Taviaux, and C. Theillet, and C. Sardet. 2000. Human E2F5 gene is oncogenic in primary rodent cells and is amplified in human breast tumors. *Genes Chromosomes Cancer.* **28**: 126-30.

Prives, C. 1998. Signaling to p53: breaking the MDM2-p53 circuit. **95:** 5-8

Pulverer B.J., J.M. Kyriakis, J. Avruch, E. Nikolakaki, and J.R. Woodgett. 1991. Phosphorylation of c-jun mediated by MAP kinases. *Nature* 353: 670-674

Qin, X.-Q., D.M. Livingston, W.G. Kaelin Jr., and P.D. Adams. 1994. Deregulated transcription factor E2F-1 expression leads to S-phase entry and p53-mediated apoptosis. *Proc. Natl. Acad. Sci. USA.* **91:**10918-10922.

Quelle, D.E., R.A. Ashmun, S.E. Shurtleff, J.-Y. Kato, D. Bar-Sagi, M. Roussel, and C.J. Sherr. 1993. Overexpression of mouse D-type cyclins accelerates G_1 phase in rodent fibroblasts. *Genes Dev.* **7:** 1559-1571.

Quelle, D.E., F. Zindy, R.A. Ashmun, and C.J. Sherr. 1995. Alternative reading frames of the INK4a tumor suppressor gene encode two unrelated proteins capable of inducing cell cycle arrest. *Cell* **83:** 993-1000

Reynisdóttir, I. and J. Massagué. 1997. The subcellular locations of $p15^{INK4b}$ and $p27^{Kip1}$ coordinate their inhibitory interactions with cdk4 and cdk2. *Genes Dev.* **11:** 492-503.

Reynisdóttir, I., K. Polyak, A. Iavarone, and J. Massagué. 1995. Kip/Cip and Ink4 cdk inhibitors cooperate to induce cell cycle arrest in response to TGFß. *Genes Dev.* **9:** 1831-1845.

Rogers, K.T., P.D.R. Higgins, M.M. Milla, R.S. Phillips, and J.M. Horowitz. 1996. DP-2, a heterodimeric partner of E2F: Identification and characterization of DP-2 proteins expressed *in vivo*. *Proc. Natl. Acad. Sci USA.* **93**:7594-7599.

Royzman, I., A.J. Whittaker, and T.L. Orr-Weaver. 1997. Mutations in Drosophila DP and E2F distinguish G_1-S progression from an associated transcriptional program. *Genes Dev.* **11:** 1999-2011.

Ruas, M., and G. Peters. 1998. The p16INK4a/CDKN2A tumor suppressor and its relatives. *Biochim Biophys Acta* **1378:** F115-77

Saito, M., K. Helin, M.B. Valentine, B.B. Griffith, C.L. Willman, E. Harlow, and A.T. Look. 1995. Amplification of the E2F1 transcription factor gene in the HEL erythroleukemia cell line. *Genomics* **25:** 130-138.

Sambrook, J., Fritsch, E.F. & Maniatis, T. 1989. Molecular cloning. A laboratory manual. 2nd ed. Cold Spring Harbour Press, New York. .

Sardet, C., M. Vidal, D Cobrinik, Y. Geng, C. Onufryk, A. Chen, and R.A. Weinberg. 1995. E2F-4 and E2F-5, two novel members of the E2F family, are expressed in the early phases of the cell cycle. *Proc. Natl. Acad. Sci. USA.* **92**: 2403-2407.

Sartorelli, V., Puri, P.L., Hamamori, Y., Ogryzko, V., Chung, G., Nakatani, Y., Wang, J.Y., and Kedes, L. 1999. Acetylation of MyoD directed by P/CAF is necessary for the execution of the muscle program. *Mol. Cell.* **4:** 725-734

Sellers, W.R., J.W. Rodgers, and W.G. Kaelin, Jr. 1995. A potent transrepression domain in the retinoblastoma protein induces a cell cycle arrest when bound to E2F sites. *Proc. Natl. Acad. Sci.* **92:** 11544-11548

Serizawa, H., Makela, T.P., Conaway, J.W., Conaway, R.C., Weinberg, R.A.and Young, R.A. 1995. Transcription factor TFIIH contains MO15 and cyclin H subunits of CDK-activating kinase. *Nature*, **374:** 280-282.

Shan, B., X. Zhu, P-L. Chen, T. Durfee,Y. Yang, D. Sharp and W.-H. Lee. 1992. Molecular cloning of cellular genes encoding retinoblastmoma-associated proteins: Identification of a gene with properties of the transcription factor E2F. *Mol Cell. Biol.* **12**:5620-5631.

Shan, B. and W.-H. Lee. 1994. Deregulated expression of E2F-1 induces S phase entry and leads to apoptosis. *Mol. Cell. Biol.* **14**:8166-8173.

Sheaff, R.J., M. Groudine, M. Gordon, J.M. Roberts, and B.E. Clurman. 1997. Cyclin E-CDK2 is a regulator of p27Kip1. *Genes Dev.* **11**:1464-1478.

Sherr, C.J. 1993. Mammalian G1 cyclins. *Cell* **73:**1059-1065.

Sherr, C.J. 1996. Cancer Cell Cycles. *Science* **274:** 1672-1677.

Sherr, C.J. and J.M. Roberts. 1999. CDK inhibitors: positive and negative regulators of G_1-phase progression. *Genes Dev.* **13**: 1501-1512

Shieh, S.-Y., M. Ikeda, Y. Taya, and C. Prives. 1997. DNA damage-induced phosphorylation of p53 alleviates inhibition by MDM2. *Cell* **91**:325-334.

Shikama, N., J. Lyon, and N.B. La Thangue. 1997. The p300/CBP family: integrating signals with transcription factors and chromatin. *Trends Cell. Biol.* 7:230-236.

Shikama, N., C.-W. Lee, S. France, L. Delavaine, J. Lyon, M. Krstic-Demonacos, and N.B. La Thangue. 1999. A novel cofactor for p300 that regulates the p53 response. *Mol Cell* **4**:365-376.

Shikama., N. Chan., H. Smith., L. Krstic-Demonacos., M. Lee., C. Cairns., W. La Thangue., N. 2000. Functional interaction between nucleosome assembly proteins and p300/CBP co-activators. *Mol. Cell.Bio* (in press)

Shirodkar, S., M. Ewen, J.A. DeCaprio, J. Morgan, D.M. Livingston, and T. Chittenden. 1992. The transcription factor E2F interacts with the retinoblastoma product and a p107-cyclin A complex in a cell cycle-regulated manner. *Cell.* **68**:157-166.

Siliciano J.D., C.E. Canman, Y. Taya, K. Sakaguchi, E. Appella, and M.B. Kastan. 1997. DNA damage induces phosphorylation of the amino terminus of p53. *Genes Dev* **11:** 3471-3481

Singh, P., S.H. Wong, and W. Hong. 1994. Overexpression of E2F-1 in rat embryo fibroblasts leads to neoplastic transformation. *EMBO J.* **13**:3329-3338.

Slansky, J.E., Li, Y., Kaelin, W.G. and Farnham, P.J. 1993. A protein synthesis-dependent increase in E2F1 mRNA correlates with growth regulation of the dihydrofolate reductase promoter. *Mol. Cell. Biol.* **13**:1610-1618.

Slansky, J.E. and P.J Farnham. 1996. Introduction to the E2F family: protein structure and gene regulation. *Curr. Top. Microbiol. Immunol.* **208:** 1-30

Smith, E.J., G. Leone, J. DeGregori, L. Jakoi, and J.R. Nevins. 1996. The accumulation of an E2F-p130 transcriptional repressor distinguishes a G0 cell state from a G1 phase state. *Mol. Cell. Biol.* **16**:6965-6976.

Smith E.J, G. Leone, J.R. Nevins. 1998. Distinct mechanisms control the accumulation of the Rb-related p107 and p130 proteins during cell growth. *Cell Growth Differ* **9:** 297-303.

Songyang Z., K.P. Lu, Y.T. Kwon, L.H. Tsai, O. Filhol O, C.Cochet, D.A. Brickey, T.R. Soderling, C. Bartleson, D.J. Graves, A.J. DeMaggio, M.F. Hoekstra, J. Blenis, T. Hunter and L.C. Cantley. 1996. A structural basis for substrate specificities of protein Ser/Thr kinases: primary sequence preference of casein kinases I and II, NIMA, phosphorylase kinase, calmodulin-dependent kinase II, CDK5, and Erk1. *Mol Cell Biol* **16:** 6486-6493

Sørensen, T.S., R. Girling, C.-W. Lee, J. Gannon, L.R. Bandara, and N.B. La Thangue. 1996. Functional interaction between DP-1 and p53. *Mol. Cell. Biol.* **16:**5888-5895.

Swope, D.L., Mueller, C.L., and Chrivia, J.C. 1996. CREB-binding protein activates transcription through multiple domains. *J. Biol. Chem.* **271:** 28138-28145

Tanaka, Y., Naruse, I., Maekawa, T., Masuya, H., Shiroshi, T., and Ishii, S. (1997). Abnormal skeletal patterning in embryos lacking a single Cbp allele: a partial similarity with Rubinstein-Taybi syndrome. *Proc. Natl. Acad. Sci. USA* **94**, 10215-10220.

Trimarchi, J.M., B. Fairchild, R. Verona, K. Moberg, N. Andon, and J.A. Lees. 1998. E2F-6, a member of the E2F family that can behave as a transcriptional repressor. *Proc. Natl. Acad. Sci. USA* **95**:2850-2855.

Trouche, D., A. Cook, and T. Kouzarides. 1996. The CBP co-activator stimulates E2F1/DP1 activity. *Nucleic Acids Res.* **24:**4139-4145.

Trouche, D. and T. Kouzarides. 1996. E2F1 and E1A12S have a homologous activation domain regulated by RB and CBP. *Proc. Natl. Acad. Sci. USA* **93**:1439-1442.

Vairo, G., D.M. Livingston, and D. Ginsberg. 1995. Functional interaction between E2F-4 and p130: evidence for distinct mechanisms underlying growth suppression by different retinoblastoma protein family members. *Genes Dev.* **9:**869-881.

Vandel, L. and T. Kouzarides. 1999. Residues phosphorylated by TFIIH are required for E2F-1 degradation during S-phase. *EMBO J.* **18:** 4280-4291

Waga, S., G.J. Hannon, D. Beach, and B. Stillman. 1994. The p21 inhibitor of cyclin-dependent kinases controls DNA replication by interaction with PCNA. *Nature* **369**:574-578.

Wang, Y. and C. Prives. 1995. Increased and altered DNA binding of human p53 by S and G2/M but not G1 cyclin-dependent kinases. *Nature* **376**:88-91.

Wang, Z.M., H. Yang, and D.M. Livingston. 1998. Endogenous E2F-1 promotes timely G0 exit of resting mouse embryo fibroblasts. *Proc. Natl. Acad. Sci.* **95**:15583-15586.

Wang, D., J. Russell, and D. Johnson. 2000. E2F4 and E2F1 have similar proliferative properties but different apoptotic properties in vivo. *Mol. Cell. Biol.* **20**:3471-3424.

Weinberg, R.A. 1995. The retinoblastoma protein and cell cycle control. *Cell* **81:**323-330.

Weintraub, S.J., C.A Prater, and D.C.Dean. 1992. Retinoblastoma protein switches the E2F site from positive to negative element. *Nature* **358:** 259-261.

Weintraub, S.J., K.N.B. Chow, R.X. Luo, S.H. Zhang, S. He, and D.C. Dean. 1995. Mechanism of active transcriptional repression by the retinoblastoma protein. *Nature* **375**:812-815.

Whyte, P. 1995. The retinoblastoma protein and its relatives. *Seminars in Cancer Biology* **6**:83-90.

Won, K.-A. and S.I. Reed. 1996. Activation of cyclin E/CDK2 is coupled to site-specific autophosphorylation and ubiquitin-dependent degradation of cyclin E. *EMBO J.* **15:** 4182-4193

Wu, C.-L., L.R. Zukerberg, C. Ngwu, E. Harlow, and J. A. Lees. 1995. *In vivo* association of E2F and DP family proteins. *Mol. Cell. Biol.* **15**:2536-2546.

Wu, C.-L., M. Classon, N. Dyson, and E. Harlow. 1996. Expression of dominant-negative mutant DP-1 blocks cell cycle progression in G1. *Mol. Cell. Biol.* **16**:3698-3706

Xiao Z.X., D. Ginsberg , M. Ewen, and D.M. Livingston. 1996. Regulation of the retinoblastoma protein-related protein p107 by G1 cyclin-associated kinases *Proc. Natl. Acad. Sci. USA.* **93**:4633-4637.

Xu, M., K.A. Sheppard, C.Y. Peng, A.S. Yee, and H. Piwnica-Worms. 1994. Cyclin A/CDK2 binds directly to E2F-1 and inhibits the DNA-binding activity of E2F-1/DP-1 by phosphorylation. *Mol. Cell. Biol.* **14**:8420-8431.

Xu, G., D.M.Livingston, and W. Krek. 1995. Multiple members of the E2F family are the products of oncogenes. *Proc. Natl. Acad. Sci.USA* **92**:1357-1361.

Yaciuk, P. and E. Moran. 1991. Analysis with specific polyclonal antiserum indicates that the E1A-associated 300KDa product is a stable nucleur phosphoprotein that undergoes cell cycle phase-specfic modification. *Mol. Cell. Biol.* **11**:5389-5397.

Yamasaki, L., T. Jacks, R. Bronson, E. Goillot, E. Harlow, and N. Dyson. 1996. Tumour induction and tissue atrophy in mice lacking E2F-1. *Cell* **85:**537-547.

Yang, X.-J., V.V. Ogryzko, J.-I. Nishikawa, B.H. Howard, and Y. Nakatani. 1996. A p300/CBP-associated factor that competes with the adenoviral oncoprotein E1A. *Nature* **382**:319-324.

Yao, T.-P., S.P. Oh, M. Fuchs, N.-D. Zhou, L.-E. Ch'ng, D. Newsome, R.T. Bronson, E. Li, D.M. Livingston, and R. Eckner. 1998. Gene dosage-development embryonic development and proliferation defects in mice lacking the transcriptional integrator p300. *Cell* **93**:361-372.

Yuan, W., G. Condorelli, M. Caruso, A. Felsani, and A. Giordano. 1996. Human p300 protein is a co-activator for transcription factor MyoD. *J. Biol. Chem.* **271**:9009-9013.

Zamanian, M. and N.B. La Thangue. 1992. Adenovirus E1a prevents the retinoblastoma gene product from repressing the activity of a cellular transcription factor. *EMBO J.* **11**:2603-2610.

Zarkowska T and S. Mittnacht. 1997. Differential phosphorylation of the retinoblastoma protein by G1/S cyclin-dependent kinases. *J Biol Chem* **272:**12738-12746

Zerfass-Thome K., Schulze, A., Zwerschke, W., Vogt, B., Helin, K., Bartek, J., Henglein, B. & Jansen-Dürr, P. 1997. p27(KIP1) blocks cyclin E-dependent transactivation of cyclin A gene expression. *Mol Cell Biol* **17:**407-415.

Zhang, Y.H. & Chellappan, S.P. 1995. Cloning and Characterization of Human DP-2, a Novel Dimerization Partner of E2F. *Oncogene*, **10:** 2085-2093.

Zhang, H.S., A.A Postigo and D.C. Dean.1999. Active transcriptional repression by the Rb E2F complex mediates G1 arrest triggered by $p16^{INK4a}$, TGF ß, and contact inhibition. *Cell* **97:** 53-61.

Zhang, H.S., M. Gavin, A. Dahiya, A.A Postigo D. Ma, R.X. Luo, J.W Harbour, and D.C. Dean. 2000. Exit from G1 and S Phase of the Cell Cycle is regulated by repressor complexes containing HDAC-Rb-hSWI/SNF and Rb-hSWI/SNF. *Cell* **101:** 79-89.

Zheng, N., E. Fraenkel, C.O. Pabo, and N.P. Pavletich. 1999. Structural basis of DNA recognition by the heterodimeric cell cycle transcription factor E2F-DP. *Genes Dev.* **13:**666-674.

Zhong H., H. SuYang H, B.H. Erdjument Bromage H, P. Tempst P, and S. Ghosh. 1997. The transcriptional activity of NF-kappaB is regulated by the IkappaB-associated PKAc subunit through a cyclic AMP-independent mechanism. *Cell* **89:** 413-424

Zhong H., R.E. Voll, and S. Ghosh. 1998. Phosphorylation of NF-kappa B p65 by PKA stimulates transcriptional activity by promoting a novel bivalent interaction with the coactivator CBP/p300. *Mol Cell* **1:** 661-71

Zhu, L., S. van den Heuvel, K. helin, A. Fattaey, M. Ewen, D. Livingston, N. Dyson, and E. Harlow. 1993. Inhibition of cell proliferation by p107, a relative of the retinoblastoma protein. *Genes Dev.* **7**:1111-1125.

Zhu, L., G. Enders, J.A. Lees, R.L. Beijersbergen, R. Bernards, E. Harlow. 1995. The pRB-related protein p107 contains two growth suppression domains: independent interactions with E2F and cyclin/cdk complexes. *EMBO J* **14:** 1904-13

Acknowledgements

I would like to thank my supervisor, Professor Nick La Thangue for his encouragement and guidance throughout this PhD. All members of cathcart lab have been an immense help to me over the years and I thank them for making my time here both fun and interesting. Laurent, in particular taught me some invaluable techniques and Noriko was a constant source of inspiration.

Liz Allen initiated many of the ideas at the beginning of this research and her continued support and advice have been invaluable. Thanks to Liz also for proofreading. I would like to express a special thank you to Amanda Cruickshank and Linda Smith for their encouragement and generosity. I don't think I would have finished this thesis so soon had it not been for their help.

Thanks to Vicky, Andrew and mum for providing a welcome diversion from the lab. Finally, I thank Matloob for helping me to put things into perspective and knowing just how to make me happy after a long days work.

This research was supported by the Medical Research Council (MRC) and the Institute of Biomedical and Life Science (IBLS) of the University of Glasgow.