This work is dedicated to my family.

BIOCHEMICAL CHARACTERISATION OF THE EUKARYOTIC CELL CYCLE REGULATORY PROTEINS, E2F AND pRB

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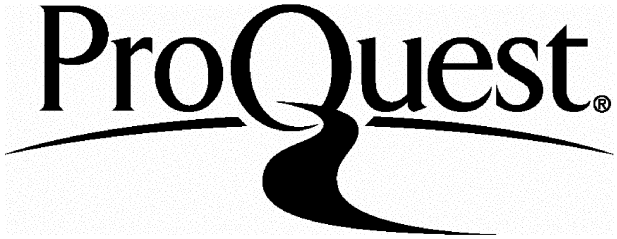

ABSTRACT

Control of the cell cycle is partly mediated by a transcriptional regulatory mechanism whose components include the pRb family of tumour suppressors (pRb, p130, p107) and the E2F/DP heterodimeric transcription factors. Each of these heterodimers consists of one member of the E2F family of proteins (E2Fs 1-6) and one of the DP family (DPs 1 and 2). E2F/DP activation of cell cycle genes is negatively regulated by cyclin A-CDK2-mediated phosphorylation of DP. The formation of a complex between E2F/DP and a pRb family protein leads to anti-proliferative transcriptional repression. Binding of the Human Papillomavirus (HPV) E7 oncoprotein to pRb blocks the interaction of the tumour suppressor with E2F/DP as part of a viral cell transformation mechanism.

Fragments of pRb, E2F-1 and DP-1 were over-expressed and purified by chromatographic means prior to their biochemical characterisation. Using the SELEX (Systematic Evolution of Ligands by Exponential Enrichment) procedure, attempts were made to determine high affinity DNA-binding sites for E2F homodimers. Although such sites were not identified, important considerations relating to the SELEX protocol are highlighted by these experiments.

Electrophoretic Mobility Shift Assays were used to demonstrate that the interaction of fragments of E2F-1 and DP-1 with their cognate DNA could be inhibited by phosphorylation by cyclin A-CDK2. Furthermore, mutation of one of two putative phosphorylation sites in DP-1 resulted in a reduced rate of cyclin A-CDK2-dependent loss of DNA binding.

Isothermal titration calorimetry studies revealed that not only does pRb interact with the minimal pRb-binding region of E2F-1, but also with additional regions outside of the transactivation domain. I present data showing that HPV E7 competes for binding to pRb with constructs of E2F-1 incorporating these additional regions. Our results suggest that the CR3 domain of E7 competes with the marked box region of E2F-1 for binding to pRb.

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ABBREVIATIONS

βME	β-mercaptoethanol
Ad	Adenovirus
ATP	Adenosine 5'-triphosphate
AU	Absorbance units
CDK	Cyclin-dependent kinase
CDKI	Cyclin-dependent kinase inhibitor
conOLIGO	Control oligonculeotide
CR(1-3)	Conserved region (1-3)
Da	Daltons
degOLIGO	Degenerate oligonucleotide
DHFR	Dihydrofolate Reductase
dNTP	2'-deoxynucleotide 5'-triphosphate
DP	Differentiation Protein
dsDNA	Double-stranded DNA
DTT	Dithiothreitol
E1A	Early 1 A Protein
E2A	Early 2 A gene
E2F	Early 2 Factor (Also a generic term for E2F/DP Heterodimeric Transcription Factors
E4	Early 4 protein
E7	Early 7 protein
EDTA	Ethylenediaminetetra acetic acid
EMSA	Electrophoretic mobility shift assay
ESMS	Electrospray mass spectrometry
G	Gap phase
GST	Glutathione-S-transferase
HPV	Human Papillomavirus
IPTG	Isopropyl-β-D-thiogalactopyranoside
ITC	Isothermal Titration Calorimetry
K_d	Dissociation constant
kDa	Kilo daltons
M	Mitotic Phase
N	Stoichiometry
PAGE	Polyacrylamide gel electrophoresis

PIC	Pre-initiation complex
pol	RNA polymerase
PP	Pocket protein
pRb	Retinoblastoma tumour suppressor gene product
recOLIGO	Recovered oligonucleotide
S	Synthesis Phase
SDS	Sodium dodecyl sulphate
SELEX	Systematic Evolution of Ligands by Exponential Enrichment
selOLIGO	Selected oligonucleotide
SV40	Simian virus 40
TAE	Tris acetate EDTA
TAg	SV40 Large T Antigen
TBP	TATA-binding protein
TFII	Transcription Factor II
Tris	Tris(hydroxymethyl)methylamine
UV	Ultra-violet
XGAL	5-bromo-4-chloro-3-indoyl-β-D-galactoside

CHAPTER 1

INTRODUCTION

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The cell cycle is a process comprising distinct and ordered cellular events, culminating in the duplication of a cell. This process is necessary for growth, development and homeostasis. The cell cycle is regulated by a plethora of complex, partially overlapping and degenerate mechanisms that seek to maintain strict control of cellular division. A devastating consequence of uncontrolled cell proliferation is cancer, responsible for one in six deaths in the developed world.

Cell cycle regulation is intimately linked to a transcriptional control mechanism. This involves the appropriate activation and repression of genes encoding proteins that regulate cell cycle progression and those that mediate basic replicative processes such as DNA synthesis. This chapter begins with an overview of the cell cycle, followed by an outline of its regulation at the transcriptional level. I will then provide a more detailed account of specific transcriptional regulatory proteins that have been investigated during this study.

1.1.

THE EUKARYOTIC CELL CYCLE

Figure 1 depicts the four main successive phases into which the cell cycle is generally divided. The G_0 phase (G for "gap") is a quiescent state in which a cell does not divide. Certain terminally differentiated cells assume this state permanently and thus never replicate, e.g. neurons. G_0 is sustained when there are low levels of nutrients or through contact inhibition, when the cell is in touch physically with other ones.

The cell's entry into G_1 can be induced by extracellular signals (such as growth factors and hormones). The G_1 phase is the longest in the cycle and is a period of growth. The cycle's duration in a multicellular organism ranges between eight hours and over one hundred days depending on the cell type, with most of this variation being attributable to the length of G_1.

The S phase (S for "synthesis") is the period in which DNA is replicated such that the number of chromosomes in the cell (compared to that in G_0/G_1) is doubled. The S phase is followed by another period of growth, the G_2 phase (Alberts *et al.*, 1994; Voet and Voet, 1995).

Nuclear and cell division take place in the M phase (M for "mitosis") that comprises the following stages. The prophase stage is characterised by the condensation of chromosomes. This is followed by metaphase during which the

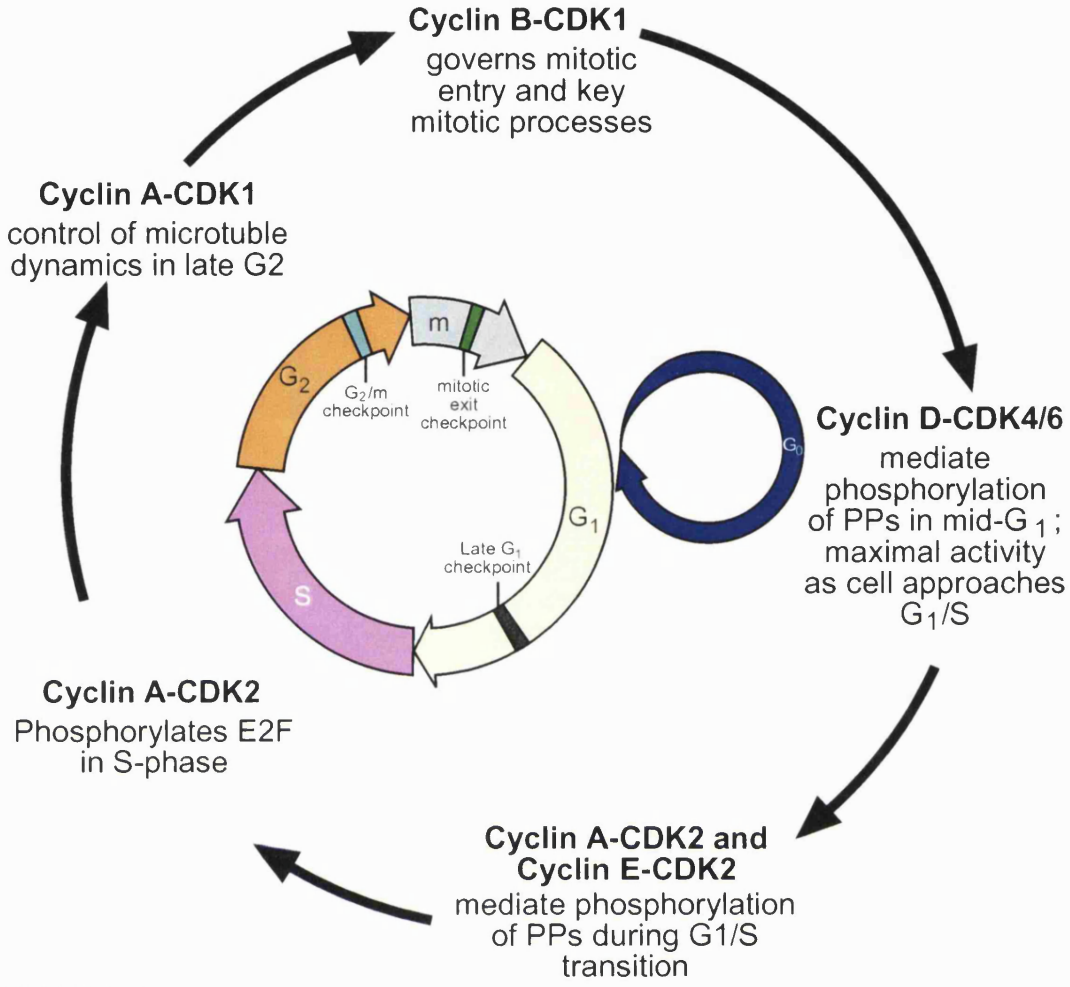


FIGURE 1

THE EUKARYOTIC CELL CYCLE

(Adapted from Lodish *et. al.*, 1999)

The four main successive phases of the cell cycle are:

G_1 phase (G for "gap") : a growth period.

S phase (S for "synthesis") : in which DNA is replicated.

G2 phase : a second growth period.

M phase (M for "mitosis") : in which nuclear and cell division take place.

A cell does not divide in **G_0** , the quiescent state.

Progression of the cycle is effected by distinct cyclin-kinase complexes. The cycle can be arrested at checkpoints if the cell is not in a satisfactory physiological state (see text).

PPs : pocket proteins

chromosomes align on the mitotic spindle, a structure formed by the reorganisation of microtubules. In anaphase, the chromosomes move to opposite poles of this spindle such that the original and duplicate DNA are segregated. Soon after the onset of mitosis in most higher eukaryotes, the nuclear envelope disintegrates into several vesicles and then reassembles around these separated chromosomes while they undergo decondensation in telophase. Two daughter cells are produced at the end of mitosis by the division of the cytoplasm, a process known as cytokinesis. However in yeast, the nucleus divides at the time of cytokinesis without disintegration of the nuclear envelope (Alberts *et al.*, 1994; Lodish *et al.*, 1999).

1.1.1

Checkpoints and Feedback Controls

Internal signal transduction pathways that constitute feedback controls, allow the cell to determine whether it is in a satisfactory physiological state to continue through the replicative cycle. If it is not, the cycle can be arrested at specific junctures known as checkpoints. Cell cycle arrest can be induced at a checkpoint in late G_1 if one of these pathways signals the presence of damaged DNA. This aborts entry into the S phase and allows the cell time to undertake the necessary repairs before chromosome duplication. Thus the integrity of the DNA in successive generations of cells is maintained. After passing through the late G_1 checkpoint, the cell becomes committed to undertaking DNA synthesis. Feedback controls also signal the completion of DNA replication. Unreplicated or damaged DNA can result in the arrest of the cycle in G_2, prior to entry into M phase at a

G_2/M checkpoint. Exit from mitosis represents another juncture that is subject to feedback control. Mitotic exit can be prevented in response to incomplete spindle assembly (Murray, 1992; Lees and Harlow, 1995; Fussenegger and Bailey, 1998; Dictor *et al.*, 1999).

1.2

TRANSCRIPTIONAL REGULATION OF THE CELL CYCLE

The repression or activation of genes whose products are involved in cell cycle progression often depends upon the phosphorylation status of the proteins that regulate the transcription of these genes, that is, whether they are hypo- or hyperphosphorylated. Such transcriptional control proteins include the retinoblastoma (Rb) family of repressors and activators as well as transcription factors such as E2F. Phosphorylation and dephosphorylation can enhance or inhibit interactions between these proteins and can affect the localisation of some of them. The phosphorylation status of many proteins involved in the cell cycle is regulated by cyclin-CDK (cyclin-dependent kinase) complexes, CDKIs (cyclin-dependent kinase inhibitors) and phosphatases.

Different cyclin-kinase complexes function during distinct phases of the cell cycle in order to control the entry of the cell into the next phase (**figure 1**). Each complex consists of a member of the cyclin family bound to a catalytic subunit from the CDK family (see **table 1**). The cyclin-CDK association, as well as phosphorylation of a conserved threonine residue in the catalytic subunit (Thr160 in CDK2), are both required to activate the kinase. Temporal regulation of cyclin levels (and hence of cyclin-CDKs to an extent) is achieved through transcriptional control of cyclins and their ubiquitin-dependent degradation. The stimulatory threonine phosphorylation is carried out by the CDK-activating kinase (CAK) that is itself a cyclin-CDK complex (Morgan, 1995).

CYCLIN FAMILY MEMBER	CYCLIN-DEPENDENT KINASE (CDK) FAMILY MEMBER	FUNCTION
Cyclin A	CDK1	Late G_2 phase
	CDK2	G_1/S and S phase
Cyclin B	CDK1	M phase
Cyclin C	CDK8	Transcription
Cyclin D	CDK4 and CDK6	Mid-G_1 phase and G_1/S transition
Cyclin E	CDK2	G_1/S transition
Cyclin F	Unknown	Unknown
Cyclin G	Unknown	Unknown
Cyclin H	CDK7	CDK-activating kinase (CAK) and transcription
p35	CDK5	Neural differentiation
Unknown	CDK3	Possibly G_1 phase

TABLE 1
MAJOR HUMAN CYCLINS AND CYCLIN-DEPENDENT KINASES
(Adapted from (Morgan, 1997))

Cyclin-kinase complexes each consist of a member of the cyclin family bound to a member of the CDK family as shown in the table above. Different complexes function during distinct phases of the cell cycle and regulate the entry of the cell into the next phase. The functions of the individual complexes are discussed in more detail in the text. Not all cyclin-kinases are involved in cell cycle regulation. Cyclin C-CDK8, for example, phosphorylates the C-terminal domain (CTD) of RNA polymerase II as part of the process of transcription (Morgan, 1995; Morgan, 1997).

The synthesis of D-type cyclins, as well as their association with CDK4 or 6, is reliant on growth factor stimulation that causes quiescent cells to enter G_1. These cyclin-kinase pairs are active in mid-G_1, with maximal activity occurring as the cell approaches the G_1/S transition. Cyclin D-kinase activity is positively and negatively regulated by cytokines and thus these particular cyclin-kinase pairs couple extracellular signals to the transcriptional control mechanism (Reynisdottir *et al.*, 1995; Sherr, 1996).

Studies suggest that members of the retinoblastoma (Rb) family of tumour suppressors (pRb, p107 and p130) are phosphorylated by cyclin D-CDK4/6 in mid-to-late G_1 and cyclin E/A-CDK2 during the G_1/S transition (Fussenegger and Bailey, 1998). An important feature of the hypophosphorylated pRb family members (also referred to as pocket proteins) is their ability to bind E2F transcription factors, resulting in the repression of E2F-responsive genes (Dyson, 1998). This in turn represses cell cycle progression since such genes encode cell cycle regulatory proteins (e.g. N-myc, b-myb) as well as proteins required for DNA synthesis (e.g. dihydrofolate reductase, DNA polymerase α) (Lavia and Jansen-Durr, 1999). Phosphorylation of the pocket proteins (PPs) by the cyclin-kinases causes their dissociation from E2F, leading to derepression of genes under the transcription factor's control (Helin, 1998). The cyclin E gene is also E2F-responsive and thus a positive feedback loop may exist in which the induction of this gene promotes cyclin E-CDK2 activity that leads to further production of cyclin E. This ensures continued cyclin E expression and PP inactivation in late G_1 (Geng *et al.*, 1996).

The pRb-E2F association results in the inhibition of RNA polymerase II (pol II)-directed mRNA synthesis, as discussed above. pRb is also able to repress pol I and III that mediate rRNA and tRNA production respectively. The mechanisms that have been proposed regarding pRb-induced transcriptional repression are described later (**section 1.3.3**). It is sufficient to point out at this stage that negative regulation of the cell cycle by pRb involves its suppression of the three RNA polymerases and consequent restraint of protein biosynthesis (Sanchez and Dynlacht, 1996; Fussenegger and Bailey, 1998). Furthermore, pRb plays a crucial role at the late G_1 checkpoint, when the cell's entry into S phase can be aborted in response to DNA damage that is detected by the p53 tumour suppressor. p53 induces the p21 CDK inhibitor whose abolition of cyclin-kinase activity prevents the phosphorylation of pRb family proteins, resulting in continued gene repression and arrest of the cell cycle in the G_1 phase (Dictor *et al.*, 1999).

CDKIs belong to either the INK4 or CIP families. Expression of specific family members is induced by contact inhibition, senescence, transforming growth factor (TGFβ, an anti-mitogenic cytokine) as well as p53. CIP family members bind to and inhibit cyclin-kinase pairs and restrain a wider range of CDKs than INK4 proteins. Members of the INK4 family abolish the activity of CDKs active in G_1. They are able to prevent cyclin-kinase complex formation by binding to solitary CDK and have also been shown to bind and inhibit intact cyclin-kinase pairs (Pavletich, 1999).

Once cells pass through the late G_1 checkpoint, they become committed to undertaking DNA synthesis. Degradation of cyclin E ensues after the cell's entry

into S phase and CDK2 binds to cyclin A. The cyclin A-CDK2 complex is responsible for S-phase progression and its phosphorylation of pRb renders the tumour suppressor inactive from the S-phase onward. The same cyclin-kinase pair also phosphorylates E2F during the S-phase, causing its dissociation from promoters and consequently the abolition of transcription from genes under its control. Since the cyclin A gene is among these, cyclin A-CDK2 complex formation is promoted by E2F, culminating in the deactivation of the transcription factor by a negative feedback loop mechanism (Dynlacht *et al.*, 1994; Krek *et al.*, 1994; Xu *et al.*, 1994; Fussenegger and Bailey, 1998).

Cyclin A binds to CDK1 during the late G_2 phase. This complex may contribute to the control of microtubule dynamics as part of the cell's preparation for mitosis. Another checkpoint occurs at the G_2/M transition with entry into the M phase being governed by the activity of cyclin B-CDK1. This cyclin-kinase pair regulates key mitotic processes including mitotic spindle formation, chromosome condensation and disintegration of the nuclear envelope. For example, cyclin B-CDK1-mediated phosphorylation of the lamin proteins, that comprise the nuclear lamina, causes them to depolymerise (Fussenegger and Bailey, 1998).

Premature mitotic entry is avoided by the inhibition of cyclin B-CDK1. This occurs through phosphorylation of a threonine and a tyrosine residue, both of which are conserved among the CDK family members (Thr 14 and Tyr 15 in CDK1) (Morgan, 1995; Fussenegger and Bailey, 1998). During S and G_2, phosphorylation of Tyr15 in CDK1 is mediated by the Wee1 kinase, found in a variety of organisms. Myt1, a membrane-associated kinase in Xenopus, has been

shown to phosphorylate both Thr14 and Tyr15 (Fussenegger and Bailey, 1998). Dephosphorylation of these residues is brought about by the CDC25 phosphatase, resulting in activation of cyclin B-CDK1 (Morgan, 1995). It is thought that this cyclin-kinase mediates inhibitory and stimulatory phosphorylation of Wee1 and CDC25 respectively, forming a positive feedback loop (Coleman and Dunphy, 1994; Morgan, 1995). The Chk1 protein kinase and p53 tumour suppressor participate in the inhibition of CDC25 activity in response to DNA damage, resulting in cell cycle arrest at the G_2/M checkpoint (Adams and Kaelin, 1998).

Cyclins A and B are degraded shortly before the onset of anaphase, with cyclin A degradation preceding that of cyclin B. It is thought that incomplete spindle assembly inhibits the ubiquitin-dependent destruction of cyclin B, thus preventing mitotic exit (Murray, 1992; Fussenegger and Bailey, 1998).

1.2.1

Cancer and the Transcriptional Regulatory Mechanism

Given their role in regulating the cell cycle, it is not surprising that the transcriptional regulators described in this section are subject to alterations that are associated with carcinogenesis. The gene encoding p53 is the most commonly mutated one in human cancer. The next most frequent genetic alterations relate to the p16 gene, encoding a CDKI that targets CDK4. p16 abnormalities are found in approximately one third of human cancers. One cell cycle regulator among pRb, cyclin D1, p16 or CDK4 is aberrant in the majority of malignant tumours (Dictor *et al.*, 1999; Pavletich, 1999). Furthermore, DNA tumour viruses subvert the cell

cycle control mechanism by virtue of oncoproteins that inactivate pRb (discussed later) and p53.

Inactivation of pRB following the overexpression of pRb-inactivating viral oncoproteins or disruption of the RB-1 (pRb gene) locus, is known to induce apoptosis. Cell death has also been shown to occur as a result of E2F-1 overexpression. Studies reveal the involvement of the p53 tumour suppressor in apoptotic induction and some details regarding the relevant mechanism have emerged. The fact that p53 plays a role in apoptosis may be the basis of the following observations. Both p53 and pRb, rather than either protein alone, are inactivated by tumour viruses. Inactivation of both proteins, by direct or indirect means, is also a frequent characteristic of other human tumours. It is interesting to note, however, that while E2F-1 overexpression can lead to p53-dependent apoptosis, E2F-1 can also induce cell death in the absence of p53 (Kaelin, 1999). The latter is partly mediated by the p53 homologue, p73 (Irwin *et al.*, 2000; Lissy *et al.*, 2000).

1.3

THE RETINOBLASTOMA TUMOUR SUPPRESSOR GENE PRODUCT (pRb)

The retinoblastoma tumour suppressor gene product (pRb) was so-named because alterations in its encoding gene were correlated with a cancer of the developing retina (retinoblastoma) (Benedict *et al.*, 1983; Cavenee *et al.*, 1983; Godbout *et al.*, 1983; Sparkes *et al.*, 1983). However, this name is perhaps misleading, since it does not convey the extent to which the protein is associated with other human cancers and cellular mechanisms in general. pRb is a component of the systems governing cell cycle control and differentiation. We have seen in **section 1.2** how current knowledge pertaining to cell cycle regulation partly attributes pRb's growth repressive function to its association with E2F. This section will describe pRb in more detail, including structural aspects of the protein related to its activities and proposed mechanisms for the transcriptional repression it induces.

1.3.1

Structural Aspects of pRb

The term "pocket proteins" is applied to members of the pRb family that comprises p107, p130 and pRb, since they possess a common structural feature known as the pocket domain (Hu *et al.*, 1990; Huang *et al.*, 1990; Kaelin *et al.*, 1990; Ewen *et al.*, 1992; Hannon *et al.*, 1993; Huang *et al.*, 1993; Mayol *et al.*, 1993; Chow and Dean, 1996; Lee *et al.*, 1998). This is divided into A and B subdomains that

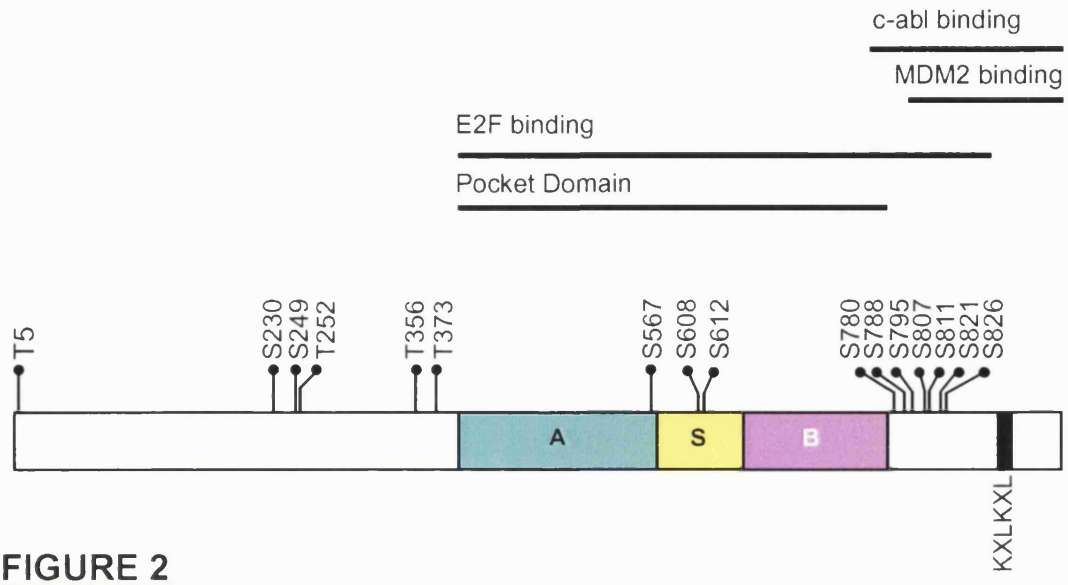


FIGURE 2

FUNCTIONAL REGIONS OF THE RETINOBLASTOMA TUMOUR SUPPRESSOR GENE PRODUCT pRb

(Adapted from Kaelin, 1999)

A and B: subdomains of the pocket domain

S: spacer region

KXLKXL: cyclin-CDK docking site
(single letter amino acid code where x is any residue)

: potential serine/threonine phosphoacceptor site

are connected by intramolecular associations along a common interface and, covalently, by a spacer region (Lee *et al.*, 1998) **(figure 2)**. The pocket region is the main focus of tumour-associated genetic changes in pRb (Hu *et al.*, 1990; Huang *et al.*, 1990; Kaelin *et al.*, 1990). This region is sufficient and necessary for the induction of transcriptional repression when fused to a heterologous DNA-binding domain (Sellers *et al.*, 1995; Weintraub *et al.*, 1995). Restraint of cell growth depends on the pocket, although an additional region of pRb is also required in this context (Hiebert *et al.*, 1992; Qin *et al.*, 1992). The principal interactions, between pRb and most of the viral and cellular proteins to which it binds, involve the pocket region (Hu *et al.*, 1990; Huang *et al.*, 1990; Kaelin *et al.*, 1990; Helin *et al.*, 1992; Kaelin *et al.*, 1992).

Among the viral proteins able to interact with pRb (discussed in **section 1.5**) is the human papillomavirus (HPV) E7 oncoprotein (Dyson *et al.*, 1989). E7 is able to disrupt pRb-E2F complexes (Chellappan *et al.*, 1992) and cooperate with an activated *ras* oncogene to transform primary rodent cells (Matlashewski *et al.*, 1987). The regions of pRb and E7 that are sufficient and necessary for complex formation are the tumour suppressor's pocket (Hu *et al.*, 1990; Huang *et al.*, 1990; Kaelin *et al.*, 1990) and a nine-residue peptide from the oncoprotein (Munger *et al.*, 1989; Jones *et al.*, 1990). This peptide incorporates an LXCXE motif (single letter amino acid code where X is any residue) found in other pRb-binding viral oncoproteins (Jones and Munger, 1996). The crystal structure of a complex comprising these minimal regions from both proteins has been solved. It reveals that the E7 peptide binds to a groove on the B subdomain of the pRb pocket. This groove is highly conserved across species as well as in the p107 and p130 family

members. It also appears from this structure that the A-B interface of the pocket is necessary for stable folding of the B subdomain (Lee *et al.*, 1998). This is probably the reason why the A subdomain is required for the binding of proteins containing LXCXE sequences to pRb (Hu *et al.*, 1990; Huang *et al.*, 1990; Kaelin *et al.*, 1990). Some cellular proteins that interact with pRb also possess an LXCXE-like motif, including HDAC1 (Brehm *et al.*, 1998; Luo *et al.*, 1998; Magnaghi-Jaulin *et al.*, 1998).

All tumour-associated pRb mutants investigated so far are unable to bind to E2F (Sellers *et al.*, 1998). E2F does not possess an LXCXE motif in its pRb-binding region (Helin *et al.*, 1992) (**figure 46**). Furthermore, it has been demonstrated that certain mutant viral oncoproteins that still possess the LXCXE motif, are able to bind pRb without inhibiting the formation of a pRb-E2F complex (Fattaey *et al.*, 1993; Huang *et al.*, 1993; Ikeda and Nevins, 1993). Such evidence indicates the presence of a region in pRb where E2F interacts, that is distinct from the groove of the B domain that binds LXCXE motifs. The interface between the A and B subdomains of pRb has emerged as a candidate for the principal binding site of E2F for the following reasons. The crystal structure of pRb-E7 reveals the presence of a groove extending half way around the interface that could be a protein binding site (Lee *et al.*, 1998). Some tumour-associated mutations map to the A-B interface (Horowitz *et al.*, 1989; Yandell *et al.*, 1989; Kaye *et al.*, 1990; Onadim *et al.*, 1992) that is also highly conserved among the pocket proteins and between pRb homologues from various species (Lee *et al.*, 1998). In keeping with these lines of evidence, the crystal structure of the pocket bound to an E2F peptide (S. Gamblin *personal communication*), shows that the transcription factor does

indeed bind to the A-B interface. This structure will be discussed in more detail later.

The N-terminus of pRb as well as the pocket domain contain BRCT-related motifs (Yamane *et al.*, 2000). BRCT regions bind double-strand breaks of DNA and are found in several proteins involved in DNA repair and cell cycle checkpoints (Yamane *et al.*, 2000).

The region C-terminal to the pocket of pRb interacts with the c-Abl tyrosine kinase. This is an S phase-activated kinase that can enhance transcription and is inhibited by the tumour suppressor (Welch and Wang, 1993; Welch and Wang, 1995). The C-terminal region of pRb also associates with MDM2, a cellular oncoprotein that can negatively regulate p53 and pRb (Xiao *et al.*, 1995).

1.3.2

Regulation of pRb

pRb is phosphorylated on amino acid residues within consensus target sites for cyclin-CDKs (Lees *et al.*, 1991) (Lin *et al.*, 1991) (**figure 2**). Upon phosphorylation by the latter, pRb is inactivated, resulting in cell cycle progression (Chen *et al.*, 1989; Hinds *et al.*, 1992; Lundberg and Weinberg, 1998). The tumour suppressor is initially phosphorylated in mid-G1 by cyclin D-CDK4/6 (Baldin *et al.*, 1993; Ewen *et al.*, 1993; Kato *et al.*, 1993; Lundberg and Weinberg, 1998). This promotes further phosphorylation by cyclin E-CDK2 in late G1, resulting in disruption of the pRb-E2F interaction (Dulic *et al.*, 1992;

Ewen *et al.*, 1993; Dynlacht *et al.*, 1994; Lundberg and Weinberg, 1998; Harbour *et al.*, 1999; Ezhevsky *et al.*, 2001). When the cell enters S phase, cyclin A-CDK2 continues to phosphorylate pRb to disrupt the association of the tumour suppressor with E2F (Pines and Hunter, 1990; Ewen *et al.*, 1993; Dynlacht *et al.*, 1994).

Each of the cyclin-kinases phosphorylates distinct sites on the tumour suppressor (Kitagawa *et al.*, 1996; Zarkowska and Mittnacht, 1997; Zarkowska *et al.*, 1997). The phosphorylation of specific sites appears to regulate distinct activities of pRb. For example, the phosphorylation of threonine 821 and 826 in pRb inhibits its interaction with proteins containing the LXCXE motif (Knudsen and Wang, 1996). The binding of E2F to pRb can be inhibited by phosphorylation of serine 608 and 612 in the spacer region of the tumour suppressor. The phosphorylation of several sites in pRb's C-terminus can also achieve the same effect (Knudsen and Wang, 1997).

Overexpression of gankyrin, an oncogenic protein, results in increased phosphorylation of pRb and activation of E2F-1 (Higashitsuji *et al.*, 2000). Gankyrin binds to pRb through an LXCXE motif (**section 1.3.1**) and accelerates the degradation of the tumour suppressor *in vitro* and *in vivo* (Higashitsuji *et al.*, 2000). Li and Tsai have demonstrated that gankyrin also binds to CDK4. This binding does not abolish CDK4-dependent phosphorylation of pRb. However, it does allow gankyrin to compete with p16 (**sections 1.2** and **1.2.1**) for binding to CDK4 and to consequently counteract p16-mediated inhibition of the kinase (Li and Tsai, 2002).

Internal cleavage of pRb abolishes its ability to bind E2F-1 (Fattman *et al.*, 1997). It has been demonstrated that such cleavage can be brought about during apoptosis by caspase-3 and –7 (Fattman *et al.*, 2001; Katsuda *et al.*, 2002). Taken together, these results suggest the following model for the regulation of pRb during apoptosis. pRb is functionally inactivated during apoptosis by caspases that cleave the tumour suppressor internally, leading to the release of E2F. The latter is then able to participate in the apoptotic process (section **1.4.3.4**).

1.3.3

Mechanisms of pRb-induced Transcriptional Repression

pRb is able to negatively regulate transcription by repressing all three RNA polymerases as outlined below.

1.3.3.1

Repression of RNA polymerase II-directed transcription

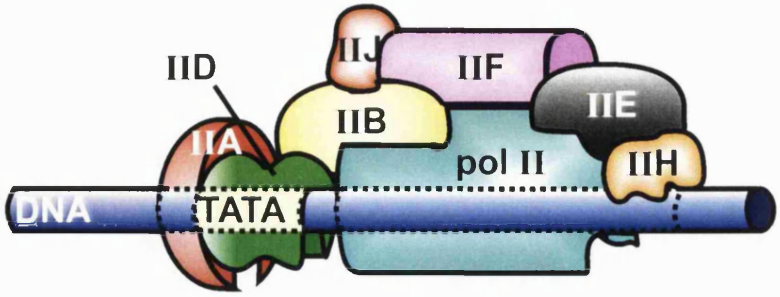
The binding of pocket proteins (PPs) to E2F results in the repression of E2F-responsive genes (Hiebert *et al.*, 1992; Weintraub *et al.*, 1992; Hagemeier *et al.*, 1993; Helin *et al.*, 1993a; Chow and Dean, 1996; Chow *et al.*, 1996; Ferreira *et al.*, 1998).

As shown in **section 1.4.1**, the pRb-binding and transactivation domains of E2Fs 1-5 overlap. It appears that the tumour suppressor is able to inhibit E2F by

masking its transactivation domain, thereby interfering with E2F's interaction with components of the basal transcriptional machinery (Pearson and Greenblatt, 1997; Ross *et al.*, 1999) (**figures 3 and 4**). Such repression of E2F is termed direct inhibition. *In vitro* transcription assays show that E2F-induced transcription is repressed by pRb if the tumour suppressor is present before PIC assembly (Ross *et al.*, 1999; Ross *et al.*, 2001). pRb's ability to repress such transcription however, is greatly diminished if the tumour suppressor is present after formation of a partial (TFIIA-TFIID) or complete PIC (Ross *et al.*, 1999; Ross *et al.*, 2001).

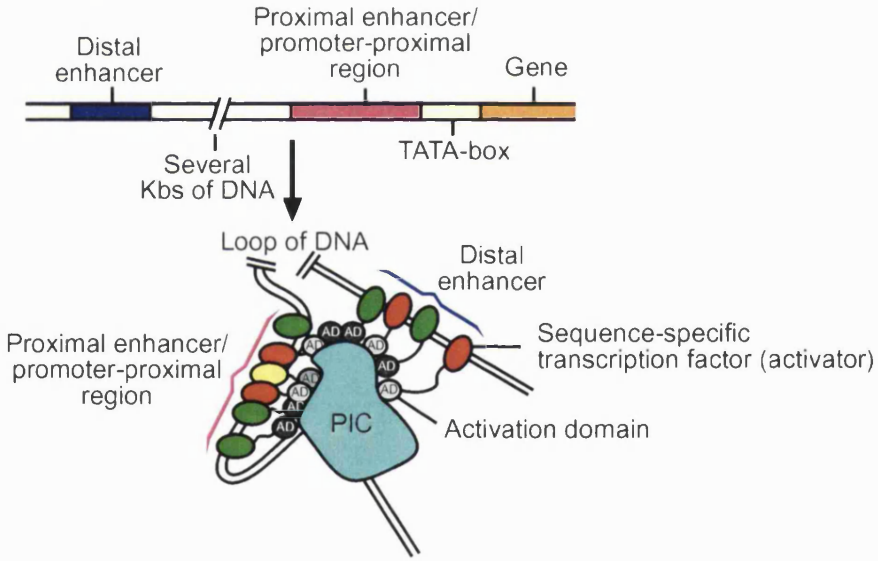
The tethering of pRb to a promoter (either through association of the tumour suppressor with E2F or through fusion of pRb to a heterologous DNA binding domain) results in the repression of transcriptional activators that have proximal DNA binding sites (Weintraub *et al.*, 1992; Adnane *et al.*, 1995; Bremner *et al.*, 1995; Sellers *et al.*, 1995; Weintraub *et al.*, 1995; Zhang *et al.*, 1999) (**figure 4**). The term active repression refers to this particular ability of the tumour suppressor. However, the mechanism by which this is achieved is distinct from that of pRb-mediated direct inhibition of E2F. Ross and colleagues have shown that active repression by pRb occurs whether the tumour suppressor is present before or after PIC assembly. This suggests that in contrast to direct E2F inhibition, active repression by pRb may involve perturbation of a process that takes place after PIC assembly, such as recruitment of cofactors (Ross *et al.*, 2001). Furthermore, pRb-mediated active repression is dependent upon the presence of chromatin, unlike pRb-induced direct inhibition of E2F which takes place whether chromatin is present or not (Ross *et al.*, 1999; Ross *et al.*, 2001).

A The Pre-initiation Complex (Adapted from Voet and Voet, 1995)



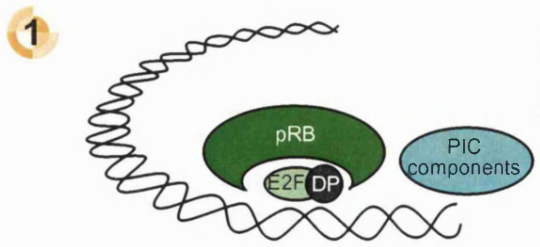
In order to find the correct transcriptional start site and initiate transcription, RNA polymerase II (pol II) requires General Transcription Factors (GTFs), of which many are multiprotein complexes. These are known as TFIIA, TFIIB etc. (TF: Transcription Factor). Pol II and the GTFs sequentially associate to form a pre-initiation complex (PIC), also referred to as the basal transcriptional machinery, that allows transcription to take place at a "basal" rate (Lodish *et al.*, 1999) (Voet and Voet, 1995). In the illustration above, the gene's promoter has a TATA-box, a conserved DNA sequence commonly found in the core promoters of genes transcribed by pol II (Lemon and Tjian, 2000) (Lodish *et al.*, 1999). The TATA-box binding protein (TBP) binds to this sequence and interacts with TBP-associated factors (TAFs) to form TFIID (Voet and Voet, 1995). TFIIH incorporates enzymatic activities that are involved in steps of transcription occurring soon after the initiation of mRNA synthesis. The kinase activity of this GTF may serve to regulate elongation of the mRNA chain (Blau *et al.*, 1996).

B Activators (Adapted from Lodish *et al.*, 1999)

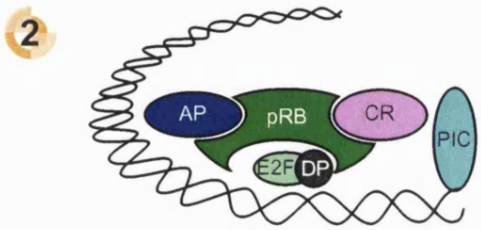


Transcription is stimulated by transcription factors known as activators (Lodish *et al.*, 1999). A simplified model for the role of these proteins is illustrated above. Activators bind specific nucleic acid sequences in enhancer regions of DNA (Lemon and Tjian, 2000). It is thought that such transcription factors stimulate the assembly of the PIC and control the frequency of transcriptional re-initiation by new pol II molecules. Their activation domains (ADs) associate with components of the basal machinery. These interactions are partly responsible for looping of the DNA and formation of a stable functional PIC (Lodish *et al.*, 1999). Several co-regulators (e.g. CBP, HDAC1- see text) are required to mediate the effects of sequence-specific transcription factors on the basal machinery (Lemon and Tjian, 2000). Co-regulators and other elements of pol II-directed transcription are described in detail elsewhere (Lodish *et al.*, 1999) (Lemon and Tjian, 2000) (Voet and Voet, 1995).

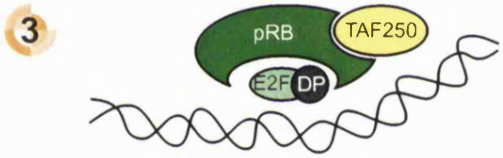
FIGURE 3: ELEMENTS OF EUKARYOTIC TRANSCRIPTIONAL INITIATION FOR GENES TRANSCRIBED BY RNA POLYMERASE II



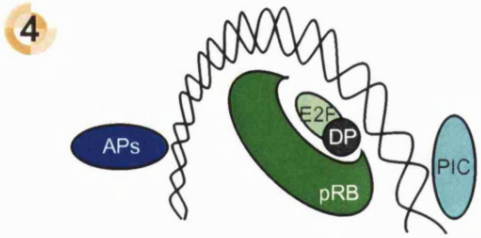
pRb masks the transactivation domain of E2F. This interferes with E2F's interaction with components of the PIC (pre-initiation complex.)



pRb represses proximally-binding activator proteins (APs), once tethered to a promoter. pRb recruits co-repressors (CRs) such as HDACs. It can also block the activator-PIC interaction.



pRb binds and inhibits TAF(II)250 kinase, a component of the PIC.



pRb partially reverses E2F-induced changes in DNA topography, disrupting interaction between PIC and transcriptional activators.

FIGURE 4

PROPOSED MECHANISMS FOR REPRESSION OF RNA POLYMERASE II-DIRECTED TRANSCRIPTION BY pRb

(Adapted from Kaelin, 1999)

In the context of the mechanism of pRb-mediated active repression, chromatin structure is thought to be of particular importance. pRb is able to recruit different types of chromatin remodeling factors, that is, factors that alter chromatin structure to facilitate or prevent access of transcription factors to nucleosomal DNA (Workman and Kingston, 1998). Among the chromatin remodeling factors with which pRb interacts are histone deacetylase 1 (HDAC1) (Brehm *et al.*, 1998; Luo *et al.*, 1998; Magnaghi-Jaulin *et al.*, 1998) and HDACs 2 and 3 (Dahiya *et al.*, 2000). These enzymes catalyse the removal of acetyl groups from histone, enhancing the interactions between DNA and nucleosomes and consequently restricting the access of transcriptional proteins to the promoter (Hassig and Schreiber, 1997). Transfection assays show that the deacetylase inhibitor trichostatin A (TSA) abrogates the ability of pRb to actively repress the USF transcription factor (Luo *et al.*, 1998). This suggests that active repression by the tumour suppressor is HDAC-dependent in this setting. Furthermore, pRb-mediated active repression is impaired by mutations in the tumour suppressor that decrease the level of HDAC activity it can bind (Dahiya *et al.*, 2000). Since pRb recruits HDAC to E2F (Brehm *et al.*, 1998; Magnaghi-Jaulin *et al.*, 1998), E2F's DNA binding activity serves to localise the deacetylase (via pRb) to promoters. Indeed in proliferating cells, HDAC1 is stably bound to an E2F target promoter (dihydrofolate reductase) during early G1 and released at the G1/S transition, as demonstrated by formaldehyde cross-linked chromatin immunoprecipitation assays (Ferreira *et al.*, 2001).

However, pRb-mediated repression of a proximal SV40 enhancer occurs independently of HDAC (Luo *et al.*, 1998). Furthermore, the study conducted by Ross and colleagues referred to earlier, showed that although pRb-mediated active repression was chromatin-dependent, it was not HDAC-dependent in their in vitro assay (Ross *et al.*, 2001). Thus in such settings, HDAC-independent mechanisms of pRb-mediated active repression must be invoked. In this context, pRb's ability to recruit other co-repressors may be the means by which the tumour suppressor achieves its effect. For example, pRb is able to recruit the CtBP co-repressor via CtIP (Meloni *et al.*, 1999). CtBP possesses both HDAC-dependent (Criqui-Filipe *et al.*, 1999; Postigo and Dean, 1999) and –independent (Meloni *et al.*, 1999; Koipally and Georgopoulos, 2000; Phippen *et al.*, 2000) repressive activities. The co-repressor interacts with the hPc2 polycomb group protein (Sewalt *et al.*, 1999) and recruits the latter to pRb (Dahiya *et al.*, 2001). It has been postulated that polycomb proteins repress gene expression by co-ordinating the formation of densely-packaged, inactive chromatin that is inaccessible to transcription factors (heterochromatin) (Pirrotta, 1997). Thus formation of the pRb-CtBP-hPc2 complex may, at least in part, be responsible for pRb-mediated active repression by HDAC-independent means. However, much further work is required to elucidate the mechanisms of CtBP-mediated repression (Chinnadurai, 2002). HDAC-dependent and –independent repressive activities are also exhibited by another pRb-binding co-repressor, RBP1 (Lai *et al.*, 1999; Lai *et al.*, 2001).

There are also other chromatin remodeling factors (apart from HDACs 1-3) that repress transcription, to which pRb binds. These include certain protein methyltransferases, such as SUV39H1. The latter is a histone methyltransferase

that interacts with pRb's pocket domain *in vivo* and cooperates with the tumour suppressor to repress the cyclin E promoter (Nielsen *et al.*, 2001). pRb also associates with BRG1 and BRM, two components of human SWI/SNF (hSWI/SNF) that belongs to a family of ATP-dependent nucleosome remodeling complexes (Dunaief *et al.*, 1994; Strober *et al.*, 1996; Trouche *et al.*, 1997). Expression of BRG1 and BRM in cells that are deficient for both of these proteins results in pRb-dependent growth arrest (Dunaief *et al.*, 1994; Strober *et al.*, 1996). pRb-mediated repression of the cyclin A promoter is compromised in cell lines lacking BRG1, while ectopic expression of BRG1 in these cells restores the tumour suppressor's ability to effect such repression (Strobeck *et al.*, 2000). BRM has been shown to cooperate with pRb, both to repress cyclin A (Reisman *et al.*, 2002), and to repress E2F-1 activity in transient transfection assays (Trouche *et al.*, 1997). A study conducted by Zhang and colleagues (Zhang *et al.*, 2000) suggests that pRb-HDAC-hSWI/SNF and pRb-hSWI/SNF complexes contribute to the temporal regulation (**section 1.2**) of cyclins A and E. pRb also binds DNMT1, a DNA methyltransferase that cooperates with the tumour suppressor to repress transcription in an HDAC-dependent and –independent manner (Robertson *et al.*, 2000).

Results from experiments conducted by Weintraub and colleagues suggest that when pRb is tethered to a promoter (e.g. by E2F), the tumour suppressor is able to repress certain proximally bound activators (PU.1, c-myc, Elf-1) by interacting with them and blocking their association with the PIC (Weintraub *et al.*, 1995) (**figure 4**). Active repression of PU.1 has also been shown to be HDAC-independent (Luo *et al.*, 1998). Repression of these proximal activators however,

requires the recruitment of pRb to the promoter by E2F, or by means of a DNA binding domain fused to the tumour suppressor (Weintraub *et al.*, 1995; Luo *et al.*, 1998). It appears that the affinity of the proximal activators for pRb is not high enough to permit them to recruit the tumour suppressor (Weintraub *et al.*, 1995; Luo *et al.*, 1998). Thus it seems that pRb has to be concentrated at the promoter by a relatively high affinity interaction with E2F, before the comparatively low affinity interaction between pRb and the proximal activator can be established (Weintraub *et al.*, 1995; Luo *et al.*, 1998).

In accordance with the majority of the current literature, I have defined active repression (above) as the repression of activators that have proximal DNA-binding sites (i.e. proximal to the site to which pRb is tethered). However, Ross and colleagues (Ross *et al.*, 2001) define the term as the repression of proximal activators to which pRb does not bind directly. Indeed these researchers studied pRb-mediated chromatin-dependent active repression of Sp1 with which the tumour suppressor does not interact to a significant extent *in vitro* (Weintraub *et al.*, 1995; Ross *et al.*, 2001). Regardless of this difference in definition, current reports (see above) do indicate that pRb is able to repress proximally binding activators. The mode of repression in this case appears to be attributable to the recruitment of co-repressors by pRb, particularly chromatin remodeling factors. However, repression of certain proximal activators may be due to an interaction with pRb that blocks their access to the PIC (once pRb has been localised to the promoter by E2F). It may be that chromatin remodeling allows repression of proximally binding activators to which pRb does not bind, while those proximal

activators, for which pRb has some affinity, are repressed by pRb-binding after E2F-mediated recruitment of the tumour suppressor.

Other mechanisms by which pRb may repress transcription include a partial reversal in E2F-induced DNA bending (Huber *et al.*, 1994), possibly perturbing interactions between transcriptional activators and the PIC. Furthermore, the tumour suppressor has been shown to bind and inhibit TAF(II)250 kinase (Siegert and Robbins, 1999), a component of the basal transcriptional machinery (**figure 4**). Defective TAF(II)250 appears to cause arrest of the cell cycle in G1 (Hayashida *et al.*, 1994).

1.3.3.2

pRb-induced repression of RNA polymerases I and III

pRb represses pol I-directed transcription by interacting with the architectural upstream binding factor (UBF) (Cavanaugh *et al.*, 1995; Voit *et al.*, 1997). The latter enhances pol I-directed transcription (Smith *et al.*, 1990; Hannan *et al.*, 1996; Hannan *et al.*, 1999). UBF is acetylated by the CBP acetyltransferase, promoting pol I-directed transcription (Pelletier *et al.*, 2000). pRb is able to repress pol I by binding to UBF and preventing its association with CBP (Pelletier *et al.*, 2000). The tumour suppressor also recruits HDAC1 (as discussed previously) that deacetylates UBF, further contributing to the suppression of pol I activity (Pelletier *et al.*, 2000). UBF-mediated activation of pol I is dependent upon the interaction of the architectural factor with the SL-1 transcription factor (Learned *et al.*, 1986; Bell *et al.*, 1988; McStay *et al.*, 1991; Beckmann *et al.*,

1995; Moss and Stefanovsky, 1995; Hempel *et al.*, 1996). This interaction is also blocked by the formation of a pRb-UBF complex (Hannan *et al.*, 2000). Endogenous pRb and p130 appear to exhibit redundancy in their negative regulation of pol I, whereas endogenous p107 does not suppress rRNA synthesis (Ciarmatori *et al.*, 2001).

It has been demonstrated that pRb represses pol III activity in vivo (White *et al.*, 1996). It achieves this by inhibiting associations between components of the pol III PIC. For example, the tumour suppressor disrupts the interaction between TFIIIB and pol III, thereby preventing the recruitment of pol III to the initiation site (Sutcliffe *et al.*, 2000).

1.4

THE E2F FAMILY OF TRANSCRIPTION FACTORS

The discovery that E2F associates with pRb (Bandara and La Thangue, 1991; Chellappan *et al.*, 1991; Chittenden *et al.*, 1991) initiated extensive study of this family of transcription factors that are key regulators of cell proliferation. This has given rise to a wealth of information owing to which an intriguing picture of E2F as both an activator and repressor of transcription has begun to emerge. The apparently dual nature of E2F is discussed here along with the structure, mode of action and regulation of the family members.

1.4.1

Structural Aspects

The E2F family of transcription factors comprises heterodimeric proteins. Each heterodimer consists of one member of the E2F family of proteins and one from the DP family (Huber *et al.*, 1993; Wu *et al.*, 1995; Cartwright *et al.*, 1998). Thus the term "E2F" is used generically to signify such a heterodimer. Members of the E2F family of proteins are able to homodimerise and bind DNA, as are DP proteins, but the DNA-homodimer interaction is much weaker than that between a heterodimer and DNA (Bandara *et al.*, 1993; Girling *et al.*, 1993; Huber *et al.*, 1993; Krek *et al.*, 1993). E2F-1 and DP-1 have been shown to cooperatively transactivate an E2F-responsive promoter (Helin *et al.*, 1993b).

Six mammalian E2F proteins have been identified to date, these being E2F-1 (Helin *et al.*, 1992; Kaelin *et al.*, 1992; Shan *et al.*, 1992), E2F-2 (Ivey-Hoyle *et*

al., 1993; Lees *et al.*, 1993), E2F-3 (Lees *et al.*, 1993), E2F-4 (Beijersbergen *et al.*, 1994; Ginsberg *et al.*, 1994; Sardet *et al.*, 1995), E2F-5 (Hijmans *et al.*, 1995; Sardet *et al.*, 1995) and E2F-6 (Morkel *et al.*, 1997; Cartwright *et al.*, 1998; Trimarchi *et al.*, 1998). Two mammalian DP family members have been identified, namely DP-1 (Girling *et al.*, 1993) and DP-2 (Ormondroyd *et al.*, 1995; Wu *et al.*, 1995; Zhang and Chellappan, 1995; Rogers *et al.*, 1996). E2F proteins have a number of functional regions that are highly conserved (**figure 5**). They all possess N-terminal DNA-binding (Kaelin *et al.*, 1992; Cress *et al.*, 1993; Ivey-Hoyle *et al.*, 1993; O'Connor and Hearing, 1994) and dimerisation regions (Helin *et al.*, 1993) that precede a "marked box" (Lees *et al.*, 1993). This latter region is bound by the adenovirus E4 (ORF6/7) protein as part of the viral replication process (O'Connor and Hearing, 1994). It may also be involved in dimerisation and DNA bending (Cress and Nevins, 1996; Vidal *et al.*, 1996). All E2F proteins, with the exception of E2F-6 have a C-terminal transactivation region in which a section for binding pocket proteins is embedded (Helin *et al.*, 1992; Kaelin *et al.*, 1992; Cress *et al.*, 1993; Hagemeier *et al.*, 1993). E2Fs 1-3 each possess a nuclear localisation signal and a cyclin A-binding region in their N-terminal portions (Adams *et al.*, 1996; Muller *et al.*, 1997; Verona *et al.*, 1997). E2F-3 is also referred to as E2F-3A, owing to the identification of an alternatively spliced form of this protein (He *et al.*, 2000; Leone *et al.*, 2000), termed E2F-3B. The latter is truncated at its N-terminus (relative to E2F-3A) but still incorporates a nuclear localisation signal (He *et al.*, 2000; Leone *et al.*, 2000) and cyclin A-binding region (He and Cress, 2002). As in the literature, the terms E2F-3 and E2F-3A refer to the same protein throughout this thesis.

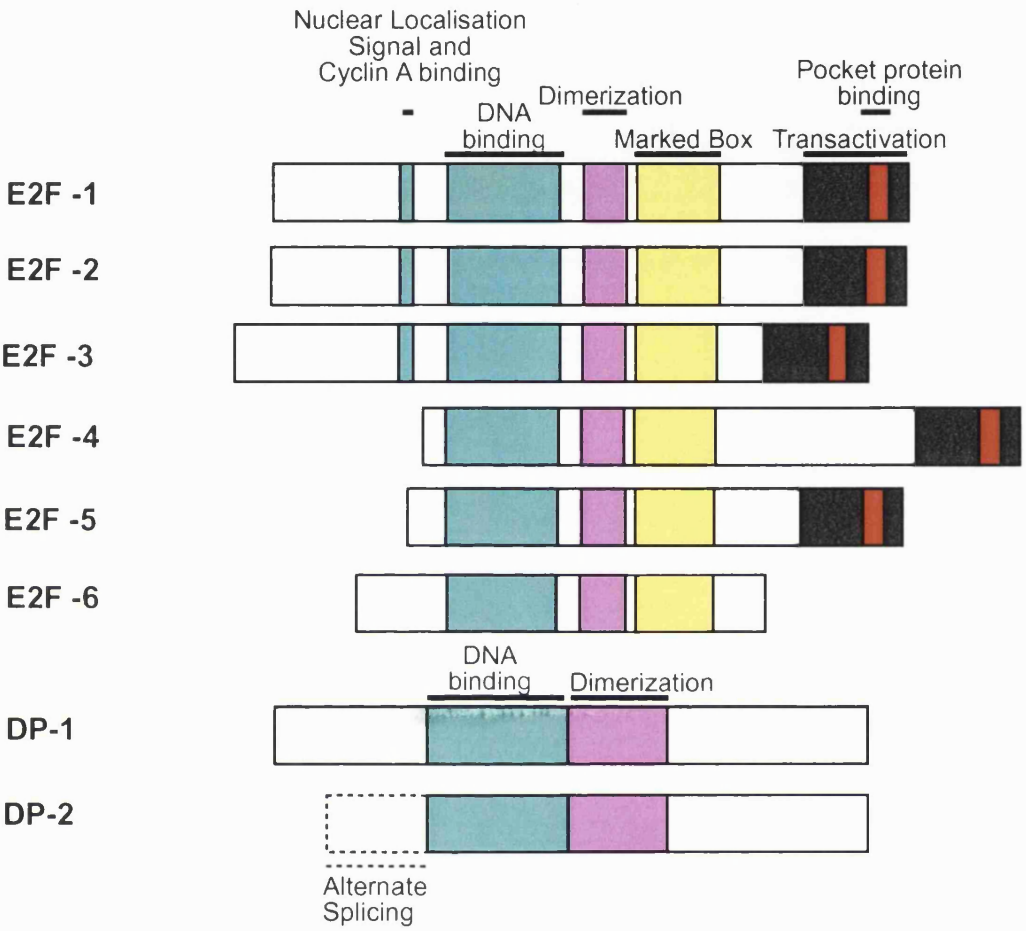


FIGURE 5

FUNCTIONAL REGIONS OF THE MAMMALIAN E2F AND DP PROTEINS.

(Adapted from Black et al., 1999)

The DP family members (**figure 5**) are distantly related to the E2F proteins, with the greatest homology occurring in the DNA binding and dimerisation regions (Girling *et al.*, 1993). Furthermore, DP-2 variants are produced through alternative splicing of the encoding gene, some of which have a nuclear localisation signal (Ormondroyd *et al.*, 1995; Zhang and Chellappan, 1995; de la Luna *et al.*, 1996; Rogers *et al.*, 1996). The different characteristics of the DP and E2F proteins, together with the ability of any E2F to dimerise with any DP (Wu *et al.*, 1995; Cartwright *et al.*, 1998), gives rise to heterodimers that are distinct in terms of how they are regulated and the functions they perform.

1.4.2

The Regulation of E2F

A number of inter-related mechanisms have been identified to date that contribute to the control of E2F activity. Their elucidation has helped to construct a model of E2F function that will be discussed later.

1.4.2.1

Synthesis of E2F Transcription Factors

Transcriptional and protein levels of E2Fs 4 and 5 are, on the whole, constant throughout the cell cycle while transcription and translation of E2Fs 1-3 occur in mid-late G_1 (Kaelin *et al.*, 1992; Slansky *et al.*, 1993; Ginsberg *et al.*, 1994; Sardet *et al.*, 1995; Moberg *et al.*, 1996; Sears *et al.*, 1997; Leone *et al.*, 1998). Activated Ras, which mediates mitogenic signals (Cahill *et al.*, 1996), has been shown to

induce an increase in the levels of E2F-1 protein and mRNA (Berkovich and Ginsberg, 2001). In contrast to E2Fs 1-3, E2F-3B (**section 1.4.1**) is detectable throughout the cell cycle and its levels peak in the G_O phase, during which it is bound to pRb (He *et al.*, 2000; Leone *et al.*, 2000; He and Cress, 2002). It is therefore thought that the primary function of E2F-3B may be to mediate transcriptional repression (in conjunction with pRb). Expression of DPs 1 and 2 does not fluctuate to a great extent during the replicative cycle (Bandara *et al.*, 1994).

1.4.2.2

Pocket Proteins and C/EBPα

The binding of pocket proteins (PPs) to E2F results in the repression of E2F-responsive genes (Hiebert *et al.*, 1992; Weintraub *et al.*, 1992; Helin *et al.*, 1993a; Chow and Dean, 1996; Chow *et al.*, 1996; Ferreira *et al.*, 1998). The ability of pRb to both inhibit E2F-induced transcription, as well as to actively repress proximally-binding activators, is discussed fully in **section 1.3.3.1**. E2Fs 1-3 bind preferentially to pRb, E2F-4 interacts with greatest affinity with p107 and p130 (as well as pRb under certain conditions) and E2F-5 binds only to p130 (Lees *et al.*, 1993; Beijersbergen *et al.*, 1994; Ginsberg *et al.*, 1994; Hijmans *et al.*, 1995; Vairo *et al.*, 1995; Wu *et al.*, 1995; Moberg *et al.*, 1996). Different complexes predominate in different phases of the cell cycle. Quiescent and differentiated cells principally contain p130-E2F complexes, pRb-E2F is most evident as cells progress from G1 to S and p107-E2F is most prevalent in S phase (Mudryj *et al.*, 1991; Cao *et al.*, 1992; Lees *et al.*, 1992; Shirodkar *et al.*, 1992; Chittenden *et al.*,

1993; Cobrinik *et al.*, 1993; Ikeda *et al.*, 1996; Moberg *et al.*, 1996). Cyclin-CDK-mediated phosphorylation of the PPs, that begins in mid-G1, results in disruption of the PP-E2F interaction (Beijersbergen *et al.*, 1995; Smith *et al.*, 1996; Xiao *et al.*, 1996; Alevizopoulos *et al.*, 1997) (also see **section 1.3.2**).

The C/EBPα transcription factor is required for the differentiation of white adipose tissue (Wang *et al.*, 1995) and neutrophil granulocytes (Zhang *et al.*, 1997). It has been found in complexes (from nuclear extracts) that contain E2F (Slomiany *et al.*, 2000; Porse *et al.*, 2001). C/EBPα inhibits the induction of an E2F/DP-responsive promoter in transient transfection assays (Slomiany *et al.*, 2000) and negatively regulates c-Myc in a manner that depends on an E2F-binding site in the *c-myc* promoter (Johansen *et al.*, 2001). Repression of E2F by C/EBPα can take place independently of pRb (Slomiany *et al.*, 2000; Johansen *et al.*, 2001; Porse *et al.*, 2001). The ability of C/EBPα to repress E2F is crucial for C/EBPα-induced terminal differentiation (Porse *et al.*, 2001). The most commonly observed C/EBPα mutation in victims of acute myeloid leukaemia, results in the overexpression of a form of C/EBPα which does not repress E2F (Pabst *et al.*, 2001; Porse *et al.*, 2001).

1.4.2.3

Phosphorylation of E2F

A conserved N-terminal region in E2Fs 1-3 allows them to interact with cyclin A-CDK2 (Krek *et al.*, 1994; Xu *et al.*, 1994). When associated with an E2F1-DP1 heterodimer in such a way, cyclin A-CDK2 phosphorylates both proteins comprising the dimer and abolishes its DNA-binding activity (Dynlacht *et al.*,

1994; Krek *et al.*, 1994; Xu *et al.*, 1994; Kitagawa *et al.*, 1995). E2F-1 DNA binding is downregulated in S phase by cyclin A-kinase-mediated phosphorylation (Krek *et al.*, 1994) (as discussed previously in **section 1.2**). The physiological importance of this was demonstrated by the expression of mutant E2F-1, defective in cyclin A binding, that caused cells to arrest in S phase and induced apoptosis (Krek *et al.*, 1995). Consistent with the absence of a cyclin A binding region in E2Fs 4 and 5 (**section 1.4.1**), cyclin A-CDK2 does not promote a loss of DNA binding by heterodimers comprising these E2Fs (Dynlacht *et al.*, 1997).

1.4.2.4

Degradation and Stabilisation of E2F

Overexpressed E2F-1 and 4 are subject to degradation by the ubiquitin-proteasome pathway for which they are targeted by sequences in their C-terminus (Hateboer *et al.*, 1996; Hofmann *et al.*, 1996). It is thought that this may provide an additional way to downregulate E2F activity. The binding of pocket proteins to E2Fs 1 and 4 stabilises E2F since it increases its half-life from approximately two hours to over eight hours, as shown by pulse-chase experiments (Hateboer *et al.*, 1996; Hofmann *et al.*, 1996). Studies conducted by Martinez-Balbas and colleagues (Martinez-Balbas *et al.*, 2000) suggest that the half-life of E2F-1 is also increased when the protein is acetylated by PCAF, an acetyltransferase (Schiltz and Nakatani, 2000).

1.4.2.5

Subcellular Localisation of E2F

In G_0 and early G_1, E2Fs-4 and 5 are present in the nucleus and cytoplasm but during other periods of the cell cycle, they are predominantly located in the cytoplasm (Allen *et al.*, 1997; Lindeman *et al.*, 1997; Muller *et al.*, 1997; Verona *et al.*, 1997). Consistent with the finding that E2Fs-1 to 3 possess a nuclear localisation signal (**section 1.4.1**), these proteins are predominantly nuclear (Magae *et al.*, 1996; Muller *et al.*, 1997; Verona *et al.*, 1997). The mechanisms governing the localisation of E2Fs-4 and 5 still require elucidation and may involve DP-2 splice variants possessing a nuclear localisation signal (de la Luna *et al.*, 1996), as well as the formation of E2F-PP complexes (Allen *et al.*, 1997; Lindeman *et al.*, 1997).

1.4.2.6

Acetylation of E2F

It has been demonstrated that acetylation of E2F-1 augments its affinity for an E2F DNA binding site and enhances transcriptional activation of an E2F-responsive promoter (Martinez-Balbas *et al.*, 2000; Marzio *et al.*, 2000). Among the E2F family members, only E2Fs 1-3 interact with and are acetylated by p300 and CBP (Marzio *et al.*, 2000), two highly-related transcriptional co-activators (Chan and La Thangue, 2001). The p300/CBP-associated factor (PCAF), previously mentioned in **section 1.4.2.4**, also associates with and acetylates E2F-1 (Martinez-Balbas *et al.*, 2000). PCAF exhibits histone acetylase activity and can

be recruited by p300 and CBP (Yang *et al.*, 1996), which also display intrinsic histone acetylase activity themselves (Bannister and Kouzarides, 1996; Ogryzko *et al.*, 1996). However, in addition to acetylating E2F, p300 CBP and PCAF also acetylate other transcription factors, demonstrating that their substrate specificity is not limited to histones (Gu and Roeder, 1997; Imhof *et al.*, 1997; Boyes *et al.*, 1998; Munshi *et al.*, 1998; Zhang and Bieker, 1998). HDAC1, (the histone deacetylase mentioned in **section 1.3.3.1**), can reverse the acetylation of E2F-1 (Martinez-Balbas *et al.*, 2000; Marzio *et al.*, 2000). This suggests that HDAC1-mediated deacetylation of E2F contributes to the downregulation of the transcription factor, given the effect acetylation has upon E2F's activity (this section) and its stability (**section 1.4.2.4**).

1.4.3

Functions of E2F

1.4.3.1

E2F's Target Genes

E2F was initially shown to be a cellular factor required for transactivation of the adenovirus E2 promoter (Kovesdi *et al.*, 1986), hence the term E2F (E2 Factor). Following the identification of the first E2F DNA-binding sites in the E2 promoter (Kovesdi *et al.*, 1986), sequence inspection and mutational analyses allowed the presence of such sites to be determined within cellular promoters, the consensus E2F site being TTTc/gGCGCc/g (**table 2**).

Genes whose promoters contain E2F-binding sites can be broadly categorised as those involved in cell cycle control as well as DNA synthesis (**table 2**). Current data portray E2F as a regulator that activates or, when associated with a pocket protein, actively represses transcription of a target gene. This is discussed in the next section (**1.4.3.2**).

Table 2 : E2F-REGULATED GENES

Adapted from Helin, 1998.

GENE/PRODUCT	NUMBER OF E2F SITES	EFFECT OF E2F SITES	REFERENCES
Growth Regulatory Genes			
Cyclin E	One to six	Repressing	(Ohtani *et al.*, 1995; Botz *et al.*, 1996; Geng *et al.*, 1996)
p107	Two	Activating	(Zhu *et al.*, 1995)
B-Myb	One palindrome	Repressing	(Lam and Watson, 1993; Zwicker *et al.*, 1996)
E2F-1	Two palindromes	Repressing	(Hsiao *et al.*, 1994; Johnson *et al.*, 1994; Neuman *et al.*, 1994)
CDK1	One	Repressing	(Tommasi and Pfeifer, 1995)
Cyclin A	One	Repressing	(Schulze *et al.*, 1995; Huet *et al.*, 1996)
c-myc	One	Not reported	(Thalmeier *et al.*, 1989; Plet *et al.*, 1992)
Genes Essential for DNA Replication			
ORC1	One palindrome	Repressing	(Ohtani *et al.*, 1996)
Thymidylate Synthase	One	Not reported	(Jenh *et al.*, 1985; Ayusawa *et al.*, 1986; Jolliff *et al.*, 1991)
Dihydrofolate Reductase	One palindrome	Activating	(Wells *et al.*, 1996; Fry *et al.*, 1997)
Thymidine kinase	One palindrome	Activating	(Karlseder *et al.*, 1996)
DNA polymerase α	One	Not reported	(Pearson *et al.*, 1991)

The list of genes in the above table is not exhaustive and is only intended to demonstrate the nature and range of promoters containing E2F sites and the effect of such sites. Further information in this context can be found elsewhere (Helin, 1998; Lavia and Jansen-Durr, 1999). Evidence is also beginning to emerge that E2F plays a role in the regulation of other genes whose products are involved in mitosis, apoptosis, differentiation and development (Ishida *et al.*, 2001; Muller *et al.*, 2001), as well as DNA repair and checkpoints (Ren *et al.*, 2002).

1.4.3.2

E2F as an Activator and Repressor of Transcription

Several lines of evidence demonstrate that E2F acts as a transcriptional activator. For example, E2F has been shown to activate transcription of reporter constructs harbouring its cognate binding sites (Helin *et al.*, 1992; Shan *et al.*, 1992). A close correlation between E2F's ability to effect transcription and to promote cell cycle progression has been demonstrated (Johnson *et al.*, 1993; Shan and Lee, 1994; Qin *et al.*, 1995). The transactivation regions of the E2F proteins are conserved (**section 1.4.1**) and functional when fused to other DNA-binding regions (Kaelin *et al.*, 1992). Furthermore, the combined loss of E2Fs 1-3 in mouse embryonic fibroblasts, severely impairs the induction of several E2F target genes (Wu *et al.*, 2001).

Other studies indicate that E2F also plays a role in transcriptional repression. Mutation of E2F binding sites in a number of promoters, such as B-myb and E2F-1, that abolish E2F binding, were found to result in increased promoter activity in G_0/G_1 (Lam and Watson, 1993; Hsiao *et al.*, 1994; Johnson *et al.*, 1994; Neuman

et al., 1994). This suggests that the transcription factor participates in transcriptional repression when bound to the DNA. These results are consistent with those obtained from in vivo footprinting analysis. For example such analysis of the B-myb promoter showed that the E2F site is specifically occupied in early G1 when the B-myb promoter is inactive (Zwicker *et al.*, 1996). The two E2F proteins expressed in *Drosophila*, dE2F-1 and dE2F-2, exhibit opposing functions. dE2F-1 serves as an activator of transcription while dE2F-2 acts as a repressor (Frolov *et al.*, 2001).

Prevailing models of E2F-mediated transcriptional regulation reconcile these two activities. A simplified model, based on the experimental evidence discussed previously, is as follows (also see **figure 6**). Heterodimers comprising E2Fs 4 and 5 repress transcription in association with PPs during G_0 and early G_1. In mid-late G_1, the PPs are phosphorylated by cyclin-CDKs and these E2Fs are no longer present in the nucleus. It may be that the release of E2Fs 4 and 5 from PPs targets them for ubiquitin-proteasome degradation, thereby decreasing their nuclear levels. This results in derepression of E2F-responsive genes. Protein levels of E2Fs 1-3 also increase in mid-late G_1 and these proteins particpate in transcriptional activation as the cycle progresses. In late S phase this transcriptional activity is abolished by cyclin A-CDK2-mediated phosphorylation of the heterodimers and perhaps by degradation of E2Fs 1-3, allowing the cell's entry into G_2.

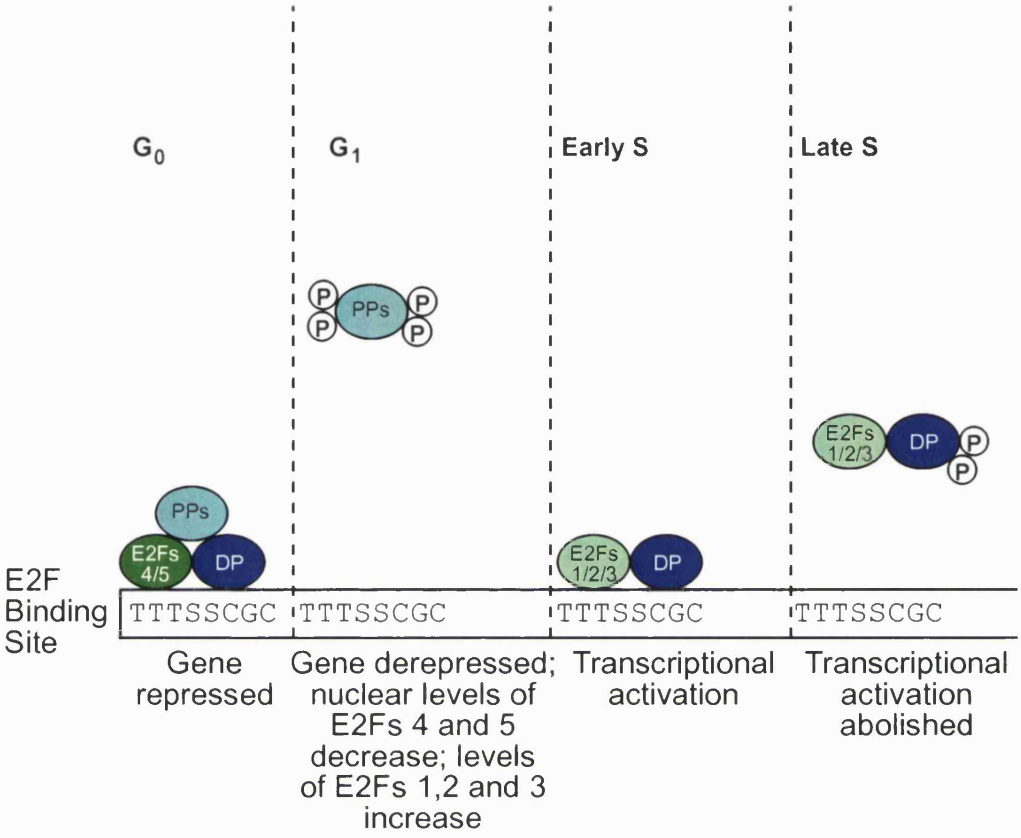


FIGURE 6

MODEL FOR E2F-MEDIATED TRANSCRIPTIONAL REGULATION

(Adapted from Helin, 1998)

TTTSSCGC : the E2F DNA-binding consensus site where S denotes C or G bases

PPs : pocket proteins

Ⓟ : phosphorylation by cyclin-dependent kinases

1.4.3.3

Specificity of E2F Family Members

The existence of several E2F family members and the differences in their regulation (as discussed previously) implies that distinct heterodimers target specific genes. Accordingly, variations were observed with regard to gene induction when the individual E2F proteins were overexpressed in cells. For example, upon overexpression in REF52 cells, E2F-2 emerged as the most potent activator of the *DHFR* and *thymidine kinase* genes (as determined by mRNA levels) compared to the other E2F proteins (DeGregori *et al.*, 1997). Furthermore, experiments involving formaldehyde cross-linking followed by immunoprecipitation have revealed that different E2F proteins interact preferentially with specific promoters (Takahashi *et al.*, 2000; Wells *et al.*, 2000; Kel *et al.*, 2001).

1.4.3.4

Oncogenic, Tumour Suppressive and Apoptotic Functions of E2F

Several studies suggest that E2F is oncogenic. For example, E2Fs-1 to 3 have been shown to induce S-phase in serum-starved REF52 cells and in serum-starved or p16-arrested Rat1 fibroblasts (Lukas *et al.*, 1996; DeGregori *et al.*, 1997). The combined loss of E2Fs 1-3 in mouse embryonic fibroblasts renders these cells unable to enter S phase, progress through mitosis and proliferate (Wu *et al.*, 2001). However, E2F can also be regarded as a tumour suppressor since there is an increased frequency of tumours in *E2F-1* knockout mice (Field *et al.*, 1996;

Yamasaki *et al.*, 1996), as well as a decrease in expression of E2F-1 and E2F-4 in primary and metastatic breast carcinomas (Ho *et al.*, 2001).

There is also compelling evidence implicating E2F in the induction of apoptosis. For example, increased apoptosis has been observed in transgenic megakaryocytes overexpressing E2F-1 (Guy *et al.*, 1996) while reduced apoptosis was reported in *E2F-1* knockout mice (Field *et al.*, 1996). It has been suggested that among the E2F family members, apoptotic induction is a property unique to E2F-1 since overexpression of other E2Fs does not induce apoptosis in serum-starved fibroblasts (DeGregori *et al.*, 1997). E2F-1 can either cooperate with p53 to induce apoptosis (Qin *et al.*, 1994; Wu and Levine, 1994; Pan *et al.*, 1998) or perform the same function in a p53-independent manner (Hsieh *et al.*, 1997; Nip *et al.*, 1997; Phillips *et al.*, 1997). Furthermore, pRb has been shown to suppress E2F-1-mediated apoptosis (Qin *et al.*, 1994). One could propose that the activating and repressing properties of E2F binding sites manifest themselves as oncogenic and tumour suppressive characteristics of E2F. It may be that tumour suppression occurs as a result of E2F's ability to induce apoptosis.

1.4.4

Mechanism of E2F Activity

1.4.4.1

Transcriptional Activation

The activation domain of E2F interacts with the basal transcription factors TBP and TFIIH (**figure 3**) (Hagemeier *et al.*, 1993; Emili and Ingles, 1995; Blau *et al.*,

1996; Pearson and Greenblatt, 1997). Furthermore, DNAse I footprinting studies suggest that E2F recruits TFIID to a promoter in a TFIIA-dependent manner (Ross *et al.*, 1999). Consistent with the involvement of the E2F-binding basal factors in PIC formation and transcriptional elongation (**figure 3**), it has been demonstrated that E2F enhances the initiation and elongation phases of transcription (Blau *et al.*, 1996).

E2F also interacts with proteins that do not form part of the pre-initiation complex, such as the transcription factor Sp1 (Karlseder *et al.*, 1996; Lin *et al.*, 1996), whose binding sites are found in several growth-regulated genes that also have E2F sites (Azizkhan *et al.*, 1993). Analysis of the *c-myc*, *DHFR* and *thymidine kinase* promoters has shown that the E2F and Sp1 sites are involved in cooperative activation of transcription (Majello *et al.*, 1995; Karlseder *et al.*, 1996; Lin *et al.*, 1996). As discussed in **section 1.4.2.6**, E2Fs 1-3 interact with proteins that exhibit histone acetylase activity. This may counteract the repressive effect of a deacetylase tethered to E2F via pRb (**section 1.3.3.1**).

It has also been demonstrated that an E2F DNA-binding site can cooperate with the cell cycle genes homology region (CHR) of the cdc25C phosphatase promoter, resulting in the up-regulation of transcription (Haugwitz *et al.*, 2002).

Studies indicate that E2F is able to induce bending of DNA and that bent DNA structure can contribute to the activation of the *E2F-1* promoter (Cress and Nevins, 1996). It may be that E2F-induced DNA bending allows components of

the transcriptional apparatus to interact productively with each other (**see figure 3**).

1.4.4.2

Transcriptional Repression

In the context of E2F-mediated transcriptional repression, mechanisms other than masking of E2F's transactivation domain (**section 1.3.3.1**) must be invoked, since preventing the binding of E2F has been shown to increase the activity of certain promoters (**section 1.4.3.2**). The mechanism of transcriptional repression by E2F, in conjunction with pRb, was described in **section 1.3.3.1**.

E2F-6 is a transcriptional repressor that lacks pocket protein binding and transactivation domains (Morkel *et al.*, 1997; Cartwright *et al.*, 1998; Gaubatz *et al.*, 1998; Trimarchi *et al.*, 1998). However, this E2F family member does incorporate a C-terminal modular repression domain (Gaubatz *et al.*, 1998) and is found in a multimeric complex containing chromatin remodeling factors (Ogawa *et al.*, 2002). Chromatin remodeling factors were discussed in **section 1.3.3.1**. It is conceivable that other proteins in the multimeric complex recruit such factors while E2F-6/DP serves to localise the repressive complex to a promoter.

1.4.4.3

Promoter Specificity

The apparent specificity exhibited by individual E2F family members, with respect to the genes they regulate (**section 1.4.3.3**), may be partly attributable to their distinct preferences for particular DNA sequences. This is supported by studies carried out by Tao and colleagues (Tao *et al.*, 1997) in which different E2F heterodimers selected distinct (albeit similar) DNA sequences from a pool of degenerate oligonucleotides. This study also showed that the binding of pRb altered the binding-site preference of E2F. Thus the selection of genes that are actively repressed may be partly dependent on the predilection of distinct pRb-E2F complexes for particular DNA sequences.

The crystal structure has been solved of a complex comprising the minimal DNA-binding domains of E2F-4 and DP-2 bound to an E2F site (Zheng *et al.*, 1999). This does not provide any evidence to support indications that E2F proteins preferentially bind specific sequences. The structure reveals that all of the E2F-4 and DP-2 residues contacting the DNA bases, as well as most of the residues that contact the DNA's phosphodiester backbone, are conserved among E2F and DP family members. However, the differences in sequence specificity may be partly due to residues outside the minimal DNA-binding domains (that are not in the crystal structure) that make additional contacts with the DNA. In the cellular environment, it is possible that sequence specificity is modulated by the interaction of E2F with other proteins that contact bases outside the core binding

site (Zheng *et al.*, 1999). The possible alteration of E2F's target specificity by other proteins in a transcriptional complex, might also explain how E2F can apparently bind a non-consensus DNA site (Yamada *et al.*, 2002).

Other mechanisms that may contribute to the selective regulation of promoters by specific E2F heterodimers are also coming to light. For example, it has been shown that E2F sites have different intrinsic bend angles and E2F family members induce distinct degrees of DNA-bending (Tao *et al.*, 1997). Taken together with data that shows DNA-bending affects promoter activation (Cress and Nevins, 1996), it appears that these differences in intrinsic and induced bending may play a role in selective promoter activation.

1.4.4.4

Apoptosis

One of the proposed mechanisms by which E2F induces p53-dependent apoptosis involves E2F's ability to transactivate $p14^{ARF}$ (Bates *et al.*, 1998). The latter stabilises p53 by preventing its degradation (by MDM2), thereby allowing p53 to induce apoptosis (Pomerantz *et al.*, 1998; Stott *et al.*, 1998; Zhang *et al.*, 1998). However, through its cyclin A binding site, E2F-1 can interact with and promote the apoptotic activity of p53 in a manner that is independent both of MDM2 and the transcriptional activity of E2F-1 (Hsieh *et al.*, 2002).

E2F-1 stimulates p53-independent apoptosis by inducing the transcription of the p53 homologue, p73 (Irwin *et al.*, 2000; Lissy *et al.*, 2000; Zaika *et al.*, 2001). A

study by Seelan and colleagues suggests that E2F-1-induced p73 expression is primarily mediated through binding of E2F-1 to two target sites in the p73 promoter (Seelan *et al.*, 2002). Another mechanism for E2F-induced p53-independent apoptosis involves Apaf-1. The latter stimulates caspase activity and is required for stress- and oncogene-induced apoptosis (Cecconi *et al.*, 1998; Yoshida *et al.*, 1998; Soengas *et al.*, 1999). While both p53 and E2F-1 can transactivate the promoter of the *Apaf-1* gene, E2F-1 is able to upregulate the Apaf-1 protein in the absence of p53 (Moroni *et al.*, 2001). Furthermore, overexpression of E2F-1 results in the downregulation of certain anti-apoptotic Bcl-2 family members in a p53-independent manner (Eischen *et al.*, 2001; Elliott *et al.*, 2001).

1.5

INACTIVATION OF pRb BY VIRAL ONCOPROTEINS

DNA tumour viruses induce cellular transformation as part of the viral replicative process (Graham *et al.*, 1975; Gluzman *et al.*, 1977; Dvoretzky *et al.*, 1980; Lowy *et al.*, 1980; Manos and Gluzman, 1985). Much of the current data pertaining to viral oncogenic activity is derived from the study of the small DNA tumour viruses including members of the adenovirus, polyomavirus and papillomavirus families (Nevins and Vogt, 1996).

Of particular importance, in the context of human health, is the papillomavirus family. Seventy seven distinct genotypes of human papillomaviruses (HPVs) have been identified (de Villiers, 1989; zur Hausen, 1996). The benign proliferations caused by HPVs described as "low-risk" include warts (Gissmann *et al.*, 1977; Orth *et al.*, 1977), while high-risk HPVs (HPV 16, 18 and others) are frequently found in cervical carcinoma (van den Brule *et al.*, 1990; Das *et al.*, 1992; Bosch *et al.*, 1995).

Genes whose products induce cell transformation are known as oncogenes (Voet and Voet, 1995) while the products themselves are termed oncoproteins. In this section, I will describe viral oncoproteins that target the pRb-E2F interaction described earlier. The emphasis will be on the HPV16 E7 oncoprotein and its relationship with pRb and E2F, since this is addressed by the experiments I carried out during the course of this study.

1.5.1

Structure

The E7 oncoprotein produced by high-risk HPVs is a small nuclear protein consisting of approximately one hundred residues (Phelps *et al.*, 1988; Greenfield *et al.*, 1991). The constituent regions of HPV16 E7 are named after the domains of the adenovirus E1A oncoprotein with which they share sequence similarity (Phelps *et al.*, 1988). Two of the E7 domains also have homology with regions of a polyomavirus oncoprotein, the SV40 large T antigen (TAg) (Stabel *et al.*, 1985; Figge *et al.*, 1988) (see **figure 7**).

The E7 domains designated conserved region 1 and 2 (CR1 & 2) are crucial for the induction of cellular transformation, since mutations in these regions abolish transforming activity (Edmonds and Vousden, 1989; Phelps *et al.*, 1992). The CR2 domain of E7 and the corresponding regions of E1A and TAg each encompass the LXCXE motif (discussed earlier) (Stabel *et al.*, 1985; Figge *et al.*, 1988; Phelps *et al.*, 1988). This motif is critical, both for the transforming activity of the three oncoproteins and for their ability to associate with pRb (DeCaprio *et al.*, 1988; Whyte *et al.*, 1988; Ewen *et al.*, 1989; Munger *et al.*, 1989; Whyte *et al.*, 1989; Dyson *et al.*, 1992). It has been shown by X-ray crystallography that a nine-residue E7 peptide containing the LXCXE sequence interacts with a groove on the B subdomain of the pRb pocket (Lee *et al.*, 1998).

There exists only a weak homology between the CR3 domains of E7 and E1A but in both proteins, this region contains two CXXC motifs that bind zinc

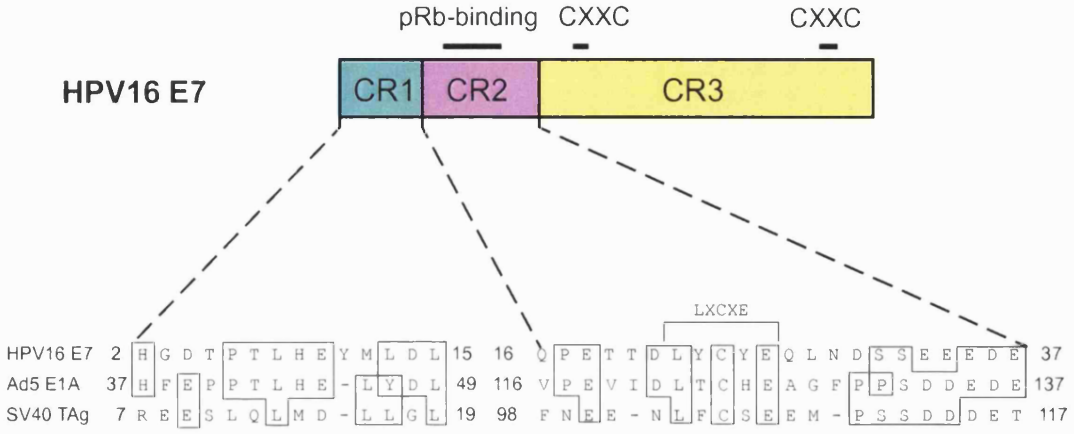


FIGURE 7

STRUCTURAL ORGANISATION OF THE HPV E7 ONCOPROTEIN

(Adapted from Jones and Munger, *1996*)

The human papillomavirus (HPV) E7 oncoprotein is divided into three domains, conserved regions (CRs) 1, 2 and 3. CR2 encompasses the pRb-binding site whose core consists of the LXCXE motif. CR3 contains two zinc-binding motifs (CXXC).

CR1 and 2 of HPV E7 share sequence similarity with portions of the adenovirus (Ad) E1A oncoprotein and the simian virus 40 large T antigen (SV40 TAg). An amino acid sequence alignment is shown of the homologous regions of these oncoproteins. The standard one letter code for amino acids is used and identical residues are boxed.

(Culp *et al.*, 1988; Phelps *et al.*, 1988; Barbosa *et al.*, 1989). In E7, these motifs are responsible for the dimerisation of the protein (McIntyre *et al.*, 1993). E7 exists primarily as a dimer under physiological protein concentrations (Clements *et al.*, 2000) and this dimerisation appears to be a requirement for the induction of cellular transformation, since mutation in one of the CXXC motifs abrogates the oncoprotein's transforming activity (McIntyre *et al.*, 1993).

1.5.2

pRb-inactivating Function

E7 can drive quiescent cells into S-phase (Sato *et al.*, 1989) and participates with an activated *ras* oncogene to transform primary rodent cells (Matlashewski *et al.*, 1987; Phelps *et al.*, 1988). As discussed in **section 1.5.1**, the cell-transforming capacity of E7, E1A and TAg correlates with their ability to bind pocket proteins.

pRb-E2F complexes in U937 cell extracts can be disrupted by each of the three oncoproteins under consideration (Chellappan *et al.*, 1991; Chellappan *et al.*, 1992). Furthermore, these complexes are absent from the majority of tested cervical carcinoma cell lines expressing E7 (Chellappan *et al.*, 1992) and from cervical carcinoma tissues harbouring HPV16 DNA (Wang *et al.*, 2000). Binding to high-risk HPV E7 leads to the destabilisation of all three pocket proteins (Boyer *et al.*, 1996; Berezutskaya *et al.*, 1997; Jones and Munger, 1997; Smith-McCune *et al.*, 1999; Giarre *et al.*, 2001; Gonzalez *et al.*, 2001; Helt and Galloway, 2001).

The targeting of pRb by E7 can be understood in the light of studies that have demonstrated the key role of the tumour suppressor in restraining cellular

proliferation (as discussed in **sections 1.2-1.4**). It is probable that E7 (and the other oncoproteins mentioned here) contribute to the creation of a cellular environment that is conducive to the replication of viral DNA (Howley, 1996). HPV does not possess its own replication enzymes and must, as a consequence, induce S-phase entry of the host cell in order to utilise the host's cellular replicative machinery (Howley, 1996; zur Hausen, 1996).

Some of the promoters of genes whose products constitute this machinery incorporate E2F sites (**table 2**). Indeed it has been shown that E7 stimulates promoters via E2F DNA binding sites (Phelps *et al.*, 1991; Zerfass *et al.*, 1995). Although the oncoprotein inactivates pRb (as discussed earlier), E7-induced upregulation of E2F-responsive genes does not appear to be solely attributable to the inactivation of the tumour suppressor. Using E7 and E2F-1 mutants that cannot bind to pRb, as well as pRb-negative cells, Hwang and colleagues have shown that E7 binds to E2F-1 and activates E2F-1-driven transcription in a pRb-independent manner (Hwang *et al.*, 2002). Thus, E7 can inactivate pRb (and hence deregulate pRb-mediated repression of E2F) and it possesses the additional ability to stimulate E2F-1-dependent transcription, the latter involving more than simply the elimination of pRb.

1.5.3

Mechanism of pRb-inactivation

Although the CR2 domain of HPV16 E7 and E1A is required for pRb-binding, peptides based on this region alone cannot disrupt pRb-E2F complexes (Huang *et*

al., 1993; Ikeda and Nevins, 1993). Furthermore, a peptide derived from the CR2 domain of HPV16 E7 has a lower binding affinity for pRb (the apparent Kd being 110 nM) than the full-length oncoprotein (apparent Kd is 1.3 nM) (Jones *et al.*, 1992; Lee *et al.*, 1998). Taken together, these findings indicate that regions outside the CR2 domain of E7 interact with pRb and bring about disruption of the pRb-E2F complex. Accordingly, it has been demonstrated that E7's CR3 domain interacts with pRb and is necessary for dissociation of the pRb-E2F complex (Stirdivant *et al.*, 1992; Huang *et al.*, 1993; Patrick *et al.*, 1994; Helt and Galloway, 2001).

Further study is required to elucidate the mechanism of E7-mediated degradation of pRb. However, the ubiquitin-proteasome pathway appears to be involved since inhibitors of the 26S proteasome interfere with this function of E7 (Boyer *et al.*, 1996; Gonzalez *et al.*, 2001).

Regarding E7-mediated (pRb-independent) activation of E2F-1-driven transcription, the oncoprotein binds E2F-1-DNA complexes but not DNA alone (Hwang *et al.*, 2002). One could speculate that E7 recruits a co-activator to E2F-1, such as P/CAF (**sections 1.4.2.4, 1.4.2.6 and 1.4.4.1**). Alternatively, the binding of the oncoprotein to other transcription factors may underpin activation of E2F-1. E7 has been shown, for example, to associate with TBP (Massimi *et al.*, 1996; Massimi *et al.*, 1997; Phillips and Vousden, 1997).

1.6

PRESENTATION OF EXPERIMENTAL WORK

The experiments presented here are divided into three areas of study, each of which has a dedicated chapter:

1) The Identification of Aptamers (DNA ligands) for the E2F-1 Homodimer (**Chapter 3**).
2) Regulation of E2F/DP DNA-Binding by Cyclin-CDK Dependent Phosphorylation (**Chapter 4**).
3) Investigation of the Interactions Between pRb, E2F and HPV E7 (**Chapter 5**).

Each of the above chapters begins with an outline of the specialised technique used in the particular area of study. This is followed by an introduction to the experiments undertaken to place the work in its proper context, after which the results are shown. The chapter ends with a discussion of the latter.

The methods (specialised and general techniques) and materials for all of the experiments are described in **Chapter 2**. A general conclusion and references are given at the end of the thesis.

CHAPTER 2

METHODS AND MATERIALS

CHAPTER 2

METHODS AND MATERIALS

2.1

GENERAL

This section outlines the general biochemical and molecular biological techniques and materials used during the course of this study. Specific experiments are described in subsequent sections of this chapter.

2.1.1

Media for Bacterial Cell Growth

The compositions of the media used for growing cells are outlined in **table 3** below.

	Luria-Bertani (LB) Broth	**Terrific Broth**	**L-agar**
Bacto Tryptone	10 g	10 g	10 g
Yeast Extract	5 g	24 g	5 g
NaCl	10 g	-	10 g
Difco Agar	-	-	15 g
Glycerol	-	4 ml	-
K_2HPO_4	6.3 g	12.54 g	-
KH_2PO_4	-	2.31 g	-
Sodium Citrate	0.45 g	-	-
$MgSO_4.7H_2O$	0.09 g	-	-
$(NH_4)_2SO_4$	0.9 g	-	-

Table 3: Compositions of the Media Used for Bacterial Cell Growth

The quantities listed above are for 1 l of medium prepared using distilled water.

2.1.2

Cloning

2.1.2.1

Restriction Enzyme Digestions

Restriction enzymes and buffers were supplied by New England Biolabs Inc. The quantity of DNA to be digested was estimated by agarose gel electrophoresis (**section 2.1.4.1**). The general rule followed was that 1 µl of enzyme was added to the reaction mixture for every microgram of DNA, as suggested by the supplier. Double digests were performed so that the mixture contained 2 restriction enzymes in a final volume of 50 µl. The buffer in the reaction mixture was optimal for digestion by both enzymes and was selected according to the supplier's recommendation. The mixture was incubated in a water-bath (Grant) at 37°C for 3 hours. Digested dsDNA oligonucleotides were purified using the Qiaquick PCR purification kit (Qiagen), according to the manufacturer's instructions.

In the case of vector DNA, 1 µl (1 U) of calf intestinal alkaline phosphatase (Promega) was added to the reaction mixture (after completion of the restriction enzyme digest) and incubation at 37°C took place for a further 30 mins. This was to prevent religation of the vector (Sambrook *et al.*, 1989). Following alkaline phosphatase treatment, the vector DNA was separated on a 1 % agarose gel

(**section 2.1.4.1**) and extracted from the matrix using a Qiaquick gel extraction kit (Qiagen) according to the manufacturer's instructions.

2.1.2.2

Ligation

The dsDNA oligonucleotides were ligated into the pUC19 (Amersham Pharmacia Biotech) vector. The quantities of vector and insert to be added to the ligation reaction mixture were estimated by agarose gel electrophoresis (**section 2.1.4.1**). A vector:insert molar ratio of 1:3 was used. The amount of insert to be added to the reaction mixture was calculated by means of the following formula (Beckler *et al.*, 1996):

$$\frac{\text{ng of vector} \times \text{size (kb) of insert}}{\text{size (kb) of vector}} \times \text{molar ratio of } \frac{\text{insert}}{\text{vector}} = \text{ng of insert}$$

A 10 µl ligation reaction mixture was set up containing 1 µl 10 x T4 ligation buffer (New England Biolabs), 50 ng pUC19 vector, 1 µl (400 U) T4 ligase (New England Biolabs) and 4 ng insert. The mixture was incubated at 16°C overnight (Grant water-bath). The ligation product was purified by ethanol precipitation (protocol follows) and resuspended in 10 µl dH_2O.

2.1.2.3

Transformation

Cells were kept on ice at all times. The ligation products (1 µl) were added to 40 µl of electrocompetent XL1B cells (Stratagene), mixed gently and transferred to a Bio-Rad 0.2 cm gap electroporation cuvette. Electroporation was carried out by means of the Bio-Rad Gene Pulsar at 2.5 kV, 200 Ω and 25 µF. Immediately after electroporation, 1 ml of sterile LB broth was added to the cuvette. The cells were incubated with agitation at 37°C for 1 hr (New Brunswick Scientific incubator-shaker).

2.1.2.4

Selection and Purification of Recombinant Plasmids

200 µl of transformed cells were plated onto an L-agar plate containing ampicillin (2.2 µg/ml), IPTG (0.5 mM) and XGAL (1.5 µg/ml). The plate was incubated at 37°C overnight. XLIB cells harbouring recombinant plasmids appeared as white colonies on the agar plate (Ruther, 1980). Each white colony was inoculated into 5 ml LB broth containing 100 µg/ml amipicillin. The cultures were grown for 6 hrs. at 37°C in an incubator-shaker (New Brunswick Scientific) at 280 rpm. Cells were harvested by centrifugation (Sorvall Legend RT) at 4000 rpm for 15 mins. and plasmid DNA was extracted from each pellet using the QIAprep Spin Miniprep Kit (Qiagen) according to the manufacturer's instructions. In order to verify the presence of inserted DNA in the purified plasmids, restriction enzyme digests

were performed (as described above) using the Bgl *II* enzyme whose cleavage site was only present in the inserts and is not found in pUC19. The digested vector was analysed on a 1 % agarose gel (**section 2.1.4.1**).

2.1.3

Protein Analysis

2.1.3.1

Polyacrylamide Gel Electrophoresis (PAGE)

Recombinant proteins were analysed by SDS-PAGE (Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis) according to the method described by Laemmli (Laemmli, 1970). The Mini-Protean II apparatus (Bio-Rad) was used except in the case of Novex™ Pre-Cast 4-12 % Tris-Glycine gels (Invitrogen). The Xcell Surelock Mini-Cell (Invitrogen) was used with the latter.

2.1.3.2

Determination of Protein Concentration

The concentration of recombinant protein was determined by UV spectrophotometry. The Beer-Lambert law (Voet and Voet, 1995) describes the amount of light absorbed by a substance at a given wavelength thus:

$$A = \varepsilon cl$$

where **A** is the absorbance, **c** is the molar concentration of the sample, **l** is the length of the light path through the sample (cm) and ε is the molar extinction coefficient (M^{-1} cm^{-1}).

The absorbance of the protein was measured at 280 nm (Shimazu UV-160A spectrophotometer) while the molar extinction coefficient was calculated by means of the Protean program (DNA star). The formula above was then used to calculate the protein concentration.

2.1.3.3

Electrospray Mass Spectrometry

The molecular mass of the recombinant protein was determined using a Fisons VG/Masslab Platform instrument. These experiments were carried out by Dr. S. Howell (Division of Protein Structure, NIMR). The determined mass was compared to the calculated value, obtained by means of the PeptideMass program (Swiss Institute of Bioinformatics) that can be accessed via the internet (http://www.expasy.ch/).

2.1.4

DNA Analysis and Purification

2.1.4.1

Agarose Gel Electrophoresis

DNA was analysed using 1 % (in the case of plasmid DNA) or 4 % (for ds DNA oligonucleotides) agarose gels according to the method described by Sambrook and colleagues (Sambrook *et al.*, 1989). Gels contained ethidium bromide at a final concentration of 0.5 μg/ml. Electrophoresis was carried out in a 1 x Tris-acetate (1 x TAE) buffer system (40 mM Tris acetate pH 7.7, 1 mM EDTA) and DNA visualised under ultra-violet illumination.

2.1.4.2

Nondenaturing Polyacrylamide Gel Electrophoresis (PAGE)

PCR products were also analysed using 12 % non-denaturing PAGE gels according to the method described by Sambrook and colleagues (Sambrook *et al.*, 1989) with the exception that the procedure was performed with TAE buffer (see above). The Mini-Protean II (Bio-Rad) apparatus was used. After electrophoresis, the gel was bathed for 30 mins. at room temperature in 50 ml 1 x TAE containing 0.5 μg/ml ethidium bromide. DNA was visualised under ultra-violet illumination.

Nondenaturing PAGE, as described above, was also used for the Electrophoretic Mobility Shift Assay (EMSA) with the modifications outlined in **section 2.3.3.2.**

2.1.4.3

Phenol/chloroform Extraction

DNA was separated from protein by mixing the sample with an equal volume of phenol-chloroform (a 25:24:1 phenol/chloroform/isoamyl alcohol mixture from Sigma). The sample was then centrifuged (IEC Micromax) at 13 000 rpm for 15 secs. and the upper aqueous layer was transferred to a clean tube. An equal volume of chloroform was mixed with the latter, followed by centrifugation (as before). The aqueous phase from this step was transferred to another clean tube and the DNA was further purified by ethanol precipitation.

2.1.4.4

Ethanol Precipitation

A one-tenth volume of 3 M sodium acetate and 2.5 volumes of absolute ethanol were added to the DNA. The sample was chilled to –20°C for 2 hrs. and the DNA precipitate was pelleted by centrifugation (IEC Micromax) at 13 000 rpm for 15 mins. at 4°C. The supernatant was discarded and the pellet washed with 70 % ethanol. Following centrifugation (as before), the supernatant was discarded and the DNA dried using a Speedvac instrument (Savant) for 10 mins. at medium heat.

2.1.4.5

Determination of DNA Concentration

Concentrations of single- and double-stranded oligonucleotides were determined by UV spectrophotometry in the manner discussed previously for proteins (**section 2.1.3.2**). However, in this case the absorbance value used in the equation was measured at 260 nm.

Owing to their random nature, a reliable molar extinction coefficient could not be established for the degenerate oligonucleotides. When preparing a degenerate oligonculeotide probe for SELEX (**section 2.2.3.1**), the concentration of DNA was estimated by UV spectrophotometry using the following formulae (Beckler *et al.*, 1996):

OD_{260} of 50 μg/ml double-stranded DNA =1 AU

OD_{260} of 33 μg/ml single-stranded DNA = 1 AU

where **OD_{260}** is the optical density of the DNA measured at 260 nm and **AU** denotes absorbance units.

2.2

IDENTIFICATION OF APTAMERS FOR THE E2F-1 HOMODIMER (METHODS AND MATERIALS)

2.2.1

Systematic Evolution of Ligands by Exponential Enrichment (SELEX)

General procedures such as cloning, restriction enzyme digestion etc. are described in **section 2.1**. The current section (**2.2**) is solely concerned with the two SELEX methods (methods 1 and 2) used in this study. The main difference between these methods was the means by which protein-DNA complexes were isolated. An outline of the SELEX technique (including the protein-DNA isolation strategies) is given in **section 3.1**.

The single-stranded oligonucleotide template (Oswel) used in the SELEX procedures (methods 1 and 2) is illustrated in **figure 12**, along with the two primers that were employed, P1 and P2 (Oswel). The template comprised a degenerate core of 20 bases that is flanked by *Eco*R I and *Bgl* II restriction enzyme sites at its 5' end and a *Hin*d III site at the 3' end. The *Bgl* II site was incorporated to verify the presence of a SELEX-derived sequence in the plasmid (that does not otherwise contain this site) prior to sequencing.

2.2.1.1

Preparation of Double-Stranded Degenerate Oligonucleotide (degOLIGO)

In order to anneal P2 to the 3' end of the template, a reaction was set up comprising 2 µl 10X Klenow buffer (1X buffer= 50 mM Tris-HCl pH 7.2, 10 mM $MgSO_4$, 50 mM KCl, 0.1 mM DTT) (New England Biolabs Inc.-NEB), 5 µl 40 µM single-stranded template, 4 µl 100 µM P2 primer, 1 µl 1 M KCl, 1 µl 10 mM DTT. This reaction was incubated at 95°C for 10 mins in a water-bath (Grant). After this period of time had elapsed, the water-bath was switched off and the reaction tube left to cool in the water until its temperature decreased to 37°C.

After cooling, 2 µl 10 mM dNTP mix (containing 2 mM each dNTP), 4 µl dH20 and 5 U (1 µl) Klenow enzyme (NEB) were added to the reaction. The synthesis of the second DNA strand was then left to proceed for 30 mins. in the water-bath at 37°C. The enzyme was deactivated by heating the reaction mixture for 20 mins. at 75°C.

2.2.2

SELEX Method 1

2.2.2.1

Selection and Amplification

20 pmol of homodimeric recombinant histidine-tagged E2F-1Δ_{cyc} (residues 92-195 of E2F-1; supplied by Dr. I. Tews; see **figure 10**) were mixed with the

Klenow reaction described earlier (containing approximately 200 pmol degOLIGO; $1.2x10^{14}$ sequences) and binding was allowed to proceed for 1 hr. at room temperature. The binding reaction was passed through a Ni-NTA Agarose spin column (Qiagen) that had been pre-equilibrated with binding buffer (50 mM Tris-HCl 7.2, 10 mM $MgSO_4$, 50 mM KCl, 0.1 mM DTT). The column was washed twice with binding buffer before elution buffer (50 mM Tris 7.8, 100 mM NaCl, 0.5 M imidazole, 20 % glycerol) was passed through it twice (2 X 100 µL). The combined eluate (200 µl) was subjected to phenol/chloroform extraction to remove protein, followed by ethanol precipitation to obtain pure DNA. The recovered DNA was resuspended in 20 µl dH2O.

Two PCR reaction mixtures were prepared, each containing 10 µl of the recovered DNA as template. Each mixture also contained 10 µl 10X PCR buffer (NEB), 200 µM each dNTP, 4 U (2 µl) Deep Vent DNA polymerase (NEB) and 10 µM of each of the P1 and P2 primers in a final volume of 100 µl. The mixtures were placed in a thermal cycler (Techne) that had been programmed to carry out denaturation for 1 min at 95°C, annealing for 1 min. at 64°C and extension for 1 min. at 72°C, for 30 cycles. The PCR product was analysed by agarose gel electrophoresis. Amplified DNA was subjected to phenol/chloroform extraction followed by ethanol precipitation and then resuspended in 20 µl binding buffer. This DNA was used for the next round of selection and amplification instead of the Klenow reaction. The subsequent rounds were performed as described above except that the number of PCR cycles in the 3^{rd} and 4^{th} rounds was reduced to 25 and then to 20 in rounds 5 and 6. This reduction was in anticipation of an increase in high-affinity binding sites being selected in each round that would serve as

templates in the PCR reactions. Since too much template can lead to the loss of the desired amplified oligonucleotide and the appearance of non-specific PCR products, the number of cycles was reduced as a counteractive measure (Pierrou *et al.*, 1995).

2.2.2.2

Cloning and Sequencing

After the sixth and final PCR, amplified DNA was purified by phenol/chloroform extraction and ethanol precipitation. The DNA (selOLIGO) was digested with *Eco*R I and *Hin*d III before being ligated into pUC19 (Amersham Pharmacia Biotech). Recombinant plasmids appeared as white colonies on IPTG-XGal-Amp plates. Plasmids isolated from cultures of these recombinants were digested with *Bgl* II to check that inserts were present. DNA sequences were determined by means of the ABI Prism™ BigDye™ Terminator Cycle Sequencing kit (PE Applied Biosystems) and ABI Prism™ 377 sequencing apparatus according to the manufacturer's instructions.

2.2.2.3

EMSA (Electrophoretic Mobility Shift Assay)

An EMSA was carried out to assess the binding of E2F-1Δ_{cyc} to the oligonucleotides recovered after the 6th round of SELEX (selOLIGO). The procedure was essentially the same as that described in **section 2.3.3** except that approximately 10 pmol each of degOLIGO and selOLIGO were end-labeled.

Final concentrations of protein and probe in the reaction mixtures are given in **section 3.3.1**.

2.2.3

SELEX Method 2

Since the SELEX procedure described earlier (method 1) failed to yield a consensus binding site for the E2F-1Δ_{cyc} homodimer, the protocol was altered. Method 2 comprised those changes that would conceivably eliminate the problems experienced with method 1 (see **section 3.4.1**). To this end, the isolation of protein-DNA complexes was attempted using EMSA, as outlined in **section 3.1.2**, rather than the Ni-Agarose matrix used in method 1. Furthermore, DNA was analysed using non-denaturing polyacrylamide gels.

2.2.3.1

Preparation of Control Oligonucleotide (conOLIGO)

As illustrated in **figure 15**, conOLIGO comprised a core sequence from the adenovirus E2A promoter containing an E2F-binding site (La Thangue *et al.*, 1990). The binding site was flanked by sequences that were not complementary to primers P1 and P2 and were devoid of *Eco*R I, Bgl *II* or *Hin*d III restriction enzyme sites to prevent the control DNA from contaminating PCRs and from being cloned.

Synthetic complementary single-stranded oligonucleotides (Oswel) were hybridised to produce conOLIGO. The strands were mixed in equimolar amounts (13.8 μM final concentration of each strand) in a reaction containing 40 mM Tris 8.0, 50 mM NaCl, 10 mM $MgCl_2$. The reaction was incubated at 95°C for 10 mins in a heating block (Grant). The block was switched off after this period had elapsed and the DNA left to cool slowly in the block to 30°C. The double-stranded DNA was concentrated to 100 μM using a microcon 10 centrifugal concentration device (Amicon). A control oligonucleotide probe for EMSA was prepared from this solution according to the protocol described in **section 2.3.3.1 (B)** and the degOLIGO was labeled in the same manner.

2.2.3.2

Selection and Amplification

The control binding reaction comprised 10 μM conOLIGO, 2 μM (24 pmol) E2F-1Δ_{cyc} homodimer, 50 mM Tris 8.2, 6 mM $MgCl_2$, 3 mM DTT, 15 % glycerol in a final volume of 10 μl. The selection reaction had the same components except that conOLIGO was replaced with approximately 146 pmol labeled degOLIGO (about $9x10^{13}$ molecules). Reactions were incubated for 10 mins. at room temperature and then separated using an 8 % native polyacrylamide gel according to the method described in **section 2.3.3.2 (B)**.

A slice of gel from the degOLIGO lane adjacent to the band of complexed conOLIGO was excised, crushed and immersed overnight at room temperature in 0.5 M ammonium acetate, 10 mM $MgCl_2$, 1mM EDTA. After centrifugation at

13000 rpm for 15 mins. (IEC Micromax), DNA in the supernatant was subjected to phenol/chloroform extraction followed by ethanol precipitation. The recovered DNA (recOLIGO) was resuspended in 20 µl dH20 and used as template in several PCR reactions. The conditions used for each of these reactions were varied and are summarised below.

All PCR reaction mixtures contained 10 µl 10X PCR buffer (Qiagen), 200 µM each dNTP, 0.5 µM P1 primer, 0.5 µM P2 primer, 2.5 U HotStarTaq DNA polymerase (Qiagen) in a final volume of 100 µl. 1 µl recOLIGO or 1 µl of a recOLIGO dilution (1 in 10, 1 in 100 or 1 in 1000) were used as template in the PCRs. The final $MgCl_2$ concentration ranged between 1.5-5 mM. Thermal cycling conditions were as follows. 95°C, 15 mins. (initial activation), 95°C, 50 s (denaturation), 50-68°C, 0.5 or 1 min (annealing), 72°C, 1 min. (extension), for 20-30 cycles.

2.3

REGULATION OF E2F/DP DNA-BINDING BY CYCLIN-CDK DEPENDENT PHOSPHORYLATION (METHODS AND MATERIALS)

2.3.1

Purification of E2F-1_{cyc}

2.3.1.1

Strategy

E2F-1_{cyc} (residues 84-195 of human E2F-1) was subcloned into the pGEX6P-1 vector (Amersham Pharmacia Biotech). The latter allows the expression of the protein of interest as a GST-fusion with a protease (human rhinovirus type 14 3C protease) cleavage site between the GST-tag and the N-terminus of the cloned fusion partner (E2F-1_{cyc} in this case). The GST-fusion protein is first bound to glutathione sepharose and then treated with the protease (supplied as PreScission™ protease by Amersham Pharmacia Biotech). This results in cleavage of the GST-tag from the fusion partner. The tag remains bound to the matrix (as does the enzyme that is itself a GST-fusion protein) while the eluate contains the fusion partner.

2.3.1.2

Protocol

E2F-1_{cyc} was subcloned into pGEX6P-1 by Dr. I. Tews while the GST-fusion protein was expressed in BL21 (DE3) cells by Ms. V. Ennis-Adeniran.

7 g of pelleted cells were resuspended, on ice, in 400 ml buffer A (20 mM BisTris Propane pH 7.5, 300 mM NaCl, 10 % glycerol, 2 mM βME, 1 mM EDTA) in which 8 Complete™ protease inhibitor tablets (Roche) had been dissolved. The suspension was sonicated on ice (Branson sonifier 450) 5 times for 1 min. at 50 % output with a 1 min. cooling period between sonications. Subsequent steps were carried out at 4°C. The lysate was centrifuged (Beckman Avanti J25) at 20 000 rpm for 30 mins. and the supernatant was ultracentrifuged (Beckman L8-70M) at 40 000 rpm for 1 hr. The supernatant from the latter step was loaded onto a 4 ml glutathione sepharose (Amersham Pharmacia Biotech) column at 1 ml/min by means of a P1 peristaltic pump (Amersham Pharmacia Biotech). A GradiFRAC system (Amersham Pharmacia Biotech) was used to measure the absorbance of the eluate (at 280 nm) during the loading and the subsequent washing step. The beads were washed in buffer B (20 mM BisTris Propane pH 7.5, 1.2 M NaCl, 10 % glycerol, 2 mM βME) since the higher NaCl concentration had been found to be effective in removing DNA that co-purified with the protein. Following re-equilibration of the glutathione sepharose in buffer A, the beads were transferred to an Econo-Pac gravity flow column (Bio-Rad) and resuspended in 4 ml buffer A containing 320 U PreScission™ protease (Amersham Pharmacia Biotech). The cleavage reaction was allowed to proceed for 4 hrs after which the eluate was collected from the column and the beads washed with 3 bed volumes of buffer A. The washes and eluate were combined and passed through 1 ml glutathione sepharose to remove some slight contamination by the GST-tag. The pure E2F-1_{cyc} was then concentrated using a Centricon 3 (Amicon) centrifugal concentration

device (according to the supplied instructions), aliquoted, analysed by mass spectrometry (**section 2.1.3.3**), frozen in liquid nitrogen and stored at -80°C.

2.3.2

Purification of DP-1_{S98} and DP-$1\Delta_{S98}$

2.3.2.1

Strategy

DP-1_{S98} (residues 84-194 of murine DP-1) and DP-$1\Delta_{S98}$ (same as DP-1_{S98} but in which the serine 98 residue was mutated to alanine) were also subcloned into the pGEX6P-1 vector, described earlier (**section 2.3.1.1**). However, using PCR, the recombinant DP-1 proteins were engineered such that their C-termini each incorporated a His-tag. The DP-1 fragments were therefore purified using glutathione sepharose (as described in **section 2.3.1.1**) and Ni-agarose. DP-1_{S98} and DP-$1\Delta_{S98}$ were each purified by the same method, as outlined below.

2.3.2.2

Protocol

DNA manipulations (subcloning and mutation of DP-1_{S98}, incorporation of His-tag) were carried out by Dr. I. Tews. Both recombinant DP-1 fragments were expressed in BL21 (DE3) cells by Ms. V. Ennis-Adeniran.

30 g of pelleted cells were resuspended on ice in 300 ml buffer C (50 mM Tris pH 7.0, 300 mM NaCl, 10 % glycerol, 2 mM βME) in which 6 Complete™ protease inhibitor tablets (Roche) had been dissolved. The suspension was sonicated, centrifuged and ultracentrifuged as described for E2F-$1_{cyc.}$ The following steps were carried out at 4°C and the chromatographic separations outlined below were monitored using the GradiFRAC system, as described for E2F-1_{cyc}. After ultracentrifugation, the supernatant was loaded at 1 ml/min onto a 5 ml glutathione sepharose (Amersham Pharmacia Biotech) column by means of a P1 peristaltic pump (Amersham Pharmacia Biotech). The beads were then washed with buffer C, transferred to an Econo-Pac gravity flow column (Bio-Rad) and resuspended in 5 ml buffer C containing 400 U PreScission™ protease in order to cleave the GST-tag (see E2F-1_{cyc} purification). After 4 hrs., the eluate was collected from the column and the glutathione sepharose was washed with 3 bed volumes buffer C. The washes and eluate were combined and applied at 1 ml/min (P1 pump) to a 3 ml Ni-agarose (Qiagen) column connected to the GradiFRAC system. The Ni-agarose was washed with buffer C and then with the same buffer containing 80 mM imidazole to remove contaminating proteins. The pure DP-1_{S98} (or DP-$1\Delta_{S98}$) remaining on the beads was subsequently eluted using buffer C containing 300 mM imidazole. The pure protein was concentrated using a Centricon 3 (Amicon) centrifugal concentration device (according to the supplied instructions), aliquoted, analysed by mass spectrometry (**section 2.1.3.3**), frozen in liquid nitrogen and stored at -80°C.

2.3.3

Electrophoretic Mobility Shift Assays (EMSAs)

2.3.3.1

Probe

(A) Choice of Oligonucleotide

The oligonucleotide used as a probe in the EMSAs (**figure 11**) contained a high-affinity E2F DNA-binding site from the adenovirus E2A promoter (La Thangue *et al.*, 1990). Studies had previously been carried out in our laboratory involving co-crystallisation of recombinant E2F homodimers and various E2A-based oligonucleotides, as well as EMSAs to assess the interactions between them (Dr. I. Tews). The results indicated that the oligonucleotide shown in **figure 11** was bound by E2F homodimers with high affinity and was also promising in terms of co-crystallisation. It was for this reason that I decided to use the same probe for my studies. The double-stranded oligonucleotide was prepared (Dr. I. Tews) by mixing complementary single-stranded synthetic DNA (Oswel) in equimolar amounts and carrying out an annealing procedure (as described in **section 2.2.3.1**).

(B) Radiolabeling

Radiolabeled probe was prepared by end-labeling the DNA duplex (**figure 11**) with ^{32}P in the following manner. An end-labeling reaction mixture was set up containing approximately 1 nmol of the DNA duplex, 50 U T_4 polynucleotide kinase (NEB), 3 MBq [γ-^{32}P]ATP (Amersham Pharmacia Biotech) and 1X T_4 buffer (NEB) in a final volume of 50 µl. The mixture was incubated at 37°C (Grant heating block) for 30 mins. after which the enzyme was inactivated by incubation at 65°C for 20 mins. The labeled DNA was purified from unincorporated ATP by applying the reaction mixture to a Sephadex MicroSpin™ G-25 column (Amersham Pharmacia Biotech) according to the manufacturer's recommended protocol. The labeled duplex was further purified by ethanol precipitation and resuspended in a solution of unlabeled duplex to give a final concentration between 50-100 µM, as determined by measuring the absorbance of the DNA (see **section 2.1.4.5**) at 260 nm (Shimazu UV-160A spectrophotometer).

2.3.3.2

Assay

(A) Preparation of Reaction Mixtures

The following steps were carried out at room temperature. EMSA reaction mixtures were set up, each of which contained gel retardation buffer (50 mM Tris pH 8.2, 6 mM $MgCl_2$, 3 mM DTT, 15 % glycerol), probe, ATP (where required),

cyclin A-CDK2 (where required) and either a recombinant E2F/DP complex, an E2F-1 fragment or a DP-1 fragment. The final concentrations of the components in each reaction are given in **chapter 4** (**section 4.3.3**) in which the recombinant proteins used are also described (**section 4.2**).

In the case of reaction mixtures to which an E2F/DP complex was added, the appropriate recombinant E2F-1 and DP-1 proteins (see results) were first mixed in equimolar amounts to make a stock solution that was left to stand for 30 mins. Aliquots from this stock were subsequently added to reaction mixtures to give the desired final concentration of E2F/DP complex. EMSA reaction mixtures were left to incubate for 10 mins. (to allow the protein and DNA to form a complex) prior to separation of the reaction products by electrophoresis. In the case of reactions containing cyclin A-CDK2, the kinase was added after the 10 min-incubation period had elapsed. Following addition of the kinase, separation by electrophoresis was performed immediately or after specific periods of time, as outlined in **section 4.3.3**.

(B) Electrophoresis

8 % nondenaturing TAE (40 mM Tris-acetate pH 8.5, 1 mM EDTA) polyacrylamide gels (also see **section 2.1.4.2**) were used to separate the EMSA reaction products. Each gel was pre-electrophoresed (4°C) at 100 V (constant voltage) until the current dropped to a constant value. After loading the samples, electrophoresis was carried out at 11 mA (constant current) for 1 hr. (4°C). The gel was then dried at 80°C for 1 hr. (Bio-Rad gel dryer model 583) and labeled

components were visualised by exposing the dried gel to autoradiographic film (X-Ograph Ltd.).

2.4

INVESTIGATION OF THE INTERACTIONS BETWEEN pRb, E2F AND HPV E7 (METHODS AND MATERIALS)

2.4.1

Purification of pRb_{AB}

2.4.1.1

Strategy

pRb_{AB} (residues 372 to 787) was subcloned into the pGEX-2T vector (Amersham Pharmacia Biotech). As with pGEX6P-1 (**section 2.3.1.1**), this vector also allows the expression of the protein of interest with a GST-tag that can be removed using PreScission™ protease, in the manner described in **section 2.3.1.1**. The pRb_{AB} construct was engineered such that two thrombin cleavage sites flanked the spacer between the A and B subdomains of pRb_{AB} (see **figure 34**). Treatment of the GST-pRb_{AB} construct (bound to glutathione sepharose) with thrombin results in the removal of most of the flexible spacer region (whose absence promotes crystallisation) although the A and B subdomains remain tightly associated. pRb_{AB} was further purified by gel filtration chromatography.

2.4.1.2

Protocol

DNA manipulations were carried out by Dr. J. Spencer. Cells were transformed with recombinant plasmid according to the method outlined in **section 2.1.2.3**, with the exception that BL21 cells (see below) were used.

(A) Production of GST-pRb$_{AB}$

E. coli BL21 (DE3) cells (Novagen) harbouring the vector described above were grown in 10 ml terrific broth, containing 100 ug/ml ampicillin, at 37°C for 6 hours in an incubator-shaker (New Brunswick Scientific, model G25) at 280 rpm. The whole culture was introduced into 500 ml of the same type of growth medium and grown overnight under the same conditions. Each of six flasks containing 750 ml of the medium was inoculated with 10 ml of the overnight culture, before being placed in the incubator as before with the temperature reduced to 28°C. Cell growth was monitored by spectrophotometry (Shimazu UV-160A spectrophotometer) and when an optical density at 600 nm (OD_{600}) between 0.75 and 0.85 AU had been attained, protein expression was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) (Biogene) to a final concentration of 0.1 mM. The cells were grown under the same conditions for 3 hrs. and harvested by centrifugation at 4000 rpm for 20 mins. (Beckman J6) before being stored at –80°C.

(B) Purification of pRb_{AB}

The following steps were carried out on ice. The harvested cells (see above) were resuspended in 300 ml buffer D (50 mM Tris pH 7, 300 mM NaCl, 1 mM EDTA, 5 mM DTT) in which 6 Complete™ protease inhibitor tablets (Roche) had been dissolved. The suspension was sonicated and centrifuged as described for E2F-1_{cyc}. The following steps were carried out at 4°C. After centrifugation, the supernatant was passed through a 0.45 μm filter (Sartorius). The filtrate was applied to 5 ml glutathione sepharose (Amersham Pharmacia Biotech) at 0.3 ml/min using a P1 peristaltic pump (Amersham Pharmacia Biotech). This loading step and the subsequent washing (outlined below) were monitored using the GradiFRAC system as described for E2F-1_{cyc} (**section 2.3.1.2**). After loading was complete, the unbound proteins were washed out of the column with buffer E (50 mM Tris pH 7, 500 mM NaCl, 5 mM DTT) at 0.5 ml/min. The glutathione sepharose was then resuspended in 10 ml of the same buffer containing 400 U thrombin (Haematologic Technologies Inc.). The beads were agitated and then left to incubate with the thrombin-buffer mixture for one hour. The buffer was then drained out and the beads were washed with buffer E at 0.5 ml/min to remove the flexible spacer described earlier. The glutathione sepharose was resuspended in 10 ml buffer E containing 400 U PreScission™ protease (Amersham Pharmacia Biotech) and 2 μM "PPACK" thrombin inhibitor (Calbiochem) before being left on a rotary shaker (Luckham Multimix MM1) overnight. The slurry was then transferred to an Econo-Pac gravity flow column (Bio-Rad) and the eluate, containing the GST-free protein, was collected. The beads were washed with 3

bed volumes buffer E and the washes and eluate were combined. The protein solution was concentrated to 5 ml, using a Vivaspin centrifugal concentration device (Sartorius), before being loaded at 1 ml/min (P1 pump) onto a Superdex 200 gel filtration column (Amersham Pharmacia Biotech) that was connected to the GradiFRAC system. Proteins were eluted using buffer F (20 mM Tris pH 7, 100 mM NaCl, 2 mM DTT) and 3 ml fractions were collected from the column. Fractions containing pure pRb_{AB} were identified by SDS-PAGE and pooled. The protein was concentrated (Vivaspin) to 10-25 μM, analysed by mass spectrometry (**section 2.1.3.3**), and stored at 4°C.

2.4.2

Isothermal Titration Calorimetry (ITC)

2.4.2.1

General Points

(A) Proteins and Peptides

ITC experiments were performed using pRb_{AB} (prepared according to the method in **section 2.4.1**), HPV16 $E7_{(17-98)}$ (supplied by Professor Marmorstein, University of Pennsylvania), E2F-$1_{(409-426)}$, E2F-$1_{(380-437)}$ and E2F-$5_{(323-340)}$ (synthesised by Dr. W. Mawby, University of Bristol), as well as pRb_{ABC} and E2F-$1_{(243-437)}$ (purified by Dr. B. Xiao). A schematic representation of these proteins is shown in **figures 33-35**.

(B) Instrumentation and Software Settings

Binding of the various peptides and proteins to each other (as outlined below) was measured by means of the VP-ITC microcalorimeter (MicroCal Inc.). The instrument was controlled using the VPViewer program (MicroCal Inc.). For the set of titrations designated A and C (see below), the syringe was programmed to deliver 29 or 30 injections (the first being 2 µl and the rest 10 µl each) of peptide/protein solution, with a gap of 200 s between injections. For set B, 29 injections (the first being 2 µl and the rest 10 µl each) of peptide solution were delivered, with a gap of 300 s between injections. Subtraction of dilution heats (see below) and analysis of the calorimetric data were carried out by means of the Origin program (MicroCal Inc.). The instrumentation and software are described in **section 5.1**. The Origin program allows fitting of isotherm curves (see **section 5.1.2**) according to three models, these being "One Set of Sites," "Two Sets of Sites," and "Sequential Binding Sites." All isotherm curves presented in this thesis were fitted according to the single site model (One Set of Sites).

(C) Sample Preparation

Prior to performing the titrations, protein samples ($E7_{(17\text{-}98)}$, $E2F\text{-}1_{(243\text{-}437)}$, pRb_{AB}, pRb_{ABC}) were dialysed against the buffers in which the titration was to be carried out (described below). 3 ml of the appropriate protein were dialysed against 2 l of buffer overnight at room temperature. The anhydrous synthetic peptides ($E2F\text{-}1_{(409\text{-}426)}$, $E2F\text{-}1_{(380\text{-}437)}$ and $E2F\text{-}5_{(323\text{-}340)}$), however, were not dialysed owing to their low molecular weights. Thus for each titration, the buffer in which the

protein had been dialysed was recovered and used to dissolve the peptide. This ensured that the samples in each ITC experiment were in exactly the same buffer. Following dialysis of the protein and preparation of the peptide solution, samples were degassed for 10 mins. at the temperature of the intended titration using the ThermoVac degassing device (MicroCal Inc.).

2.4.2.2

Titrations

Each experiment was carried out more than once. Experiments were performed using the same protein stocks throughout.

(A) Determination of Minimal Fragments of pRb and E2F-1 Required for Binding

Titrations were carried out in 50 mM Tris pH 7.6, 100 mM NaCl and 1 mM TCEP (Pierce). Each of the E2F constructs (E2F-$1_{(409-426)}$, E2F-$1_{(380-437)}$, E2F-$1_{(243-437)}$), at a concentration between 100-150 μM, was titrated into 12-15 μM pRb_{AB} or pRb_{ABC} at a temperature of 22°C.

(B) Investigation of the Specificity of the E2F/pRb Interaction

Titrations were carried out in 50 mM Tris pH 7.3, 100 mM NaCl, 2 mM βME, 1 mM EDTA. Each of the E2F peptides (E2F-$1_{(409-426)}$ and E2F-$5_{(323-340)}$), at a concentration between 220-223 μM, was titrated into 22-25 μM pRb_{AB} at a temperature of 16°C.

(C) Investigation of HPV E7-mediated Inhibition of pRb-E2F Complex Formation

Titrations were carried out in 50 mM Tris pH 7.6, 100 mM NaCl and 1 mM TCEP. To prepare E7/pRb complexes, the $E7_{(17-98)}$ protein fragment and either pRb_{AB} or pRb_{ABC} were mixed in equimolar amounts.

(i): HPV16 $E7_{(17-98)}$, at a concentration between 100-150 μM, was titrated into 10-15 μM of each of the pRb constructs.

(ii): Each of the E2F constructs ($E2F\text{-}1_{(409-426)}$, $E2F\text{-}1_{(380-437)}$, $E2F\text{-}1_{(243-437)}$), at a concentration between 100-150 μM, was titrated into 10-15 μM of a stoichiometric $E7/pRb_{AB}$ or $E7/pRb_{ABC}$ complex. Another ITC experiment was carried out as part of this set of titrations (see **section 5.4.3**). $E2F\text{-}1_{(243-437)}$, at a concentration between 100-150 μM, was titrated into a protein solution containing 30-45 μM $E7_{(17-98)}$ and 10-15 μM pRb_{AB} (3:1 ratio of $E7_{(17-98)}$ to pRb_{AB}).

(D) Determination of Dilution Heats

For each of the above titrations, an identical titration was carried out except that the macromolecule in the sample cell was replaced with buffer. This allowed the "dilution heat" of each injection to be measured. This value was subtracted from the heat associated with the corresponding injection in the protein-ligand titration, using the Origin program. Enthalpic contributions, due to events other than protein-ligand binding, are discussed in **section 5.1.1**.

CHAPTER 3

IDENTIFICATION OF APTAMERS FOR THE E2F-1 HOMODIMER

CHAPTER 3

IDENTIFICATION OF APTAMERS FOR THE E2F-1 HOMODIMER

3.1

OUTLINE OF THE SYSTEMATIC EVOLUTION OF LIGANDS BY EXPONENTIAL ENRICHMENT (SELEX)

This in vitro procedure, based on combinatorial chemistry, is a means to identify nucleic acid ligands that bind a target molecule with high affinity and specificity. Selected ligands (referred to as aptamers) may be single-stranded RNA and DNA or double-stranded DNA. Examples of targets amenable to this procedure include nucleic acid binding proteins, nucleic acid enzymes and small organic molecules such as theophylline (Tuerk, 1997; Kusser, 2000). While SELEX has provided insights into the mechanisms of some cellular processes, its potential contribution in the fields of therapeutics and diagnostics is also being explored (Osborne *et al.*, 1997; Famulok and Jenne, 1998).

3.1.1

General Procedure

Figure 8 illustrates the general procedure followed to isolate double-stranded DNA sequences that interact with a target molecule such as a transcription factor:

(1) A pool of oligonucleotides is synthesised that comprise a degenerate core flanked by defined regions, whose sequences allow amplification by PCR and

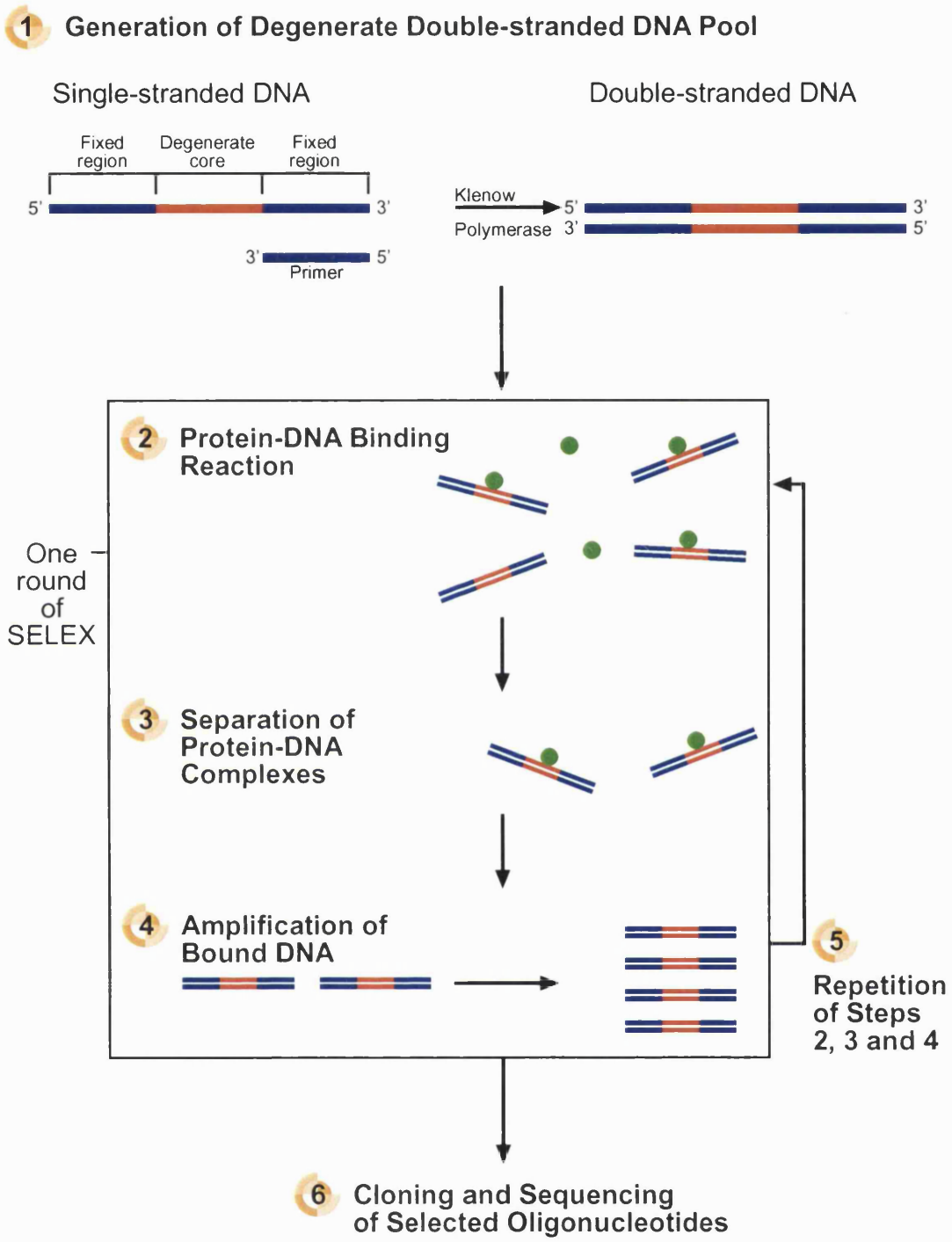


FIGURE 8

GENERAL SELEX PROCEDURE

The SELEX protocol is described in the text. Primers that correspond to the fixed DNA sequences are used for the generation of double-stranded DNA (step 1) and amplification of the oligonucleotides by PCR (step 4). The fixed regions also consist of restriction enzyme sites, allowing the oligonucleotides to be cloned into a plasmid prior to sequencing (step 6).

cloning into a plasmid. A primer is annealed to the 3' end of these single-stranded oligonucleotides and Klenow polymerase is used to catalyse the synthesis of complementary strands, thereby generating a library of degenerate double-stranded DNA sequences.

(2) A binding reaction is set up comprising the target molecule (i.e. the protein of interest) and the degenerate pool. The number of DNA sequences in the reaction is between 10^{14} and 10^{15}, the latter being close to the practical limit of saturation (Gold *et al.*, 1995).

(3) Protein-DNA complexes are separated from unbound DNA e.g. by affinity chromatography or EMSA (see below).

(4) The bound DNA is amplified by PCR and these products are then used in another binding reaction.

(5) The binding, separation and amplification steps are repeated, exponentially increasing the proportion of DNA sequences that specifically bind the target with high affinity, until such oligonucleotides become the prevalent species.

(6) The selected sequences are cloned and sequenced.

3.1.2

Isolation of Protein-Aptamer Complexes

In this study, the target molecule used in the SELEX experiments was recombinant histidine-tagged E2F-1. Thus attempts were made to isolate protein-DNA complexes using a Ni-Agarose spin column (Qiagen). A similar separation method was successfully employed in a SELEX experiment performed to isolate

aptamers of the LexA protein from *Mycobacterium tuberculosis* (Dullaghan, 1999).

Another separation technique employed in the present study is based on the EMSA (Electrophoretic Mobility Shift Assay) (**figure 9**; EMSA technique outlined in **section 4.1**) which has also been successfully employed before in the context of the SELEX procedure (Blackwell and Weintraub, 1990; Xu and Strauch, 1996). In this case, use is made of a control oligonucleotide that has approximately the same molecular weight as the degenerate DNA. However, instead of a random core, the control oligonucleotide contains a sequence to which the protein of interest is known to bind. The control and degenerate oligonucleotides are both radiolabeled and each is mixed with the protein of interest. The reactions are separated by electrophoresis in adjacent lanes of the gel so that the band representing bound DNA in the control lane can be used as a marker to estimate the position of bound DNA in the experimental lane of the gel. A slice of gel containing DNA from the experimental lane is excised. The DNA is extracted, amplified and subjected to another round of SELEX. As the high affinity sequences become more abundant, the bound degenerate DNA becomes increasingly visible on the autoradiograph.

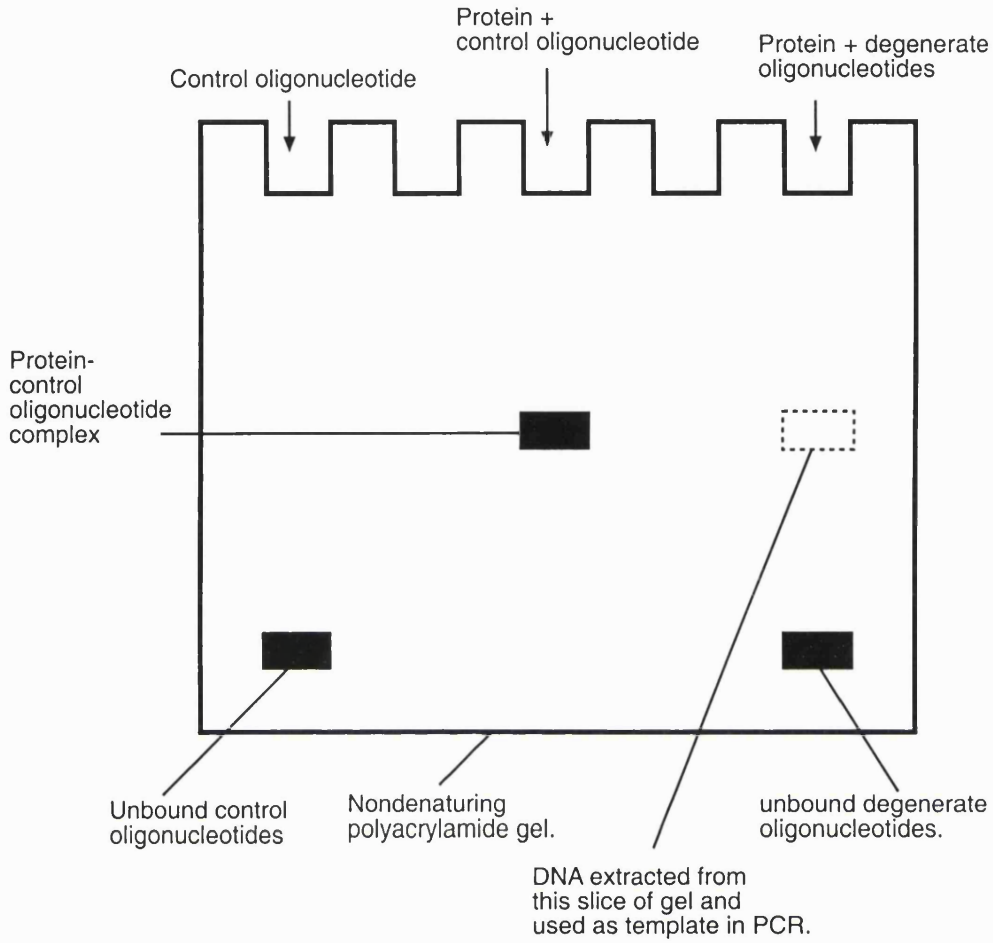


FIGURE 9

SEPARATION OF PROTEIN-APTAMER COMPLEXES USING THE ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

The band corresponding to the protein-control oligonucleotide complex is used as a marker to estimate the position of complexes formed between the protein and DNA from the degenerate oligonucleotide pool (see text).

IDENTIFICATION OF APTAMERS FOR THE E2F-1 HOMODIMER

3.2

INTRODUCTION TO EXPERIMENTS

When these experiments were undertaken, attempts were being made by colleagues to obtain crystals of the E2F-1Δ_{cyc} homodimer (residues 92-195 of E2F-1; **figure 10**) bound to DNA that were suitable for X-ray crystallographic studies. The oligonucleotide sequence used in these attempts was derived from the adenovirus E2A promoter (**figure 11**) and contains an asymmetric E2F binding site. This suggests that a heterodimer might have greater affinity for the site than a homodimer. Indeed, other researchers have shown that E2F heterodimers bind more strongly to sequences based on wild-type promoters than E2F or DP homodimers (Bandara *et al.*, 1993).

The discovery of DNA sequences for which the E2F homodimer had greater affinity was considered to be useful for the intended structural work, since the tighter binding might promote the formation of well-ordered crystals. Furthermore, crystallographic analysis would yield more informative results if the oligonucleotide in the crystal were specific for the homodimer rather than the heterodimer. The SELEX technique was employed to find such oligonucleotides whose sequences would themselves provide some insight into DNA binding by E2F transcription factors.

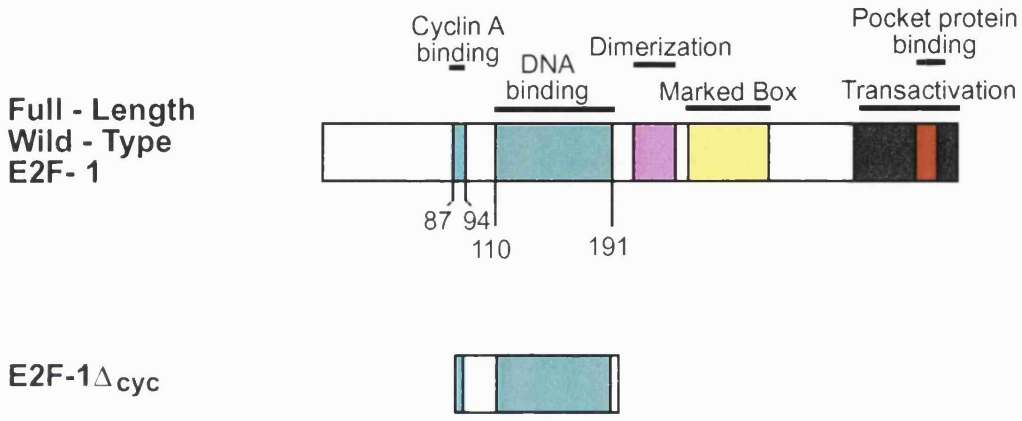


FIGURE 10

E2F-1 PROTEIN FRAGMENT USED IN SELEX EXPERIMENTS (METHODS 1 AND 2)

(Adapted from Black *et al.*,1999)

E2F-1Δ_{cyc}(residues 92-195 of E2F-1) consists of a truncated cyclin A-binding region (Adams et al.,1996) and the DNA-binding domain (Ivey-Hoyle et al.,1993).

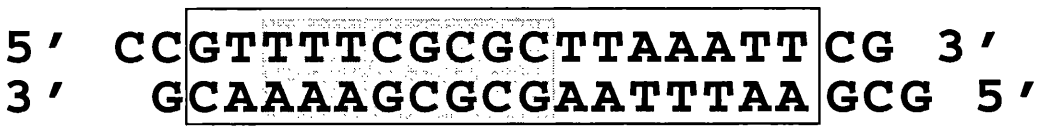


FIGURE 11

OLIGONUCLEOTIDE DERIVED FROM ADENOVIRAL PROMOTER

The boxed DNA sequence is from the adenovirus E2A promoter (-70 to –54) and the shaded sequence is the E2F binding site (La Thangue *et al.*, 1990).

This oligonucleotide was used for co-crystallisation with E2F-1Δ_{cyc} (see text).

(The same oligonucleotide was end-labeled with [γ-^{32}P]ATP for use as a probe in the EMSA experiments described later in **Chapter 4, section 4.3.3**).

IDENTIFICATION OF APTAMERS FOR THE E2F-1 HOMODIMER

3.3

RESULTS

Two approaches (referred to as method 1 and 2) were used to identify aptamers for E2F-1 by means of the SELEX technique. In order to address some problems I encountered concerning method 1 (that will be fully discussed later in this chapter), changes in the protocol were incorporated into method 2. The main difference between the two approaches was the manner in which protein-aptamer complexes were isolated. This is explained in greater detail in **section 3.1.2** and is briefly described below.

3.3.1

Method 1

Histidine-tagged E2F-1Δ_{cyc} (purified by Dr. I. Tews) (**figure 10**) was mixed with a pool of oligonucleotides comprising degenerate cores flanked by restriction enzyme sites and sequences complementary to PCR primers (**figure 12**). The binding reaction mixture was applied to a Ni-agarose spin-column to isolate protein-aptamer complexes. The bound DNA was purified by phenol/chloroform extraction and ethanol precipitation and used as a template in a PCR reaction. Amplified DNA was also purified in the same manner and used for the next binding reaction in place of the degenerate pool.

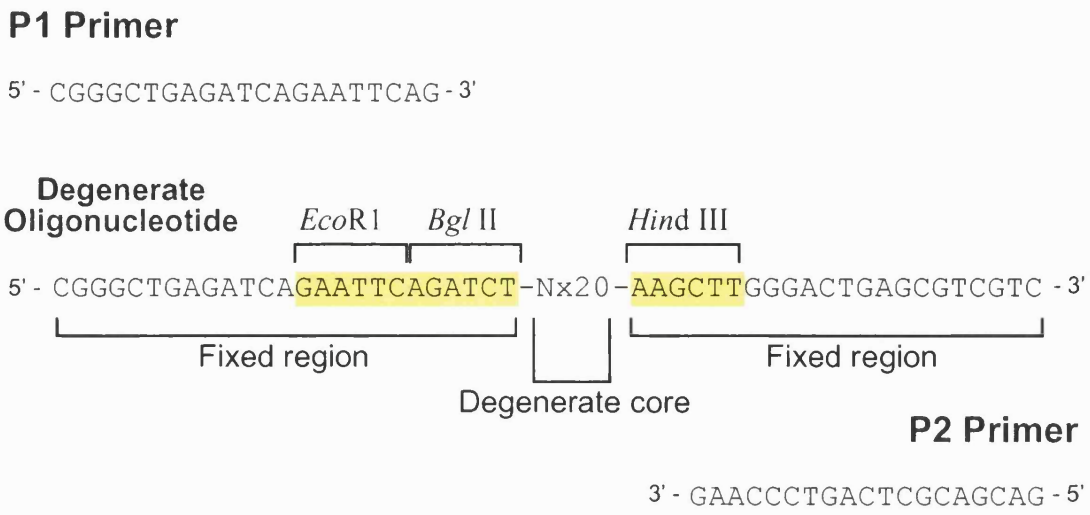


FIGURE 12

POOL OF DEGENERATE OLIGONUCLEOTIDES USED IN SELEX PROCEDURE (METHODS 1 AND 2)

Each of the degenerate oligonucleotides consisted of a degenerate core of 20 bases flanked by fixed regions. The latter incorporated restriction enzyme sites (shown in yellow) for cloning, as well as sequences corresponding to the P1 and P2 primers for amplification by PCR. Klenow polymerase was used to catalyse the synthesis of complementary strands, thereby generating a pool of degenerate DNA duplexes (degOLIGO).

After six rounds of binding and amplification, the interaction of the recovered DNA (selOLIGO) with E2F-1Δ_{cyc} was assessed by means of an EMSA (EMSA technique outlined in **section 4.1**). As shown in **figure 13**, a probe consisting of the original degenerate oligonucleotides (degOLIGO) was not retarded in the assay (gel A, lane 2), while a shift was apparently produced when selOLIGO was used as the probe (gel B, lane 2). This suggested that oligonucleotides that bind to E2F-1Δ_{cyc} had been isolated by the SELEX procedure.

Following the cloning and sequencing of selOLIGO, the DNA sequences were aligned (**figure 14**) using the MegAlign program (DNA Star). Bases constituting the restriction enzyme sites in each oligonucleotide were removed prior to the alignment procedure in order to allow the identification of a consensus (if any) within the degenerate region comprised of 20 bp. A single DNA sequence that is common to the majority of oligonucleotides did not emerge.

16 out of the 53 oligonucleotides shown in **figure 14** were anomalous in that the 3' end of their degenerate cores comprised a DNA sequence corresponding to that of the P2 primer used in the PCR reactions (**figure 12**). This spurious region was also removed prior to alignment to facilitate the determination of sequences selected by the E2F homodimer. These truncated sequences do not consist of 20 bp. The other oligonucleotides comprised 20 bp (after removal of the restriction enzyme sites) except two that consisted of 19 bp and one consisting of 22 bp. These anomalies are discussed later in this chapter.

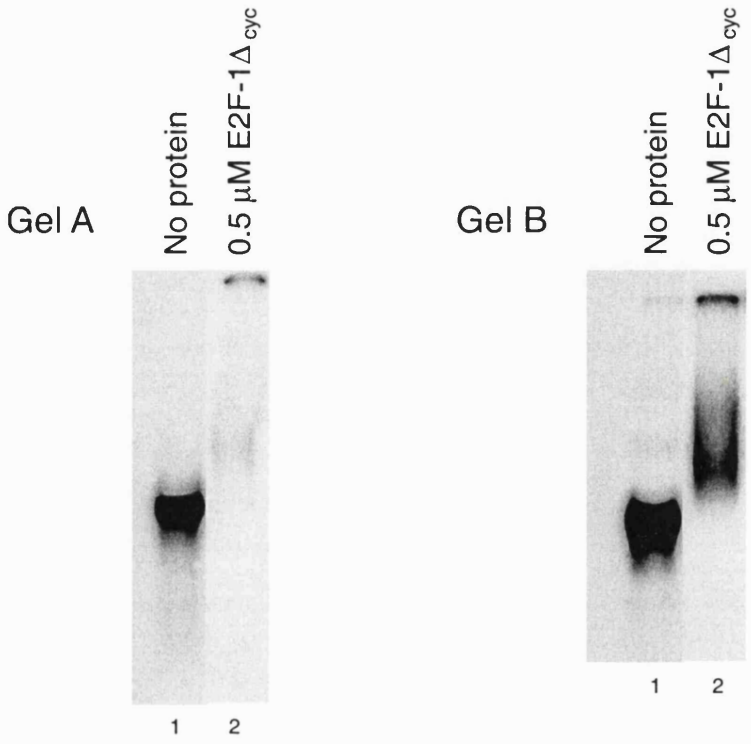


Figure 13

ASSESSMENT OF SELEX (METHOD 1) PROCEDURE BY EMSA

Each reaction mixture contained 50 mM Tris pH 8.2, 6 mM $MgCl_2$, 3 mM DTT and 15 % glycerol. The concentrations of the other components in the mixtures are listed below. The concentration of E2F-1Δ_{cyc} is expressed as the molarity of dimeric protein. Each reaction mixture was loaded onto a non-denaturing 8 % polyacrylamide gel (see methods).

Gel A: reaction mixtures contained approximately 10 nM degenerate oligonucleotide (degOLIGO) probe and either no protein (lane 1) or 0.5 μM E2F-1Δ_{cyc}, (lane 2).

Gel B: reaction mixtures contained approximately 10 nM oligonucleotide recovered after the sixth round of SELEX (selOLIGO) as probe and either no protein (lane 1) or 0.5 μM E2F-1Δ_{cyc} (lane 2).

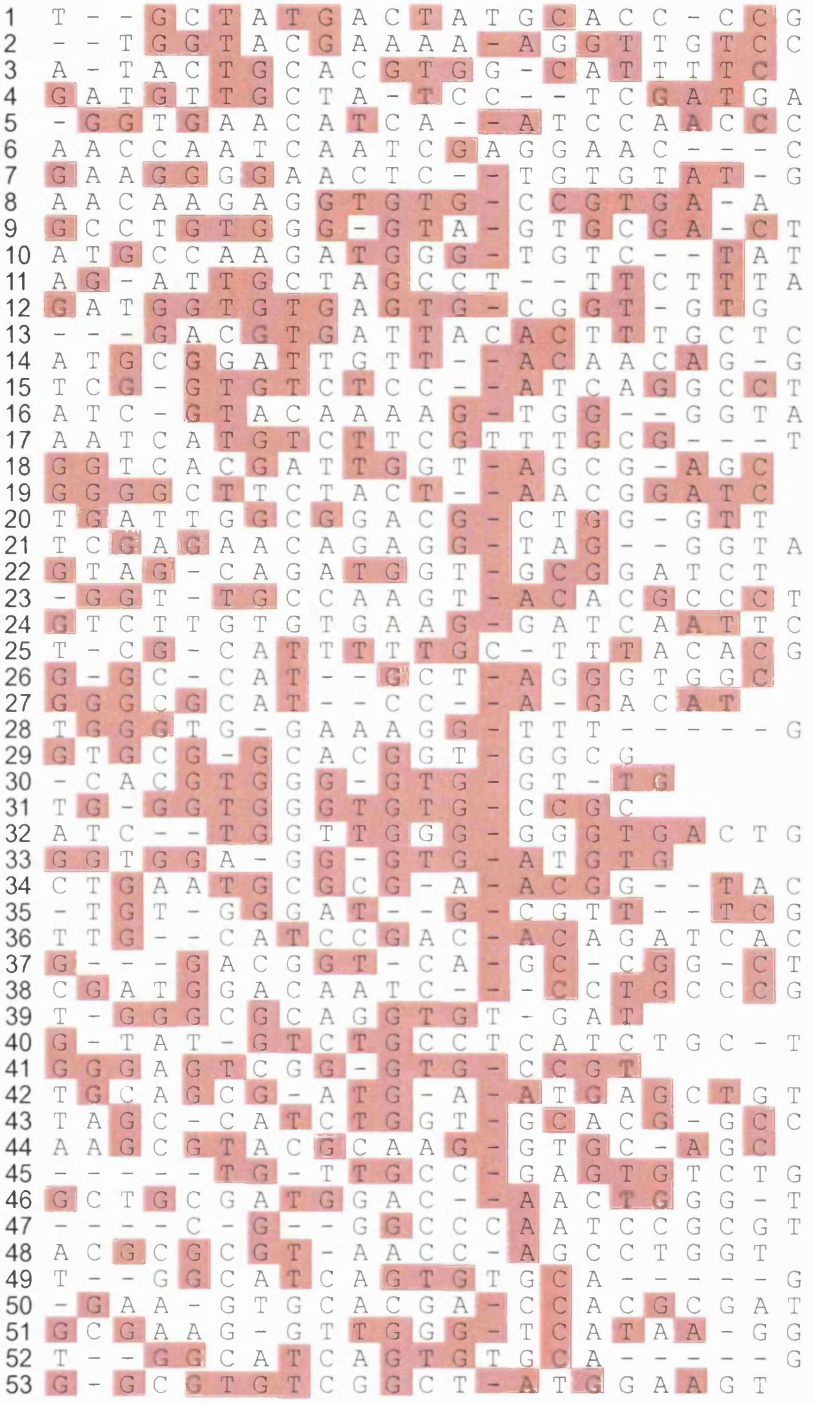


FIGURE 14

ALIGNMENT OF DNA SEQUENCES OBTAINED FROM SELEX (METHOD 1) PROCEDURE

pUC19 plasmids harbouring the oligonucleotides recovered after the sixth round of SELEX were subjected to DNA sequencing. The sequences were aligned by means of the MegAlign program (DNA Star) using the Clustal Method. The program highlights the most frequently occurring sequences in red.

3.3.2

Method 2

This method was essentially the same as that described above except that the protein-aptamer complexes were isolated using a variation of the EMSA technique (previously described in **section 3.1.2**). Briefly, the binding reaction mixture was subjected to electrophoresis along with another mixture containing the homodimer and a control oligonucleotide (conOLIGO) (**figure 15**) to which the protein is known to bind. Using the position of the retarded conOLIGO as a marker, a slice of gel thought to contain complexes of protein and DNA from the degenerate pool was excised (**figure 16**). DNA was extracted from the gel slice and used as a template for PCR.

Amplification of this recovered DNA (recOLIGO) proved to be a persistent problem despite alterations in the conditions (thermal cycling conditions, template concentration) of several PCRs. When analysed by polyacrylamide gel electrophoresis, PCR products typically had smears that were of a higher molecular weight than the desired product (**figure 17A**, lane 2). Other reactions produced very little or no product and reactions were not reproducible. The reasons behind these problems are discussed later in this section.

5′ CGGGCTGAGATCAGAATATATAATACGTTTTCGCGCTTAAATTCGTTACATAAGACTGAGCGTCGTC 3′

FIGURE 15

CONTROL OLIGONUCLEOTIDE (conOLIGO) USED IN SELEX (METHOD 2)

The core of the oligonucleotide (boxed) consists of a sequence from the adenovirus E2A promoter (-70 to –54). This contains an E2F DNA-binding site (shaded) (La Thangue *et al.*, 1990). ConOLIGO has approximately the same molecular weight as the degenerate oligonucleotides (degOLIGO) used in the SELEX procedure. Only one strand of conOLIGO is shown.

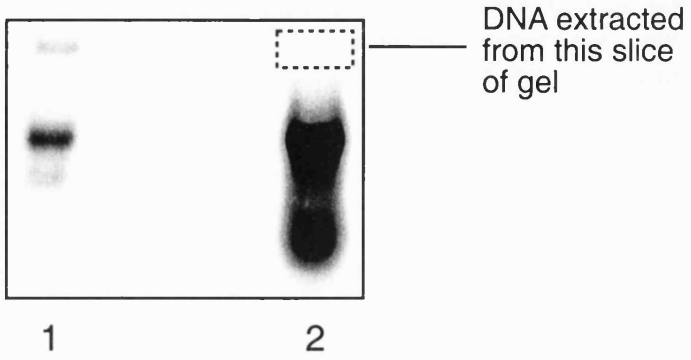


Figure 16

SELEX (METHOD 2): ISOLATION OF E2F-DNA COMPLEXES

The electrophoretic mobility shift assay was used to isolate complexes of DNA and E2F-1Δ_{cyc}

Each reaction mixture contained 50 mM Tris 8.2, 6 mM $MgC1_2$,3 mM DTT, 15% glycerol, 24 pmol E2F-1Δ_{cyc} and either 100 pmol of the control oligonucleotide (conOLIGO) probe (lane 1) or 146 pmol of the degenerate oligonucleotide (degOLIGO) probe (lane 2).

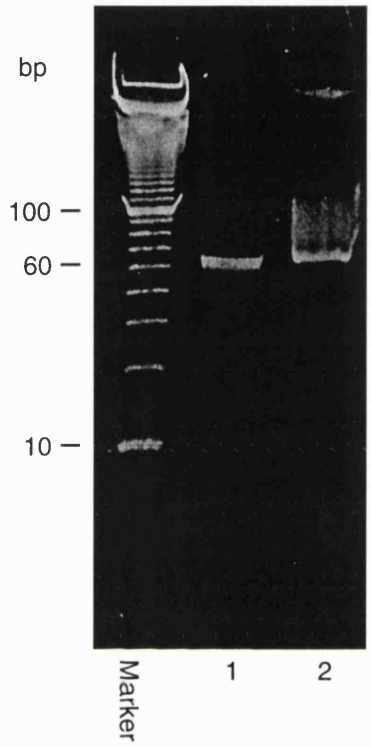


Figure 17A

NON-DENATURING P.A.G.E. ANALYSIS: Amplification of Recovered DNA (Selex Method 2)

Samples were loaded onto a 12% non-denaturing polyacrylamide gel. The gel was stained with ethidium bromide and DNA visualised under UV light (see methods).

Each of the degenerate oligonucleotides in the initial binding reaction with E2F-1Δ_{cyc} consisted of 67 bp (lane 1). The PCR product typically obtained after attempts to amplify the bound DNA (recovered from the EMSA gel) is shown in lane 2.

3.4

DISCUSSION

Specific problems that culminated in the failure to isolate high-affinity aptamers bound by the E2F-1Δ_{cyc} homodimer are discussed here in addition to some general improvements to the SELEX protocols.

3.4.1

SELEX Method 1

The amplified DNA obtained from each PCR reaction during the SELEX procedure was analysed on a 4 % agarose gel and appeared as a discrete band. However, such a gel would not sufficiently resolve an oligonucleotide comprising 67 bp and a non-denaturing polyacrylamide gel should have been used. Indeed, when the amplified DNA used for cloning was later analysed using the latter type of gel, spurious bands and a smear were seen, indicating that PCR optimisation was actually required. The poor quality of the DNA probably gave rise to the anomalous sequences obtained (as described in **section 3.3.1**), and may have caused the retardation of radiolabeled DNA in the bandshift (**figure 13**). Nevertheless, DNA sequencing revealed that inserts had been cloned that were of the correct size (20 bp). Thus some "normal" PCR products had been produced. However, aberrant PCR would have reduced the number of sequences available to

the protein for binding and may have prevented the faithful amplification of some, or all of, the oligonucleotides bound by the protein.

The use of Ni-Agarose spin columns to isolate E2F-DNA complexes may also have contributed to the failure of the SELEX experiment. A control reaction comprising the oligonucleotide pool without any added protein, was passed through a spin column in the same manner as a binding reaction. The solution recovered from the elution step was found to yield the same PCR product (in terms of size, amount and quality), as a binding reaction containing protein.

During a SELEX experiment, oligonucleotides that bind to the immobilisation matrix as well as those that bind to the protein undergo amplification. If these matrix-binding sequences outnumber the protein-binding ones, then isolation of the former is favoured. This may also occur if the chosen experimental conditions lead to preferential matrix-binding compared to protein-binding. In either case, the selection of protein-specific aptamers is compromised (Conrad *et al.*, 1996). Conrad and colleagues also state that in their experience, the failure of a SELEX experiment is often attributable to the accumulation of matrix-binding oligonucleotides.

According to other researchers, this problem can be avoided by applying the oligonucleotide pool to the matrix before mixing it with protein. Matrix-binding sequences are thus pre-adsorbed onto the matrix and the recovered DNA is used for the binding reaction. This step can be repeated before each round of selection (Conrad *et al.*, 1996; Tuerk, 1997; Kusser, 2000). Other isolation techniques

involving immunoaffinity and glutathione-sepharose matrices have been successfully employed to determine the DNA binding sites of E2F heterodimers from cell or nuclear extracts (Chittenden *et al.*, 1991; Ouellette *et al.*, 1992; Tao *et al.*, 1997). Xu and Strauch (Xu and Strauch, 1996) report that their attempt to isolate aptamers of the AbrB protein of *Bacillus subtilis* using nitrocellulose filters failed. They subsequently successfully employed an EMSA-based isolation method that we also tried after our first SELEX attempt using a spin column was unsuccessful.

3.4.2

SELEX Method 2

Non-denaturing polyacrylamide gels were used for analysing DNA in the second SELEX attempt (method 2). Amplification of DNA recovered after the first binding reaction (recOLIGO) proved to be a persistent problem despite alterations in the conditions (thermal cycling conditions, template and $MgCl_2$ concentrations). PCR products typically consisted of smears of higher molecular weight than the desired product when analysed using a non-denaturing polyacrylamide gel. This caused the second SELEX attempt to be abandoned. SELEX-related PCR problems have been experienced by other researchers (Crameri and Stemmer, 1993) and some remedial measures have been suggested as outlined below.

In SELEX, the presence of a large number of random sequences increases the potential for spurious priming events (Crameri and Stemmer, 1993; Conrad *et al.*,

1996). In method 2, since only one round of SELEX had been performed, several different DNA sequences may have been recovered from the excised gel. The results of SELEX-related PCRs performed by Crameri and Stemmer (Crameri and Stemmer, 1993), suggest that the complementary strands of a template/PCR product that comprise slightly GC-rich 3' ends (like degOLIGO), may act as primers themselves by hybridising to the degenerate region of other sequences. This is one possible explanation for the evolution of a smear of products of higher molecular weight than the template (**figure 17A**).

The solution to this problem appears partly to lie in re-designing the template so the complementary strands comprise AT-rich 3' ends (and by making the corresponding modifications in the primers). Since shorter products and primer dimers can form as a result of using primers with GC-rich 3' ends, it would be beneficial to ensure that these ends are also AT-rich. Indeed, it is recommended with regard to PCRs in general that primers should not contain three G/C nuleotides in a row at their 3' ends (Beckler *et al.*, 1996).

Crameri and Stemmer found that although re-designing their primers improved their PCR results, larger products still accumulated as the SELEX rounds continued and standard PCR optimisation attempts failed to eradicate them. Supplementing the PCR reaction with *E. coli* single strand binding protein (SSB), known to destabilise mispairing, allowed them to obtain the desired products after each round of nine selections (Crameri and Stemmer, 1993).

Other researchers have performed the SELEX procedure using gel-purified PCR products (Chittenden *et al.*, 1991; Tao *et al.*, 1997) but this is not practical if the yield of the desired product is low and the reactions are not reproducible, as was the case in my experiments. The quality and amount of gel-purified DNA may also be poor and variable (Crameri and Stemmer, 1993).

3.4.3

General Points Concerning SELEX

There are other general considerations for improvement of the SELEX protocol. Since the E2F-1Δ_{cyc} homodimer is able to interact with the consensus DNA binding site of the heterodimer, it would have been possible to use partially degenerate versions of the known sequence as templates (Conrad *et al.*, 1996). This may have improved chances of success as the level of degeneracy is reduced. Blackwell and Weintraub use this type of approach in their SELEX experiment to determine the different binding sequence preferences of homo- and heterodimers of the eukaryotic MyoD and E2A DNA-binding proteins (the E2A proteins referred to here are distinct from the adenoviral promoter also known as E2A)(Blackwell and Weintraub, 1990).

Other parameters that are of importance in SELEX include the pool:target ratio. In method 1, I used a 10:1 ratio for each of the six rounds of SELEX while for the single binding reaction in method 2, a 6:1 ratio was used. It has been suggested by others that a low ratio (between 1:1 and 10:1) should be used during the early rounds of selection to promote sequestration of aptamers. However, the

pool:target ratio should be increased in subsequent rounds to encourage competition between the oligonucleotides (Conrad *et al.*, 1996; Fitzwater and Polisky, 1996). Competitors, such as poly(dI-dC) (Blackwell and Weintraub, 1990; Chittenden *et al.*, 1991) or sonicated salmon sperm DNA (Ouellette *et al.*, 1992) can also be added to the binding reactions to promote the selection of aptamers (Conrad *et al.*, 1996). Other conditions that may be altered include the amount of protein used in the binding reactions (since this partly affects the number of DNA sequences that are bound), equilibration times for these reactions, as well as the constituents of the binding buffer and their concentrations (Conrad *et al.*, 1996; Fitzwater and Polisky, 1996). For example, non-specific binding of DNA to a target can be suppressed by increasing the concentration of monovalent cations in the binding buffer (Conrad *et al.*, 1996).

3.4.4

Crystal Structure of the E2F/DP-DNA Complex

Pavletich and colleagues have solved the crystal structure of a complex comprising an E2F-4/DP-2 heterodimer bound to an E2F site derived from the adenovirus E2 promoter (Zheng *et al.*, 1999). The minimal DNA binding domains of the dimer's constituent proteins were used in this study and each was shown to adopt a winged-helix fold (**figure 17B**) that is also found in other eukaryotic transcription factors such as HNF-3γ (Clark *et al.*, 1993). This fold incorporates three α helices and a β sheet, each of which participates in the formation of a compact hydrophobic core. However, neither E2F-4 nor DP-2 possesses the carboxy-terminal wing extension that is found in the winged-helix domain of

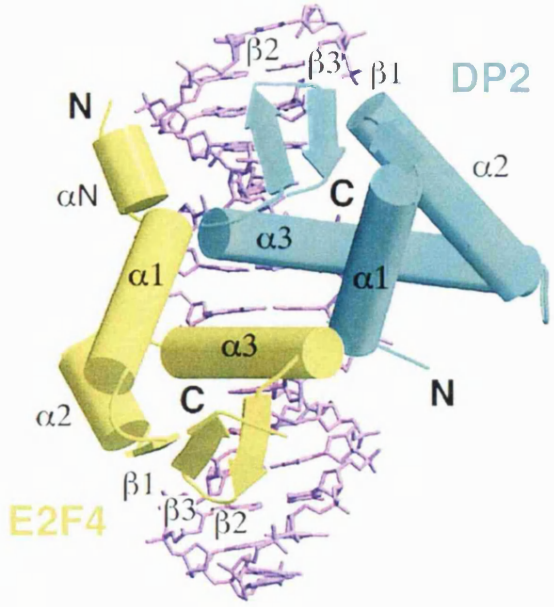


FIGURE 17B

CRYSTAL STRUCTURE OF THE E2F-4/DP-2 HETERODIMER BOUND TO DNA

(Zheng et al., 1999).

Above is a view of the complex as it appears when looking down the axis of approximate two-fold symmetry in the heterodimer (see text). The minimal DNA binding domain of each protein monomer adopts a winged-helix fold (ribbons representation).

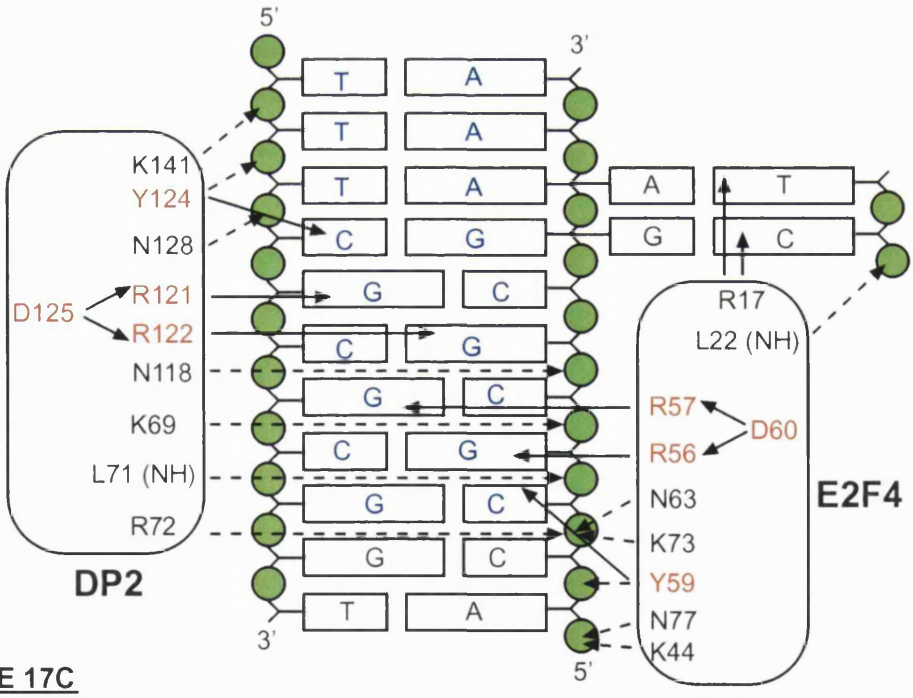


FIGURE 17C

SCHEMATIC SHOWING INTERACTIONS BETWEEN E2F-4/DP-2 AND DNA

(adapted from Zheng et al., 1999).

The single letter amino acid code is used for residues of E2F-4 and DP-2. Residues labelled in red are found within the RRXYD motif (see text). Bases of the E2F DNA binding site (in adenovirus E2 promoter) are labelled in blue while green circles connected by black lines represent the DNA's sugar-phospate backbone.

HNF-3γ (Clark *et al.*, 1993). Furthermore, there are some differences between the structures of E2F-4 and DP-2, although their individual conformations are largely the same. E2F-4 has an amino-terminal helical extension (αN) (**figure 17B**) that is conserved in the E2F family but not in the DP family. This feature is important in DNA sequence recognition, as we shall see later. In addition, each of the α2 and α3 helices of DP-2 are longer than the corresponding motifs in E2F-4 (**figure 17B**) by approximately two turns.

Protein-DNA Interactions

The phosphodiester backbone of the DNA is contacted by the amino terminus of each protein monomer's α1 helix and by segments of its β sheet (**figure 17B**). The α3 helix of each protein partner binds in the major groove of the DNA and participates in critical interactions with the edges of the bases (**figures 17B and 17C**). The contacts made by residues comprising the α3 and αN helices with the DNA are discussed below.

Recognition of the T-rich segment of the DNA-binding site (TTTCGCGCG) is related to the αN helical extension that is exclusive to the E2F partner (**figure 17B**). αN incorporates an invariant arginine residue (R17 in E2F-4) whose deletion abrogates DNA binding (Jordan *et al.*, 1994). The side chain of R17-E2F penetrates deep inside the DNA's minor groove, close to its T-rich segment. This side chain makes contacts with the O2 group of a thymidine base (TTTCGCGCG), as well as with the O2 and sugar groups of the neighbouring cytosine (TTTCGCGCG) (**figure 17C**).

E2F-4 and DP-2 interact in a fundamentally symmetric manner with the palindromic portion of the DNA binding site (i.e. CGCGCG), both proteins making very similar contacts to the bases. Within this portion of DNA, all of the protein-base and most of the protein-phosphate contacts are made by residues conserved in the E2F and DP families. The individual α3 helices of both proteins incorporate a conserved RRXYD motif through which E2F-4 and DP-2 each contact half of the palindromic DNA segment (i.e. CGCGCG and CGCGCG) (**figure 17C**). Each of the arginine residues within the motif (R56-E2F, R57-E2F and R121-DP, R122-DP) makes two hydrogen bonds with a guanine. The individual guanine bases contacted in this manner are located in adjacent base pairs and are on opposite strands (CGC) (**figure 17C**). From the crystal structure, it is conceivable that the arginine residue (**R**RXYD) contacting the central base of the half-site (CGC), could interact with a guanine located on either strand of the DNA at this position. This reflects reports showing that some E2F DNA binding sites incorporate a cytosine base at this point in the sequence (i.e. c/gCC instead of c/gGC) (Slansky and Farnham, 1996).

In the crystal structure, the contacts made by the motif's arginine residues to the guanine bases appear to be stabilised by interactions between amino acids within the motif. The aspartic acid residue (RRXY**D**, i.e. D60-E2F and D125-DP) makes a hydrogen bond with each of the motif's arginine residues. In DP-2, a residue outside the motif also contributes to the stabilisation of these arginine residues. The side-chain of N118-DP forms a bridge between the DNA's phosphodiester backbone and the guanidinium group of R122-DP (**R**RXYD) (**figure 17C**).

The tyrosine residue in the motif (RRXYD, i.e. Y59-E2F and Y124-DP) interacts with one of the cytosine bases in the half-site (CGC) (**figure 17C**). The residue makes several van der Waals contacts with the C5 and C6 atoms of the base, by virtue of its phenyl group. These close contacts reflect the preference for a cytosine or guanine base at this position in the consensus sequence (c/gGC). The tyrosine side chain's hydroxyl group makes contacts with phosphate and sugar groups of the DNA.

Protein-protein Interactions

An extensive interface exists between E2F-4 and DP-2 that is mainly hydrophobic, involving the α1 and α3 helices of each protein (**figure 17D**). α3-E2F packs between α1-DP and α3-DP, while α3-DP packs with the interface's two E2F helices in a reciprocal manner. The arrangement of the helices of both proteins thus confers an approximate two-fold symmetry upon the interface. This underlies observations that the protein partners can also homodimerise (**section 1.4.1**). Since residues in this region are highly conserved within the individual protein families, one would expect other combinations of E2F and DP family members to incorporate similar interfaces.

The imprecise symmetry at the interface is attributable to variations in the reciprocal intermolecular associations between the E2F and DP helices. In this context, differences in contact density are of particular significance. α3-E2F and α1-DP associate through 70 van der Waals contacts. In contrast, only 20 corresponding van der Waals interactions exist between α3-DP and α1-E2F.

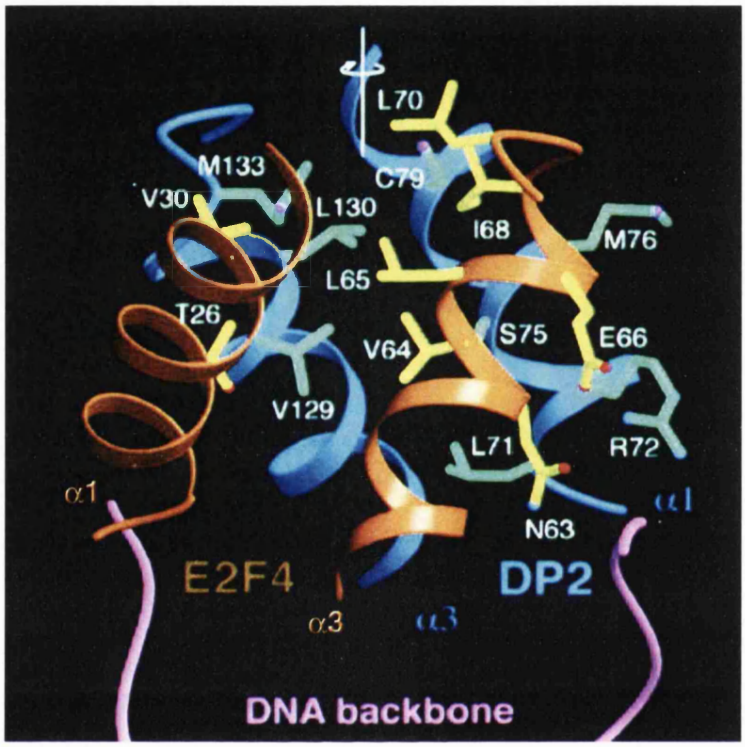


FIGURE 17D

RIBBONS REPRESENTATION OF THE INTERFACE BETWEEN E2F-4 AND DP-2
(Zheng et al., 1999).

The interface is formed by the α1 and α3 helices of both proteins. In the interests of clarity, only those residues are shown that participate in making multiple van der Waals contacts.

Among the residues at the densely-packed half of the interface, for example, are V64-E2F (α3) and L71-DP (α1) (**figure 17D**). Their side chains contact each other, as well as additional interface residues, through a total of 21 intermolecular interactions. However, the number of contacts made by the corresponding residues in α3-DP and α1-E2F, in the sparsely-packed half of the interface, is significantly lower. V129-DP (α3) is involved in six intermolecular interactions and L22-E2F (α1) in only one.

The asymmetry discussed above arises from the distinct inter-helical distances within each protein. The distance between α1-DP and α3-DP is about 3 Å shorter than that between the equivalent E2F helices. This is due to the relative size of the hydrophobic residues in the core of each protein. Small hydrophobic residues, for example V78-DP and A126-DP, interact with each other at the α1-α3 interface of DP-2. The analogous helices in E2F-4, however, pack against each other (in general) through larger residues. For example, F29-E2F and I61-E2F correspond to V78-DP and A126-DP respectively. Residues at the α1-α3 interface within the individual proteins are conserved in their respective families. The structures of other E2F and DP family members therefore, will probably differ in a manner comparable to that discussed here. One can also conclude, from the difference in inter-helical distances described above, that the protein-protein interfaces within the homodimers would be distinct from that within a heterodimer.

A comparison of the interface residues belonging to the monomers reveals that approximately two thirds of these amino acids differ between E2F-4 and DP-2. These differences also confer asymmetry upon the interactions at the interface. At

the densely-packed half of the interface for example, a salt bridge is formed between E66-E2F and R72-DP (**figure 17D**), both of which are conserved in their respective families. In contrast, the corresponding residues (G72-E2F and M131-DP) in the sparsely-packed interface portion do not interact. Furthermore, the salt bridge between arginine and glutamic acid described earlier, could not form between monomers of the same protein family. The asymmetric features of the interface outlined above, contribute to the preference exhibited by E2F and DP to bind DNA as heterodimers.

Implications for Aptamers of the E2F-1Δ_{cyc} Homodimer

The structure described above shows that all of the residues of E2F-4 and DP-2 that interact with the DNA bases, as well as most of those that associate with the phosphodiester backbone, are conserved within the individual protein families. Combinations of winged helix domains from other E2F and DP family members therefore, would be expected to interact with the DNA binding site in a very similar manner. The case of the E2F-1Δ_{cyc} homodimer, however, would be somewhat different. Although the SELEX experiments described in this chapter did not generate aptamers for E2F-1Δ_{cyc}, I will outline here how the crystal structure might relate to DNA sequence selection by this recombinant protein.

The winged-helix domain of E2F-1 (equivalent to the domain of E2F-4 in the crystal structure) encompasses residues 112-195 (Zheng *et al.*, 1999). However, E2F-1Δ_{cyc} contains an additional region outside this domain, since it incorporates residues 95-195. It is possible that the additional residues partly modulate

sequence specificity and that as a result, E2F-1Δ_{cyc} would interact preferentially with a slightly different sequence compared to that recognised by the E2F-4 fragment in the crystal structure. This point is even more relevant in the context of full-length E2F and DP heterodimers. The different preferences of such heterodimers for specific sequences (**section 1.4.4.3**) may also be attributable to residues outside the winged-helix domains. It is worth mentioning at this point that in the cellular environment, other proteins bound to the heterodimers may also play a role in modulating sequence specificity by interacting with bases outside the consensus site.

The crystallographic data shows that while each winged-helix domain contacts half of the palindromic DNA sequence (CGCGCG), the asymmetry in the extended binding-site (TTTCGCGCG) is exclusively associated with the αN helical extension of E2F-4, that is conserved in the E2F family. It is conceivable that the E2F-1Δ_{cyc} homodimer, that also incorporates the αN component, would preferentially select an entirely palindromic site, perhaps with a central core comprising C/G bases, flanked by a T-rich segment at the 5' end and a corresponding A-rich segment at the 3' end.

As explained above, the protein-protein interface within a homodimer would differ from that within a heterodimer, owing to the distinct interhelical arrangement associated with the winged helix-domain of each protein partner. This might generate subtle differences between the DNA sequences recognised by E2F homodimers, DP homodimers and heterodimers.

3.4.5

SELEX and E2F – The Next Step

Although elucidation of an E2F heterodimer's crystal structure rendered that of the homodimer somewhat redundant, and SELEX experiments have been successfully performed with E2F heterodimers, there may yet be a future role for SELEX. This pertains to the specificity of individual E2F heterodimers for distinct genes. I have already discussed the current data regarding such specificity (**sections 1.4.3.3** and **1.4.4.3**). However, there are caveats associated with the experimental approaches employed to obtain these results, examples of which follow.

The experiments conducted by DeGregori and colleagues (DeGregori *et al.*, 1997) involved the infection of quiescent REF52 cells with adenoviral vectors expressing each of the E2F proteins. Since the latter are not at physiological levels, they may bind to promoters owing to their increased concentration. The abnormally high concentration may also abolish significant protein-protein interactions. Furthermore, the overexpression of a certain E2F protein during quiescence does not necessarily reflect the normal timing of its expression in the cell cycle (Farnham, 1996; Slansky and Farnham, 1996; Wells *et al.*, 2000).

The SELEX experiments performed by Tao and colleagues demonstrate that different E2F heterodimers have distinct preferences for the precise sequences they bind (Tao *et al.*, 1997). However, since the evolved sequences are not those

of natural promoters, a link between a specific heterodimer and a certain promoter cannot be established.

In the context of E2F specificity, a recent study may represent an improvement in experimental design (Wells *et al.*, 2000). Wells and co-workers treated NIH 3T3 cells with formaldehyde in order to cross-link transcription complexes to the promoter DNA. This was followed by immunoprecipitation with antibodies against specific E2F proteins or members of the pRb family. The co-precipitating DNA was analysed by promoter-specific PCR primers allowing specific proteins and DNA sequences to be matched according to their interactions *in vivo*. However, it appears that the use of different antibodies that are specific for the same protein may generate conflicting results (perhaps due to the variation in epitope accessibility) (Wells *et al.*, 2000).

A modified form of SELEX, known as Genomic SELEX, may also prove to be informative in these specificity studies. In this case, a genomic DNA library replaces the degenerate pool of oligonucleotides. This variation of the SELEX procedure thus allows the identification of sequences bound with highest affinity *in vivo* by a protein (Gold *et al.*, 1995). However, since the binding reaction conditions that are chosen could affect the affinities of the individual E2F proteins for specific promoters, the results obtained from such an approach would only serve to complement studies carried out using other methods.

CHAPTER 4

REGULATION OF E2F/DP DNA-BINDING BY CYCLIN-CDK DEPENDENT PHOSPHORYLATION

CHAPTER 4

REGULATION OF E2F/DP DNA-BINDING BY CYCLIN-CDK DEPENDENT PHOSPHORYLATION

4.1

OUTLINE OF THE ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

EMSA has been used extensively in this thesis to investigate protein-DNA interactions. This technique can yield a considerable range of information including binding affinity and stoichiometry and these aspects of EMSA have been discussed in detail elsewhere (Dent and Latchman, 1994; Taylor *et al.*, 1994).

4.1.1

Principle of the EMSA

Nucleic acid bound to protein migrates more slowly through a nondenaturing polyacrylamide gel than that which is unbound. This change in electrophoretic mobility underpins the EMSA technique, also known as the gel-shift or gel retardation assay (Fried and Crothers, 1981; Garner and Revzin, 1981). The DNA is radioactively labeled, allowing the bands corresponding to free DNA and the retarded or shifted ones of complexed DNA, to be visualised by autoradiography or phosphorimaging (see **figure 18**).

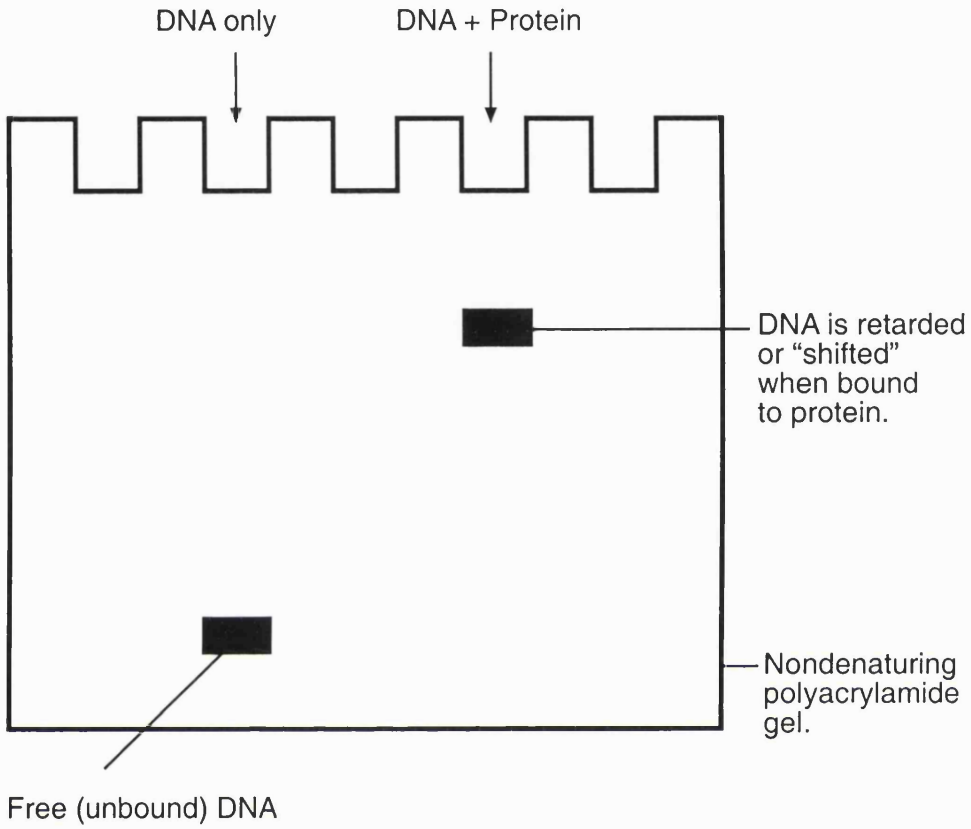


FIGURE 18

PRINCIPLE OF THE ELECTROPHORETIC MOBILITY SHIFT ASSAY

Radioactively - labeled DNA ("probe") and a mixture of the DNA and protein of interest are subjected to electrophoresis. The positions of the bands are visualised by autoradiography or phosphorimaging (see text).

The time required to complete an EMSA experiment greatly exceeds the half-life of typical protein-DNA complexes and yet they still form discrete bands. This observation is attributable to caging (Fried and Crothers, 1981) caused by the polyacrylamide gel. After dissociation of a complex, the rate of dispersal of its components is reduced due to the gel matrix, thus favouring reassociation (Carey, 1991; Cann, 1998).

REGULATION OF E2F/DP DNA-BINDING BY CYCLIN-CDK DEPENDENT PHOSPHORYLATION

4.2

INTRODUCTION TO EXPERIMENTS

Cyclin-CDK phosphorylation of cell cycle proteins probably represents the primary mechanism for regulating the life cycle of a eukaryotic cell. As I have outlined in **Chapter 1 (section 1.2)**, cyclins (and their associated kinases) are the time-keepers of the cell cycle. Different cyclin-CDK complexes become active during various points in the cell cycle and are then responsible for altering the properties of other cell-cycle proteins by phosphorylation.

The E2F/DP transcription factor is a particularly important target, both directly and indirectly, of cyclin-cdk activity. One of the primary events responsible for the G1 to S transition is the release of transcriptionally-active E2F/DP following phosphorylation of pRb. These particular modifications occur mainly through the actions of cyclins D & E. Later in the cell cycle, during S-phase, cyclin A-CDK2-mediated phosphorylation of E2F/DP inhibits its DNA-binding activity and prevents the transcription of E2F/DP responsive genes.

A number of experiments suggest that it is the phosphorylation of the DP component of the heterodimer that is of particular importance for down-regulation of the transcription factor. For example, Krek and colleagues (Krek *et al.*, 1994) have demonstrated that the phosphorylation of DP-1 is S-phase specific and only

occurs in the presence of an E2F-1 partner that is capable of binding cyclin A. Furthermore, it has been demonstrated that the expression of DP-1 protein, whose potential CDK phosphorylation sites have been mutated, causes NIH 3T3 cells to accumulate in S phase. These sites are serine and threonine residues in SP (serine-proline) and TP (threonine-proline) motifs that are found in DP-1's N-terminal region (Krek *et al.*, 1995). However, these experiments did not determine the contribution made by individual DP phosphorylation sites to inhibition of DNA-binding.

It is well established that efficient substrate phosphorylation by CyclinA-CDK2 often involves both an appropriate substrate sequence and the presence of a cyclin recognition motif. This motif is characterised by the sequence RXL, which may either be supplied *in cis* or, *in trans*, to the phosphoacceptor site (Brown *et al.*, 1999). In the case of E2F/DP, as described above, it is phosphorylation of the DP which is thought to mediate the effect of CyclinA-CDK2-dependent phosphorylation, but the E2F which supplies the recognition site. The relevant site on E2F-1 occurs between residues 87-94 (PVKRRLDL) (Adams *et al.*, 1996). The X-ray structure of CyclinA-CDK2/E2F-$1_{(87\text{-}94)}$ was solved in the laboratory (S. Gamblin *personal communication*) as part of a program to understand the cell cycle regulation of E2F/DP. Although I was not involved in the structure determination, I include here a short description of this complex because it provides a structural framework for the biochemical experiments I have carried out.

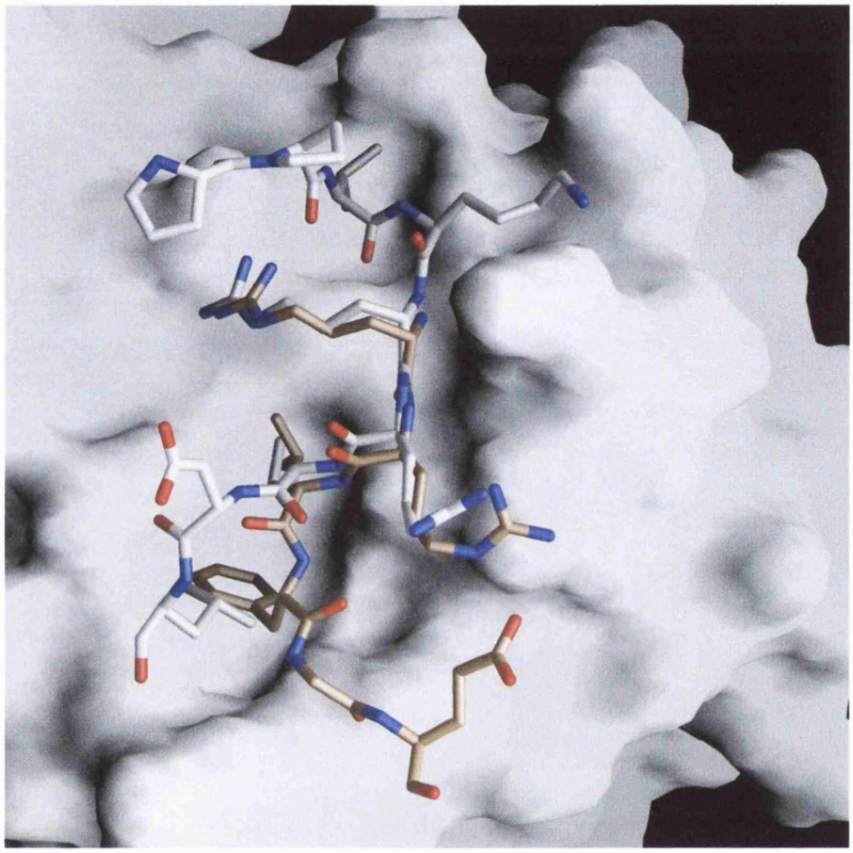


FIGURE 19

SURFACE REPRESENTATION OF RECRUITMENT PEPTIDE-BINDING SITE OF CYCLIN A

(S. Gamblin, *personal communication*; Brown *et al.*, 1999)

Part of the surface of Cyclin A is shown as a solid grey surface. The E2F-1 recruitment peptide is shown as white ball-and-stick and that of p107 as brown ball-and-stick.

The CyclinA-CDK2/E2F$_{(87\text{-}94)}$ structure (see **figure 19)** reveals that the recruitment peptide of E2F binds to a hydrophobic site on the surface of the cyclin. This result is not surprising given that the structures of related recruitment peptides from p27^{KIP1} and p107 have also been shown to bind to this site (Russo *et al.*, 1996; Brown *et al.*, 1999). The conformation of the bound peptide is such that several of the side chains point away from the surface of the cyclin and play no role in binding. The part of the peptide which is best defined in the X-ray analysis, and hence the part that interacts tightly with the cyclin, is centered around the essential RRL motif. However, there is defined electron density, albeit somewhat weaker, for several residues n-terminal to this conserved motif. This situation contrasts with that of p107 bound to cyclin A (Brown *et al.*, 1999), where electron density starts very close to the core motif but extends rather further at the C-terminal end.

R90-E2F (**R**XL) forms salt bridges with both carboxyl oxygens of E220 of the cyclin and explains the specificity for the arginine of the RXL motif. The side chain of R91-E2F (R**X**L) lies on the surface of the cyclin but does not apparently make any interactions. This observation is again consistent with this residue not being important for the overall interaction. L92-E2F (RX**L**) sits in a deep hydrophobic pocket on the surface of the cyclin. The cyclin contributes the side chains of I213, L214 and W217 to the formation of the pocket while the E2F peptide also contributes 94L. The side chain of 93D-E2F is oriented away from the cyclin and makes no interactions. As mentioned above, 94L packs against the cyclin and 92L. After this residue the electron density for the peptide is relatively poor.

Comparison of the structure of E2F-$1_{(87\text{-}94)}$ bound to cyclin A-CDK2 with that of the recruitment peptide of p107 (Brown *et al.*, 1999) reveals several interesting features (**figure 19**). The p107 peptide is only ordered from the arginine at the beginning of the recognition motif. The conformation of the peptide backbone and the side chains of the first three amino acids, spanning the recognition motif, is very similar between the two structures. The next residue in p107 is F33 (RRL**F**), this residue contributes to the hydrophobic pocket which accommodates L32-p107. The side chain of F33-p107 is, to a certain extent, mimicked by 94L-E2F. In other words, the E2F-1 recognition peptide is able to accommodate an acidic residue after the core leucine residue (RX**L**) by looping it out and bringing the next, hydrophobic residue, to pack against the leucine. Thereafter, the chains of the E2F-1 and p107 peptides exit the cyclin binding sites in different directions. It is remarkable to observe how the cyclin binding site is able to achieve a very similar set of interactions with the core of the recognition peptides in spite of the variation in sequence. These observations are further supported by recent experiments carried out in the laboratory which reveal that the binding constants of E2F- and p107-derived peptides for cyclin A-CDK2 are quite similar (Sheraz Gul *personal communication*).

The hydrophobic patch on the surface of cyclin A that contacts RXL, is located approximately 35 Å away from the active site of CDK2. The function of the RXL motif may be to increase the local concentration of the substrate relative to the enzyme's active site (Schulman *et al.*, 1998). However, the current crystallographic information (discussed earlier), relating to the association of

cyclin A-CDK2 with E2F/DP, is limited to the interaction between cyclin A and E2F-1's cyclin-binding domain. The second crucial interaction between the enzyme's active site and DP-1's SP/TP motifs, as well as its relationship with the RXL-cyclin A association, has yet to be analysed by X-ray crystallography.

In order to better understand the structural basis of cyclin A-CDK2-mediated phosphorylation of E2F/DP, attempts were being made in our laboratory to co-crystallise fragments of this cyclin-kinase pair with a recombinant E2F/DP heterodimer. In these studies, promising results had begun to emerge with a heterodimer consisting of residues 84-195 of E2F-1 ($E2F\text{-}1_{cyc}$) and residues 84-194 of DP-1 ($DP\text{-}1_{S98}$). The E2F-1 fragment contains the cyclin A-binding region, incorporating the RXL motif discussed earlier. The DP-1 fragment contains only one of the potential phosphoacceptor sites (S98) that occur at the N-terminus of the wild-type protein.

I decided therefore to carry out experiments to determine the physiological relevance of such a crystal structure. The question that had to be addressed in this context was whether the cyclin-kinase abolished the DNA-binding activity of the recombinant heterodimer, containing only one of the putative phosphoacceptor sites found in wild-type DP-1. The information thus gained would be an important first step in working out the mechanism by which phosphorylation leads to a loss of DNA binding by the transcription factor. For example, it would allow us to determine whether the disruption of E2F/DP binding to DNA could be brought about by a single phosphorylation event as opposed to concerted modifications at several sites on DP-1.

For these experiments, I used E2F/DP heterodimers that were formed by mixing purified E2F-1_{cyc} and either DP-1_{S98} or DP-$1\Delta_{S98}$ (in which S98 had been mutated to alanine). These protein fragments are shown schematically in **figure 20**. The effect of cyclin A-CDK2-mediated phosphorylation on the DNA binding activity of these heterodimers was assessed using electrophoretic mobility shift assays (EMSAs).

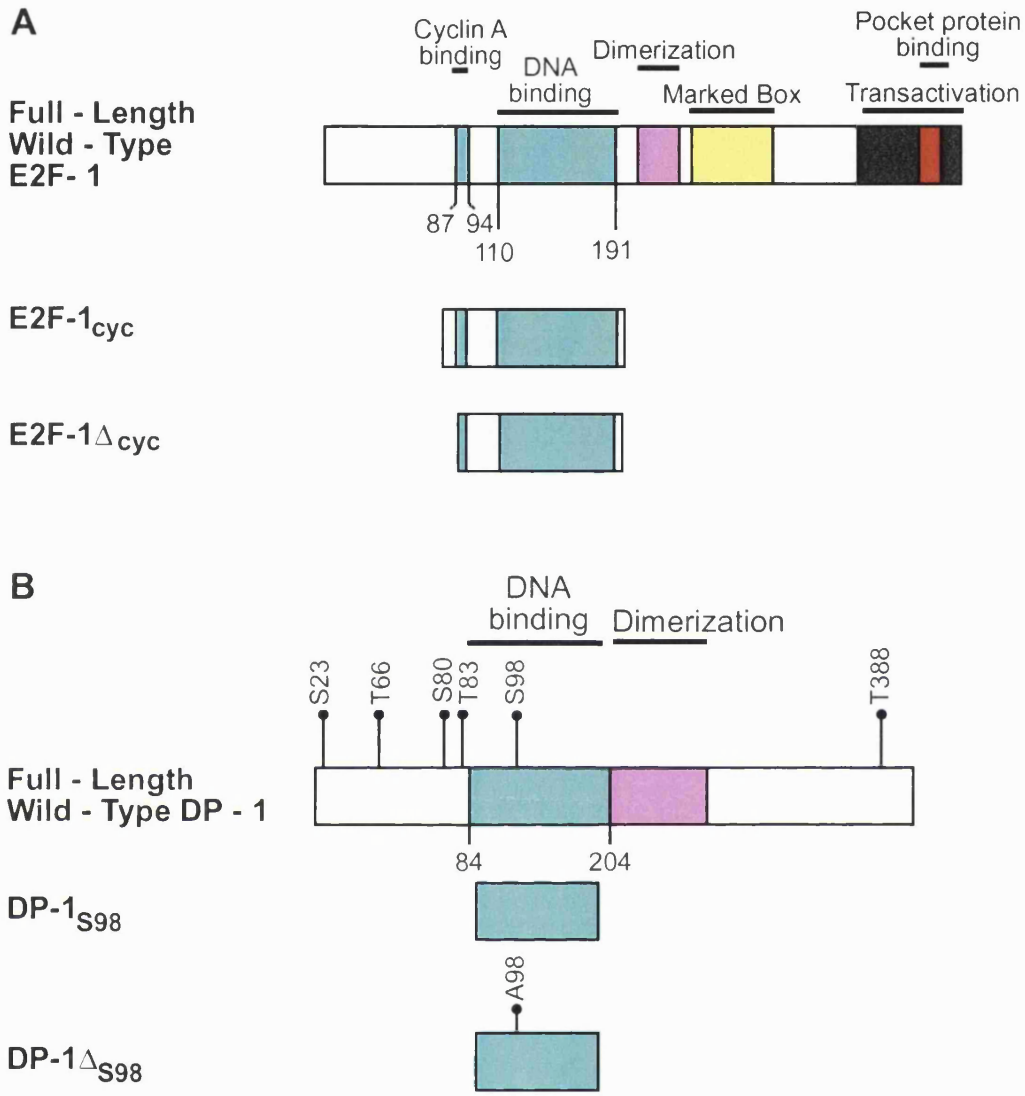


FIGURE 20

E2F AND DP FRAGMENTS USED IN EMSA EXPERIMENTS

(Adapted from Black *et al.*,1999)

A: E2F-1_{cyc} (residues 84-195 of E2F-1) consists of the minimal cyclin A-binding region (Adams *et al.*, 1996) and the DNA -binding domain (Ivey-Hoyle *et al.*, 1993). E2F-$1\Delta_{cyc}$ (residues 92-195 of E2F-1) has an incomplete minimal cyclin A-binding region but is otherwise the same as E2F-1_{cyc} .

B: S and T denote the serine and threonine residues (found in SP/TP motifs) that are potential CDK phosphorylation sites (Krek *et al.*, 1995). DP-1_{S98} (residues 84-194 of DP-1) consists of most of the DNA-binding domain (Girling *et al.*, 1993), as well as one of the putative phosphoacceptor sites (serine 98). DP-$1\Delta_{S98}$ is the same as DP-1_{S98} except that the serine 98 residue is mutated to alanine.

REGULATION OF E2F/DP DNA-BINDING BY CYCLIN-CDK DEPENDENT PHOSPHORYLATION

4.3

RESULTS

4.3.1

Purification of E2F-1_{cyc}

E2F-1_{cyc} (the fragment of E2F-1 comprising residues 84-194) was expressed in *E. coli* as a GST-fusion protein, with a PreScission™ protease cleavage site located between the affinity tag and the N-terminus of the E2F-1 fragment. The following purification steps were assessed by SDS-PAGE. After binding of the GST-fusion to glutathione sepharose (**figure 21**, Gel A, lane 1), PreScission™ protease was added to the matrix, resulting in the elution of E2F-1_{cyc} (**figure 21**, Gel B, lane 1) while the GST-tag remained bound to the beads. Electrospray mass spectrometry (ESMS) (**figure 22**) revealed that the molecular mass of the protein was 13020.44 Da, close to the calculated value of 13019.82 Da, confirming the identity and integrity of the recombinant protein (i.e. ESMS allowed us to verify that the construct had not been truncated, for example). ESMS also showed that the E2F fragment was extremely pure since it was the only species detected in the analysis. Each preparation typically yielded 5-6 mg of pure E2F-1_{cyc}.

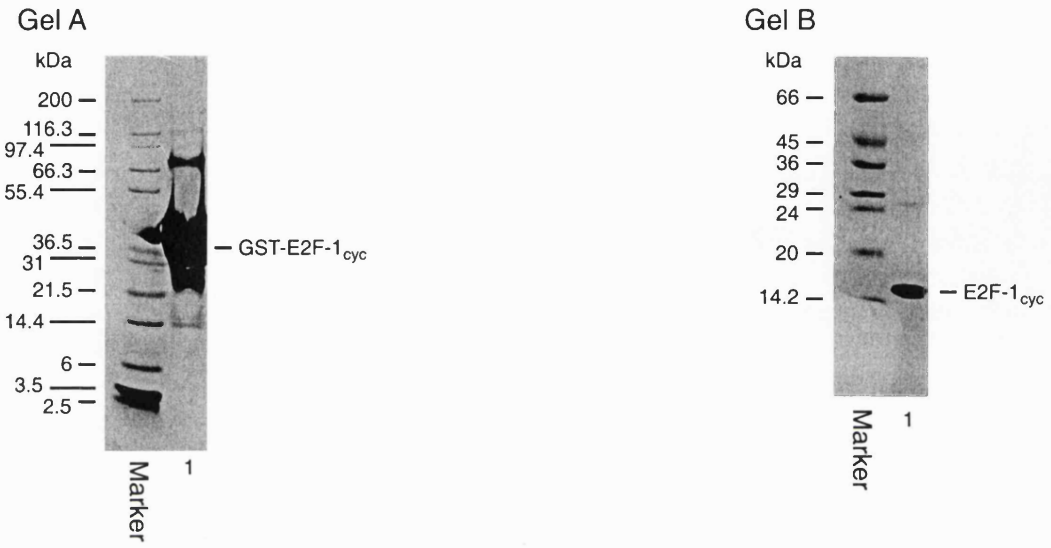


Figure 21

PURIFICATION of E2F-1_{cyc}: SDS-PAGE ANALYSIS:
The calculated molecular weight of GST-E2F-1_{cyc} is 39.4 kDa while that of E2F-1_{cyc} is 13.0 kDa.

Gel A (4-12 % gel): Glutathione sepharose beads were boiled in SDS loading buffer after application of the *E. coli* lysate (lane 1). **Gel B (12.5 % gel):** The eluate from glutathione sepharose, obtained after treating the matrix-bound GST-E2F-1_{cyc} with PreScission™ protease, is shown in lane 1.

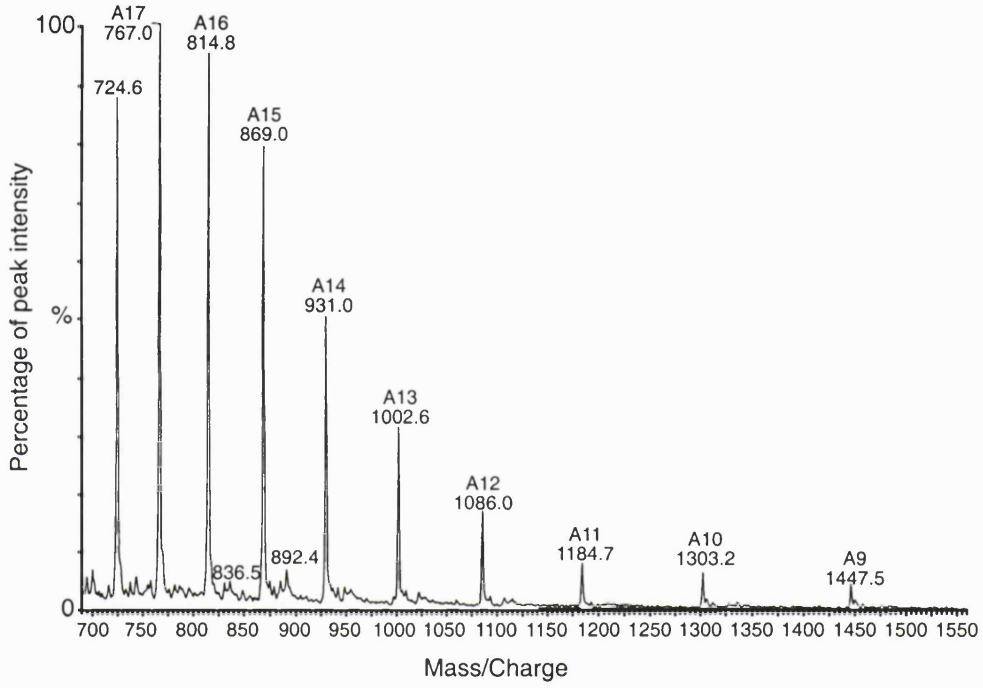


FIGURE 22

MOLECULAR MASS DETERMINATION OF PURIFIED E2F-1cyc BY ESMS

One species was detected whose mass was determined to be 13020.44±0.81 Da.

Calculated mass of E2F-1_{cyc} : 13019.82 Da.

4.3.2

Purification of DP-1_{S98} and DP$1\Delta_{S98}$

DP-1_{S98} (fragment of DP-1 comprising residues 84-194) was also expressed in *E.coli* as a GST-fusion protein whose GST-tag could be removed using PreScission™ protease. This tag was at the N-terminus of DP-1_{S98} while the C-terminus incorporated a His-tag. This "double-tagging" approach was employed to eliminate truncated DP fragments that had appeared during previous purifications of other DP constructs. Only DP fragments containing both tags (i.e. intact constructs) would be present after the two affinity chromatography steps.

As with the E2F-1_{cyc} fragment, the purification steps were assessed by SDS-PAGE. After immobilisation of the GST-fusion on a glutathione sepharose matrix (**figure 23**, Gel A, lane 1), the eluate obtained after addition of PreScission™ protease was applied to Ni-agarose and a step-elution gradient followed. Contaminating proteins, the most prominent of which was smaller than DP-1_{S98} (**figure 23**, Gel B, lane 1), were removed using a wash buffer containing 80 mM imidazole. Following the wash, the pure DP1_{S98} remaining on the beads (**figure 23**, Gel B, lane 2) was released using an elution buffer containing 300 mM imidazole. 8-9 mg of pure DP-1_{S98} were typically obtained by this method. The expression and purification of DP-$1\Delta_{S98}$ (identical to DP-1_{S98} except that serine 98 was mutated to alanine) proceeded in essentially the same manner (SDS-PAGE analysis not shown).

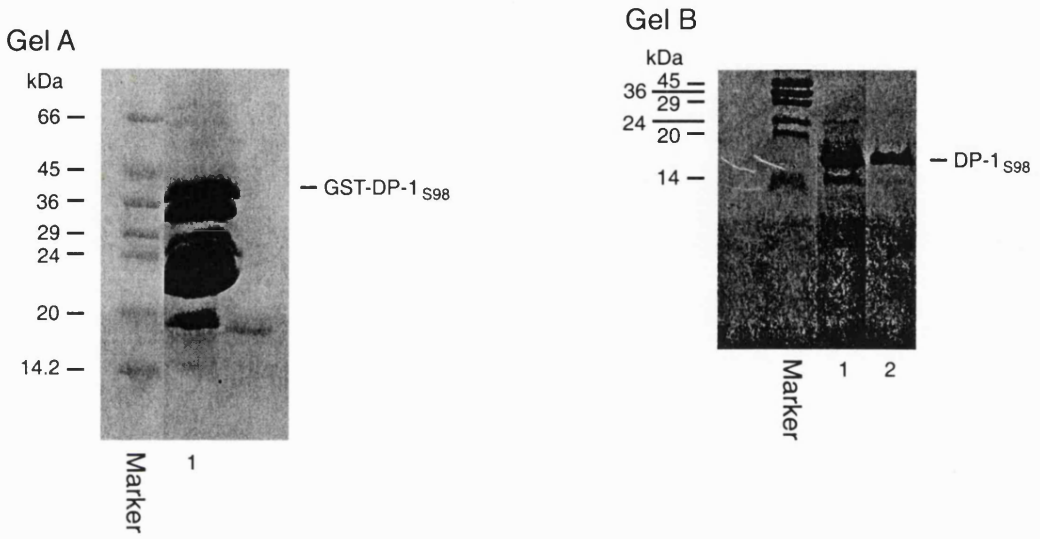


Figure 23

PURIFICATION OF DP-1$_{S98}$: SDS-PAGE ANALYSIS
The calculated molecular weight of GST-DP-1 $_{S98}$ is 40.5 kDa, while that of DP-1 $_{S98}$ is 14.1 kDa.

Gel A (12.5 % gel): Glutathione sepharose beads were boiled in SDS loading buffer after application of the *E. coli* lysate (lane 1). **Gel B (15 % gel):** Ni-agarose beads were boiled in SDS loading buffer after application of the eluate from glutathione sepharose (lane 1) and then after the application of the wash buffer containing 80 mM imidazole (lane 2). The pure DP-1$_{S98}$ bound to the Ni-agarose (lane 2) was eluted using a buffer containing 300 mM imidazole.

Each of the ESMS traces for DP-1_{S98} and DP-$1\Delta_{S98}$ show two species (**figures 24** and **25**). In each trace, one species has a molecular mass close to the calculated value of the desired recombinant protein while the second species has a mass that is approximately 75 Da greater. The increased mass is most likely attributable to the presence of βME in the protein's buffer. This reducing agent forms an adduct with a given protein and consequently increases its molecular mass by approximately 75 Da.

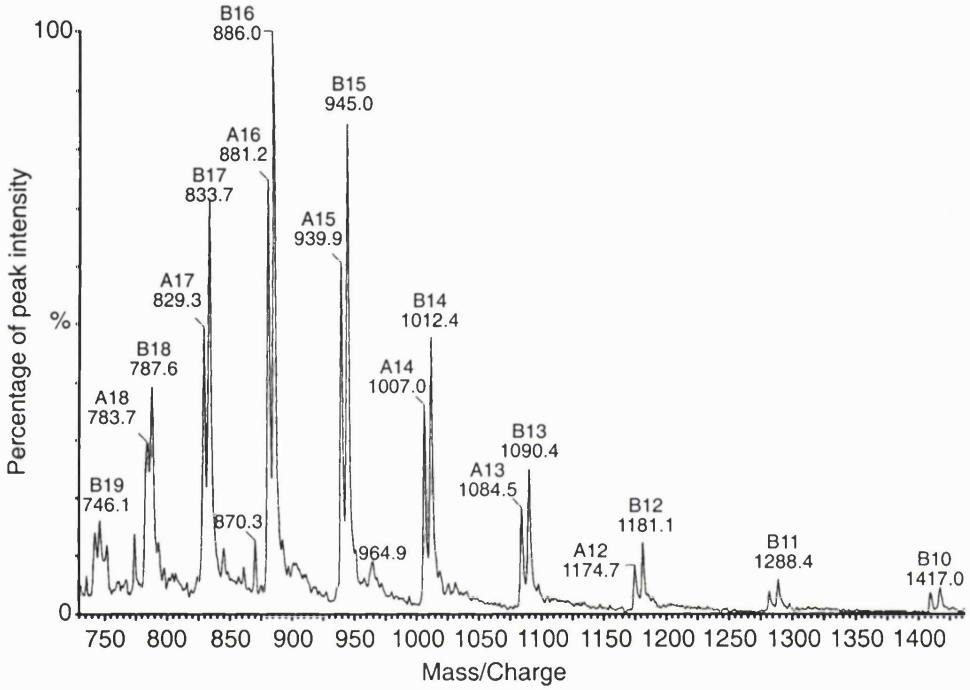


FIGURE 24

MOLECULAR MASS DETERMINATION OF PURIFIED DP-1_{S98} BY ESMS

Two species were detected whose masses were determined to be:

A: 14175.06±1.31 Da
B: 14099.77±2.58 Da

Calculated mass of DP-1_{S98}: 14101.82 Da.

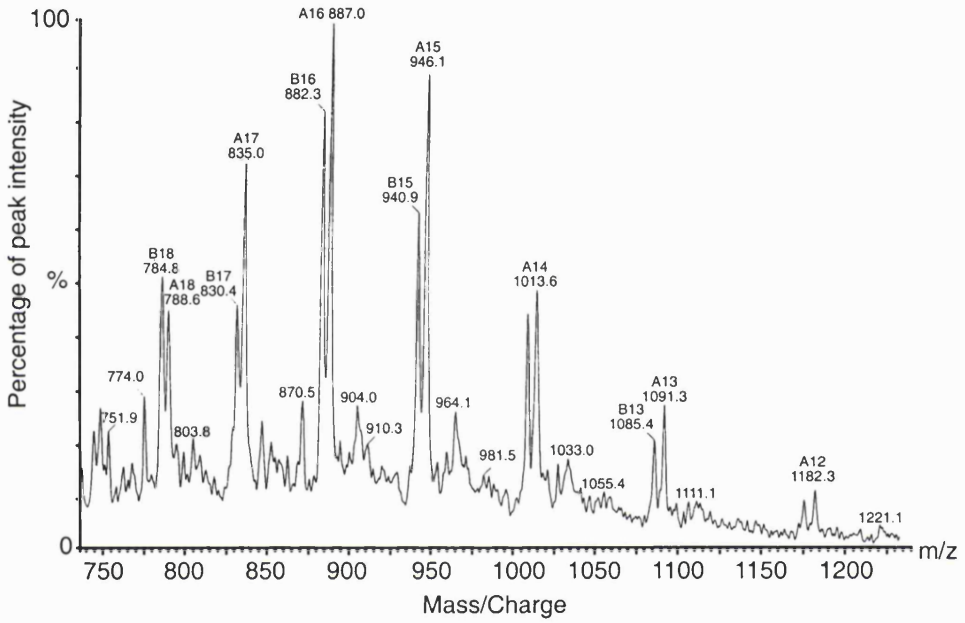


FIGURE 25

MOLECULAR MASS DETERMINATION OF PURIFIED DP-1Δ_{S98} BY ESMS

Two species were detected whose masses were determined to be:

A: 14085.35±1.66 Da
B: 14160.52±1.94 Da

Calculated mass of DP-1Δ_{S98} : 14085.77 Da.

4.3.3

Phosphorylation of the E2F Transcription Factor by Cyclin A-CDK2

In conformity with previous studies (Girling *et al.*, 1993; Krek *et al.*, 1993; Jordan *et al.*, 1994), the DNA-binding domains of E2F-1 and DP-1 each interacted *in vitro* with an oligonucleotide consisting of the E2F DNA-binding site (**figure 26**, lanes 2 and 3). The oligonucleotide sequence was shown previously in **figure 11**, **section 3.2**. The DP-1 fragment exhibited greater DNA-binding activity than the recombinant E2F-1 (**figure 26**), in contrast to reports by others (Bandara *et al.*, 1993; Helin *et al.*, 1993). This may be attributable to the latter's use of different protein constructs and/or the respective stability of these constructs.

Nevertheless, the E2F-1 and DP-1 constructs that I purified formed a complex that bound to the E2F site (**figure 27**). In agreement with earlier studies (Bandara *et al.*, 1993), increased DNA-binding activity was observed when the E2F-1 and DP-1 fragments were present in the same binding reaction (lane 4), compared to those of the separate proteins (lanes 2 and 3). This demonstrates that the two proteins interact synergistically to bind DNA (Bandara *et al.*, 1993). This cooperative binding to DNA occurred despite the absence of the dimerisation domains of E2F-1 and DP-1, in accordance with previous observations (Fraenkel, 1998; Zheng *et al.*, 1999). Other reports indicate that the dimerisation regions of the two proteins are required for their association in the absence of DNA (Helin *et al.*, 1993; Krek *et al.*, 1993).

ELECTROPHORETIC MOBILITY SHIFT ASSAYS (Figures 26 - 30)

Each reaction mixture contained 50 mM Tris 8.2, 6 mM $MgCl_2$, 3 mM DTT, and 15 % glycerol. The concentrations of the other components in the mixtures are listed below the corresponding autoradiographs. The DNA probe was derived from the E2F-binding site of the adenovirus E2 promotor. In the case of reaction mixtures to which an E2F/DP complex was added, the appropriate recombinant E2F-1 and DP-1 proteins were first mixed in equimolar amounts to make a stock solution. Aliquots from this stock were added to reaction mixtures to give the desired final concentration of the E2F/DP complex. The concentration (see below) of recombinant E2F-1, DP-1 or E2F/DP complex in each mixture is expressed as the molarity of dimeric protein. Each reaction mixture was applied to a non-denaturing 8 % polyacrylamide gel. Further details of the protocol are given in the Methods and Materials section.

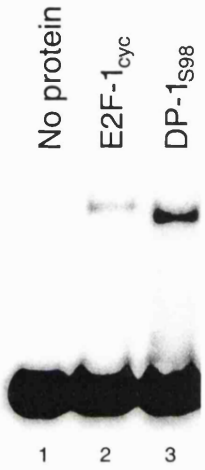


Figure 26 Binding of Recombinant E2F-1 and DP-1 to DNA

Reaction mixtures each contained 3 μM DNA probe and either no protein (lane 1), 3 μM $E2F\text{-}1_{cyc}$ (lane 2) or 3 μM $DP\text{-}1_{S98}$ (lane 3).

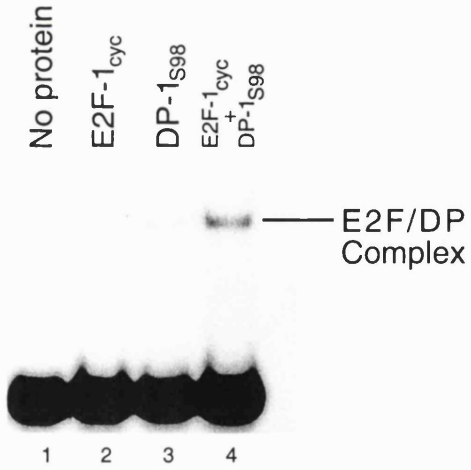


Figure 27 E2F-1/DP-1 Complex Formation

Reaction mixtures each contained 3 μM DNA probe and either no protein (lane 1), 1.5 μM $E2F\text{-}1_{cyc}$ (lane 2) , 1.5 μM $DP\text{-}1_{S98}$ (lane 3) or 1.5 μM of each recombinant protein (lane 4).

An E2F/DP complex, that comprised the cyclin A-binding E2F protein fragment (E2F-1_{cyc}), interacted with cyclin A-CDK2 producing a supershift (**figure 28**, lane 3). This interaction was specific since a supershift was not observed with a heterodimer comprising E2F-$1\Delta_{cyc}$ (fragment of E2F-1 comprising residues 92-195; purified by Dr. I Tews), whose cyclin A-binding region is truncated (**figure 28**, lanes 4 and 5). These data support observations reported by Krek and colleagues (Krek *et al.*, 1994).

It has been shown previously that the addition of cyclin A-CDK2 to an EMSA reaction supplemented with ATP results in the inhibition of the DNA-binding activity of a heterodimer comprising E2F and DP proteins (Dynlacht *et al.*, 1994; Krek *et al.*, 1994; Xu *et al.*, 1994; Dynlacht *et al.*, 1997). I demonstrate here that the same effect is produced with a DP-1 fragment (DP-1_{S98}) lacking all but one (serine 98) of the putative CDK phosphoacceptor sites found in full-length wild-type DP-1. This is apparent from **figure 29** that shows the DNA-binding activity of a heterodimer comprising E2F-1_{cyc} /DP-1_{S98} is abrogated when the kinase and ATP are present in the reaction (lane 4). However, cyclin A-CDK2 has the same effect on a heterodimer comprising E2F-1_{cyc}/DP-$1\Delta_{S98}$ in which the serine 98 residue of DP-1 has been mutated to alanine (lane 7).

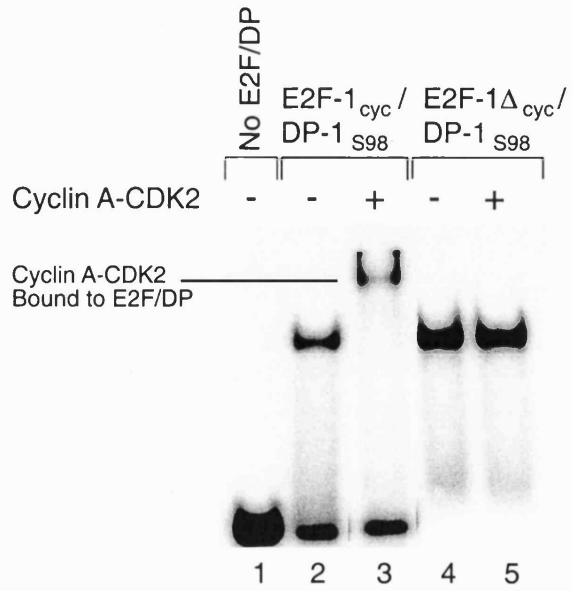


Figure 28 Interaction of Cyclin A-CDK2 with E2F/DP

Reaction mixtures each contained 0.5 μM DNA probe and either no E2F/DP complex (lane 1), 3 μM E2F-1_{cyc}/DP-1_{S98} (lanes 2 and 3) or 3 μM E2F-$1\Delta_{cyc}$ /DP-1_{S98} (lanes 4 and 5). The final concentration of cyclin A-CDK2 (where added) in each reaction was 3 μM.

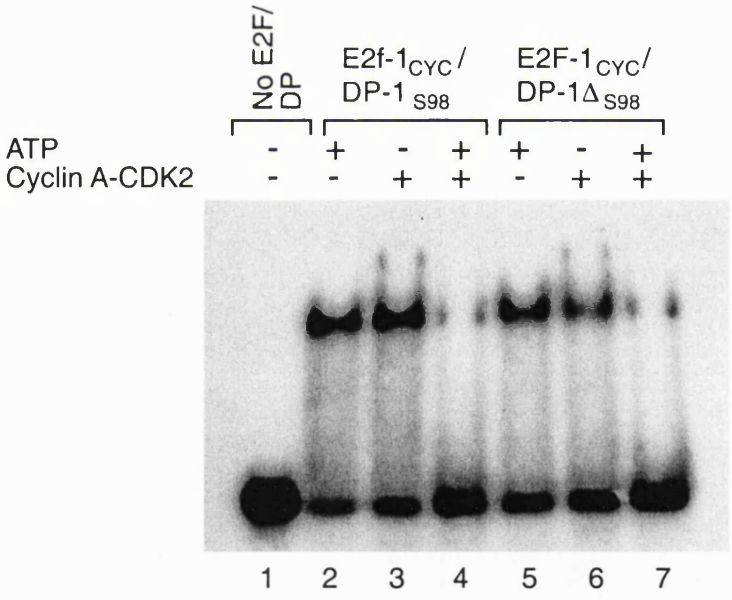


Figure 29 Abolition of E2F/DP DNA-binding Activity by Cyclin A-CDK2

Reaction mixtures each contained 0.5 μM DNA probe and either no E2F/DP (lane 1), 2.5 μM E2F-1_{cyc} /DP-1_{S98} (lanes 2, 3 and 4) or 2.5 μM E2F-1_{cyc}/DP-$1\Delta_{S98}$ (lanes 5, 6 and 7). The final concentration of ATP (where added) in each mixture was 2.5 mM and that of cyclin A-CDK2 was 0.5 μM. After addition of the kinase (the last component to be added), reaction mixtures were immediately subjected to electrophoresis.

Differences between the two heterodimers (wild-type and mutant), in the context of abolition of DNA-binding, were apparent under different assay conditions. The concentration of cyclin A-CDK2 in the reactions was reduced. For each heterodimer, identical reactions were analysed by EMSA as a function of incubation time. **Figure 30** shows that under these conditions, the DNA-binding activity of E2F-1_{cyc}/DP-$1\Delta_{S98}$ is lost at a slower rate than that of E2F-1_{cyc}/DP-1_{S98}. This difference in rate is most obvious at the 5-hour time-point at which E2F-1_{cyc}/DP-$1\Delta_{S98}$ still binds a significant amount of DNA while the binding activity of E2F-1_{cyc}/DP-1_{S98} has been abolished (compare lane 6 gel A to lane 6 gel B).

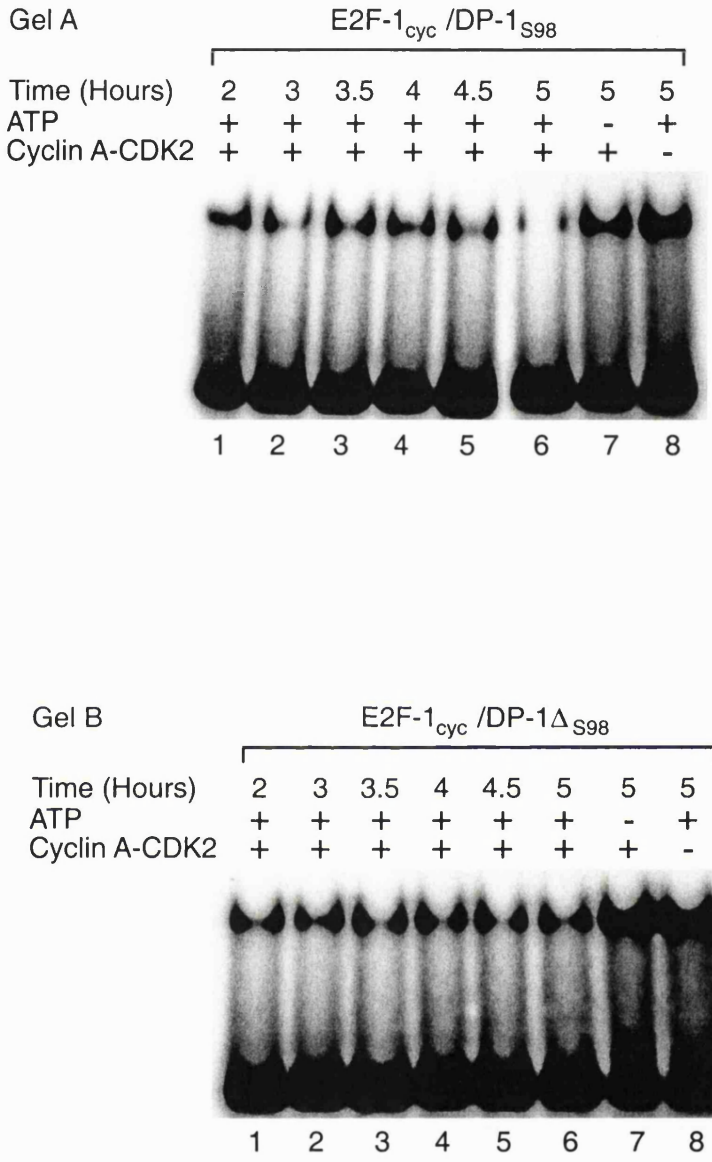


Figure 30

Effect of Serine 98 Mutation on Abolition of E2F/DP DNA-binding Activity

Reaction mixtures each contained 0.5 μM DNA probe and either 2.5 μM E2F-1_{cyc}/DP-1_{S98} (gel A) or 2.5 μM E2F-1_{cyc}/DP-$1\Delta_{S98}$ (gel B). The final concentration of ATP (where added) in each mixture was 2.5 mM while that of cyclin A-CDK2 was 40 nM. After addition of the kinase (the last component to be added), reaction mixtures were left to stand at room temperature for the number of hours indicated, prior to separation by electrophoresis.

REGULATION OF E2F/DP DNA-BINDING BY CYCLIN-CDK DEPENDENT PHOSPHORYLATION

4.4

DISCUSSION

The DNA binding activity of the recombinant heterodimer used in the EMSA experiments incorporating one of the putative DP phosphorylation sites, is abolished by cyclin A/CDK2. Furthermore, it appears that the activity of a heterodimer comprising a DP-1 mutant in which serine 98 has been mutated to an alanine residue, is not abolished as quickly as that comprising the wild-type DP-1 fragment.

There is, however, a caveat associated with these findings owing to the vector chosen to subclone the DP-1 fragment. When the GST tag is cleaved from recombinant proteins expressed using the pGEX6P1 vector, some additional residues remain attached to the N-terminus of the protein of interest. These residues belong to the linker region between the GST-tag and the protein's N-terminus (see **figure 31**, A and B). It was unfortunate that in this case, the serine residue from this linker and the proline residue of the subcloned DP combined to form an unintended SP motif. These unintended residues are in the same position as a TP motif found in the full-length DP-1 protein. Furthermore, the spurious SP motif was at a point in the recombinant DP's N-terminus that is similar to the position of an actual SP motif in full-length DP-1 (serine 80 and proline 81)

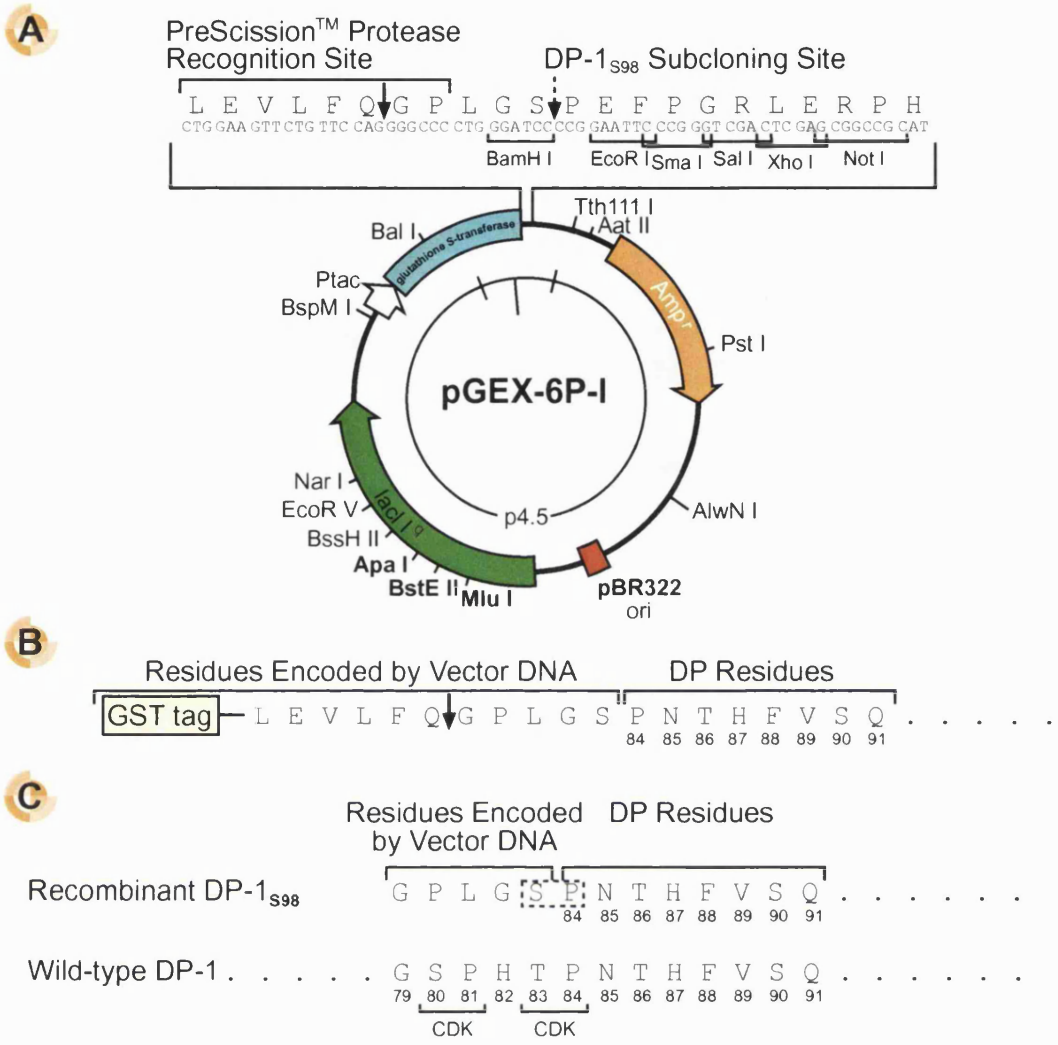


FIGURE 31: SUBCLONING STRATEGY FOR DP-1$_{S98}$

DNA encoding the DP-1$_{S98}$ fragment was generated by PCR (using the murine DP-1 gene as a template) and then inserted into the pGEX-6P-1 vector (Dr. I Tews) that allows expression of the protein as a GST-fusion.

A (adapted from the Amersham Pharmacia Biotech catalogue 1998): Multiple cloning site (MCS) of the pGEX-6P-1 vector. Restriction enzyme sites are bracketed below the DNA sequence. Amino acids (single letter code) are shown above their corresponding codons. The solid arrow indicates the point at which the PreScission™ Protease enzyme cleaves the recombinant protein, resulting in removal of the GST-tag. The dashed arrow indicates the site at which the DNA encoding DP-1$_{S98}$ was inserted.

B N-terminus of the recombinant DP-1$_{S98}$ GST fusion protein. A linker region lies between the GST tag and residue 84 of DP-1. The arrow indicates the PreScission™ protease cleavage site. Residue numbers are shown below the amino acid code.

C Comparison of the N-terminus of recombinant DP-1$_{S98}$ (after cleavage of the GST-tag using PreScission™ Protease) with the corresponding residues of wild-type murine DP-1. Bracketed residues in the wild-type protein sequence indicate the SP and TP motifs that constitute potential cyclin-CDK phosphorylation sites. The dashed box in the recombinant protein's sequence highlights the spurious SP motif introduced as a result of the subcloning strategy.

(**figure 31**, C). Thus these unintended residues may have mimicked the motifs found in full-length DP and caused the abolition of DNA binding.

Krek and colleagues (Krek *et al.*, 1995) have demonstrated that a DP-1 mutant devoid of the putative phosphorylation sites described earlier, is poorly labeled in an *in vitro* kinase reaction compared to wild-type DP-1. It may be that the mutant DP-1 we used dissociated from DNA (**figure 29**) because of phosphorylation at the introduced serine residue. The mutation of serine 98 to alanine does, however, appear to have affected the heterodimer's rate of dissociation from DNA (**figure 30**). It is conceivable that the identity and/or number of sites that are phosphorylated affect the rate at which the heterodimer dissociates from DNA, that is, the efficiency with which E2F is downregulated. However, further experiments would be required to confirm this, as discussed below.

Although it has been established that cyclin A/CDK2-mediated phosphorylation of E2F inhibits the latter's DNA-binding activity, questions remain in this context, regarding the significance of the phosphorylation of the heterodimer's individual components. In keeping with observations that the phosphorylation of DP-1 is particularly important, Dynlacht *et al.* (Dynlacht *et al.*, 1994) report that in preliminary experiments (for which the data is not shown), the prior treatment of DP-1, but not E2F-1, with cyclin A-CDK2 resulted in a marked decrease in heterodimeric DNA binding. They have also observed that the DP-1 component must be present if cyclin A-CDK2 is to inhibit DNA-binding. However, this latter finding conflicts with that of Kitagawa *et al.* (Kitagawa *et al.*, 1995) whose experiments show that the ability of E2F-1 (homodimer) to bind DNA is

significantly compromised by action of cyclin A-CDK2. Furthermore, E2F-1 has potential CDK2 phosphorylation sites (Dynlacht *et al.*, 1994) and it undergoes phosphorylation by cyclin A/CDK2 in vitro (Dynlacht *et al.*, 1994; Xu *et al.*, 1994; Dynlacht *et al.*, 1997). This suggests that the phosphorylation of the E2F protein component needs to be further investigated to ascertain its effects on the heterodimer's actvity.

In order to clarify the significance of the putative CDK phosphorylation sites of the heterodimer's components in the context of negative regulation of DNA binding, future experiments could involve the following approaches. EMSA experiments, such as those performed in this study, could be undertaken using E2F and DP proteins whose potential sites have been mutated in an alternating manner and in different combinations. In vivo studies can also be carried out in which these mutants are expressed in cells so their effects on cell cycle progression can be determined.

Structural studies are still required to provide insight into the mechanism of cyclin A-kinase action as the following observations indicate. Immunoprecipitation experiments by Guida and Zhu show that the association between cyclin A-CDK2 and E2F1-DP1 is less stable than that between this cyclin-kinase pair and a complex comprising p107/E2F4-DP1 (Guida and Zhu, 1999). However, the phosphorylation of DP-1 in the former complex is more efficient than that in the latter complex. This indicates that the arrangement of the components in a complex and not just stability of interactions is important in determining the target of cyclin A-CDK2. Studies conducted by Dynlacht *et al.* (Dynlacht *et al.*, 1997)

reveal that cyclin A-CDK2 and cyclin B-CDK2 (that does not complex with E2F) both phosphorylate E2F-1 and DP-1 and both enzymes generate the same tryptic phosphopeptides of these substrates. However, it is only cyclin A-kinase that can abolish DNA-binding by E2F1-DP1, suggesting that the formation of a stable enzyme-substrate complex is required for negative regulation of the heterodimer rather than the weaker interactions that are traditionally associated with enzymatic activity. Since E2F heterodimers comprising E2Fs 4 and 5 (that lack the cyclin A-binding region) are not inhibited by cyclin A-CDK2, it appears that the conserved N-terminal region shared by E2Fs1-3 confers substrate specificity upon the enzyme (Dynlacht *et al.*, 1997).

CHAPTER 5

INVESTIGATION OF THE INTERACTIONS BETWEEN pRb, E2F AND HPV E7

CHAPTER 5

INVESTIGATION OF THE INTERACTIONS BETWEEN pRb, E2F AND HPV E7

5.1

OUTLINE OF ISOTHERMAL TITRATION CALORIMETRY (ITC)

When molecules interact with each other there is often an accompanying absorption or generation of heat. ITC allows direct measurement of the change in heat or enthalpy (ΔH) that accompanies such interactions (Tame *et al.*, 1998). For the purposes of this thesis, we shall consider protein-peptide (protein-ligand) interactions. In the simplest case the equilibrium we are concerned with is:

$$\mathbf{P + L \Leftrightarrow PL} \qquad \text{(eq.1)}$$

where P = protein, L = ligand, PL = protein-ligand complex.

5.1.1

The ITC Apparatus and Mode of Operation

In this study, we used the VP-ITC microcalorimeter (MicroCal Inc.) whose basic components are shown in **figure 32**.

A heater on the reference cell receives a constant power supply, while the amount of power supplied to the sample cell (feedback power) is subject to continuous

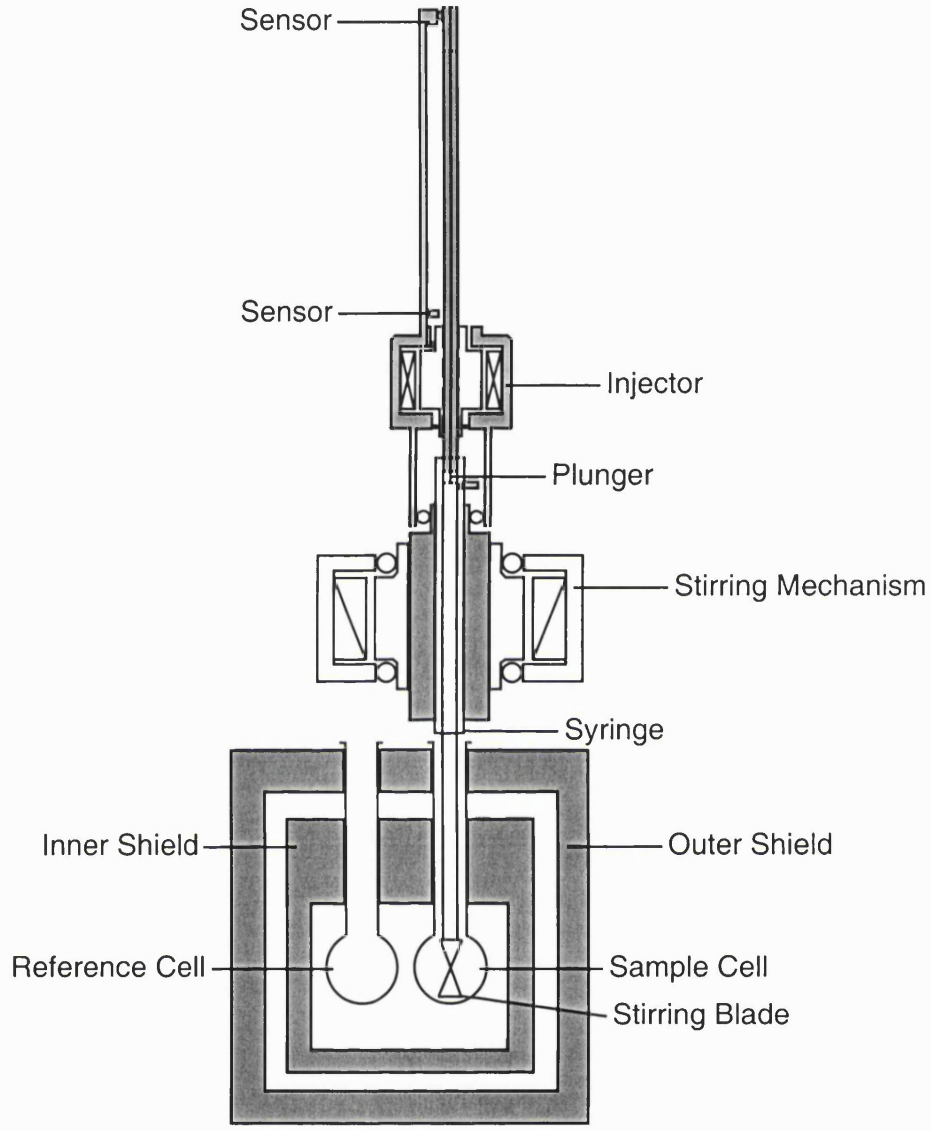


FIGURE 32

BASIC COMPONENTS OF THE VP-ITC MICROCALORIMETER
(Adapted from User's Manual)

The sample and reference cells are enclosed in two adiabatic shields. The stirring blade on the tip of the injection needle helps to mix the sample cell's contents thoroughly.

adjustment such that the two cells are maintained at the same temperature. The peptide (ligand) is introduced into the macromolecule solution in the sample cell by a number of injections of equal volume. This is carried out by the computer-controlled syringe that also spins to ensure thorough mixing of the reactants. There is a sufficient time delay between injections to allow equilibrium to be reached before more peptide is introduced. If, for example, an exothermic reaction takes place in the sample cell as a result of the introduction of ligand, the feedback power to this cell is automatically reduced to minimise the temperature difference between the two cells caused by the generation of heat. This reduction in feedback power is equal to the amount of heat released by the reaction (Wiseman *et al.*, 1989; Ladbury and Chowdhry, 1996).

Apart from the peptide and macromolecule, the solutions in the two cells should be identical to avoid enthalpy changes associated with interactions other than protein-ligand binding. Correction for superfluous enthalpic contributions (e.g. those due to dilution of solvent components) can be made by performing a titration with the solutions devoid of the species of interest. If there are any significant enthalpy changes as a result of this, they can be subtracted from the data obtained from the protein-ligand titration (Blandamer, 1998).

Nevertheless, it is important to appreciate that the enthalpy change measured is for the whole system and thus other events such as the interaction of protein or ligand with the solvent may also make an enthalpic contribution. It is thus more accurate to describe the thermodynamic binding parameters determined by ITC as being apparent or observed values (Tame *et al.*, 1998).

5.1.2

Determination of Thermodynamic Binding Parameters

Typical data obtained by the above procedure for exothermic protein-peptide associations are shown in **figure 38** (**section 5.3.2**). In the upper panel, each peak corresponds to heat output per injection and the baseline between them represents that time period where equilibrium conditions have been re-established. Heat output decreases as the binding sites on the macromolecule become occupied, hence the reduction in the size of the peaks.

Using "Origin" data analysis software (MicroCal Inc.), the area of each peak is calculated allowing the total heat per injection (kcal/mol injectant) to be determined and subsequently plotted against the molar ratio of the peptide and macromolecule (Ladbury and Chowdhry, 1996). This produces a binding isotherm of the type shown in the lower panel of **figure 38** (**section 5.3.2**).

Fitting of the isotherm curve provides a value for the change in enthalpy (ΔH) associated with the binding process (Lakey and Raggett, 1998):

$$\Delta H_{interaction} = \Delta H_{final} - \Delta H_{initial}$$

(eq.2)

Changes in enthalpy on introduction of ligand are a measure of the extent of binding and in the binding isotherm, these enthalpic changes are correlated with

the molar ratios of the interacting species. Fitting the data provides an estimate of the binding constant (K_B) (Lakey and Raggett, 1998; Tame *et al.*, 1998):

$$K_B = \frac{[PL]}{[P][L]}$$

(eq.3)

The stoichiometry of binding (N) is related to the value of the molar ratio corresponding to the inflection point of the fitted isotherm curve. The Gibbs free energy change (ΔG) and change in entropy (ΔS) are calculated using the relationship below, ΔH and K_B having already been determined (Ladbury and Chowdhry, 1996; Lakey and Raggett, 1998):

$$\Delta G^\circ = -R\,T\,\ln K_B = \Delta H^\circ - T\Delta S^\circ$$

(eq.4)

where R = gas constant, T = absolute temperature.

from changes to a system, that have almost equal and opposite effects on the free energy (Dunitz, 1995; Cooper, 1999; Sleigh *et al.*, 1999).

5.1.3

The Thermodynamic Parameters and How Interactions Influence Them

We shall see in subsequent sections of this chapter, how the determination of binding constants using ITC has provided valuable information regarding the

interactions between pRb, E2F and E7. However, additional insight into molecular associations can be derived from the other thermodynamic parameters that can be determined by ITC in the manner discussed above.

A negative value for $\Delta G°$ indicates a spontaneous interaction and as is apparent from equation 4, this value consists of enthalpic and entropic contributions. Thus a negative (exothermic) $\Delta H°$ value and a positive $\Delta S°$ value are favourable with regard to complex formation. Where an exothermic enthalpy value is dominant, an interaction is described as being "enthalpically driven" and vice versa (O'Brien *et al.*, 2001).

The binding affinity is governed by the Gibbs energy of binding since

$$K_B = e^{-\frac{\Delta G°}{RT}} \qquad \text{(eq. 5)}$$

Evidently, a given value of $\Delta G°$ (and hence the same binding affinity) can arise from different combinations of $\Delta H°$ and $\Delta S°$ values (Leavitt and Freire, 2001). In the case of different binding reactions with similar affinities therefore, $\Delta H°$ and $\Delta S°$ determinations provide an additional means by which the interactions can be characterised (Tame *et al.*, 1998).

A phenomenon known as enthalpy-entropy compensation is frequently associated with processes that involve biomolecules in solution (Gilli *et al.*, 1994) and it

appears to be a general property of weak intermolecular interactions (hydrogen bonds, van der Waals etc.) that mediate protein-ligand binding (Dunitz, 1995). This occurrence is characterised by enthalpy and entropy changes, resulting from changes to a system, that have almost equal and opposite effects on the free energy (Dunitz, 1995; Cooper, 1999; Sleigh *et al.*, 1999).

ΔH°

The value for ΔH° that is determined by ITC for an equilibrium binding event, is directly related to the number and strength of the non-covalent interactions that are formed or broken when the system undergoes a transition from one state to another (O'Brien *et al.*, 2001). The formation of such interactions (e.g. van der Waals, hydrogen bonds) is enthalpically favourable (ΔH° is negative) whereas their elimination has the opposite effect (Tame *et al.*, 1998; Leavitt and Freire, 2001). It is important to note that the measured ΔH° value does not only relate to the formation of interactions between protein and ligand (which can be referred to as the binding enthalpy or ΔH°_{bind}) but also includes contributions from other equilibria. Thus the ΔH° value obtained by ITC is the total change in enthalpy for the whole system (Tame *et al.*, 1998). For example, ΔH° may include enthalpic changes associated with conformational change of the interactants (Thomson *et al.*, 1994; Mandiyan *et al.*, 1996), ionisation of polar groups (Baker and Murphy, 1996) and changes in the associations between the solvent and interactants (Lundback and Hard, 1996). Thus the terms observed or apparent enthalpy change ($\Delta H^\circ_{obs}/\Delta H^\circ_{app}$) are used to describe the ΔH° value determined by ITC (**section 5.1.1**).

Hydrogen bonds between the solvent and side-chains of the interactants are generally likely to be weaker than those in the protein-ligand complex but, more numerous. As with the other types of interactions, it is the number and strength of hydrogen bonds in the bound and unbound states that governs the extent to which these associations contribute to binding energetics (Sleigh *et al.*, 1999). Upon complex formation, the burial of polar groups in an apolar environment would be enthalpically unfavourable since these groups could otherwise form hydrogen bonds with water molecules. The burial of charged groups in an apolar environment would also affect the ΔH° value in the same manner (Sleigh *et al.*, 1999). However, this effect can be counteracted by other favourable interactions within the complex involving these groups (Warshel, 1987; Xu *et al.*, 1997).

In the context of the effect of hydrogen bonding on the thermodynamics of binding, the following points are of interest. Owing to the distinct electronegativities and orientations of donor and acceptor atoms, as well as a lack of reliability concerning experimental protocols, values determined for the free energy change associated with hydrogen bond formation fall into a wide range (Dill, 1990). Furthermore, it has been proposed that the hydrophobic effect (discussed in the next paragraph) is the driving force that underpins protein-ligand complex formation, while hydrogen bonds participate more in determining specificity (as opposed to affinity) (Chothia and Janin, 1975). However, hydrogen bonding has been shown to make an important contribution to the free energy of binding in several systems (Fersht, 1987).

Hydrophobic interactions constitute one of the main forces that drive protein-ligand complex formation and protein folding (Kauzmann, 1959). The effect of hydrophobicity on hydrogen bonding between water molecules also demonstrates that the ΔH° value does not exclusively reflect the formation of interactions between protein and ligand. At the hydrophobic surfaces of a solute, the water molecules adopt a well-ordered state (Ladbury and Chowdhry, 1996; Tame *et al.*, 1998). The latter is characterised by stronger hydrogen bonding than that found in the bulk solvent and consequently the ordering of water molecules in this manner is enthalpically favourable (Davies *et al.*, 1999). However, upon burial of the hydrophobic surfaces when the protein-ligand complex is formed, the ordered water molecules are released into the bulk solvent. The stronger hydrogen bonding network is therefore broken which is enthalpically unfavourable (Ladbury and Chowdhry, 1996; Tame *et al.*, 1998).

Owing to the more closely packed nature of protein structures compared to liquid water, the van der Waals forces associated with the formation of a protein-ligand complex may be considerably greater than those between ligand and solvent (Richards, 1974; Page, 1976). The formation of van der Waals interactions between the protein and ligand would thus be more favourable enthalpically than those formed between ligand and solvent. Where there is a requirement for a protein to accept a range of ligands however, the latter may not fit tightly into the protein's binding site. This may render it difficult to estimate the strength of the van der Waals interactions, although ligand side-chains may form average numbers of such contacts per atom (Sleigh *et al.*, 1999).

In the case of a binding reaction that is coupled to the absorption or release of protons by the interactants, complex formation is pH-dependent and the binding enthalpy is contingent upon the ionisation enthalpy of the buffer in which the reaction takes place (Leavitt and Freire, 2001). ITC can also be used to evaluate protonation/deprotonation coupling, allowing the dissection of intrinsic binding from protonation contributions to the overall energetics (Gomez and Freire, 1995; Baker and Murphy, 1996).

ΔS°

The ΔS° value is comprised of entropic changes that are also associated with the whole system as it undergoes a transition from one state to another (Tame *et al.*, 1998). ΔS° mainly reflects two contributions, these being changes in solvation entropy and changes in conformational entropy (Leavitt and Freire, 2001).

The burial of portions of the protein and ligand upon complex formation (desolvation) results in the release of water molecules, that were interacting with these portions, into the bulk solvent. This increases the degrees of freedom of the water molecules and thus a favourable entropy is observed (ΔS° is positive) (Tame *et al.*, 1998; Leavitt and Freire, 2001). This change in solvent interactions dominates the ΔS° value in many cases of protein-ligand binding and it is particularly important with regard to hydrophobic interactions between protein and ligand (Tame *et al.*, 1998; Leavitt and Freire, 2001). As discussed earlier, water molecules surrounding hydrophobic portions of a protein or ligand adopt an organised state. The ordering of water molecules in this manner reduces their

degrees of freedom compared to those water molecules in the bulk solvent. Upon complex formation, the burial of the hydrophobic surfaces results in the breaking of the ordered hydrogen bonding network. The restricted water molecules are consequently released into the less restrictive environment of the bulk solvent, an entropically favourable event (Ladbury and Chowdhry, 1996).

Changes in translational and internal entropy also result from complex formation. Defined portions of the interactants may for example change conformation when the complex forms, becoming more tightly folded. This would be entropically unfavourable since the number of internal degrees of freedom of the system would be reduced. It would be entropically favourable, however, if portions of the interactants were to become more mobile upon protein-ligand binding (Tame *et al.*, 1998). Rather than such localised effects, an overall 'tightening' of a structure may occur when binding takes place (Cooper *et al.*, 1994).

INVESTIGATION OF THE INTERACTIONS BETWEEN pRb, E2F AND HPV E7

5.2

INTRODUCTION TO EXPERIMENTS

I have already described much of the background literature pertaining to the interaction of pRb with E2F and the effect of the HPV E7 oncoprotein in **Chapter 1**. Here, I will summarise the questions that sponsored the work presented and discussed in this section. Several of these issues are closely related to the crystallographic program being undertaken in the laboratory. I was not involved in the X-ray analysis of pRb/E2F, but I will refer to it in order to provide a structural framework and rationale for my biochemical experiments.

For the purposes of X-ray analysis, it is important to be able to define the minimal protein constructs that possess proper functionality. In a two-, or even three-component system, this analysis becomes both more complex and more critical for successful crystallisation.

The first question to be addressed was simply what are the minimal fragments of pRb and E2F required for stable, and physiologically relevant, binding. To some extent the controversy in the literature relating to this, emanates from a very obvious ambiguity. In the literature, the name E2F is used to describe members of the E2F protein family (1-6) and also to describe the physiologically relevant form of the transcription factor – that is the heterodimeric E2F/DP species. As I shall

describe more fully later in this chapter, E2F and DP proteins both have binding sites on pRb. These binding sites are, however, quite distinct and probably non-overlapping. The first part of this chapter therefore deals with the ITC experiments that I carried out to determine which parts of the C-terminal fragment of E2F-1 (**figure 33**) were required for stable binding to pRb.

These measurements were carried out against two different pRb constructs in order to determine the role played by the C-terminal domain of pRb in E2F interaction. These two constructs, pRb_{AB} (containing the A and B subdomains without the intervening spacer) and pRb_{ABC} (containing the linked A-B pocket together with the intact C-terminal domain), are shown schematically in **figure 34**.

The second issue I was interested to address was the basis of the specificity which E2F protein family members (E2Fs 1-5) exhibit towards pRb, p107 and p130 (discussed previously in **Chapter 1, section 1.4.2.2**). Although the extent of the experiments carried out was limited, the results are very interesting and certainly suggest that this will be a fruitful area for future study. The minimal pocket protein-binding domains of E2F-1 and E2F-5 that were used in this set of experiments are illustrated in **figure 33**.

Finally, I will describe the experiments that were carried out to probe the mechanism by which HPV E7 inhibits pRb/E2F complex formation. As described in **Chapter 1** (**section 1.5**), cellular transformation by DNA tumour viruses involves the production of a viral oncoprotein that binds to pRb and prevents its interaction with E2F/DP. Human papillomavirus (HPV), the etiological agent for

cervical cancer, produces the small zinc-binding protein E7 for this purpose. It has already been established (Huang *et al.*, 1993) that residues 17-98 of HPV16 E7 (**figure 35**) are necessary to interfere with E2F/DP binding to pRb. However, it has been unclear how E7 exerts its effects and, in particular, what parts of the E2F protein are required to mediate these effects.

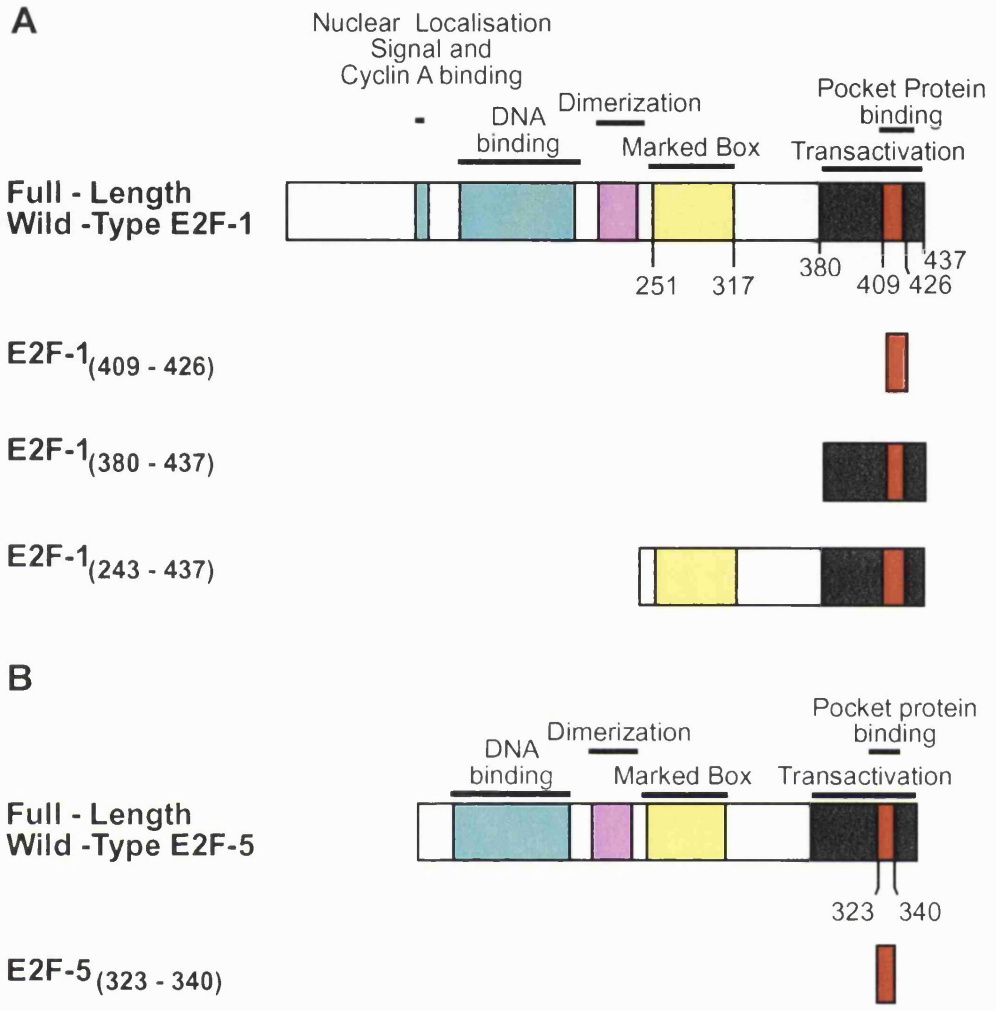


FIGURE 33

E2F PROTEIN FRAGMENTS USED IN ITC EXPERIMENTS

(Adapted from Black *et al.*, 1999)

Residue numbers, for the amino acids of which the construct consists, are in brackets.

A: E2F-1$_{(409 - 426)}$ is the minimal pRb-binding region (Helin *et al.*, 1992); E2F-1$_{(380 - 437)}$ consists of the transactivation domain (Hagemeier et al., 1993) in which the pRb-binding region is embedded; E2F-1$_{(243 - 437)}$ incorporates the marked box region (Lees *et al.*, 1993) as well as the domains in the other E2F-1 constructs.

B: E2F-5$_{(323 - 340)}$ is the pocket protein-binding region of E2F-5, equivalent to residues 409 - 426 of E2F-1(Shan *et al.*, 1996).

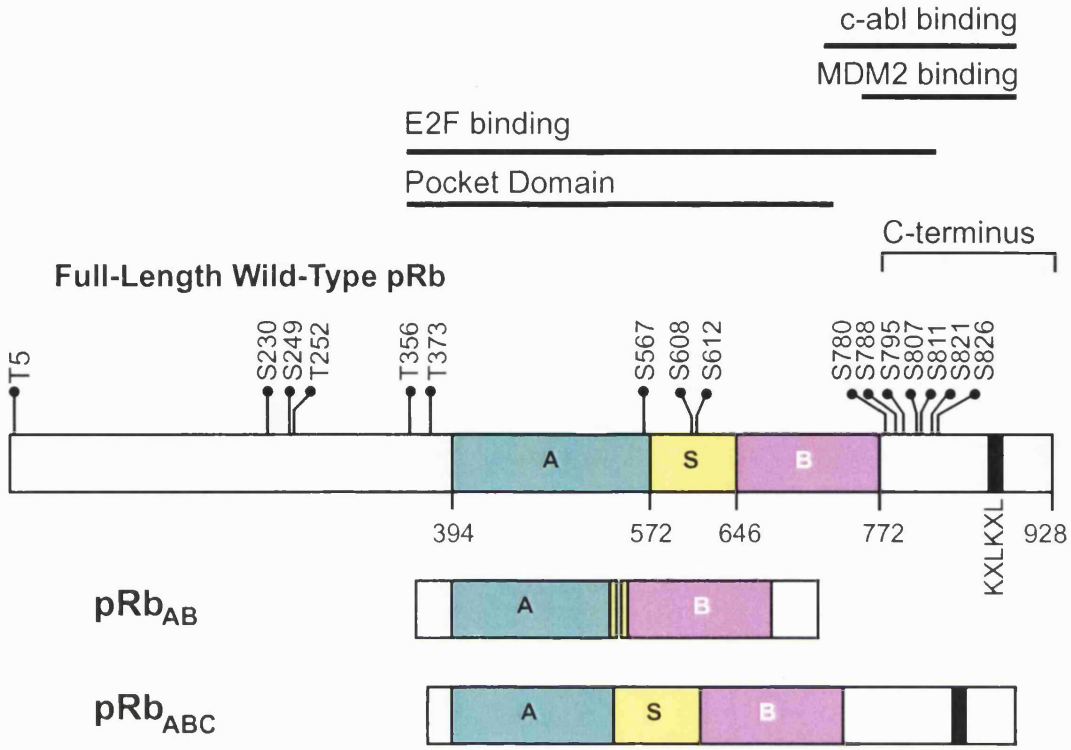


FIGURE 34

FRAGMENTS OF pRb USED IN ITC EXPERIMENTS

(Adapted from Kaelin, 1999)

A and B: subdomains of the pocket domain

S: spacer region

KXLKXL: cyclin-CDK docking site
(single letter amino acid code where x is any residue)

: potential serine/threonine phosphoacceptor site

pRb_{AB}: This construct, based on that in a previous study (Lee et al., 1998), incorporates the A and B subdomains that constitute the pocket region. Treatment of the recombinant pRb_{AB} construct with thrombin allows removal of most of the spacer region (see text). The A and B subdomains remain tightly associated despite removal of the flexible spacer (Lee et al.,1998) whose absence promotes crystallisation. Elimination of the spacer does not, however, change the binding affinity of pRb for E2F (Lee et al.,1998 and results presented in this thesis). The final purified recombinant pRb_{AB} protein consisted of residues 372 to 589 (encompassing the A subdomain) and 636 to 787 (encompassing the B subdomain).

pRb_{ABC}: This construct consists of residues 380-928 and includes the spacer region.

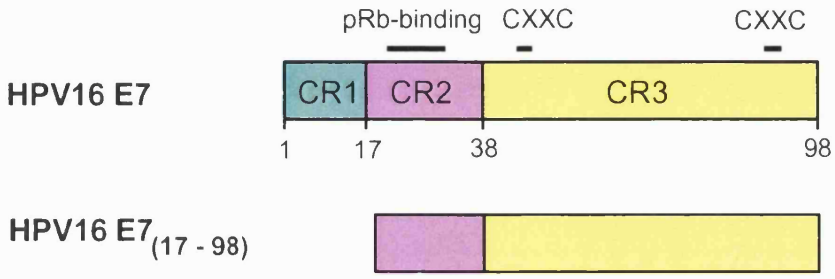


FIGURE 35

HPV16 E7 PROTEIN FRAGMENT USED IN ITC EXPERIMENTS

(Adapted from Clements *et al.*, 2000)

CR: conserved region

The HPV16 $E7_{(17-98)}$ fragment (residues 17-98 of HPV16 E7) consists of the CR2 and CR3 domains. CR2 encompasses the pRb-binding site whose core consists of the LXCXE motif. CR3 contains the two zinc-binding motifs (CXXC).

INVESTIGATION OF THE INTERACTIONS BETWEEN pRB, E2F AND HPV E7

5.3

RESULTS

5.3.1

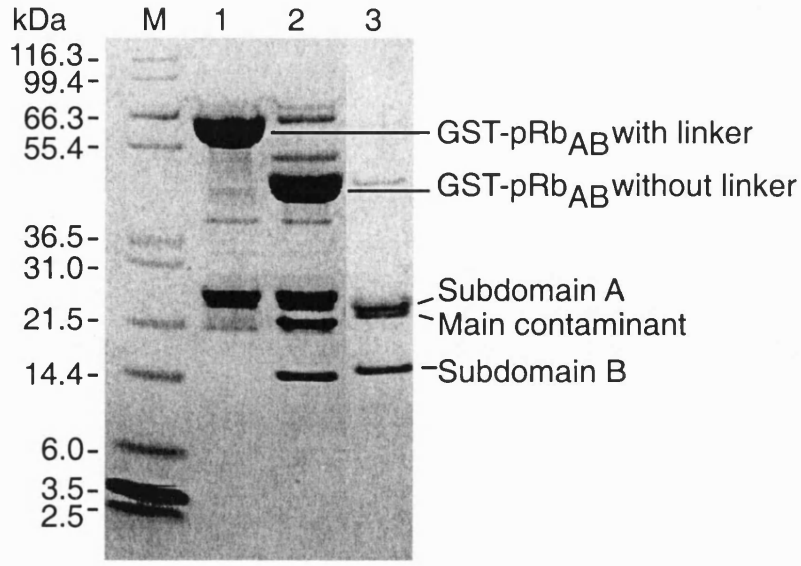
Purification of pRb_{AB}

pRb_{AB} (**figure 34**) was expressed in *E. coli* as a GST-fusion protein with a PreScission™ protease cleavage site located between the affinity tag and the N-terminus of the pRb_{AB} moiety. The linker region between the A and B subdomains of pRb_{AB} was flanked by two thrombin cleavage sites.

Steps of the purification were assessed by SDS-PAGE. After binding of the GST-fusion to glutathione sepharose (**figure 36**, gel A, lane 1), the spacer region was removed by adding thrombin to the beads, the A and B subdomains remaining bound to each other (see **figure 34** and **figure 36**, gel A, lane 2). The GST-tag was removed using PreScission™ protease, allowing the pRb_{AB} protein to elute from the glutathione sepharose while the tag remained bound to the matrix (**figure 36**, gel A, lane 3). The most prominent contaminating protein after this step appeared as a band just below that of the A subdomain on an SDS-PAGE gel (**figure 36**, gel A, lane 3). Contaminants were separated from pure pRb_{AB} by gel filtration chromatography (**figure 36**, gel B). ESMS (**figure 37**) confirmed the integrity and purity of the recombinant protein. The analysis showed that the molecular masses

of the subdomains of pRb_{AB} were close to the calculated values. The subdomains were also the only species detected in the analysis. Each preparation typically yielded 10-15 mg of pure pRb_{AB}.

Gel A



Gel B

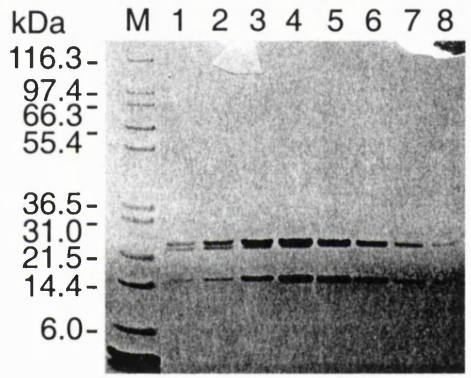


Figure 36 PURIFICATION OF pRb $_{AB}$: SDS-PAGE ANALYSIS

4-12% gels were used in the analysis. The calculated molecular weights of the recombinant proteins are:

GST-pRb$_{AB}$ with linker: 75.6 kDa
GST-pRb$_{AB}$ without linker: 70.7 kDa
Subdomain A of pRb: 25.9 kDa
Subdomain B of pRb: 18.4 kDa

Gel A

Glutathione sepharose beads were boiled in SDS loading buffer after application of the *E. coli* lysate (lane 1) and after treating the matrix-bound GST-fusion protein with thrombin (lane 2). The eluate obtained after the addition of PreScission™ protease to the glutathione sepharose (to remove the GST-tag) is shown in lane 3.

Gel B

Analysis of the fractions obtained from gel filtration chromatography (lanes 1-8) shows the separation of pure pRb$_{AB}$ (lanes 4-8) from the main contaminant (lanes 1-3; also see gel A).

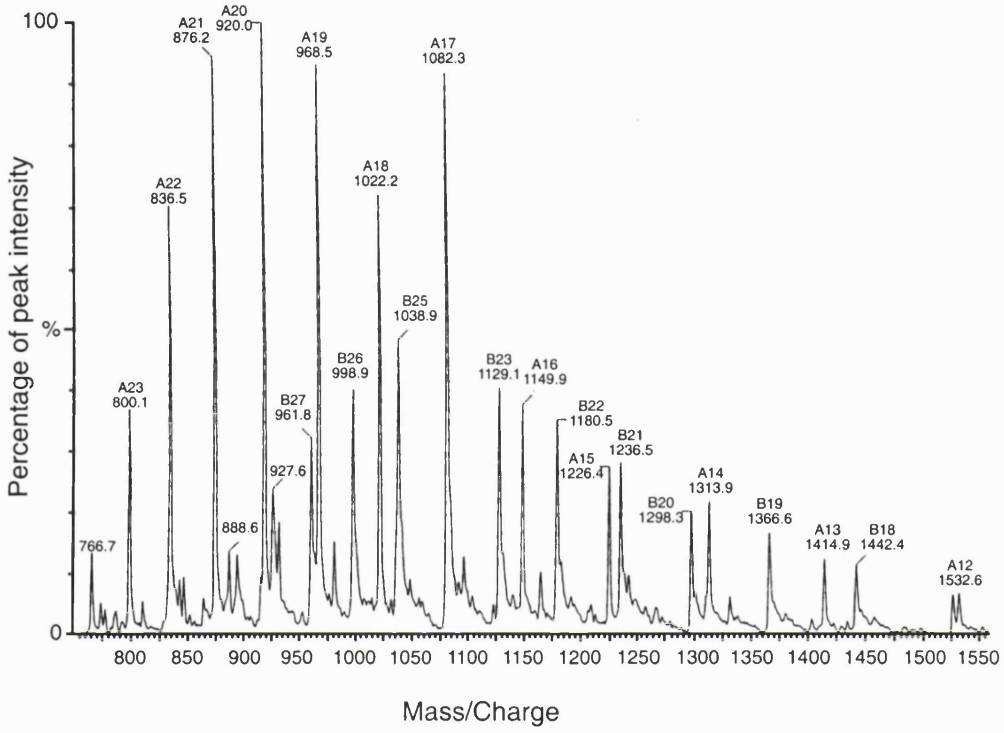


FIGURE 37

MOLECULAR MASS DETERMINATION OF PURIFIED pRb_{AB} BY ESMS

Two species were detected whose masses were determined to be:

A: 18381.08±0.77 Da
B: 25948.14±1.72 Da

The calculated masses of the pRb_{AB} subdomains are:

Subdomain A: 25.9kDa
Subdomain B: 18.4 kDa

5.3.2

Isothermal Titration Calorimetry Experiments

Three sets of ITC experiments (A, B and C) were performed, as outlined in the following pages of this section. The proteins and ligands used for these experiments were pRb_{AB} (purification described on the previous page), HPV16 $E7_{(17-98)}$ (supplied by Professor Marmorstein, University of Pennsylvania), E2F-$1_{(409-426)}$, E2F-$1_{(380-437)}$ and E2F-$5_{(323-340)}$ (synthesised by Dr. W. Mawby, University of Bristol), as well as pRb_{ABC}, and E2F-$1_{(243-437)}$ (purified by Dr. B. Xiao, NIMR). Schematic representations of these recombinant proteins are shown in **figures 33-35** (**section 5.2**).

The data for each of the titrations were processed using the Origin data analysis software (MicroCal Inc.) that was described in **section 5.1.2**. A selection of these results is presented here (**figures 38-45**). The upper panels show the heat output per injection while the lower panels show the isotherm curve, fitted according to a single site model. The results presented were obtained after correcting for heat of dilution. Values determined from the titrations are summarised in the following tables (**tables 4-10**). Experimental conditions are summarised below each isotherm and table. A detailed account of the experimental method can be found in **section 2.4.2**.

Data analysis for each experiment also yields estimated errors (quoted in the following results). This error represents the quality of the fit between the experimental data and the modelled binding isotherm. As such this error is not a measure of the error expected for multiple repeats of the same experiment. In

practice, the observed variations between repeat experiments are significantly greater than the error of the fit for good binding curves. The results presented here are those from actual experiments. In order to be confident that these results were reliable, each experiment was carried out more than once. Although insufficient repeat experiments were performed to be able to calculate an empirical mean and standard deviation for each experiment, the repeat experiments suggest that the variation in measurements (between different runs of the same experiment) is less than 25 %. Although this is a fairly large error, it does not alter the fundamental conclusions drawn from the experiments. Moreover, it is apparent that the biggest variations in measurements occur as a result of using different stocks of protein. The experiments whose results are shown here were carried out using the same protein stocks throughout. It would have been very difficult to produce sufficient protein in one batch to carry out multiple repeats of all the experiments.

Set A: Determination of Minimal Fragments of pRb and E2F-1 Required for Binding

These experiments involved the titration of either pRb_{AB} or pRb_{ABC} with each of three C-terminal E2F constructs ($E2F\text{-}1_{(409\text{-}426)}$, $E2F\text{-}1_{(380\text{-}437)}$ and $E2F\text{-}1_{(243\text{-}437)}$).

	pRb_{AB}		pRb_{ABC}	
	K_d (μM)	N	K_d (μM)	N
$E2F\text{-}1_{(409\text{-}426)}$	0.34±0.03	0.97	0.3±0.03	0.88
$E2F\text{-}1_{(380\text{-}437)}$	0.11±0.01	0.96	$0.07\pm9.8\times10^{-3}$	0.92
$E2F\text{-}1_{(243\text{-}437)}$	<0.01	1.06	<0.01	0.94

TABLE 4
DISSOCIATION CONSTANTS AND STOICHIOMETRIES DETERMINED BY ITC FOR INTERACTION OF pRb WITH E2F-1

Titrations were carried out at 22°C in 50 mM Tris pH 7.6, 100 mM NaCl and 1 mM TCEP. Each of the E2F constructs ($E2F\text{-}1_{(409\text{-}426)}$, $E2F\text{-}1_{(380\text{-}437)}$, $E2F\text{-}1_{(243\text{-}437)}$), at a concentration between 100-150 μM, was titrated into 12-15 μM pRb_{AB} or pRb_{ABC}.

Set B: Investigation of the Specificity of the E2F/pRb Interaction

pRb_{AB} was titrated with either the minimal pocket protein-binding region of E2F-1 (E2F-$1_{(409-426)}$) or that of E2F-5 (E2F-$5_{(323-340)}$).

	pRb_{AB}	
	K_d (μM)	N
E2F-$1_{(409-426)}$	0.19±0.02	0.81
E2F-$5_{(323-340)}$	0.86±0.02	0.72

TABLE 5
DISSOCIATION CONSTANTS AND STOICHIOMETRIES DETERMINED BY ITC FOR INTERACTION OF pRb WITH E2F-1 AND E2F-5

Titrations were carried out at 16°C in 50 mM Tris pH 7.3, 100 mM NaCl, 2 mM βME, 1 mM EDTA. Each of the E2F peptides (E2F-$1_{(409-426)}$ and E2F-$5_{(323-340)}$), at a concentration between 220-223 μM, was titrated into 22-25 μM pRb_{AB}.

Set C: Investigation of HPV E7-mediated Inhibition of pRb-E2F Complex Formation

(i): Either pRb_{AB} or pRb_{ABC} was titrated with HPV-$E7_{(17-98)}$ (**table 6**).

(ii): Stoichiometric complexes were made of HPV-$E7_{(17-98)}$ with pRb_{AB} and with pRb_{ABC} and each was titrated with E2F-$1_{(409-426)}$, E2F-$1_{(380-437)}$ and E2F-$1_{(243-437)}$ (**table 7**).

	pRb_{AB}		pRb_{ABC}	
	K_d (μM)	N	K_d (μM)	N
$E7_{(17-98)}$	<0.01	0.85	<0.01	1.08

TABLE 6
DISSOCIATION CONSTANTS AND STOICHIOMETRIES DETERMINED BY ITC FOR INTERACTION OF pRb WITH E7

Titrations were carried out at 22°C in 50 mM Tris pH 7.6, 100 mM NaCl and 1 mM TCEP. HPV16 $E7_{(17-98)}$, at a concentration between 100-150 μM, was titrated into 10-15 μM of each of the pRb constructs.

	pRb_{AB}/E7		pRb_{ABC}/E7	
	K_d (μM)	N	K_d (μM)	N
E2F-1$_{(409-426)}$	0.36±0.02	1.07	0.39±0.07	0.87
E2F-1$_{(380-437)}$	0.18±0.02	1.01	0.16±0.04	0.96
E2F-1$_{(243-437)}$	0.09±6.6×10^{-3}	1.06	0.36±0.13	0.7

TABLE 7
DISSOCIATION CONSTANTS AND STOICHIOMETRIES DETERMINED BY ITC FOR INTERACTION OF pRb/E7 COMPLEX WITH E2F-1

Titrations were carried out at 22°C in 50 mM Tris pH 7.6, 100 mM NaCl and 1 mM TCEP. Each of the E2F constructs (E2F-1$_{(409-426)}$, E2F-1$_{(380-437)}$, E2F-1$_{(243-437)}$), at a concentration between 100-150 μM, was titrated into 10-15 μM of a stoichiometric pRb_{AB}/E7 or pRb_{ABC}/E7 complex.

The dissociation constants (μM) shown below were presented in the previous tables. Some of the values have been merged into one table here so that comparisons between them can be made more easily during the discussion that follows (**section 5.4**).

	pRb_{AB}	$pRb_{AB}/E7$	pRb_{ABC}	$pRb_{ABC}/E7$
E2F-1$_{(409-426)}$	0.34±0.03	0.36±0.02	0.3±0.03	0.39±0.07
E2F-1$_{(380-437)}$	0.11±0.01	0.18±0.02	0.07±9.8×10^{-3}	0.16±0.04
E2F-1$_{(243-437)}$	<0.01	0.09±6.6×10^{-3}	<0.01	0.36±0.13

TABLE 8
DISSOCIATION CONSTANTS FOR INTERACTION OF pRb AND pRb/E7 COMPLEX WITH E2F-1

Titrations of E2F-1 into pRb: Titrations were carried out at 22°C in 50 mM Tris pH 7.6, 100 mM NaCl and 1 mM TCEP. Each of the E2F constructs (E2F-1$_{(409-426)}$, E2F-1$_{(380-437)}$, E2F-1$_{(243-437)}$), at a concentration between 100-150 μM, was titrated into 12-15 μM pRb_{AB} or pRb_{ABC}.

Titrations of E2F-1 into pRb/E7: These experiments were carried out at 22°C in 50 mM Tris pH 7.6, 100 mM NaCl and 1 mM TCEP. Each of the E2F constructs (E2F-1$_{(409-426)}$, E2F-1$_{(380-437)}$, E2F-1$_{(243-437)}$), at a concentration between 100-150 μM, was titrated into 10-15 μM of a stoichiometric $pRb_{AB}/E7$ or $pRb_{ABC}/E7$ complex.

Some of the K_d values shown previously are again shown here, together with the other thermodynamic measurements associated with these titrations. As with **table 8**, this table is also designed to allow an easy comparison of the data during the discussion (**section 5.4**).

	pRb_{AB}	pRb_{AB}/E7	pRb_{ABC}	pRb_{ABC}/E7
E2F-1$_{(409-426)}$	K_d= 0.34±0.03 ΔH°= −46.11±0.4 TΔS°= −8.85 ΔG°= −37.26	K_d= 0.36±0.02 ΔH°= −39.85±0.32 TΔS°= −3.42 ΔG°= −36.43	K_d= 0.3±0.03 ΔH°= −42.84±0.52 TΔS°= −5.92 ΔG°= −36.92	K_d= 0.39±0.07 ΔH°= −45.98±1.02 TΔS°= −9.71 ΔG°= −36.27
E2F-1$_{(380-437)}$	K_d= 0.11±0.01 ΔH°= −33.06±0.32 TΔS°= 6.28 ΔG°= −39.34	K_d= 0.18±0.02 ΔH°= −24.69±0.31 TΔS°= 13.39 ΔG°= −38.08	K_d= 0.07±9.8x10^{-3} ΔH°= −22.17±0.21 TΔS°= 18.44 ΔG°= −40.61	K_d= 0.16±0.04 ΔH°= −21.01±0.39 TΔS°= 17.34 ΔG°= −38.35
E2F-1$_{(243-437)}$	K_d= <0.01 ΔH°= −27.36±0.07	K_d= 0.09±6.6x10^{-3} ΔH°= −21.44±0.12 TΔS°= 18.41 ΔG°= −39.85	K_d= <0.01 ΔH°= −10.88±0.43	K_d= 0.36±0.13 ΔH°= −14.64±0.82 TΔS°= 21.80 ΔG°= −36.44
E7	K_d= <0.01 ΔH°= −55.90±0.82		K_d= <0.01 ΔH°= −31.54±0.44	

TABLE 9
THERMODYNAMIC PARAMETERS FOR THE INTERACTION OF pRb, pRb/E7 and E7 WITH E2F-1

Units: K_d (μM)
ΔG°, ΔH° and TΔS° (kJ mol^{-1})

Titrations of E2F-1 into pRb: Titrations were carried out at 22°C in 50 mM Tris pH 7.6, 100 mM NaCl and 1 mM TCEP. Each of the E2F constructs (E2F-1$_{(409-426)}$, E2F-1$_{(380-437)}$, E2F-1$_{(243-437)}$), at a concentration between 100-150 μM, was titrated into 12-15 μM pRb_{AB} or pRb_{ABC}.

Titrations of E2F-1 into pRb/E7: These experiments were carried out at 22°C in 50 mM Tris pH 7.6, 100 mM NaCl and 1 mM TCEP. Each of the E2F constructs (E2F-1$_{(409-426)}$, E2F-1$_{(380-437)}$, E2F-1$_{(243-437)}$), at a concentration between 100-150 μM, was titrated into 10-15 μM of a stoichiometric pRb_{AB}/E7 or pRb_{ABC}/E7 complex.

Titrations of E7 into pRb: These experiments were carried out at 22°C in 50 mM Tris pH 7.6, 100 mM NaCl and 1 mM TCEP. HPV16 E7$_{(17-98)}$, at a concentration between 100-150 μM, was titrated into 10-15 μM of each of the pRb constructs.

	pRb_{AB}
E2F-1$_{(409-426)}$	K_d= 0.19±0.02 μM $\Delta H°$= −27.68±0.25 kJ mol^{-1} $T\Delta S°$= 9.53 kJ mol^{-1} $\Delta G°$= −37.21 kJ mol^{-1}
E2F-5$_{(323-340)}$	K_d= 0.86±0.02 μM $\Delta H°$= −17.08±0.49 kJ mol^{-1} $T\Delta S°$= 17.35 kJ mol^{-1} $\Delta G°$= −34.43 kJ mol^{-1}

TABLE 10
THERMODYNAMIC PARAMETERS FOR INTERACTION OF pRb WITH E2F-1 AND E2F-5

Titrations were carried out at 16°C in 50 mM Tris pH 7.3, 100 mM NaCl, 2 mM βME, 1mM EDTA. Each of the E2F peptides (E2F-$1_{(409-426)}$ and E2F-$5_{(323-340)}$), at a concentration between 220-223 μM, was titrated into 22-25 μM pRb_{AB}.

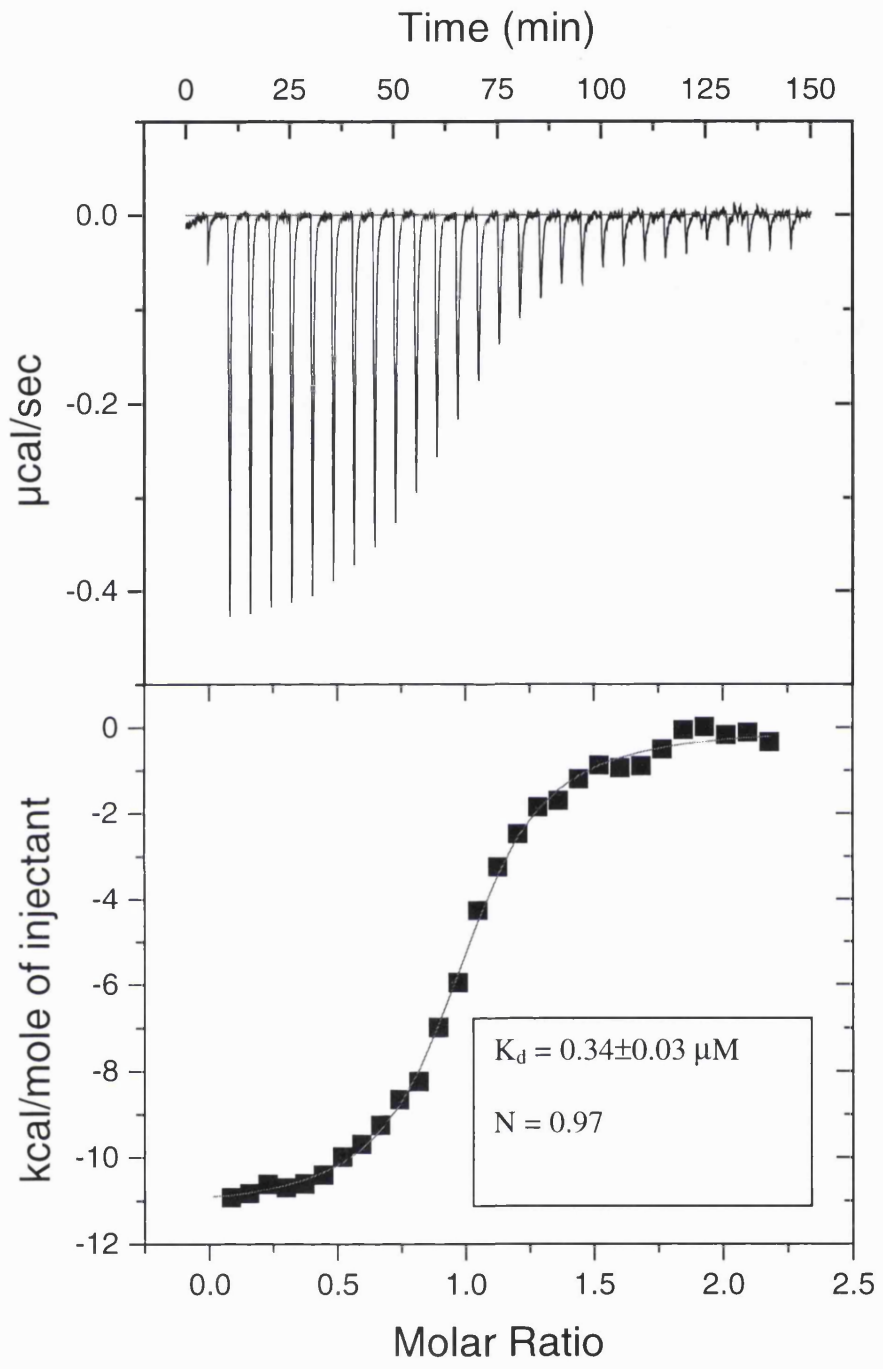


FIGURE 38
ITC TITRATION SHOWING INTERACTION OF pRb_{AB} WITH E2F-$1_{(409-426)}$

This experiment was carried out at 22°C in 50 mM Tris pH 7.6, 100 mM NaCl and 1 mM TCEP. The syringe was programmed to deliver 29 injections (the first being 2 µl and the rest 10 µl each) of E2F-$1_{(409-426)}$ (100-150 µM) into the cell containing pRb_{AB} (12-15 µM).

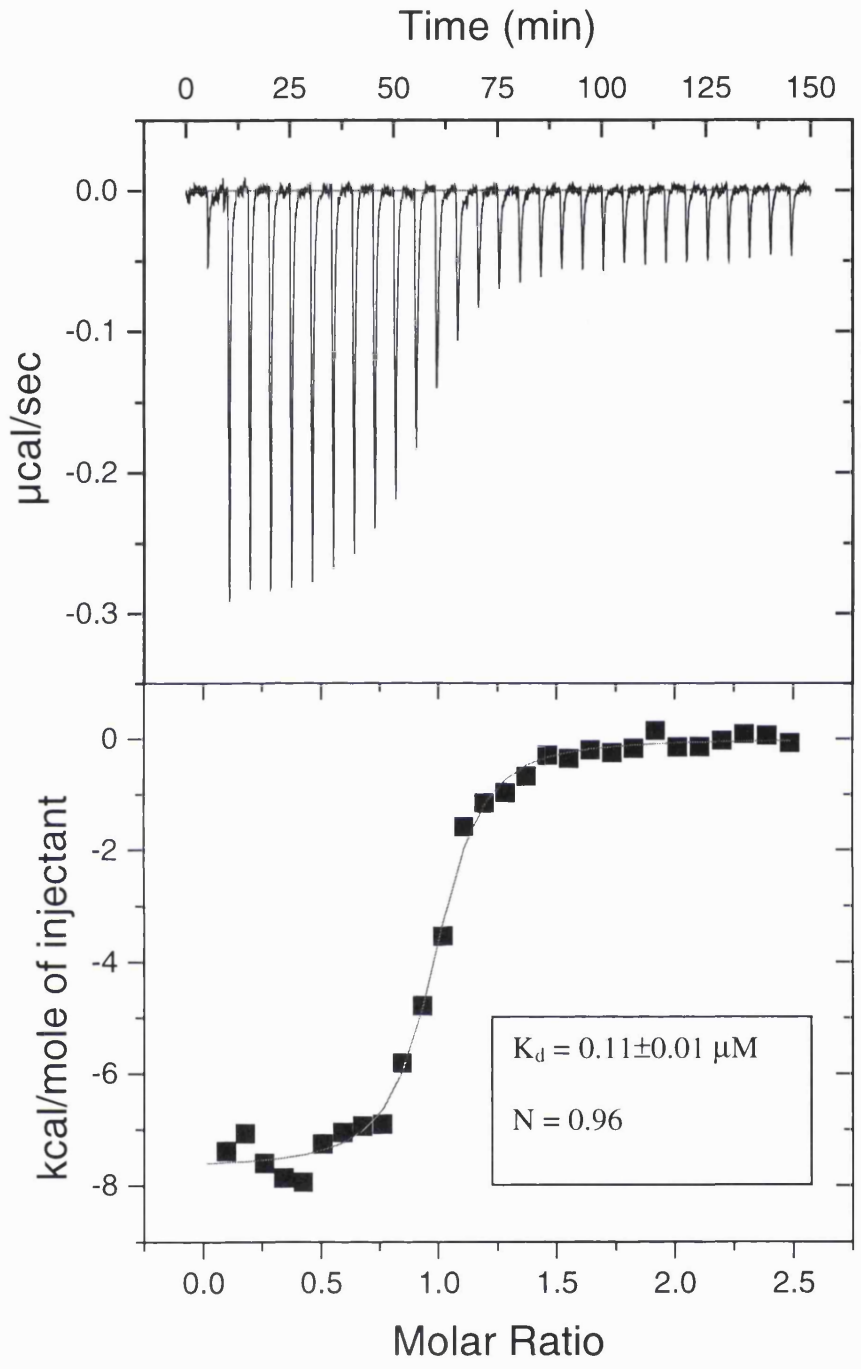


FIGURE 39
ITC TITRATION SHOWING INTERACTION OF pRb_{AB} WITH E2F-$1_{(380-437)}$

This experiment was carried out at 22°C in 50 mM Tris pH 7.6, 100 mM NaCl and 1 mM TCEP. The syringe was programmed to deliver 29 injections (the first being 2 µl and the rest 10 µl each) of E2F-$1_{(380-437)}$ (100-150 µM) into the cell containing pRb_{AB} (12-15 µM).

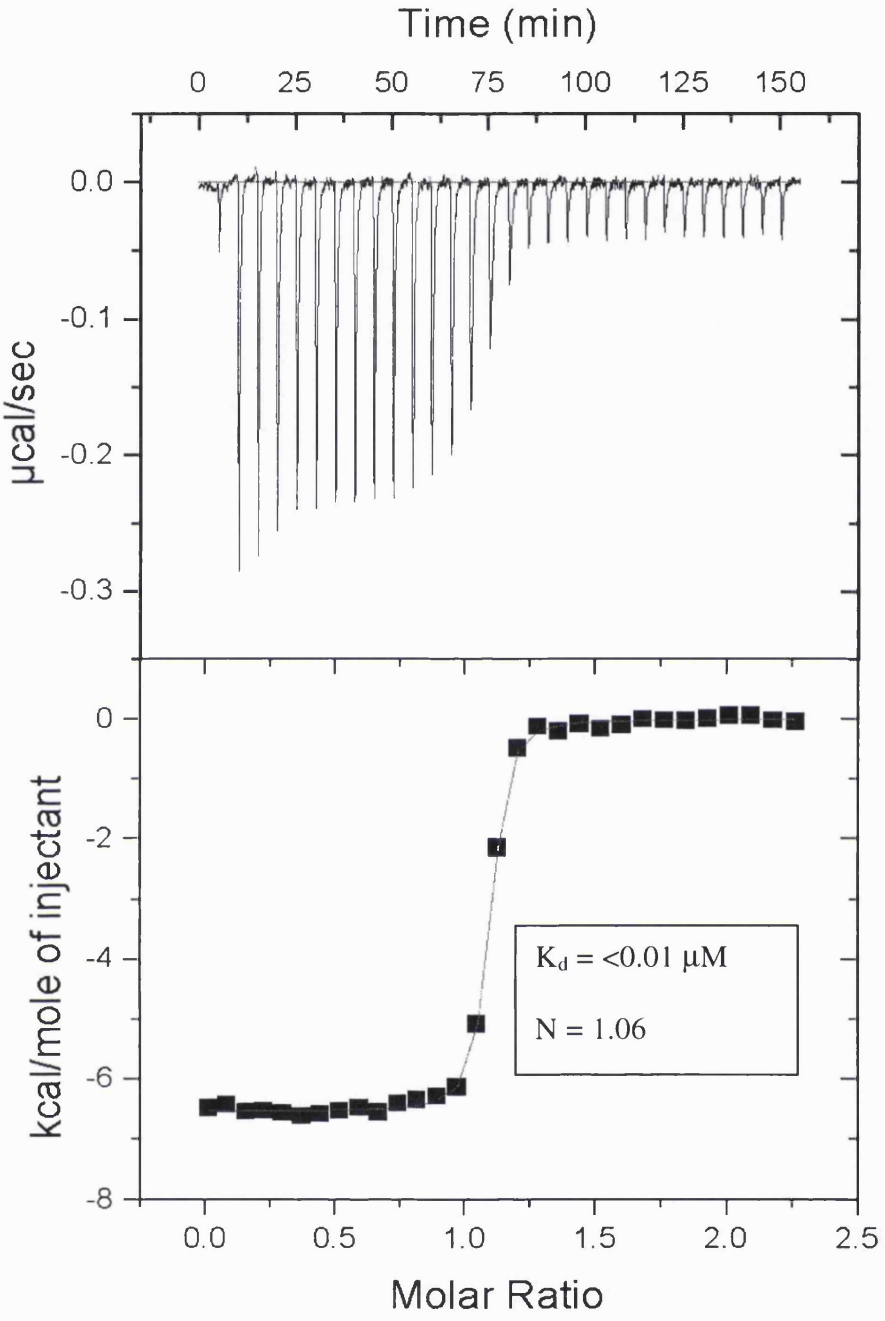


FIGURE 40
ITC TITRATION SHOWING INTERACTION OF pRb_{AB} WITH E2F-$1_{(243-437)}$

This experiment was carried out at 22°C in 50 mM Tris pH 7.6, 100 mM NaCl and 1 mM TCEP. The syringe was programmed to deliver 29 injections (the first being 2 µl and the rest 10 µl each) of E2F-$1_{(243-437)}$ (100-150 µM) into the cell containing pRb_{AB} (12-15 µM).

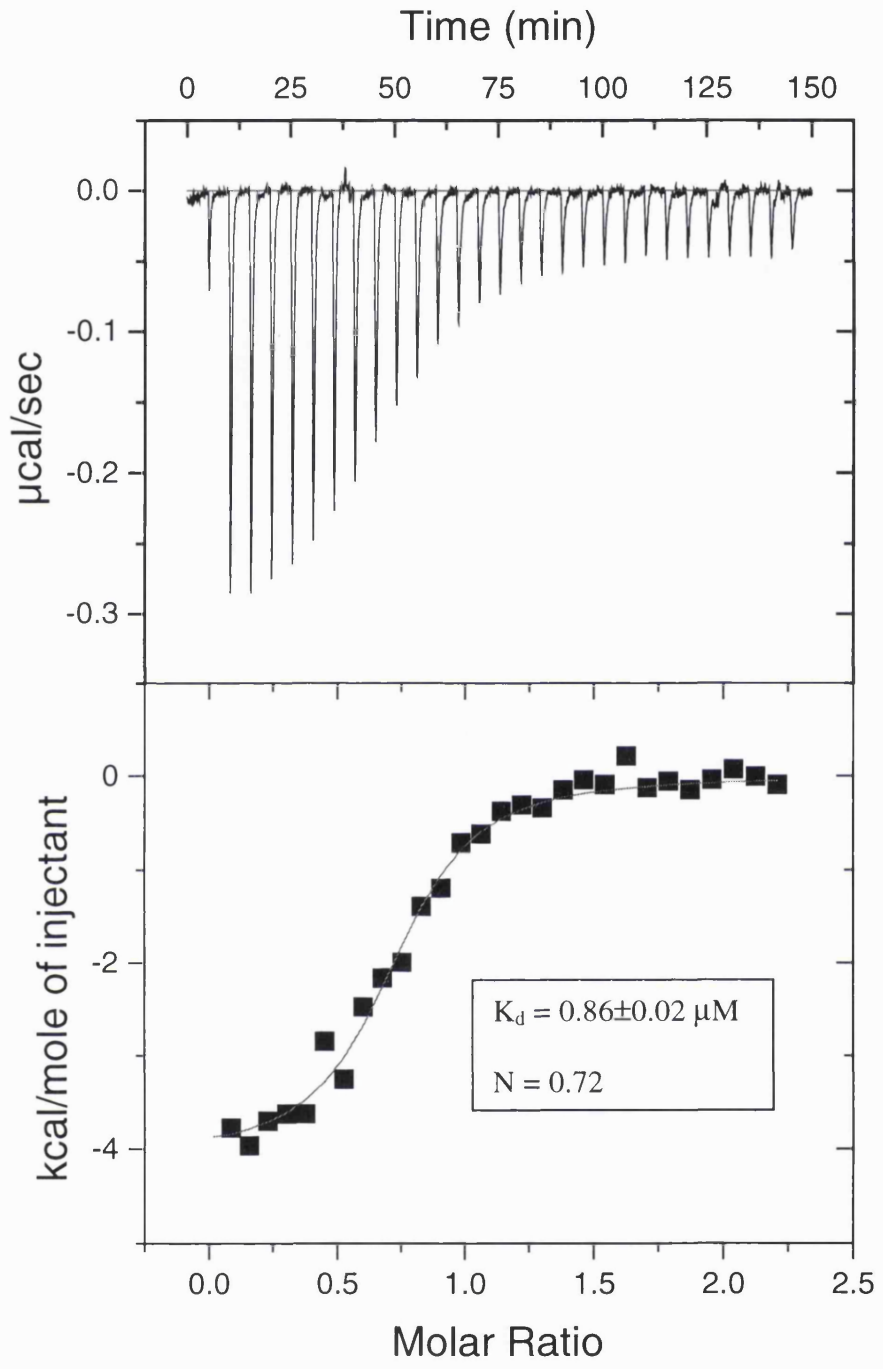


FIGURE 41
ITC TITRATION SHOWING INTERACTION OF pRb_{AB} WITH E2F-$5_{(323-340)}$

This experiment was carried out at 16°C in 50 mM Tris pH 7.3, 100 mM NaCl, 2 mM βME and 1 mM EDTA. The syringe was programmed to deliver 29 injections (the first being 2 µl and the rest 10 µl each) of E2F-$5_{(323-340)}$ (220-223 µM) into the cell containing pRb_{AB} (22-25 µM).

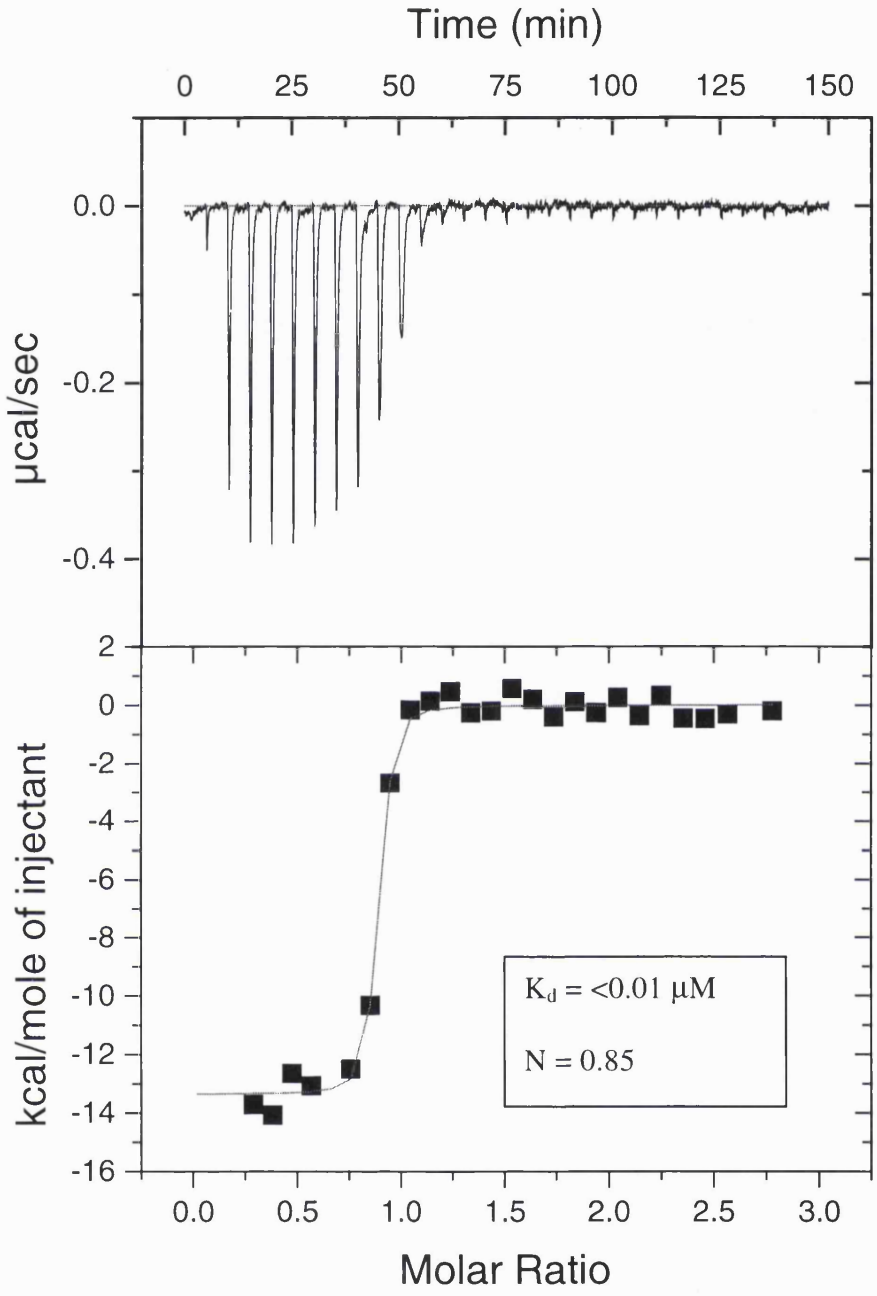


FIGURE 42
ITC TITRATION SHOWING INTERACTION OF pRb_{AB} WITH $E7_{(17-98)}$

This experiment was carried out at 22°C in 50 mM Tris pH 7.6, 100 mM NaCl and 1 mM TCEP. The syringe was programmed to deliver 30 injections (the first being 2 µl and the rest 10 µl each) of HPV16 $E7_{(17-98)}$ (100-150 µM) into the cell containing pRb_{AB} (10-15 µM).

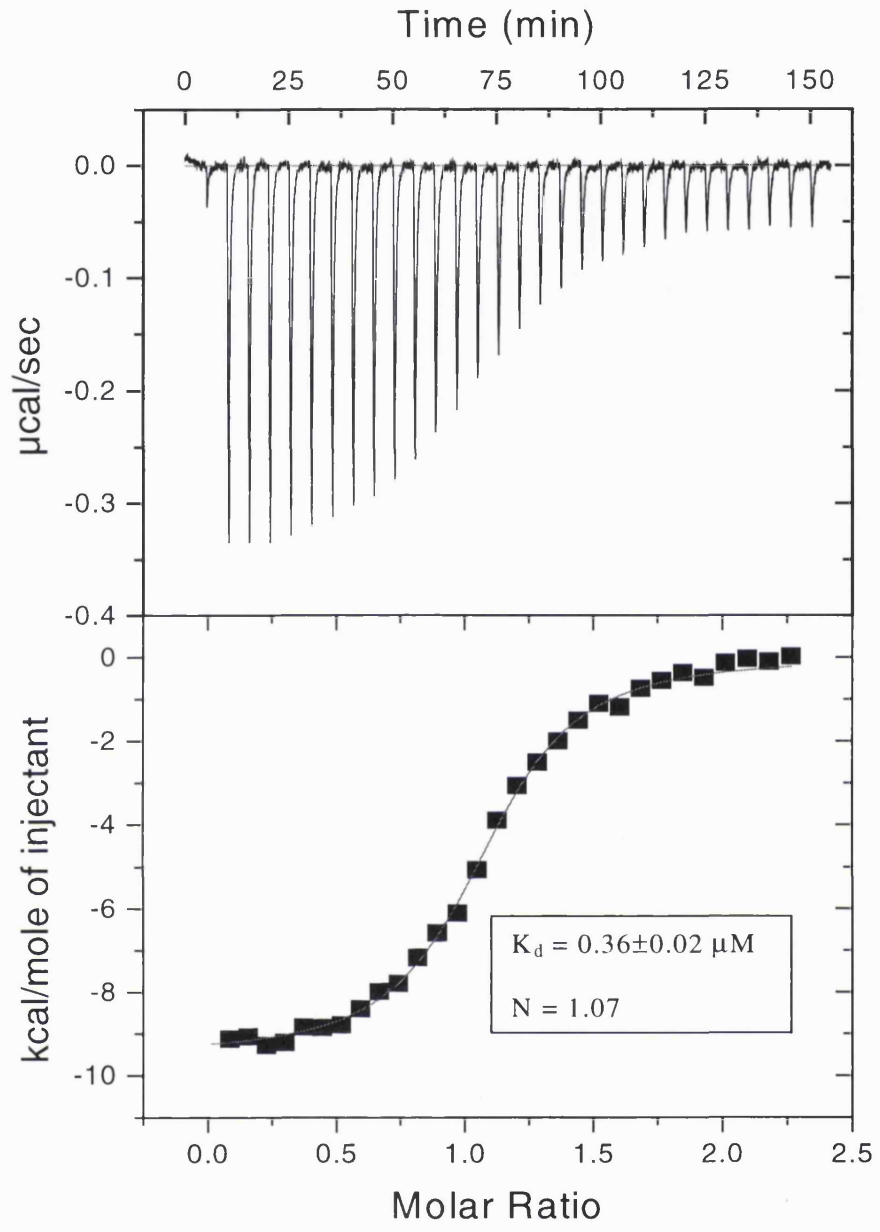


FIGURE 43
ITC TITRATION SHOWING INTERACTION OF pRb_{AB}/E7 COMPLEX WITH E2F-$1_{(409-426)}$

This experiment was carried out at 22°C in 50 mM Tris pH 7.6, 100 mM NaCl and 1 mM TCEP. The syringe was programmed to deliver 30 injections (the first being 2 µl and the rest 10 µl each) of E2F-$1_{(409-426)}$ (100-150 µM) into the cell containing the stoichiometric pRb_{AB}/E7 complex (10-15 µM).

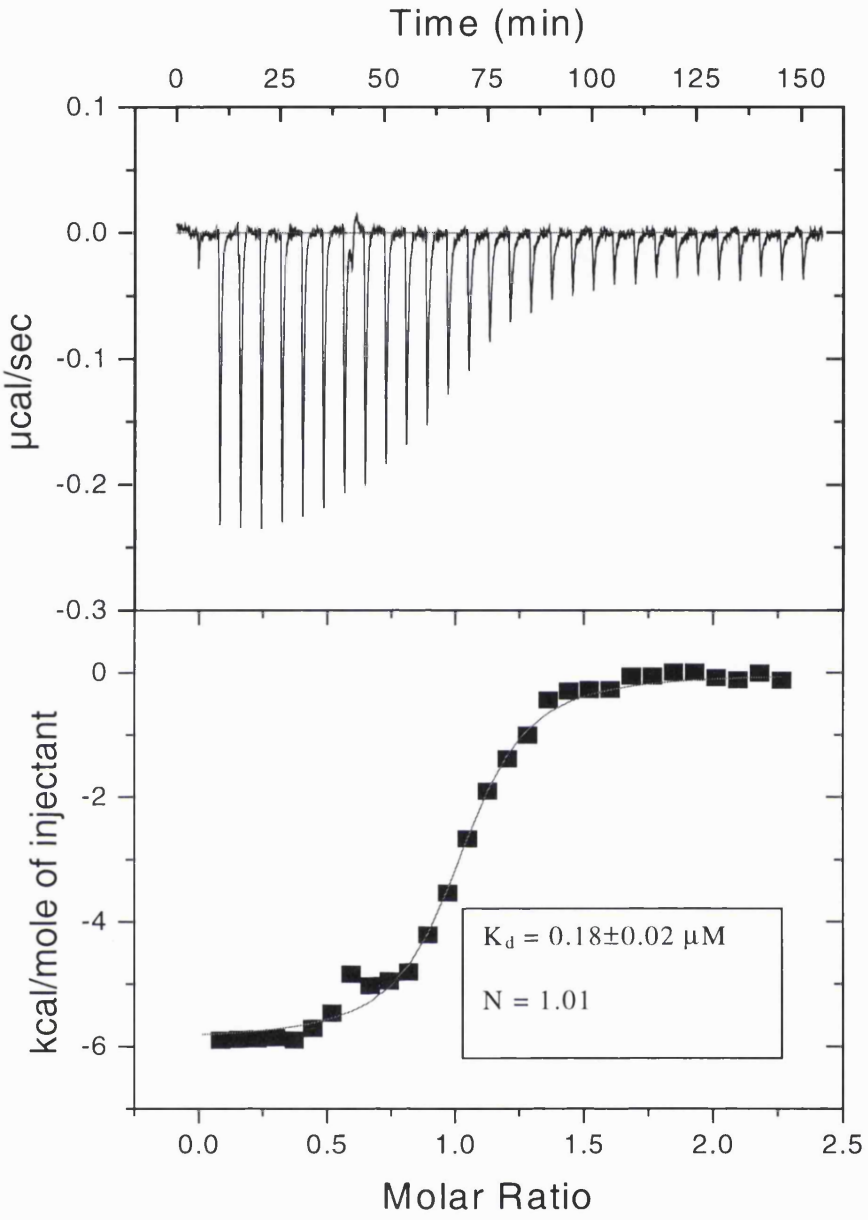


FIGURE 44
ITC TITRATION SHOWING INTERACTION OF pRb_{AB}/E7 COMPLEX WITH E2F-$1_{(380-437)}$

This experiment was carried out at 22°C in 50 mM Tris pH 7.6, 100 mM NaCl and 1 mM TCEP. The syringe was programmed to deliver 30 injections (the first being 2 µl and the rest 10 µl each) of E2F-$1_{(380-437)}$ (100-150 µM) into the cell containing the stoichiometric pRb_{AB}/E7 complex (10-15 µM).

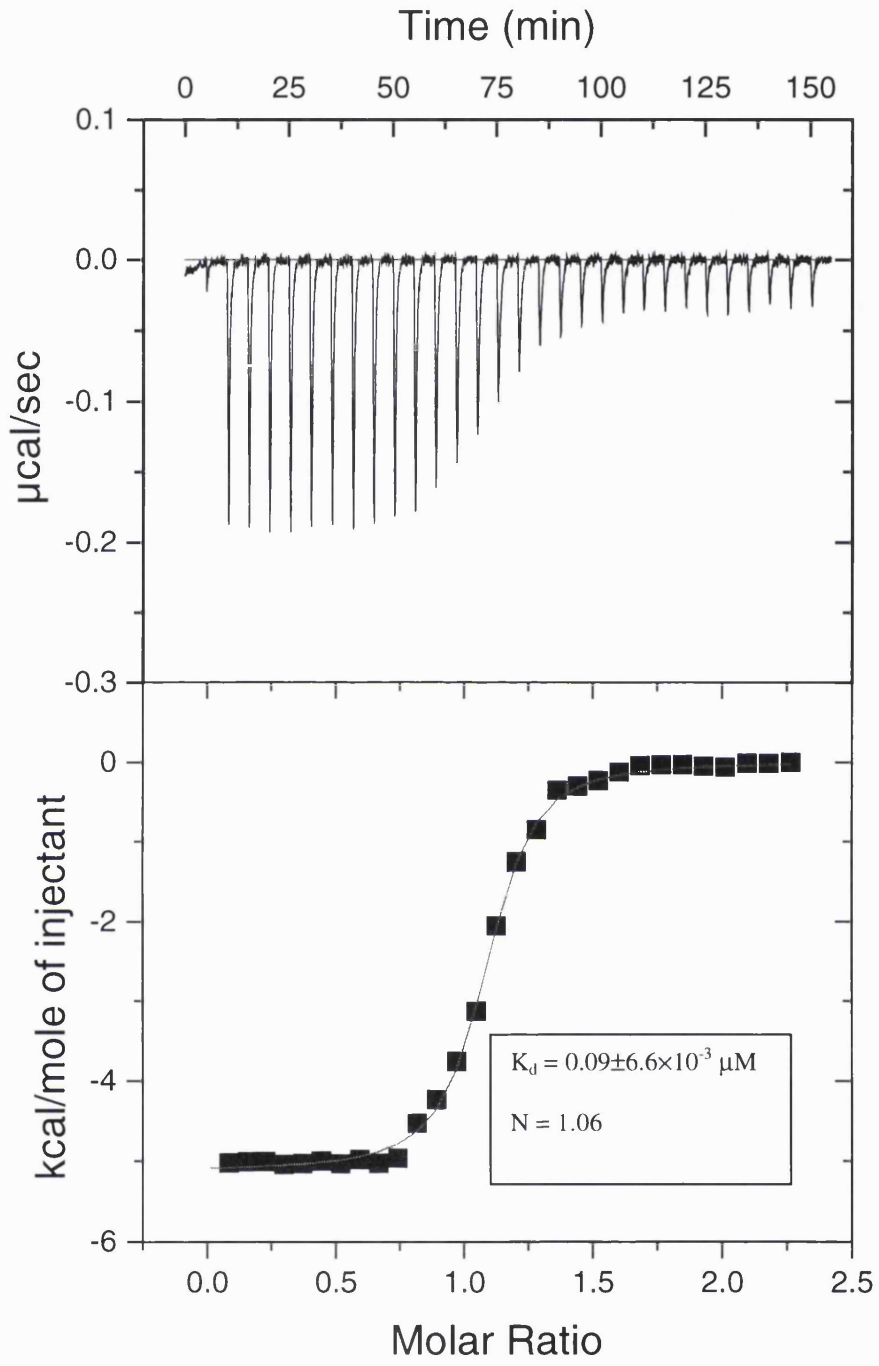


FIGURE 45
ITC TITRATION SHOWING INTERACTION OF pRb_{AB}/E7 COMPLEX WITH E2F-$1_{(243-437)}$

This experiment was carried out at 22°C in 50 mM Tris pH 7.6, 100 mM NaCl and 1 mM TCEP. The syringe was programmed to deliver 30 injections (the first being 2 µl and the rest 10 µl each) of E2F-$1_{(243-437)}$ (100-150 µM) into the cell containing the stoichiometric pRb_{AB}/E7 complex (10-15 µM).

INVESTIGATION OF THE INTERACTIONS BETWEEN pRb, E2F AND HPV E7

5.4
DISCUSSION

5.4.1
Determination of the Minimal Fragments of pRb and E2F-1 Required for Binding

The binding of three different fragments of E2F-1 against pRb_{AB} and pRb_{ABC} were assesed by ITC. The shortest of these, E2F-$1_{(409-426)}$, represents the minimal pRb-binding sequence of E2F-1. This fragment has previously been shown to be necessary and sufficient for pRb recruitment (Helin *et al.*, 1992). The binding constants obtained by titrating pRb_{AB} and pRb_{ABC} with this E2F-1 fragment are shown in **table 4** (see also **figure 38**). These results show that E2F-$1_{(409-426)}$ interacts with essentially the same binding constant to both species of pRb. This provides further evidence to support the data (reviewed in **Chapter 1, section 1.3.1**) suggesting that the AB pocket of pRb represents the primary binding site for E2F.

Next I measured the binding of a larger synthetic peptide to pRb_{AB} and pRb_{ABC}, E2F-$1_{(380-437)}$, which represents the entire activation domain of E2F-1. The binding constants obtained from these titrations are also shown in **table 4** (see also **figure 39**). This peptide bound slightly tighter than the previous one, approximately three

times more tightly to both pRb constructs. Although these differences appear significant in terms of the errors calculated from the fit of the modelled binding curves to the raw data, they represent small differences in binding energy. These small differences in binding energy in turn imply that any additional interactions between pRb and the transactivation domain, outside of the 409-426 region, must be minimal.

The next experiments measured the binding of E2F-$1_{(243-437)}$ to pRb_{AB} and pRb_{ABC}. This E2F-1 construct, unlike the previous two, was over-expressed in bacteria and the binding constants obtained from the ITC experiments are shown in **table 4** (see also **figure 40**). This fragment bound significantly tighter to both pRb constructs than the shorter E2F peptides. The binding constants obtained from fitting the ITC data were tighter than 10nM in both cases. The tightness of the binding for this construct is such that it cannot be accurately determined by ITC within the present experimental setup. Inspection of the titration curves showed that there were very few points which actually lie on the transition of the binding curve (see **figure 40** as an example). Most of the data points were on the plateau at each end of the binding reaction. What this means is that it is not possible to obtain a robust fit of the modelled binding reaction to the (sparse) data points. In order to overcome this problem it would be necessary to repeat the titrations at significantly lower (at least $1/10^{th}$) concentrations of protein in the calorimeter and syringe respectively. Unfortunately, the heat change of the reaction would then be too small to measure reliably. For the present it is only possible to say that there must be an additional interaction of E2F-1 with pRb outside of the transactivation domain which enhances the binding of the minimal

E2F-1 pocket protein-binding fragment by more than ten-fold. These additional interactions are unlikely to be very extensive however, because this region of E2F shows no binding to pRb in the absence of the E2F-$1_{(409-426)}$ segment (Helin *et al.*, 1992).

Since the K_d values for the binding of E2F-$1_{(243-437)}$ to the pRb constructs were not accurately determined, it is not possible to use these data to conclude that this E2F-1 construct has similar affinities for pRb_{AB} and pRb_{ABC}. However, direct competition experiments carried out by Dr. Bing Xiao in my laboratory (Bing Xiao *personal communication*) show approximately equal partition of E2F-$1_{(243-437)}$ binding between pRb_{AB} and pRb_{ABC}. This would be consistent with similar binding constants for the two pRb species.

It has previously been suggested that the so-called 'marked box' region of E2F-1 is required for stable binding of E2F/DP to pRb (O'Connor and Hearing, 1994). The ITC experiments described above suggest that some additional interaction between E2F-1 and pRb occurs within the fragment 243-380. The marked box region of E2F-1 spans residues 251-317 (Lees *et al.*, 1993). It seems likely then, although certainly not proven, that the additional interaction seen here does correspond to that reported by O'Connor & Hearing. To be certain which part of E2F-1 is responsible for this additional interaction will require the construction of further E2F-1 fragments to probe the role of residues between the end of the marked box and the beginning of the transactivation domain.

The results presented here also show that all three of the E2F-1 constructs tested interact with similar binding constants to pRb_{AB} and pRB_{ABC} (**table 4**). This is an important result because it establishes that the AB pocket of pRB is necessary and sufficient for E2F-1 binding. The fact that it is widely quoted in the literature that stable E2F binding to pRb requires an intact C-terminal domain on pRb (Qin *et al.*, 1992) can probably be explained in a different manner. It is the binding of the E2F/DP heterodimer to pRb, and not the E2F protein itself, that is more stable in the presence of the C-terminal domain of pRb. This idea is strongly supported by experiments that show that there is a direct interaction between a part of DP-1 with the C-terminal domain of pRb (Bandara *et al.*, 1994). This interaction occurs in the absence of a functional AB pocket and implies that the E2F/DP interaction with pRB involves a bi-dentate interaction. Thus it appears that E2F-1 interacts with the AB pocket while DP-1 interacts with some part of the C-terminal domain of pRb. Again, it will be necessary to generate expression constructs of DP, capable of producing large quantities of well behaved protein, to test and quantify the DP/pRb interaction further.

5.4.2

Investigation of the Specificity of the E2F/pRb Interaction

Since there is a well-defined preference amongst members of the E2F protein family for pRb, p107 and p130 (see Chapter 1), I carried out an ITC experiment to compare the binding of the minimal pRb-binding fragment of E2F-1 and E2F-5 to pRb_{AB}. The binding constants are shown in **table 5** (see also **figure 41**). These binding data indicate that the minimal pocket protein binding fragment of E2F-1

binds approximately 4.5 times tighter to pRb_{AB} than does the equivalent fragment of E2F-5 ($E2F\text{-}5_{(323\text{-}340)}$). Physiologically, the observation is that E2F-1 binds preferentially to pRb whereas E2F-5 is mainly found in complexes with p130 (as discussed in **Chapter 1, section 1.4.2.2**). Although the ITC data do indicate tighter binding of $E2F\text{-}1_{(409\text{-}426)}$ to pRb_{AB} than $E2F\text{-}5_{(323\text{-}340)}$, the effect seems too small to be meaningful. Inspection of the amino-acid sequences for the minimal pRb-binding fragments of E2Fs 1 to 5 shown in **figure 46**, reveals that there is a high degree of sequence conservation. In order to appreciate the significance of this pattern of conservation across the E2F family members, and its implications for specificity of binding to pRb, I will describe here the main features of the crystal structure of the complex of $pRb_{AB}/E2F\text{-}1_{(409\text{-}426)}$ (**figure 47**) that has recently been solved in the laboratory (S. Gamblin *personal communication*).

5.4.2.1

Crystal Structure of $pRb_{AB}/E2F\text{-}1_{(409\text{-}426)}$ Complex

The A and B domains of pRb_{AB} both have 'cyclin box' architectures (a five-helix structural motif found in cyclins), but only the A domain maintains a stable fold when expressed on its own. The extensive interface between the A and B domains, made up in large part by conserved hydrophobic residues, is evidently necessary to stabilise the structure of the B domain (Lee *et al.*, 1998). There is some evidence to suggest that this structural dependence of the B domain on interface contacts may represent a mechanism for regulation of pRb binding to E2F/DP. Accordingly, cyclin/cdk-dependent phosphorylation of residues in the pocket leads to disruption of certain interactions between the two domains, which

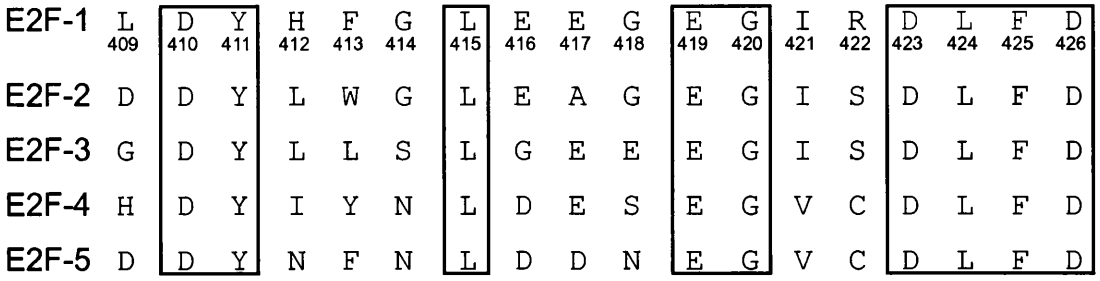


FIGURE 46

E2F RESIDUES CRITICAL FOR pRb BINDING
(Adapted from Shan *et al.*, 1996)

Amino acid sequences of the minimal pocket protein-binding regions of wild-type E2Fs 1-5 are aligned above. Conserved residues are boxed. Residue numbers (of E2F-1) are below the single letter amino acid code.

Amino acid substitutions at five residues in E2F-1 (coloured red) lead to a loss of pRb-binding (Shan et al., 1996). These residues are among those conserved across five members of the E2F protein family as shown above.

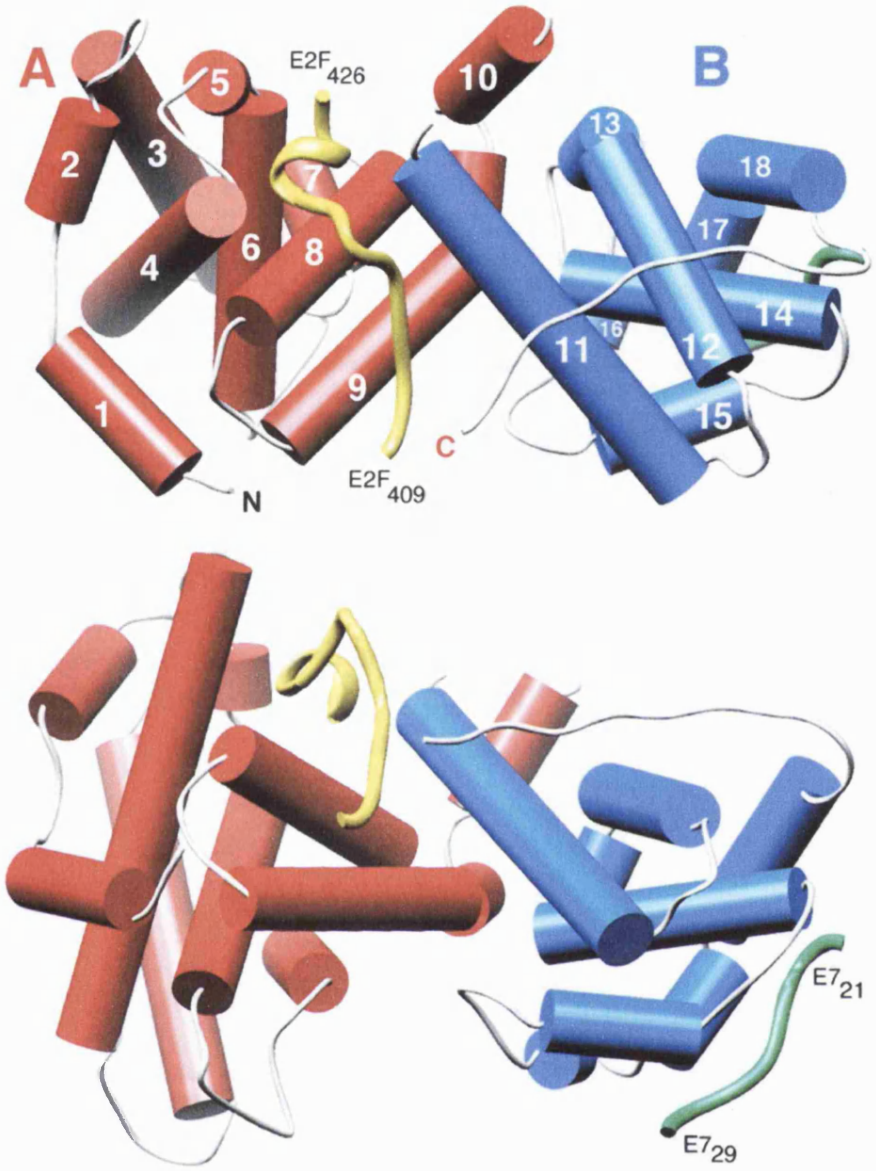


FIGURE 47

CRYSTAL STRUCTURE OF pRb POCKET DOMAIN BOUND TO E2F-1 AND E7 PEPTIDES

Ribbons representation of pRb. Subdomain A of pRb's pocket is shown in red while subdomain B is in blue. The E2F-1 peptide is coloured yellow and the E7 peptide is green. In the upper panel, helices are numbered sequentially. The lower panel is an orthogonal view to that shown above.

The E2F-$1_{(409\text{-}426)}$ peptide binds to the A-B interface of pRb's pocket (S. Gamblin, *personal communication*), while the $E7_{(21\text{-}29)}$ peptide binds to the B subdomain of the pRb pocket (Lee *et al.*, 1998).

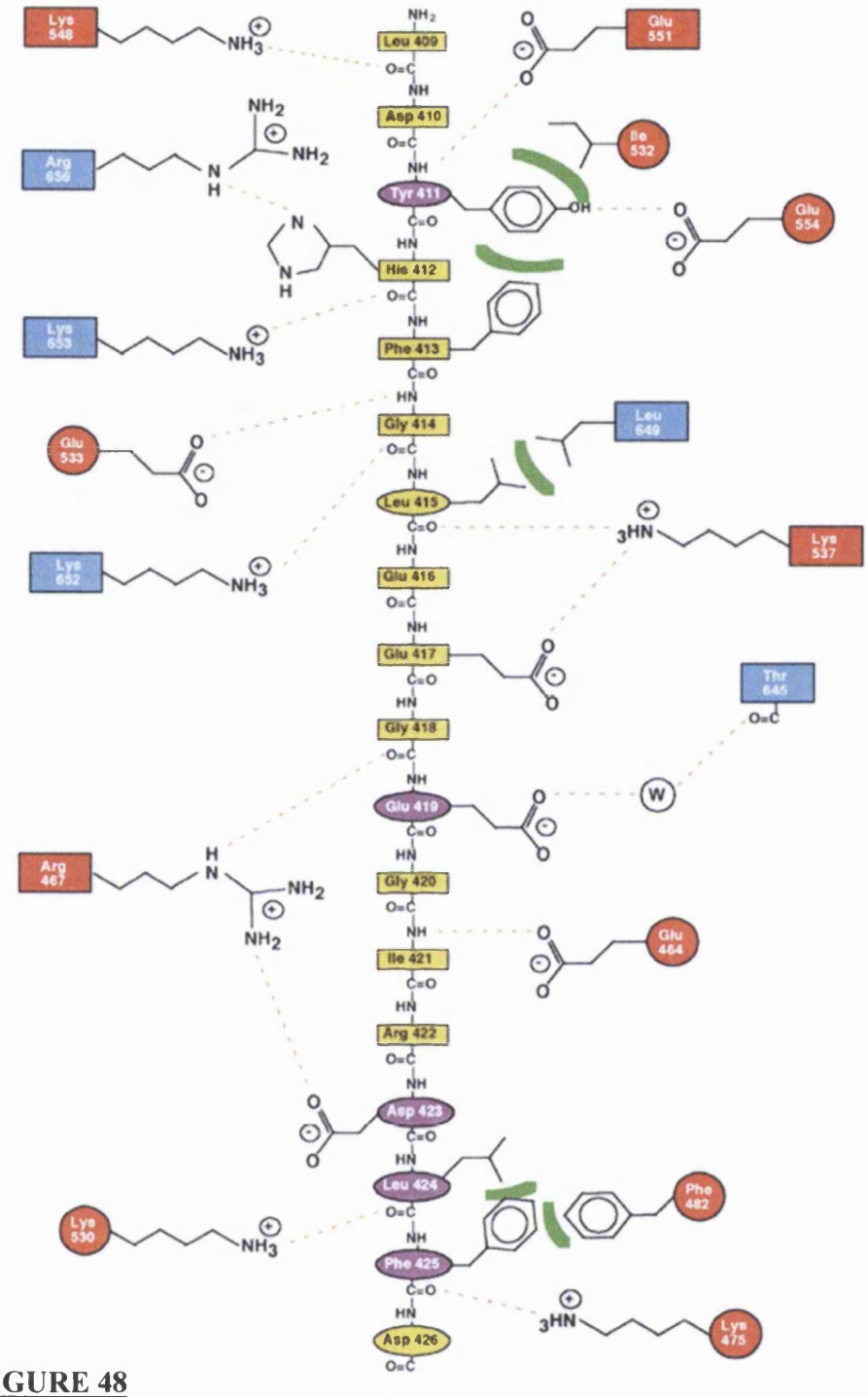


FIGURE 48

SCHEMATIC SHOWING INTERACTIONS BETWEEN E2F-1 PEPTIDE AND POCKET DOMAIN OF pRB

*(*S. Gamblin, *personal communication)*

Colours correspond to those in **figure 47** (red for pRb subdomain A, blue for subdomain B, and yellow for E2F-$1_{(409\text{-}426)}$ peptide), except residues in purple. The latter are the five residues of E2F-1 whose mutation has been shown to lead to loss of binding to pRb (see text). The pRb residues are shown as circles where invariant. The E2F-1 residues are shown as ovals for those residues invariant over E2Fs 1-5 (as shown in **figure 46**).

in turn, leads to breakdown of the AB pocket structure and loss of E2F-1 binding (Harbour *et al.*, 1999). The packing of the A and B domains generates a waist-like interface groove into which E2F-$1_{(409\text{-}426)}$ binds in a largely extended manner, apart from a single helical turn at its C-terminus (**figure 47**). The two end regions of the E2F-1 peptide make extensive contacts with pRb_{AB}, while interactions made by the middle section of E2F-$1_{(409\text{-}426)}$ (residues 416 to 420) are relatively sparse in comparison (**figure 48**). Overall a high proportion of the hydrogen bond interactions between the two molecules involves the side chains of conserved pRb residues interacting with the main chain of E2F-$1_{(409\text{-}426)}$.

Within the E2F-$1_{(409\text{-}426)}$ construct there are seven residues that are conserved across all E2F protein family members (**figure 46**). Amino-acid substitutions at five of these positions have been shown to lead to loss of binding to pRb but retention of the E2F-1 protein's transactivation potential (Shan *et al.*, 1996). The following description focuses on the structural role of these five residues. Tyr(411)-E2F appears to play an important role in peptide binding because its phenolic ring occupies a hydrophobic pocket created by Ile(536)-Rb, Ile(547)-Rb and Phe(413)-E2F, while its hydroxyl group hydrogen bonds to the invariant Glu(554)-Rb. Towards the C-terminal part of the E2F-1 peptide, Leu(424)-E2F and Phe(425)-E2F make several hydrophobic interactions, two of which are with conserved residues. Leu(424)-E2F makes contacts with the aliphatic portion of the side chain of Lys(530)-Rb while also packing against Leu(415)-E2F and Phe(425)-E2F. In addition, Phe(425)-E2F itself packs against Phe(482)-Rb (**figure 48**).

5.4.2.2

Structural Role of Non Conserved Residues between E2F-$1_{(409-426)}$ and E2F-$5_{(323-340)}$

Given the crystal structure just described (S. Gamblin, *personal communication*) it is now possible to assess the role played by those residues of E2F-$1_{(409-426)}$ that are not conserved between E2F-1 and E2F-5. These residues, and the substitutions involved between E2F-1 and E2F-5 are: L409D, H412N, G414N, E416D, E417D, G418N, I421V and R422C (**figure 46**). L409 is the first residue in the E2F-1 peptide from the complex and is not involved in any contacts with the pRb. Consequently, this residue is not well-ordered and its substitution to D in E2F-5 is unlikely to have any effect. H412 of E2F-1 is substituted for N in E2F-5 and, in certain contexts, it is possible for asparagine to make a similar hydrogen bond as histidine. However, in the pRb/E2F structure, H412 sits in a basic pocket created by R787, R656, K653, K652 and H784 of pRb_{AB} and does not seem to make a hydrogen bond through its side chain. The substitution would not therefore seem likely to influence binding. G414 of E2F-1 does not adopt particularly strained phi, psi values and mutation *in silico* does not produce any bad contacts with the pRb. The E2F residues 416-418, which are E E G in E2F-1 and D D N in E2F-5, are the most poorly ordered part of the bound peptide because this part of the structure loops out away from the pRb. Infact, comparison of the conformation of the E2F-1 peptide in the four independent copies present in the crystallographic asymmetric unit, reveals that this is the only part of the peptide where significant

variations are observed. Taken together, these observations strongly imply that differences in E2F sequence at these positions are unlikely to influence binding to pRb. The isoleucine residue at position 421 of E2F-1 is conservatively substituted for valine in E2F-5. The side chain of I421 sits in a shallow hydrophobic pocket on pRb but does not make intimate Van der Waals contacts and again *in silico* mutation does not lead to steric clashes with pRb. Finally, the side chain of R422 of E2F-1 is oriented away from the surface of pRb, with only the first aliphatic part of the side chain making contacts with pRb. Again, it seems unlikely that substitution at this position would greatly affect E2F binding to pRb.

This analysis of the structure of pRb/E2F, with respect to the role of those residues that vary between E2F-1 and -5, confirms the notion that they are not substantially involved in determining binding specificity. As described earlier, the key interactions made by the E2F-1 peptide involve invariant residues. Although this result might have been guessed at in the absence of the crystal structure, it is not a trivial point. Given that E2F functions as a transcriptional activator, there are functional restraints on sequence divergence beyond the need to interact with pRb. The results of the ITC experiment described here, taken with the crystal structure, strongly imply that the respective pocket protein preferences of E2F-1 and E2F-5 do not arise from their minimal pocket protein binding domains.

The question remains as to how different E2F family proteins specifically bind to different pocket proteins. Given the preceding results concerning the somewhat tighter binding of E2F-$1_{(243\text{-}437)}$ than E2F-$1_{(409\text{-}426)}$ to pRb, one of the next experiments will be to make expression constructs for similar fragments from the

other E2F protein family members. It is certainly possible to imagine that the 4.5-fold effect seen between the short E2F-1 and E2F-5 peptides binding to pRb could be significantly enhanced in the context of larger fragments of E2F proteins containing the marked box region.

5.4.3

Investigation of HPV E7-mediated Inhibition of pRb-E2F Complex Formation

It has been shown previously that the binding of E2F-$1_{(409-426)}$ and a peptide based on the LXCXE motif of E7 ($E7_{(21-29)}$), to the pRb pocket, are independent events (Lee *et al.*, 1998). The crystal structure of pRb/E2F-$1_{(409-426)}$ described above, taken with the structure of pRb/$E7_{(21-29)}$ (Lee *et al.*, 1998), shows that the two binding sites on pRb for the peptides, are certainly non-overlapping (**figure 47**) and are indeed more than 30 Å apart on the surface of pRb_{AB}. It has also previously been shown that binding of an E7 'LxCxE' peptide to pRb_{AB} is not sufficient to inhibit E2F/DP from binding to the tumour suppressor's pocket (Huang *et al.*, 1993). Instead, constructs of E7 which contain the zinc-binding domain as well as the 'LxCxE' motif (residues 17-98) are required to mediate this biological function (Huang *et al.*, 1993). I was therefore interested to carry out binding measurements of the three different E2F-1 constructs described above against pRb in the presence and absence of $E7_{(17-98)}$.

This part of the work was made possible by a collaboration between my laboratory and that of Professor Marmorstein at the University of Pennsylvania.

Accordingly, I was given 10's mg quantities of HPV-16 $E7_{(17-98)}$ in a highly pure, concentrated, and monodisperse condition pre-frozen in small aliquots. The first experiments I carried out were simply to measure the binding constant of this protein for the two pRb constructs. The binding constants determined from these two titrations are shown in **table 6** (see also **figure 42**). In both cases the E7 protein binds at better than 10nM to both constructs. As described previously, binding of this tightness cannot be accurately determined in the current experimental setup. Nonetheless these data indicate that the binding of $E7_{(17-98)}$ to pRb_{AB} is significantly tighter than that of the $E7_{(21-29)}$ peptide, since the K_d of the latter interaction, as determined by ITC, is 110 nM (Lee *et al.*, 1998). Other studies have also demonstrated that $E7_{(20-29)}$ has a lower binding affinity for pRb_{AB} than the full-length oncoprotein (Jones *et al.*, 1992). There has previously been some data suggesting that the C-terminal domain of pRb, as well as the A/B pocket, is required for efficient binding of full-length E7 (Patrick *et al.*, 1994). Although the ITC titrations presented above seem to suggest very similar binding of pRb_{AB} and pRb_{ABC} to $E7_{(17-98)}$, it is not possible to exclude the possibility that pRb_{ABC} does indeed bind significantly tighter than pRb_{AB}, but that these differences cannot be detected by the ITC experiments. Indeed, competition experiments (Bing Xiao *personal communication*) suggest that $E7_{(17-98)}$ does bind tighter to pRb_{ABC} than pRb_{AB}. Nonetheless, the data presented here show that there must be significant interactions between the E7 CR3 domain with pRb_{AB}.

Next, I made stoichiometric complexes of $E7_{(17-98)}$ with pRb_{AB} and with pRb_{ABC} and titrated them with E2F-$1_{(409-426)}$, E2F-$1_{(380-437)}$ and E2F-$1_{(243-437)}$. The binding constants obtained from these experiments are shown in **table 7** (see also **figures**

43-45). Three points emerge from these experiments. Firstly, there is little difference between the behaviour of pRb_{AB} and pRb_{ABC} (**table 7**). Secondly, binding of the two shorter E2F-1 constructs to pRb is largely unaltered by the presence of $E7_{(17\text{-}98)}$ bound to the pocket protein (**table 8**). Thirdly, the binding of E2F-$1_{(243\text{-}437)}$ to both pRb constructs is at least ten-fold weaker in the presence of $E7_{(17\text{-}98)}$ (**table 8**). Moreover, the presence of E7 reduces the binding constant for E2F-$1_{(243\text{-}437)}$ binding to pRb to about the value obtained for the two shorter E2F-1 constructs (**table 8**).

These data show that $E7_{(17\text{-}98)}$ competes with E2F-$1_{(243\text{-}437)}$ for binding to pRb but binds independently of either of the two shorter constructs of E2F-1. These results suggest that most likely E7 subverts the normal function of pRb by binding to it in such a way that its zinc finger containing domain (residues 38-98) spatially overlaps with the 243-380 region of E2F-1. This fragment includes the marked box region (residues 251-317) of E2F-1, whose role in pRb binding was discussed earlier in this section. The role of the E2F marked box region in pRb-binding is further supported by the fact that this region of the E2F-1 protein is also targeted by the adenovirus E4 protein. This interaction leads to dimerisation of a pair of E2F/DP heterodimers and loss of pRb binding (O'Connor and Hearing, 1994). It is intriguing that two different DNA tumour viruses should produce proteins that target a segment of the E2F protein that is distinct from the transactivation domain and which makes a much weaker interaction with pRb than the E2F-$1_{(409\text{-}426)}$ fragment. It may allude to a more significant function for the marked box region of E2F proteins than has hitherto been identified.

The fact that the presence of E7 bound to pRb reduced the apparent binding constant for E2F-$1_{(243-437)}$ to a value similar to that observed for E2F-$1_{(409-426)}$ binding to pRb alone seemed intriguing. The notion behind doing such an experiment was that there would be a competition experiment taking place in the ITC cell. Accordingly, the presence of stoichiometric E7 should reduce the apparent binding constant for the E2F-1 construct. The value thus obtained is not a true binding constant. In order to probe these ideas further, another titration of E2F-$1_{(243-437)}$ into pRb was carried out but this time with a 3:1 ratio of $E7_{(17-98)}$ to pRb_{AB}.

Interestingly, approximately the same apparent binding constant for E2F-$1_{(243-437)}$ binding to pRb_{AB} was obtained as in the previous experiment. This result strongly suggests that the binding of E2F-$1_{(243-437)}$ is bi-dentate and that the two binding components are not rigidly linked. The presence of $E7_{(17-98)}$, bound to pRb, prevents the binding of one part of the E2F-$1_{(243-437)}$ construct to pRb but not of the other. As I have already argued, it is likely that it is some part of the 243-380 region of E2F-1 that overlaps spatially with E7. Thus, it seems that the two binding sites on E2F-1 for pRb are both spatially distinct, and flexibly linked.

5.4.4

Conclusions in Terms of ΔH° and ΔS°

In **section 5.1.3**, I described how different interactions influence ΔH° and ΔS° values. In this section, I analyse the ITC results in this context (see **table 9**).

The X-ray crystal structure of the E2F-$1_{(409-426)}$-pRb_{AB} complex (**section 5.4.2.1**) shows that the prevalent contacts between protein and ligand are hydrogen bonds, van der Waals and hydrophobic interactions (S. Gamblin, *personal communication*). The thermodynamic data, summarised in **table 9**, shows that the interaction between E2F-$1_{(409-426)}$ and pRb (pRb_{AB} or pRb_{ABC}) is enthalpically-driven, with a dominant exothermic enthalpy change and an unfavourable entropy change. This suggests that the hydrophobic interactions between protein and ligand are less significant in the context of complex formation than the other non-covalent contacts. The unfavourable entropy change may reflect the restricted mobility of protein and ligand upon binding. For example, a comparison of the crystal structures of pRb's pocket, with and without the E2F-$1_{(409-426)}$ peptide, shows that the α4-α5 loop of domain A in pRb only becomes ordered when making contacts with the C-terminal end of the E2F peptide (S. Gamblin, *personal communication*) (Lee *et al.*, 1998).

There is a successively greater entropic contribution to the free energy of binding (with a correspondingly less favourable $\Delta H°$) as the E2F fragment being titrated into pRb (pRb_{AB} or pRb_{ABC}) becomes larger. As discussed above, the E2F-$1_{(409-426)}$-pRb interaction is enthalpically driven and is associated with a negative $\Delta S°$ value. Where E2F-$1_{(380-437)}$ is titrated into pRb, the enthalpy term is still dominant although the entropy term has become favourable. There is, however, little significant difference between the binding affinities associated with the interactions of these E2F constructs and pRb (discussed in **section 5.4.1**). In the case of the E2F-$1_{(243-437)}$-pRb interactions, an accurate measure of the K_d value (and hence $\Delta G°$ and $T\Delta S°$) could not be obtained for the reasons outlined earlier

(**section 5.4.1**). However, it is apparent that the enthalpy term has become less favourable and the binding has become tighter, (compared to the interactions between the other E2F fragments and pRb), as discussed in **section 5.4.1**. The tighter association thus appears to be driven by an increase in entropy, suggesting the formation of a higher proportion of hydrophobic interactions between pRb and residues 243-380 of E2F-1. This portion of E2F-1 encompasses the marked box region whose role in pRb-binding was discussed earlier (**sections 5.4.1** and **5.4.3**). In the light of the thermodynamic data, it is conceivable that the marked box is involved in hydrophobic interactions with the tumour suppressor's pocket.

Although this general trend in $\Delta H°$ and $\Delta S°$ values is observed with respect to titrations involving E2F fragments and both pRb constructs, there is some difference between the results for E2F-pRb_{AB} and E2F-pRB_{ABC} interactions. The latter titrations have more favourable $\Delta S°$ values compared to the former (at least in the case of interactions involving E2F-$1_{(409-426)}$ and E2F-$1_{(380-437)}$), with correspondingly less favourable enthalpy changes. There are not, however, any significant changes in the binding affinities whether these E2F constructs are titrated into pRb_{AB} or pRb_{ABC}. Even in the case of E2F-$1_{(243-437)}$-pRb titrations, where accurate measurements of the binding constant and change in entropy could not be made, competition experiments suggest that the binding constant is similar when either pRb construct is present (**section 5.4.1**). The less favourable enthalpy associated with E2F-$1_{(243-437)}$-pRb_{ABC} (compared to E2F-$1_{(243-437)}$-pRb_{AB}), is probably also balanced by an increase in entropy such that a similar binding affinity is maintained. This increase in entropy (with minimal change in binding affinity) that has been observed for titrations involving pRb_{ABC} (as opposed to

pRb_{AB}) and the E2F constructs, may reflect the flexibility of the C-terminus of pRb.

Other competition experiments suggest that E7 binds more tightly to pRb_{ABC} than to pRb_{AB} (**section 5.4.1**). Once again, it was not possible to determine the values for thermodynamic parameters (apart from $\Delta H°$) associated with these interactions. However, the $\Delta H°$ value related to the E7-pRb_{ABC} titration is less favourable than that involving E7 and pRb_{AB}. As with the E2F-$1_{(243-437)}$-pRb interactions discussed earlier, a gain in entropy appears to drive the tighter association between E7 and pRb_{ABC}. This suggests that hydrophobic interactions are formed between E7 and the C-terminus of the tumour suppressor that is present in the pRb_{ABC} construct.

In the case of titrations involving the E2F constructs and pRb/E7 (pRB_{AB}/E7 or pRB_{ABC}/E7), there is once again a successive increase in entropy (with correspondingly less favourable enthalpy changes) as the E2F constructs become larger. However, the K_d values for these titrations do not differ significantly. As discussed earlier (**section 5.4.3**), the presence of E7 reduces the binding affinity of E2F-$1_{(243-437)}$ for pRb to approximately that observed for the two shorter E2F constructs, suggesting that the binding of E2F-$1_{(243-437)}$ is bi-dentate and its two binding components (encompassed by residues 243-380 and 409-426) are not rigidly linked. It appears then that the more positive entropy for the interaction of pRb/E7 with E2F-$1_{(243-437)}$, compared to $\Delta S°$ values for pRb/E7 interacting with the other E2F fragments, might reflect the increased molecular flexibility

associated with E2F-$1_{(243-437)}$ when one of its binding components (between residues 243-380) is prevented from binding to pRb by E7.

The phenomenon of enthalpy-entropy compensation (described in **section 5.1.3**) is also apparent from some of the ITC results. The set of titrations involving the E2F fragments and pRb_{AB}/E7 (or pRb_{ABC}/E7), are associated with a wider variation in $\Delta H°$ and $\Delta S°$ values compared to the variation in $\Delta G°$ values which fall into a relatively narrow range. The same is true of titrations involving the two shortest E2F fragments and pRb_{AB} (or pRb_{ABC}).

Results pertaining to the thermodynamic characterisation of the interaction between E2F-$5_{(323-340)}$ and pRb are summarised in **table 10**. As discussed previously (**section 5.4.2**), E2F-$1_{(409-426)}$ binds approximately 4.5 times tighter to pRb_{AB} than E2F-$5_{(323-340)}$. The enthalpy change associated with the E2F-$5_{(323-340)}$-pRb_{AB} interaction is less favourable than that associated with the binding of E2F-$1_{(409-426)}$ to pRb_{AB}. This may reflect the reduced number and strength of non-covalent interactions formed between pRb_{AB} and the E2F-$5_{(323-340)}$ peptide, compared to those formed between the same construct of the tumour suppressor and E2F-$1_{(409-426)}$. The entropic contribution to binding that is associated with E2F-$5_{(323-340)}$-pRb_{AB} is somewhat greater than that associated with E2F-$1_{(409-426)}$-pRb_{AB}. Although this may be indicative of an increase in hydrophobic interactions in the E2F-$5_{(323-340)}$-pRb_{AB} complex, it is not possible to confirm this by simply comparing the sequences of the E2F peptides (**figure 46**) (i.e. in the absence of an E2F-$5_{(323-340)}$-pRb_{AB} structure).

CHAPTER 6

CONCLUSION

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Progression of the cell cycle from G1 to S phase is critically regulated by the interaction of pRb with E2F. In this context, the transcription factor E2F acts as the primary means of localising pRb to specific promoter sites. When bound to E2F, pRb exerts powerful anti-proliferative effects and prevents cells from entering S phase. The anti-proliferative effects of pRb are apparently mediated in two different ways. Firstly, by binding to the transcriptional activation domain of E2F, pRb prevents E2F from interacting with components of the basal transcription apparatus. Secondly, pRb recruits other factors to the E2F/pRb complexes which are then responsible for repression of transcription. This active transcriptional repression seems to be achieved by a variety of mechanisms but includes the binding and inactivation of other transcription factors as well as the recruitment of proteins which regulate chromatin structure. The activities of both E2F and pRb are regulated by cyclin-CDK-dependent phosphorylation. Phosphorylation of pRb at a number of sites by cyclin D-CDK4/6 and cyclin E-CDK2 during late G1 phase leads to loss of E2F binding. Subsequently, phosphorylation of E2F/DP by cyclin A-CDK2 leads to loss of binding of the heterodimer to promoter sites.

To a large extent both E2F and pRb are modular, that is many of their functions seem to be contained within defined domains. This feature is extremely important for studying these proteins because it means that experiments can be carried out with fragments of the two proteins which can be expressed, purified and handled

on a reasonable scale. Whilst the utility of this kind of "divide and conquer" strategy cannot be denied, it is clearly important to remain aware of the limitations of not studying the whole system.

The transcription factor E2F is made up of a heterodimer of E2F (1-6) and DP (1-2). The heterodimeric factor contains binding and phosphoacceptor sites for cyclin-CDK, DNA-binding and dimerisation domains, transcriptional activation regions and sequences with high affinity for pRb. The work described in this thesis has been concerned with characterising several of these activities.

It is apparent that different E2F proteins are differentially regulated by members of the Rb family of pocket proteins. E2F-1, 2 and 3 are bound and regulated by pRb, E2F-4 is found in complex with all three pocket proteins while E2F-5 binds only to p130. The subcellular locations of E2F proteins also vary. E2F-1, 2 and 3 are found exclusively in the nucleus presumably by virtue of their nuclear localisation signals. E2F-4 and 5, on the other hand, seem to require other nuclear factors such as their DP partners or relevant pocket proteins to recruit them to the nucleus.

The fact that at least twelve different heterodimeric combinations of E2F and DP proteins can occur suggests that these complexes may differ in their DNA sequence specificity. However, the empirical data to support this hypothesis remains ambiguous. All E2F/DP combinations bind to, and transactivate, at the consensus E2F site (Zheng *et al.*, 1999). However, *in vitro* binding site selection experiments suggest that E2F-1 and E2F-4 exhibit significant differences in their

optimal target binding sequences (Tao *et al.*, 1997). One possible rationale for these observations is that the differences in binding affinity needed to mediate a distinct biological response may be quite small. In other words a ten-fold difference in affinity between two potential promoter sequences could be sufficient to account for a particular pattern of gene expression. This level of discrimination can be achieved by very small differences in total binding energy. For example, the difference of a single hydrogen bond could produce a ten-fold difference in affinity. In this way biologically important effects can be mediated by differences in interaction affinity which may not be detectable in many assay systems.

One function of E2F is to act as an adaptor or recruitment factor for pocket proteins to particular promoters. Both aspects of the adaptor function are subject to regulation by cyclin-CDK phosphorylation. The binding of E2F/DP to its promoter sites is negatively regulated by cyclin A-CDK2-mediated phosphorylation of DP during S phase while the binding of pocket proteins to E2F is inhibited at G1-to-S by the action of cyclin D- and cyclin E-CDK complexes. However, the molecular mechanisms by which these two events occur remain unclear, although some clues to this process are beginning to emerge.

Cyclin-CDKs, which are responsible for orchestrating cell cycle events by phosphorylation of key regulatory proteins, are themselves subject to catalytic activation by phosphorylation. In this case, phosphorylation of Thr160 (CDK2) on the so-called T-loop leads to a substantial reorganisation of this loop structure. The conformational change appears to be driven by the phosphate group

interacting with three arginine residues, each of which comes from a different part of the structure. These three arginine residues in turn hydrogen bond to other CDK and cyclin groups, thus propagating the effects of the T-loop phosphorylation. The result of these changes includes movements of up to 7Å in the T-loop. Phosphorylation of Thr160 completes the reorganisation of the substrate binding site that is initiated by cyclin binding to CDK (Pavletich, 1999).

E2F/DP binds to DNA by making contacts with both the DNA bases and the phosphodiester backbone (Zheng *et al.*, 1999). It seems feasible that phosphorylation within DP's N-terminus by cyclin A-CDK2, leads to intra-molecular interactions whereby the phosphorylated DP competes with the backbone phosphate groups of the DNA for binding sites on E2F/DP. I have shown here that phosphorylation of DP at just one or two sites in the N-terminal region of DP is sufficient to substantially reduce DNA binding by E2F/DP. Further work will be required to determine if phosphorylation of S98 (DP-1) alone is sufficient to inhibit DNA binding. It may well be that there is functional redundancy amongst the potential phosphoacceptor sites in the N-terminus of DP-1 just as there appears to be in pRb. If the mechanism of negative regulation of DNA binding by DP phosphorylation is one of intramolecular competition for phosphate binding sites then the energetics of such an event are very interesting. It may seem unlikely at first glance that the extensive set of interactions made by E2F/DP with its cognate DNA can be competed by single or double phosphorylation sites in the N-terminus of DP. However, being an intramolecular event, the apparent concentration of phosphorylated DP, with respect to the phosphate binding sites on E2F/DP, is very high and could well enable the DP N-

terminus to compete very effectively with the intermolecular interaction of DNA. It may prove possible to further test these ideas at a structural level, once reasonably large amounts of phosphorylated DP are available. Either by X-ray crystallography or by NMR it may prove possible to determine changes in contacts between the N-terminus of DP and the E2F/DP DNA-binding sites upon phosphorylation of DP.

The nature of the interaction between E2F/DP and pRb, and its regulation by phosphorylation, is one of fundamental importance for understanding the eukaryotic cell cycle. Although no clear mechanism exists for this process, important insights are now available. From the studies described here, several points have emerged. The interaction of the two components of E2F/DP with pRb are distinct events. E2F interacts through its minimal pRb-binding sequence, and probably its marked box region, with the pocket domain of pRb. DP has been shown to interact with the C-terminus of pRb and not the A/B pocket (Bandara *et al.*, 1994). Taken together these observations clarify the ambiguity in the literature as to the requirement of the C-terminus of pRb (that is residues beyond 780) for physiological interaction with E2F. As discussed before, this ambiguity arises because the term E2F is sometimes used to mean members of the E2F family of proteins (1-6) and at other times to mean the heterodimeric E2F/DP transcription factor. Physiological complexes of E2F/DP involve the C-terminus of pRb. Truncation of the C-terminus of pRb leads to a loss of transcriptional repression in cells (Qin *et al.*, 1992). However, the reduction in affinity of this truncated pRb for E2F/DP has been estimated to be about ten-fold (Qin *et al.*, 1992). Whilst this is only a rough estimate of the effect it is consistent with the weak interaction of

DP alone with pRb (Bandara *et al.*, 1994). There is no reason to think that this rather modest change in binding affinity is not sufficient to account for the observed physiological effect.

Although the minimal pRb-binding fragment of E2F-1 (409-426) is sufficient to produce transcriptional repression, in the context of a Gal4 fusion construct (Helin *et al.*, 1993a), its binding to pRb is at least ten-fold weaker than a construct also containing the marked box region of E2F. The presence of an interaction between the marked box of E2F and pRb has previously been suggested (O'Connor and Hearing, 1994) but my studies have suggested a new importance for this interaction. It should be noted, however, that my studies have not ruled out the possibility that residues between the end of the marked box (E2F-1 320) and the start of the transactivation domain (E2F-1 380) are responsible for the additional interaction. Further work will be required to make a construct of E2F-1 (residues 320-437) to validate the role of the marked box. Nonetheless, it seems likely to be correct. Somewhat unexpectedly, the E2F-$1_{(243\text{-}437)}$ construct is required for the action of papillomavirus E7 on pRb. Stoichiometric complexes of pRb/E7 bind E2F-$1_{(409\text{-}426)}$ and E2F-$1_{(380\text{-}437)}$ much the same as pRb alone. However, pRb/E7 exhibits a significantly weaker interaction with E2F-$1_{(243\text{-}437)}$. The most likely explanation for this effect is that there is spatial overlap between the CR3 domain of E7 and the marked box of E2F on the surface of pRb. Interestingly, the presence of E7 bound to pRb appears to reduce the binding of E2F-$1_{(243\text{-}437)}$ by at least ten-fold. Energetically this is a relatively small effect but is apparently sufficient to enable the virus to subvert its host cell's normal cell cycle regulation. However, it should be noted that binding of E7 also leads to enhanced degradation

of pRb (Clements *et al.*, 2000). The relative significance of these two effects on viral over-ride of the cell cycle is not known.

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