CHARACTERISATION OF A CELLULAR TRANSCRIPTION FACTOR WHICH CO-ORDINATES CELL CYCLE EVENTS WITH TRANSCRIPTION

A

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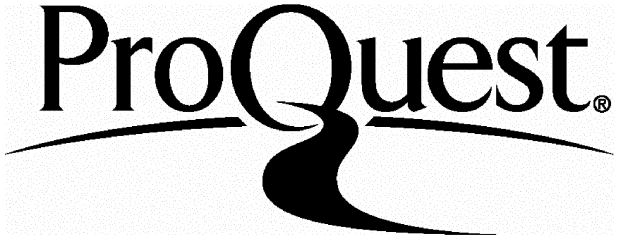

Abstract

Transcription in eukaryotes is mediated by one of three RNA polymerases. RNA polymerase II transcribes all protein coding genes and requires a number of accessory proteins termed transcription factors for the efficient initiation and stimulation of transcription. The cellular transcription factor DRTF1 was originally defined in embryonal carcinoma (EC) stem cells using the adenovirus E2a promoter and is also known to regulate transcription from a number of cellular genes. DRTF1 binds to an E2F motif and resolves as several DNA binding complexes referred to as DRTF1**a**,**b** and **c**, of which the **b**/**c** form is able to activate transcription.

In this study I have shown that DRTF1/E2F complexes vary in different cell types and that the **a** complex can be disrupted by viral oncoproteins, such as adenovirus E1a and SV40 large T antigen. This requires regions within these proteins which are also necessary for the efficient transformation and immortalisation of tissue culture cells suggesting DRTF1/E2F may mediate these processes. These viral proteins sequester a number of cellular polypeptides including the tumour suppressor retinoblastoma gene product (pRb), pRb-related p107, cyclins and cyclin-dependent kinases, which regulate cell cycle progression at a number of different levels. These proteins were also shown to be components of the **a** complex.

The pRb gene is frequently mutated in human tumours, an event which is thought to inactivate the negative growth regulatory effects of pRb. Two mutations identified in small cell lung carcinomas encoded proteins which failed to bind DRTF1/E2F suggesting that the growth suppressing properties of pRb may be mediated at the transcriptional level through DRTF1/E2F.

Cyclin-dependent kinases are believed to exert their control by regulating the activity protein substrates through phosphorylation at key points during cell cycle progression. Cyclin A binds to two kinase subunits, $p33^{cdk2}$ and $p34^{cdc2}$, but was only able to direct $p33^{cdk2}$ to DRTF1/E2F. The presence of a cdc2-like kinase in DRTF1/E2F implies that this transcription factor may play a role in cell cycle control by coupling cell cycle events with the initiation of transcription.

Recently two distinct DNA binding components of DRTF1/E2F have been isolated termed E2F-1 and DP-1. DP-1 is a common component of all E2F site: DNA-binding complexes in certain cell types whereas, based on other studies, E2F-1 is restricted to a proportion of DRTF1/E2F complexes. I have shown that DP-1 and E2F-1 are able to form heterodimers with greater DNA binding activity than either homodimer, an interaction which requires a region of similarity between both proteins. During cell cycle progression DRTF1/E2F complexes are regulated with "free" transcriptionally active DRTF1/E2F appearing in S-phase. DP-1 was found to be a component of all DRTF1/E2F complexes during the cell cycle in NIH 3T3 cells and is likely to form heterodimers with other E2F-1-like molecules during cell cycle progression.

A number of genes expressed in S-phase contain DRTF1/E2F binding sites in their promoters, such as the DHFR, DNA polymerase α, B-myb and cdc2 genes, suggesting that this transcription factor may co-ordinate the expression of these genes during the cell cycle. These results imply that DRTF1/E2F plays a pivotal role in regulating cell cycle progression by enabling cell cycle events to be integrated with transcription.

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Abbreviations:

A	adenine
AP-1	activating protein 1
ATF	activating transcription factor
ATP	adenosine 5'-triphosphate
bHLH	basic helix-loop-helix
BSA	bovine serum albumin
bzip	basic leucine zip
C	cytosine
cdc	cell division cycle
cDNA	complementary deoxyribonucleic acid
cm	centimetre
CRE	cAMP response element
CREB	CRE binding protein
CREM	CRE modulator
CTD	C-terminal domain
CTF	CCAAT-binding transcription factor
CTP	cytosine 5'-triphosphate
D	Dalton
DOC	deoxycholate
DHFR	dihydofolate reductase
DRTF1	differentiation regulated transcription factor 1
DTT	dithiothreitol

E.coli	*Eschericha coli*
E2F	E2 factor
EC	embryonal carcinoma
EDTA	ethylenediaminetetraacetic acid
FCS	foetal calf serum
g	gram
G	guanine
GST	glutathione-S-transferase
GTP	guanosine 5'-triphosphate
h	hour
H_2O	distilled water
HCF	host cell factor
HCl	hydrochloric acid
HEPES	(N-[2-Hydroxethyl]piperazine-N'-[2-ethanesulfonic acid])
HMG	high mobility group
HSV	herpes simplex virus
IPTG	isopropyl-β-D-thiogalactopyranoside
Id	Inhibitor of differentiation
Inr	Initiator
k	1000
kD	kilodalton
KLH	keyhole limpet haemocyanin
l	litre

LAP	liver activating protein
LIP	liver inhibitory protein
PAGE	polyacrylamide gel electrophoresis
PBSA	phosphate buffered saline A
PCNA	proliferating cell nuclear antigen
PE	parietal endoderm
POU	Pit1 Octamer Unc 86
M	molar
mg	milligram
min	minute(s)
ml	millilitre
mM	millimolar
mRNA	messenger RNA
mut	mutant
ng	nanogram
nm	nanometres
NP-40	Nonidet P40
NTP	nucleotide triphosphates
OD	optical density
pRb	retinoblastoma gene product
RNA	ribonucleic acid
RNAse	ribonuclease
RNAP	RNA polymerase

rpm	revolutions per minute
s	second
SDS	sodium dodecyl sulphate
SRF	serum response factor
SV40	simian virus 40
T	thymine
TAF	TBP-associated factor
TBP	TATA binding protein
TF	transcription factor
Tris	tris(hydroxymethyl)methylamine
Tween 20	polyoxyethylene sorbitan monolaurate
U	units/uracil
uv	ultra violet
V	volts
VP16	virion protein 16
v/v	volume/volume
w/v	weight/volume
wt	wild-type
μg	microgrammes
μl	microlitre
μM	micromolar
YY1	Ying Yang 1

CHAPTER ONE

Introduction

The control of transcription in eukaryotic cells

Gene expression in eukaryotes is mediated predominantly at the transcriptional level although post-transcriptional and post-translational mechanisms also play an important role (reviewed in Cleveland,1989;Peltz and Jacobson,1992;Melefors and Hentze, 1993). The initiation of transcription requires one of three RNA polymerases, each of which transcribe specific sets of genes. RNA polymerase I (RNAP I) transcribes the ribosomal RNA (rRNA) genes and RNA polymerase III (RNAP III) transcribes those genes encoding small RNAs, such as tRNA, 5S RNA and some snRNAs. RNA polymerase II (RNAP II) is responsible for transcribing all protein encoding genes and has been studied in detail. Much information on the initiation of transcription has been obtained by identifying *cis*-acting DNA sequences required for efficient transcription and characterising the protein factors which bind these sequences.

1.1 Control of transcription by RNAP II

Transcription of a typical RNAP II gene is modulated by DNA sequences which occur both upstream and downstream from the transcriptional start site (summarised in Figure 1.1a,b). Several functionally distinct groups of regulatory DNA sequences have been identified which can be divided into three classes; promoter elements,

enhancer elements and basal elements. The term promoter is used to describe sequences proximal to the transcriptional start site, whilst enhancer generally refers to sequences which activate transcription from many kilobases upstream or downstream of the start site. In contrast basal elements are required for the correct positioning and initiation of transcription. These regulatory sequences are made up of distinct DNA sequences that bind proteins, termed transcription factors, which are themselves necessary for transcription to take place. Transcription factors comprise a heterogenous group of proteins which affect transcription in a number of different ways. They can be broadly divided into two classes; those which mediate basal transcription common to all genes, and those which can specifically activate transcription of genes above basal levels and may be gene specific. In recent years progress in characterising the properties of transcription factors at the molecular level has yielded much information (reviewed in Mitchell and Tjian,1989;Zawel and Reinberg,1992).

1.1.1 Basal factors for RNAP II transcription

Basal transcription factors affect transcription through two types of DNA element. The first, termed the TATA box, is an AT rich sequence found approximately 25-30 nucleotides upstream from the transcriptional start site, whilst the second type of element, termed an initiator (Inr), overlaps the transcriptional start site (Smale and Baltimore, 1989;Smale *et al.*,1991;Carcamo *et al.*,1990). Most RNAP II promoters contain either a TATA motif or an initiator although some contain both (Smale and

Baltimore,1989). Fractionation of cell extracts has revealed several activities required to support basal transcription *in vitro* from TATA containing promoters. These have been named TFIIA,IIB,IID,IIE,IIF, IIH and IIJ and bind the TATA box together with RNAP II in an ordered and sequential manner (summarised in Figure 1.2;Reinberg *et al.*,1987;Buratowski *et al.*,1989;Flores *et al.*,1991). Originally TFIIJ was only required for transcription when pure fractions of TFIID were used, thus in earlier experiments TFIIJ may already have been associated with TFIID (Reinberg *et al.*,1987;Cortes *et al.*,1991). Of these general factors only TFIID binds directly to the TATA box in a sequence-specific manner. After binding of TFIID, TFIIA may also be required to stabilize the TFIID complex although it may not be required for subsequent steps. The IID/A complex next binds TFIIB which then recruits RNAP II together with TFIIF to form a "pre-initiation" complex. This is followed by the sequential addition of TFIIE, IIH and IIJ to complete the pre-initiation complex although RNA synthesis only occurs once nucleotide triphosphates are available (Buratowski *et al.*,1989;Flores *et al.*, 1991).

The carboxyl-terminal domain (CTD) of RNAP II contains multiple tandem repeats of the heptapeptide Tyr-Ser-Pro-Thr-Ser-Pro-Ser which are conserved in human, yeast and *Drosophila* CTDs although the number of repeats varies (Hahn *et al.*,1989;Peterson *et al.*,1990;Hoffman *et al.*,1990;Hoey *et al.*,1990). Two forms of RNAP II have been observed which are due to differential phosphorylation of the CTD; thus the RNAPIIA form is underphosphorylated and preferentially associates with the pre-initiation complex whereas, in contrast, RNAPIIO is heavily phosphorylated and is involved in transcription elongation (Bartholomew *et*

al.,1989;Payne *et al.*,1989;Laybourn and Dahmus.,1990). Recently, the general factor, TFIIH, was shown to possess a kinase activity responsible for phosphorylating RNAP II (Lu *et al.*,1991;1992). Thus, after the assembly of the pre-initiation complex with RNAPIIA, TFIIH may phosphorylate the CTD causing it to disrupt from its association with TFIID such that elongation takes place (Lu *et al.*,1991;1992).

Promoters regulated by Inr elements are thought to use the same general factors as those required for TATA containing promoters but in a different manner. Unlike the TATA motif, Inr sequences appear to be more diverse and therefore it is possible that they may function in a distinct manner . For example, some Inrs in the adenovirus major late (Ad-MLP) and terminal deoxynucleotidyl transferase (TdT) promoters contain a conserved CTCA motif that binds RNAP II weakly but can be further stabilized by providing TFIID,IIB and IIF (Carcamo *et al.*,1990;1991). Thus, in this case Inr dependent promoters use the same general factors as TATA promoters. In contrast, a novel transcription factor, TFII-I, has been identified that binds specifically to one type of Inr although the role of TFII-I is poorly understood and may or may not bind directly to general factors (Roy *et al.*,1991). Another interesting initiator, protein termed YY1 (Ying Yang 1), has been identified that binds to the adeno-associated virus P5 promoter and also to cellular promoters (Shi *et al.*,1991;Park and Atchison,1991). YY1 can bind either close to the transcriptional start site or further upstream in the P5 promoter and it represses transcription from both positions. Interestingly, the adenovirus E1a protein can activate transcription through YY1, thus changing it from a repressor to an activator (Shi *et al.*,1991).

Many TATA-less RNAP II promoters which contain binding sites for the Sp1 transcription factor also require TFIID for transcription (Pugh and Tjian,1990). Therefore, in some circumstances, activating transcription factors (discussed below) may direct TFIID to the start site by a "tethering" function (Pugh and Tjian,1991). Alternatively, it is also possible that TFIID recognises a cryptic DNA sequence within these promoters.

Further information about these basal factors has been generated via their molecular characterisation. For example,TFIID was initially characterised as a 300Kd complex although when the TATA binding protein (TBP) was cloned it encoded a 40Kd polypeptide (Peterson *et al.*,1990;Hoffman *et al.*,1990;Hoey *et al.*,1990). It was predicted therefore that TFIID would be comprised of a number of polypeptides of which TBP would be the DNA binding component. In contrast to purified TFIID, which in a reconstituted system mediates transcription by activating transcription factors such as Sp1 and CTF (CCAAT-binding transcription factor), TBP only mediates basal level transcription and is not influenced by these activating transcription factors (Pugh and Tjian,1990;Tanase *et al.*,1991). Thus, the additional proteins which make up TFIID are also required to mediate activation of transcription (Pugh and Tjian,1990;Tanese *et al.*,1991). Several of these proteins are tightly associated with TBP and have been given the name of TAFs (TBP associated factors) or co-activators (Dynlacht *et al.*,1991). Recently a number of TAFs have been isolated and characterised (Hoey *et al.*,1993;Ruppert *et al.*,1993;Hisatake *et al.*,1993;Dynlacht *et al.*,1993). Of these, only $TAF_{II}250$ appears to bind directly to TBP although it can bind several of the other TAFs such as $TAF_{II}110$ and $TAF_{II}60$

(Weinzierl *et al.*,1993). Certain TAFs, such as $TAF_{II}80$ only bind $TAF_{II}250$ when other TAFs are bound implying TAF-TAF interactions. Therefore the assembly of TAFs with TBP may be sequential and analogous to the incorporation of general factors into a transcription pre-initiation complex (Dynlacht *et al.*,1993). It is also thought that different transcription factors may utilise distinct TAFs. For example, Tanase *et al.* (1991) purified several TAFs required for CTF mediated transcription which could not support activation by Sp1. Furthermore, Sp1 binds specifically to $TAF_{II}110$ implying that the activation domains of transcription factors may bind specific TAFs (Hoey *et al.*,1993). In this manner different activating factors possessing heterogeneous activation domains can modify the common basal machinery by interacting with subsets of TAFs (Weinziel *et al.*,1993).

1.1.2 Activating transcription factors for RNAPII

Activating transcription factors consist of a heterogeneous group of proteins some of which can be cell or tissue specific such as GATA 1 (Martin *et al.*,1990) whereas others, for instance AP1, are more ubiquitous (Angel *et al.*,1988;Lee *et al.*,1988a). The cellular distribution of these transcription factors is believed to be necessary for regulating specific genes and may therefore regulate gene expression in specific cell types. These proteins are modular and can be separated into at least two domains, required for DNA binding and transcriptional activation (Mitchell and Tjian 1989;Frankel and Kim,1991). The DNA binding domain confers the sequence-specific DNA binding properties of the protein and usually contains a cluster of basic residues

capable of forming secondary structures which contact individual bases in DNA (reviewed in Mitchell and Tjian,1989). Many of these domains are predicted to form a amphipathic α helix which fits into the major groove of DNA (O'Neil *et al.*, 1990;Patel *et al.*,1991;Anthony-Cahill *et al.*,1992), although others such as AP-2 and CTF have unusual DNA binding motifs (Williams *et al.*,1988;Santoro *et al.*, 1988). Not all transcription factors bind in the major groove of DNA and some related to HMG (high mobility group) proteins, such as the HMG-I(Y) proteins, can bind AT rich sequences in the minor groove of DNA (Strauss and Varshavsky,1984;Reeves *et al.*,1987). This is mediated through amino acid residues in the DNA binding domain which form an "AT hook" structure (Reeves *et al.*,1990).

Many transcription factors are grouped together because they contain regions of significant amino acid similarity and may therefore form similar structural motifs. When this similarity occurs within the DNA binding domain it often suggests that the proteins in question bind to the same or similar DNA sequence. This is evident with the octamer family (Oct-1 to Oct-10, reviewed in Schöler,1991) which bind to the consensus sequence ATGCAAAT, although some members of the octamer family can bind to a CTCATGA sequence (Schöler *et al.*,1989;Kemler *et al.*,1989;Suzuki *et al.*,1990;Schreiber *et al.*,1990). The octamer proteins were originally identified as factors which bind to regulatory DNA sequences in the immunoglobulin heavy chain enhancer but also bind to the promoters of a number of cellular and viral genes. The octamer proteins may also play an important role during mouse embryonic development as they are differentially expressed early during murine embryogenesis and some such as Oct-4 (also known as Oct-3) are abundant in embryonic stem cells

but not their differentiated derivatives (Schöler *et al.*,1989;Lenardo *et al.*,1989;Rosner *et al.*,1990;Scöhler *et al.*,1990). They contain a conserved POU domain so called because it is conserved between the Pit-1, Octamers and Unc-86 transcription factors (Herr *et al.*,1988). Within this region is a POU specific domain and a POU homeodomain of which the POU homeodomain binds DNA, although the POU specific domain can influence DNA binding specificity through contacting DNA (Sturm *et al.*,1988). The POU homeodomain forms a structure similar to the homeodomain of other transcription factors, such as some *Drosophila* developmental control genes and mouse PAX (paired-box) genes (Hoey and Levine,1988;Schöler,1991;Gruss and Walther,1992). This domain is predicted to form three α helices of which helix two and three form a helix-turn-helix motif similar to bacterial DNA binding proteins (Pabo and Sauer,1984).

In contrast, Sp1, Krox 20 and TFIIIA contain a motif known as a zinc finger which is necessary for these proteins to bind DNA (Kadonaga *et al.*,1987;Chavier *et al.*,1988;Miller *et al.*,1985). The zinc finger is formed by two cysteine and two histidines residues tetrahedrally co-ordinating zinc to form a structure which fits into the major groove of DNA (Miller *et al.*,1985). Within this region are scattered basic residues which may also contribute to DNA binding. A similar structure can also be achieved by four correctly positioned cysteine residues and is found in the DNA binding domain of the steroid hormone receptor family of transcription factors (Krust *et al.*,1986;Kumar *et al.*,1987).

To summarise therefore, many different DNA binding domains have been identified in activating transcription factors which form a variety of structures necessary for

interacting with DNA.

Some transcription factors bind DNA as dimers and are capable of forming either homodimers or heterodimers. This is exemplified by the AP-1 transcription factor which was originally defined on the human metallothionein$_{IIA}$ promoter but also binds to the promoter of a number other cellular and viral genes (Angel *et al.*,1987;Lee *et al.*,1987a;Jones *et al.*,1988). AP-1 was initially found to contain a number of polypeptides, one of which was the c-fos protein and another was the product of the c-jun gene (Bohmann *et al.*,1987;Angel *et al.*,1988;Raucher *et al.*,1988). These two proteins contained regions of amino acid similarity in their DNA binding domain and also in a region adjacent to this was a conserved motif containing several leucines equally spaced by six amino acid residues first noted in C/EBP (Landschulz *et al.*,1988a;b). This was referred to as a leucine zip and was subsequently shown to be a dimerisation domain, although sequences in the DNA binding domain influence the stability of the heterodimer (Neuberg *et al.*,1989;Kouzarides and Ziff,1989). In addition, this region has been predicted to form a coiled coil structure and is thought to undergo a conformational change upon binding DNA which may in turn lead to greater affinity for the AP-1 site (reviewed in O'Shea *et al.*,1991;Kerpola and Curran,1991). Both c-jun and c-fos are members of families of related proteins which may dimerise with each other thus expanding their regulatory potential. Although jun can form dimers with other members of the jun family, fos only forms heterodimers with jun proteins. The jun and fos families can also dimerise with members of the CREB/ATF family, sometimes resulting in a change in DNA specificity and thus extending the number of potential target genes (Hai and Curran,1991). These

transcription factors together are referred to as bzip proteins as they all contain a basic region adjacent to the leucine repeat (Landshulz *et al.*,1988a).

Another common structural motif is seen in a group of transcription factors termed the bHLH proteins, which contain a basic region adjacent to a helix-loop-helix motif. This was originally noted in a number of different transcription factors, such as E12 and E47, which bind to the E box sequences in the immunoglobulin enhancer (Murre *et al.*,1989a). A number of muscle regulatory transcription factors such as MyoD, Myf5 and myogenin contain a similar motif and like the bzip proteins they too can form homo and heterodimers, interactions which require the bHLH region (Murre *et al.*,1989b;Davis *et al.*,1990). The myogenic factors are capable of heterodimerising with the E12 and E47 factors and therefore these proteins may have very different roles depending on the partners they choose (Lasser *et al.*,1991). Some transcription factors contain both bHLH and leucine zip motifs such as c-myc, AP-4 and USF, although the necessities for each structure in DNA binding is not clear (reviewed in Lüscher and Eisenman,1990). C-myc binds as a heterodimer with a specific partner, termed max or myn (Blackwood and Eisenman,1991;Prendergast *et al.*,1991). Dimerisation in this case requires both the bzip and bHLH region of both proteins furthermore, mutation of either the bHLH or the leucine zip impairs the ability of myc to transform cells (Prendergast *et al.*,1991;Lüscher and Eisenman,1990). Therefore, for c-myc at least, both domains are required for the activity of this transcription factor. To further complicate matters Bengal *et al.* (1992) have shown that the bzip, c-jun protein can dimerise with the bHLH MyoD protein, thus a complex network of interactions between transcription factors is likely to occur within

a cell.

The transcriptional activation domains of these proteins are also of a heterogeneous nature although some common features exist. For example, some activation domains such as those in Sp1 and CREB, contain a glutamine rich region or in the case of CTF (CAAT-binding transcription factor), a proline rich region (Courey and Tjian,1988;Mermod *et al.*,1989). Other activation domains are mainly acidic such as those of Gal4 and VP16 (Hope and Struhl,1986;Gill and Ptashne,1987). These proteins are thought to function by binding DNA sequences upstream or downstream of the transcriptional start site and stabilise or concentrate basal factors at the site of initiation, presumably through binding to basal factors such as TAFs (summarised in Figure 1.1b). This may be achieved by looping of the DNA such that these transcription factors are in close proximity to the basal machinery. Indeed, this idea seems likely as it has been supported by electron microscopy data as well as the observation that DNA sequences can activate transcription in *trans* (Théveny *et al.*,1987;Müller *et al.*,1989;Li *et al.*,1991). However, this does not exclude the use of other mechanisms either independently or in combination (reviewed in Ptashne,1986;1988).

1.2.1 The role of chromatin structure in the initiation of transcription

The packaging of DNA into nucleosomes has long been thought to act as an obstacle to transcription. RNA polymerases have been shown to transcribe through chromatin templates *in vitro* indicating that chain elongation is not impeded by

assembled histones (Losa and Brown,1987;Lorch *et al.*,1987). The initiation of transcription *in vitro* however can be inhibited by bound histones probably by competing with transcription factors for DNA sites in promoters (Knezetic *et al.*,1986;Workman and Roeder,1987). For example, transcription of the β-globin promoter can be inhibited by preincubation of DNA templates with histones, although if histones are added after crude preparations of *trans*-acting factors then this does not occur (Emerson and Felsenfeld,1984). However, Taylor *et al.* (1991) showed that some transcription factors such as Gal4 can bind to DNA sites complexed to histones whereas others, like heat shock transcription factor could not implying each protein has different properties. Similar experiments were performed using histone H1 instead of the histone octamer complex and in this case both Gal4 and Sp1 could displace H1 to activate transcription (Croston *et al.*,1991;Laybourn and Kadonaga,1991). Thus, the nature of chromatin may be involved with regulating the initiation of transcription *in vitro*.

1.2.2 Regulation of transcription factors by dimerisation.

As already mentioned transcription factors can form heterodimers with each other. In some cases this can result in altered DNA binding specificity thereby influencing which genes are transcribed. Heterodimerisation can affect the activity of transcription factors in other ways. For example the cAMP response element (CRE) mediates activation by cAMP and has been shown to bind a transcription factor called CREB (CRE binding protein), a member of the bzip family of transcription factors

(Landschulz *et al.*,1988b). Another gene product called CREM (CRE modulator) which antagonises cAMP induced transcription has high amino acid sequence similarity to CREB but lacks the two glutamine rich domains required for transcriptional activation in CREB (Foulkes *et al.*,1991). CREM can down-regulate transcription induced by CREB which is believed to be mediated through creating non-functional CREM/CREB heterodimers (Foulkes *et al.*,1991).

The c-myc protein binds DNA poorly as a homodimer but binds more efficiently as a heterodimer with max (Blackwood and Eisenman,1991;Prendergast *et al.*,1991). Max homodimers can bind DNA but in contrast to myc does not possess an activation domain and hence causes repression of transcription by blocking myc binding (Kato *et al.*,1990;1992;Kretzner *et al.*,1992). When myc and max are expressed together transcription through the myc site is activated. Given the *in vitro* binding data one might assume that myc provides the transcriptional activating function in the heterodimer and max facilitates DNA binding such that the activity of myc can be controlled by limiting the availability of max protein. This is also seen with the jun and fos proteins as fos homodimers do not bind DNA whereas jun homodimers can and jun/fos heterodimers bind more efficiently than either homodimer, although unlike myc and max, both jun and fos contain activation domains. In more general terms, the bHLH proteins can be inhibited by a negative regulator, called Id, which possesses the HLH region but no basic domain (Benezra *et al.*,1990). Similarly I-POU lacks amino acid residues necessary to bind DNA and inhibits DNA binding of the POU protein Cf1-a, presumably by forming non-DNA binding heterodimers (Treacy *et al.*,1991). Therefore, in addition to changing the DNA binding specificity,

transcription can be either stimulated or antagonised by the dimerisation of transcription factors.

1.2.3 Phosphorylation of transcription factors

Phosphorylation as a means of regulating the activity of transcription factors has been studied in detail although only a few examples will be discussed (for review see Hunter and Karin,1992). The DNA binding activity of transcription factors can be both stimulated and inhibited by phosphorylation. For example, the c-jun protein is phosphorylated on several sites, two of which are mitogen induced and three of which are present in quiescent cells (Boyle *et al.*,1991;Smeal *et al.*,1991). The latter are present in the DNA binding domain of c-jun and are believed to prevent DNA binding, since dephosphorylation or mutation of the phosphorylation sites increases DNA binding activity (Boyle *et al.*,1991). The serum response factor (SRF), which binds to a SRE (serum response element) in the c-fos promoter, is stimulated by serum to bind DNA (Treisman,1987,Malik *et al.*,1991). In contrast to the situation with c-jun this increased binding can be inhibited by phosphatase treatment (Manak *et al.*,1990). Thus, the serum-induced phosphorylation of SRF stimulates DNA binding activity. One kinase which may be responsible for phosphorylation of SRF is caesin kinase II since recombinant SRF can be phosphorylated on serine residues by caesin kinase II *in vitro* leading to increased DNA binding (Janknecht *et al.*,1992;Marais *et al.*,1992). However, the relevance of this observation *in vivo* remains to be established.

Phosphorylation of transcription factors need not only affect DNA binding activity to modulate transcription. For example when cells are stimulated to divide with mitogens c-jun becomes phosphorylated on sites in the activation domain (Smeal *et al.*,1991;Pulverer *et al.*,1991). Phosphorylation contributes to increased transcription by c-jun as mutating these residues reduces *trans*activation (Smeal *et al.*,1991). The kinase responsible for this has not been identified although the MAP (mitogen activated protein) kinases are good candidates as they can phosphorylate the same sites *in vitro* that are phosphorylated *in vivo* (Pulverer *et al.*,1991). Thus, phosphorylation of c-jun probably increases the activity of the activation domain. A similar observation has been reported for SRF although in this case an accessory protein is modified. SRF forms ternary complexes on the c-fos SRE with an accessory protein, $p62^{TCF}$ (Shaw *et al.*,1989). Two related Ets domain proteins, Elk-1 and SAP-1 have been shown to possess $p62^{TCF}$-like properties (Hipskind *et al.*,1991;Dalton and Treisman,1992). Recently Elk-1 was shown to be phosphorylated by p42/p44 MAP kinase *in vitro* on sites which are also phosphorylated *in vivo* (Gille *et al.*,1992;Marais *et al.*,1993). These amino acids occur within the activation domain of Elk-1 and are necessary for the transcriptional activity of this factor. Since ternary complex formation may be essential for full SRE function and phosphorylation may stimulate ternary complex formation, one mechanism for regulating transcription could be through modulating these complexes (Gille *et al.*,1992). However Marais *et al.* (1993) have suggested that ternary complex formation is not influenced by phosphorylation but does produce a more potent activation domain in Elk-1 which may contribute to greater activation of transcription.

Another intriguing method of regulation involves controlling the cellular distribution of a factor. The NF-κB transcription factor regulates the expression of the immunoglobulin κ light chain gene and is composed of a p50 and p65 subunit (Sen and Baltimore,1986;Kawakami *et al.*,1988;Baeuerle and Baltimore,1989). Both the p50 and p65 subunits are thought to contact DNA and hence NF-κB is thought to function as a heterodimer (Urban *et al.*,1990;Schmitz and Baltimore,1991). Isolation of the gene encoding the p50 subunit gave unexpected information as it encoded a p110 precursor protein which was thought to be cleaved to generate the p50 subunit (Kieran *et al.*,1990;Ghosh *et al.*,1990;Bours *et al.*,1990). In non-stimulated cells NF-κB DNA binding activity can not be detected in the nucleus but can be released from cystolic fractions using detergents (Baeuerle and Baltimore,1988a). This is mediated through an inhibitor of NF-κB called IκB which sequesters NF-κB in the cytoplasm by binding p65 (Baeuerle and Baltimore,1988b). When IκB is phosphorylated *in vitro* by PKC it dissociates from NF-κB,Therefore *in vivo* a similar phosphorylation event could enable NF-κB to translocate from the cytoplasm to the nucleus and activate transcription (Baeuerle and Baltimore,1988b;Ghosh and Baltimore,1990). Recently, IκB has been shown to comprise a family of inhibitory proteins which may be also be regulated by both phosphorylation and dephosphorylation although the exact role of phosphorylation in the control of NF-κB remains to be elucidated (Zabel and Baeuerle,1990;Link *et al.*,1992).

In conclusion, phosphorylation plays a major role in regulating gene expression by controlling the activity of transcription factors at a number of levels. Since many of the kinases involved in these processes are activated by growth factors, it is a

mechanism which allows extracellular signals to be relayed to the nucleus and subsequently alter gene expression.

1.2.4 Alternative splicing

The splicing of genes encoding transcription factors can also be used to regulate transcription. A number of transcription factors contain inhibitory domains which when removed allow greater transcriptional activation or DNA binding. The E12 and E47 factors are generated by alternative splicing of the E2A gene and differ by one exon which encodes the bHLH domains in each. E12 binds DNA poorly when compared to E47 due to an inhibitory domain in E12 which impairs E12 homodimer, but not heterodimer DNA binding activity, thus two very different bHLH proteins can be generated through the splicing of one gene (Sun and Baltimore,1991). The mTFE3 factor binds to the μE3 site of the immunoglobulin heavy chain enhancer and is alternatively spliced to produce mTFE3-L and a shorter mTFE3-S (Roman *et al.*,1991). Both proteins contain HLH and bzip motifs although mTFE3-L activates transcription more efficiently than mTFE3-S. When both forms are expressed together mTFE3-S reduces the activation potential of mTFE3-L presumably by creating a less active heterodimer. Therefore, immunoglobulin gene expression can be controlled by regulating the levels of mTFE3-L and mTFE3-S (Roman *et al.*,1991).

As described earlier the CRE can be activated by CREB and inhibited by CREM (Foulkes *et al.*,1991). However, the CREM gene can also encode an activator of CRE mediated transcription, termed CREMτ, which contains two extra exons coding for

two glutamine rich domains and hence activates transcription (Foulkes *et al.*,1992). Thus CREM, is both an activator and repressor of CRE mediated transcription depending on the spliced variant used (Foulkes *et al.*,1992). Similarly, I-POU can inhibit the binding of Cf1-a, however a spliced variant of I-POU which has only two additional amino acids in the DNA binding domain allows this protein to bind DNA. This converts I-POU from an inhibitor to an activator of transcription (Treacy *et al.*,1992).

Another level of control is exemplified by the LAP (liver activating protein) transcription factor which is enriched in liver cells although its RNA is ubiquitous (Descombes *et al.*,1990). LAP is a member of the bzip family but its gene lacks any intervening sequences. Instead a novel method of protein translation is used to produce different forms of this protein. Sequences within the 5' region of the mRNA transcript allow translation from two initiation sites. The larger message encodes the LAP protein whilst an internal site encodes for LIP (liver inhibitor protein) which inhibits transcription (Descombes and Schibler,1991). The shorter LIP lacks an activation domain but retains the bzip region and can therefore dimerise with LAP. Thus, LIP is able to inhibit LAP mediated transcription and may play an important role in liver development.

1.3 Viral transcription factors

A number of virally encoded proteins regulate the transcription of both viral and cellular genes. For example, the herpes simplex virus type 1 VP16 and adenovirus

type 5 E1a protein are able to regulate transcription without directly binding to DNA in a sequence specific manner (Sadowski *et al.*,1988;Lillie and Green,1989). However, when these proteins are provided with a foreign sequence-specific DNA binding domain, such as the Gal4 DNA-binding domain, they are then able to activate transcription from GAL4 reporter genes indicating that both VP16 and E1a contain potent activation domains (Sadowski *et al.*,1988,Lillie and Green,1989). VP16 has been shown to bind to the RNAP II basal factors TFIID and TFIIB and the region of E1a responsible for transcriptional activation binds TBP. Therefore these proteins can contact the general transcriptional machinery (Stinger *et al.*,1990;Lin *et al.*,1991a;Lee *et al.*,1991).

How do these viral proteins regulate transcription specifically? E1a has been shown to bind to the cellular transcription factor ATF-2 and in so doing may bridge this transcription factor with the basal factors (Liu and Green,1990). Therefore, ATF-2 would provide DNA sequence-specificity for E1a although ATF-2 has an activation domain and activates transcription in the absence of E1a. Nevertheless in adenovirus infected cells one could imagine that E1a has evolved to target proteins like ATF-2 to select for the transcription of viral genes. This is particularly appealing as many of the adenovirus genes activated by E1a contain binding sites for the ATF family although cellular genes regulated by ATF-2 may also be activated (reviewed in Jones *et al.*,1988). Oct-1 has been shown to bind VP16 in the regulation of transcription through TAATGARAT sequences which is related to the octamer binding site and important for regulation of the viral ICP4 gene (O'Hare and Goding,1988;Stern *et al.*,1989;Stern and Herr,1991). Again, VP16 appears to bridge Oct-1 with the basal

machinery although a third protein, termed HCF (host cell factor), is required for VP16 to bind Oct 1. Thus, these viral proteins have evolved to sequester cellular proteins such that they can transcribe their own genes rather than cellular ones.

1.4 RNAP I transcribed genes

Transcription of the rRNA genes by RNAP I is mediated through one type of promoter element which requires at least two transcription factors: promoter selectivity factor (SL1) and upstream binding factor (UBF) to direct accurate promoter-specific transcription (Learned *et al.*,1985;1986;Bell *et al.*,1988). UBF binds specifically to a GC sequence in the human rRNA promoter and is the only protein necessary for promoter binding. In contrast SL1 can only bind to the promoter when UBF is present and produces a cooperative DNA binding complex with extended DNA binding properties. Thus UBF is analogous to the RNAP II activating transcription factors and SL1 similar to the basal factors. Surprisingly, the RNAPII TFIID component TBP was also shown to be a component of SL1 suggesting that different RNA polymerases share common basal factors (Comai *et al.*,1992). The TBP isolated from SL1 contained different TAFs to those in RNAPII basal factor, TFIID, therefore both RNAP I and II utilise TBP but require different TAFs thereby maintaining promoter specificity.

1.5 RNAP III transcribed genes

Transcription from RNAP III promoters is more complex. For example, the VA_1RNA, tRNA and 5SRNA genes have promoter elements downstream of the transcriptional start site whereas the U6 and 7SK genes have upstream promoters and a TATA box. The genes with downstream promoters contain an A and B (also referred to as C) box which binds the multicomponent complex TFIIIC. TFIIIC recruits TFIIIB to a position upstream of the transcriptional start site where RNAP III may be subsequently recruited. The RNAP III promoters containing TATA motifs had been shown to utilise RNAP II activators implying they may use RNAP II basal factors (Murphy *et al.*,1987;Das *et al.*,1988). Furthermore as for RNAP I transcribed genes TBP was required for transcription of the U6,VA1,tRNA and 5S RNA genes since depletion of TBP from RNAP III transcribing extracts abolishes transcription (Lobo *et al.*,1991;Margottin *et al.*,1991;Simmen *et al.*,1991;White *et al.*,1992). In addition the TFIIIB fraction was shown to contain TBP together with some novel TAFs (Taggart *et al.*,1992). Thus as for RNAP I and RNAP II mediated transcription, TBP is an essential component although it appears to be associated with polymerase specific TAFs (reviewed in Rigby,1993).

1.6.1 Cell cycle regulation of transcription

The eukaryotic cell cycle is composed of two main phases, DNA replication (S-phase) and mitosis (M-phase) which are separated by two gap phases (G1 and G2).

During the gap phases it is believed that the status of the cell is checked before proceeding through to the next phase (Pardee,1989). The decision to divide is thought to be made at a specific point in G1 termed the restriction point (Pardee,1989). This was originally defined in cultured cells which, when deprived of growth factors were committed to divide after passing the restriction point but not before. This biological phenomena may be analogous to a similar event in yeast called START (Pardee,1989;Hartwell,1974). Since events such as DNA replication require a high degree of organisation and co-ordination, the expression of specific genes encoding proteins involved in these processes must be tightly controlled to ensure cell cycle progression occurs in the correct manner. Many of the genes necessary for DNA synthesis, such as the dihydrofolate reductase (DHFR), thymidine kinase, DNA polymerase α, PCNA (proliferating cell nuclear antigen) and thymidylate synthase genes are transcribed in a cell cycle regulated manner (Farnham and Schimke, 1985;Stewart *et al.*,1987;Bravo and Macdonald-Bravo.,1985;Pearson *et al.*,1991). The mRNA for these genes peaks at the G1/S boundary, consistent with a role in DNA synthesis. Therefore these genes are likely to be coordinately expressed in a dividing cell after receiving the appropriate cellular signals. Ultimately these signals are likely to alter gene expression by modulating the activity of transcription factors.

1.6.2 Cell cycle regulated transcription factors

A number of transcription factors are regulated during the cell cycle and as cells differentiate. For example the c-myb transcription factor is predominantly expressed

in haematopoietic cells and is down-regulated during the differentiation of certain cell types (Clarke *et al.*,1988;Kirsch *et al.*,1986). In addition the c-myb gene is transcribed in a cell cycle dependent manner with a peak of expression at the G1/S boundary suggesting therefore that it may be required for DNA synthesis in haematopoietic cells (Thompson *et al.*,1986;Golay *et al.*,1991). The myb protein can also bind DNA and activate transcription through a myb responsive element (MRE) although specific target genes have not so far been identified (Biedenkapp *et al.*,1988;Weston and Bishop,1989). Myb is also an oncogene and this combined with the above observations imply that myb regulates genes required for cell cycle progression in haematopoietic cells.

The histone genes are also periodically transcribed during the cell cycle (Heintz *et al.*,1983;Plumb *et al.*,1983). The promoters of these genes do not contain common DNA elements and hence transcription is likely to be regulated by many different transcription factors (Daily *et al.*,1986;Fletcher *et al.*,1987;Gallinari *et al.*,1989). For example, the Oct-1 transcription factor has been implicated in the regulation of the H2B gene (La Bella *et al.*,1988;1989). Although the levels of Oct-1 protein do not vary through the cell cycle it is phosphorylated in a cell cycle-dependent manner being maximally phosphorylated at M-phase (Roberts *et al.*,1991). Since Oct-1 phosphorylation inhibits DNA binding activity the lack of transcription of the H2B gene in M-phase may be partly attributed to reduced binding of Oct-1 (Segil *et al.*,1991).

Several genes encoding transcription factors are transcribed in response to serum stimulation of quiescent cells. For example, transcription from the c-myc gene is

induced after serum stimulation although in cycling cells RNA expression remains constant suggesting that the gene product may be involved in initiating cell division rather than co-ordinating later events (Müller *et al.*,1984). The c-fos and c-jun genes are also induced by serum, although their transcripts appear earlier than myc (Greenberg and Ziff,1984;Müller *et al.*,1984). AP-1 (jun/fos heterodimers) can bind a DNA sequence called the TRE (TPA responsive element) following treatment of cells with phorbol esters (Angel *et al.*,1987;Lee *et al.*,1987a). These chemicals are believed to produce this effect by activating cellular signalling pathways through proteins such as PKC (reviewed in Karin and Smeal,1992). Therefore this signalling may result in the activation of genes containing TREs which in turn could mediate the proliferative functions of the AP-1 transcription factor.

1.6.3 Control of transcription by anti-oncogenes

Recently a number of genes which control the cell cycle have been shown to encode transcription factors. Of particular interest are the products of tumour suppressor genes or anti-oncogenes (Weinberg,1992). Tumour suppressor genes in contrast to the growth promoting oncogenes were originally identified because they are frequently deleted or mutated in human tumours and were therefore thought to negatively regulate the cell cycle.

The p53 gene is a particularly good example as it frequently mutated in a variety of human tumours and expression of the wild-type p53 gene in tumour cells reduces their tumourigenic potential (Levine *et al.*,1991;Chen *et al.*,1990). Microinjection of

p53 protein into tumour cells can also cause growth arrest in G1 suggesting p53 functions as a negative regulator of proliferation (Goodrich and Lee,1992). In addition, a number of viral oncoproteins encoded by DNA tumour viruses, such as adenovirus E1b, SV40 large T antigen and the human papilloma virus E6 protein sequester p53 which could mediate some of the tumourigenic properties of these proteins (Linzer and Levine,1979;Lane and Crawford;1979;Werness *et al.*,1982; Sarnow *et al.*,1990). The p53 protein has been implicated in a number of biological processes such as apoptosis, DNA replication and DNA repair. Recently p53 was shown to bind DNA in sequence-specific manner and activate transcription whereas some mutant p53 proteins were unable to do so (Kern *et al.*,1991;El-Deiry *et al.*,1991;Funk *et al.*,1992;Farmer *et al.*,1992). Furthermore, a transcription activation domain exists in p53 when assayed using the Gal4 DNA binding domain indicating that it has some of the properties expected of a sequence-specific transcription factor (Fields and Jang,1990;Raycroft *et al.*,1990). In addition specific transcription by p53 can be antagonised by expression of the adenovirus E1b and papilloma virus E6 proteins.Therefore it is likely that p53 functions as a transcription factor (Yew and Berk.,1992;Lechner *et al.*,1992). p53 also binds to and is regulated by the product of the mdm2 (murine double minute 2) oncogene which is able to inhibit p53 transactivation and thus, behaves as a cellular equivalent of the above viral oncoproteins by modulating transcription (Momand *et al.*,1992). Since microinjected p53 protein arrests cells in G1 one might predict that this is mediated through the regulation of genes important in cell cycle progression. Therefore oncogenic proteins, such as mdm2 and E1b, may function by inhibiting the genes activated by p53.

Surprisingly, transgenic mice lacking a functional p53 gene are phenotypically normal although they develop tumours more frequently (Donehower *et al.*,1992). Therefore the role of p53 in cell cycle control is not essential. However since these mice are more susceptible to tumour formation it is probable that the growth suppressing effects of p53 are relevant in the later stages of carcinogenesis in adult mice.

Another example of a tumour suppressor is the retinoblastoma gene product (pRb) which was originally identified in retinoblastomas but was subsequently found to be mutated in a wide variety of other human tumours (reviewed in Weinberg,1992). As for p53, expression of pRb in tumour cells reduces the proliferation rate and also produces less tumourigenic cancers. Also microinjection of pRb protein can arrest cells in G1 (Huang *et al.*,1988;Bookstein *et al.*,1989;Goodrich *et al.*,1991). In addition pRb is likely to be an essential gene since transgenic mice lacking the pRb gene die during embryonic development from a variety of defects (Lee *et al.*,1992;Jacks *et al.*,1992;Clarke *et al.*,1992). As for p53, pRb is also sequestered by viral oncoproteins, such as the E1a, T antigen and E7 proteins through regions which are necessary for the oncogenic effects of these proteins. Thus it was thought that these protein inactivated the growth suppressing properties of pRb (Whyte *et al.*,1988;DeCaprio *et al.*,1988;Dyson *et al.*,1989).

The data presented in this thesis and recent work from other groups has established pRb as a regulator of transcription. A DNA sequence called the retinoblastoma control element (RCE) was first identified in the c-fos promoter and allows pRb to repress transcription (Robbins *et al.*,1990). A similar sequence was also identified in the promoters of the c-myc, insulin-like growth factor II and transforming growth

factor β1 genes which can be both repressed and activated by pRb depending on the cell type used (Kim *et al.*,1991;1992a). Unlike p53 however, pRb does not bind specifically to DNA and therefore is likely to regulate these genes indirectly. Recently, a direct interaction between the pRb and the muscle regulatory transcription factor MyoD has been shown (Gu *et al.*,1993). Since MyoD is believed to activate transcription of muscle specific genes pRb may play a role in cellular differentiation as well as cell cycle control. The role of pRb in transcriptional control will be discussed thoroughly in later chapters.

Finally, the Wilms tumour gene which was noted to be deleted in kidney tumours has also be shown to encode a transcription factor (Call *et al.*,1990,Rauscher *et al.*,1990). The Wilms tumour protein (WT1) contains amino acid sequences similar to the zinc finger motif of Sp1 and TFIIIA (section 1.1.2, Rauscher *et al.*,1990). Indeed WT1 binds to an "Sp1-like" sequence and regulates transcription through this site. As with p53 the target genes for WT1 are unknown but are likely to contribute to negative regulation of proliferation an idea which has been supported by the finding that WT1 can repress transcription from the Insulin-like growth factor II gene through a high affinity WT1 binding site (Drummond *et al.*,1992). In conclusion some of the tumour suppressor genes identified to date encode transcription factors or regulate the initiation of transcription.

1.6.4 Coordinating cell cycle control with transcription

Much has been learnt about cell cycle control from studying the yeast cell cycle as

many of the regulatory mechanisms established in yeast are conserved in the mammalian cell cycle. In the fission yeast, *Schizosaccharomyces pombe*, a number of cell division cycle (cdc) mutants have been isolated which affect specific phases of the cycle. Most notable are mutants which affect the product of the cdc2 gene, which generally cause growth arrest in G2 although some exert their effects in G1 (Nurse and Thuriaux,1980;Nurse and Bissett,1981). The product of the cdc2 gene $p34^{cdc2}$ is a serine/threonine protein kinase which is activated by a B-type cyclin subunit encoded by the cdc13 gene (Booher and Beach,1987;Hagan *et al.*,1988). During the cell cycle, $p34^{cdc2}$ protein levels remain unchanged whilst the cyclin transcript is periodically expressed in a cell cycle-dependent manner, peaking in G2 (Booher and Beach,1987). The cdc2 protein must be phosphorylated on threonine residues and dephosphorylated on a tyrosine residue by the cdc25 phosphatase before the kinase is fully active (Booher and Beach,1986;Gautier *et al.*,1989). Thus, as might be predicted cdc25 mutants which can not dephosphorylate the cdc2 protein cause G2 arrest (Fantes,1979). In *S.pombe* cyclins which are likely to control other phases of the cell cycle have also been isolated but are poorly characterised (Bueno *et al.*,1991). Similar mechanisms of control are apparent in the budding yeast, *Saccharomyces cerevisiae*, where the CDC28 gene encodes the homologue of the $p34^{cdc2}$ kinase and which is again required for both the G1/S and G2/M transitions (Piggot *et al.*,1982;Reed and Wittenberg, 1990). Two groups of cyclins are evident in budding yeast termed CLB (cyclin B) and CLN (cyclin). The CLB cyclins are expressed at the G2/M boundary and bind to CDC28 protein to form a kinase complex. In G1,the CDC28 product binds to the CLN cyclins (also known as START

cyclins) which are thought to be required for progression through G1 (Richardson *et al.*,1989, Wittenberg *et al.*,1990). It is thought that the CDC28/CLB kinase is required for the completion of mitosis whereas the CDC28/CLN complex is necessary for progression through G1, explaining why mutations in CDC28 can affect both G1 and G2 (summarised in Figure 1.3). One might predict that CDC28/CLB phosphorylates different substrates to those seen by CDC28/CLN although this remains to be established.

The roles of these proteins in yeast are well established but are they present in higher eukaryotic organisms? The answer is yes. In *Xenopus laevis* an activity required for mitosis, termed maturation promoting factor (MPF) was found to consists of homologues of cdc13 and $p34^{cdc2}$ (Arion *et al.*,1988;Dunphy *et al*,1989;Draetta *et al.*,1989), and mammalian cells also contain similar proteins (Draetta *et al.*,1987). The human homologue of $p34^{cdc2}$ has been isolated through complementation of a mutant cdc2 strain(Lee and Nurse,1987). As in yeast, mammalian $p34^{cdc2}$ protein levels remain constant through the cell cycle and kinase activity peaks during mitosis (Draetta *et al.*,1987;Pines and Hunter,1989;Solomon *et al.*,1990). Inhibition of mammalian $p34^{cdc2}$ with antibodies prevents the completion of mitosis confirming the similarity of this kinase with the yeast homologues (Riabowol *et al.*,1989). Thus for simplicity this will be referred to as $p34^{cdc2}$ although this nomenclature refers to the *S.pombe* gene product. Mammalian $p34^{cdc2}$ binds to two distinct cyclins, called cyclin A and B, both of which are cell cycle regulated (Draetta *et al.*,1989;Pines and Hunter,1989;1990). Cyclin B expression peaks at the G2/M transition whereas cyclin A peaks slightly earlier at the end of S-phase (Pines and Hunter,1990;1991). It seems

likely therefore that in higher eukaryotic cells cyclin B/p34^{cdc2} is analogous to CDC28/CLB and cdc2/cdc13 in yeast (summarised in Figure 1.3) . In contrast cyclin A appears to be required for S-phase as its depletion prevents DNA synthesis although it may also be required for mitosis (Girard *et al.*,1991;Pagano *et al.*,1991a). Rescue of the CLN mutation in budding yeast has been used to isolate G1 cyclins from mammalian cells named cyclin C and E (Lew *et al.*,1991;Koff *et al.*,1991;Xiong *et al.*,1991). Over-expression of cyclin E has been shown to shorten the length of G1 possibly due to binding of a kinase subunit and phosphorylating appropriate substrates, however the role of cyclin C is less well established (Ohtsubo and Roberts,1993). Another group of cyclins, called D-type cyclins, were identified through rescue of CLN mutations in yeast and also independently during studies of CSF-1 (colony stimulating factor 1) stimulation of haematopoietic cells (Matsushime *et al.*,1991;Lew *et al.*,1991;Xiong *et al.*,1991). Although the D-type cyclins are necessary for progression through G1 in growth stimulated cells the mRNA levels are constant during the cell cycle suggesting they may be required to initiate cell division rather than regulating later events (Baldin *et al.*,1993;Sewing *et al.*,1993). Thus, in mammalian cells it appears that several cyclins are present each performing different but overlapping functions during the cell cycle (Sherr,1993 summarised in Figure 1.3).

In contrast to yeast several cdc2-like kinase subunits have been identified in mammalian cells and of these five have been classed as cyclin-dependent kinases (cdk) as they bind to and are regulated by cyclins (Tsai *et al.*,1991;Meyerson *et al.*, 1992;Sherr,1993). Each cdk appears to bind more than one cyclin for example p34^{cdc2}

binds cyclins A and B whereas $p32^{cdk2}$ binds cyclins A, E and D. (Sherr,1993). However using a *Xenopus laevis* cell free system, depletion of $p33^{cdk2}$ and $p34^{cdc2}$ suggested they were required for S-phase and mitosis respectively (Fang and Newport,1991). Similarly, cdk2 appears to be required for S-phase in mammalian cells whereas cdc2 controls mitosis (Tsai *et al.*,1993;Riabowol *et al.*,1989). Since evidence suggests that cyclin A is required for progression through both S-phase and M-phase, it seems likely that the cdk2/cyclin A kinase regulates the former whilst cdc2/cyclin A is important for the latter. Cyclin E complexes with cdk2 in G1. Therefore the effects of cyclin E on G1 are likely to be mediated through cdk2 (Koff *et al.*,1992). It is possible that a single kinase may have different substrates depending on its cyclin partner as cdk2 can bind both cyclins A and E in different stages of the cell cycle. However, such kinase specific substrates have so far not been identified. To conclude mammalian cells possess several cyclin and kinase subunits which are likely to regulate cell cycle progression, each combination performing different or possibly overlapping functions.

Are these cell cycle regulatory kinases able to directly modulate transcription? The C-terminal domain of RNAP II contains a number of cdc2 phosphorylation sites which can be phosphorylated *in vitro* by $p34^{cdc2}$ (Cisek and Corden,1989). It is known that unphosphorylated RNAP II associates with the pre-initiation complex and that phosphorylation is subsequently required for transcription elongation to begin although it is not clear if $p34^{cdc2}$ is the kinase required for this activation step *in vivo*. In fact Lu *et al.* (1992) have shown that the general factor TFIIH possess a kinase activity responsible for phosphorylating the CTD of RNAP II, suggesting that $p34^{cdc2}$ may

have other effects or functions in combination with TFIIH.

The Oct-1 transcription factor is phosphorylated on a number of residues during the cell cycle (Roberts *et al.*,1991;Segil *et al.*,1991). Phosphorylation of Oct-1 inhibits DNA binding activity and may be mediated through the mitotic $p34^{cdc2}$ kinase which can phosphorylate Oct-1 *in vitro*. However $p34^{cdc2}$ only phosphorylates some of the residues found to be phosphorylated *in vivo* and may therefore function in concert with other kinases to regulate Oct-1 DNA binding activity (Roberts *et al.*,1991;Segil *et al.*,1991). Oct-1 is believed to be important for the transcriptional control of the histone H2B gene and one can imagine that inhibition of Oct-1 binding activity in mitosis is necessary to ensure histones genes are not transcribed in M-phase (Heintz *et al.*,1983). The HMG-I(Y) proteins can also be phosphorylated by $p34^{cdc2}$ *in vitro* on similar sites phosphorylated *in vivo* (Reeves *et al.*,1991;Nissen *et al.*,1991). This occurs within the DNA binding domain of these proteins and inhibits DNA binding activity. Thus in an analogous manner to Oct-1, phosphorylation of HMG-I(Y) proteins can be used to control DNA binding activity.

The p53 protein can form complexes with and is phosphorylated by $p34^{cdc2}$ *in vitro* (Milner *et al.*,1990;Stürzbecher *et al.*,1990). Furthermore, p53 is phosphorylated during the cell cycle at G1/S, an event which may be required for its entry into the nucleus (Addison *et al.*,1990;Bischoff *et al.*,1990). Therefore, regulation of genes activated by p53 could be exerted by controlling the cellular distribution of this transcription factor. The $p34^{cdc2}$ protein is also capable of phosphorylating pRb *in vitro* on the same ten consensus sites which are phosphorylated *in vivo* (Lin *et al.*,1991b;Lees *et al.*,1991;Hu *et al.*,1992). The pRb protein is sequentially

phosphorylated during the cell cycle being hypophosphorylated in G1 and hyperphosphorylated in G2/M (Buchkovich *et al.*,1989;DeCaprio *et al.*,1989;Mihara *et al.*,1989). Microinjected pRb protein can cause growth arrest in G1 coinciding with the appearance of hypophosphorylated pRb (Goodrich *et al.*,1991). Therefore it is believed that hypophosphorylated pRb suppresses growth whereas hyperphosphorylated pRb probably does not. How this regulation modulates transcription is currently unclear although one might predict that different genes may respond to different phosphorylated forms of the pRb protein or alternatively phosphorylation may allow pRb to bind to different transcription factors (see later chapters).

Many other activating transcription factors contain potential cdc2 phosphorylation sites but since this site is similar to that recognised by MAP kinases it is difficult to conclusively assign specific substrates to a particular kinase (Nigg,1993,Hunter and Karin,1992).

In conclusion, cell cycle progression appears to be controlled by the activity of a family of cdc2-like kinase subunits and their regulatory cyclin subunits. One important level of control is the initiation of transcription which may be modulated through directly phosphorylating transcription factors. This would allow transcription to be co-ordinated with cell cycle events, thus ensuring appropriate changes in gene expression occur during cell cycle progression.

1.7 The control of transcription from viral promoters

Much work has centred on the control of viral promoters in embryonic stem (ES) cells and in the F9 embryonal carcinoma (EC) cell line as a means for defining cellular transcription factors regulated during differentiation. F9 EC cells are pluripotential stem cells derived from an explanted teratocarcinoma (Bernstine *et al.*,1973). They can be induced to differentiate using, cAMP and retinoic acid (RA), to form several cell types similar to those in the developing mouse embryo. Treatment with RA gives rise to primitive endoderm whereas cAMP and retinoic acid together produce parietal endoderm (Strickland and Mahdavi,1978;Strickland *et al.*,1980). Some viral promoters are inefficiently transcribed in EC cells which could result from a lack of positively acting factors or from the presence of specific repressors, though it is possible that both models are applicable. For example the SV40 and polyoma virus enhancers are poorly transcribed in EC cells but more efficiently in PE cells. This in part can be explained by the cellular transcription factor PEA1 (murine AP-1) which regulates expression of the polyoma enhancer and is abundant in differentiated cells but low in EC cells (Kryske *et al.*,1987).

The presence of repressors has been implied from isolating mutant viruses which are expressed more efficiently in EC and ES cells and therefore have overcome the block to gene expression. Some of these have mutations which reduce the binding of "repressor" proteins or conversely generate binding sites for positively acting factors (Hilberg *et al.*,1987;Kovesdi *et al.*,1987a;Tsukiyama *et al.*,1989;1990;Prince and Rigby,1991)

In contrast to the polyoma enhancer some viral genes are expressed efficiently in EC cells such as those in the adenovirus type 5 (Ad5) genome. During adenovirus infection, expression of the E1a,E1b,E2a,E3 and E4 genes are dependent on the immediate early E1a protein (Berk *et al.*,1979;Nevins,1981). E1a does not bind to DNA specifically and it has been hypothesised therefore that E1a may be able to *trans*-activate these genes probably through modulating cellular factors (summarised in Figure 1.4). Analysis of the *cis*-acting DNA sequences in these viral genes did not reveal a single common motif which conferred activation by E1a thus, *trans*-activation is likely to be mediated via a number of different mechanisms (Nevins,1987).

When F9 EC cells were infected with a mutant adenovirus, dl312, which lacks the E1a gene, the E2a gene was still efficiently expressed, whereas when differentiated parietal endoderm (PE) cells were infected with same virus activation was not observed indicating an absolute requirement for E1a in the latter cell type (Imperiale *et al.*,1984). Based on this observation it was suggested that F9 EC cells contain an "E1a-like" activity which compensates for the lack of viral E1a in activation of the E2a gene. Furthermore, this activity was restricted to EC cells as PE cells were unable to produce a similar effect (Imperiale *et al.*,1984). The viral E1a protein has a variety of biological effects such as transcriptional activation and repression, immortalisation and transformation of cells which are thought to be generated through by binding cellular proteins (summarised in Figure 1.4). In contrast the properties of the cellular E1a-like activity remain to be established but may result from a single protein with the same properties as viral E1a or may be produced by a variety of different activities unique to EC cells. Genes encoding cellular proteins with

homology to E1a have been identified which may contribute to the E1a-like activity, however they remain relatively uncharacterised (Defeo-Jones *et al.*,1991).

Analysis of *trans*-acting DNA binding complexes on the E2a promoter revealed several proteins which may be responsible for E1a activation. An ATF binding site and two binding sites for the transcription factor E2F are present in an inverted repeat. The ATF site can bind a number of different related factors some of which are regulated during the differentiation of EC cells (Hai *et al.*,1990;Tassios and La Thangue,1990). E2F binds to the sequence TTTCGCGC on the E2a promoter and was originally identified in HeLa cell extracts (Kovesdi *et al.*,1986a;b). Following adenovirus infection of Hela cells E2F was found to be induced to bind both E2F sites on the E2a promoter (Kovesdi *et al.*,1986a;b;Yee *et al.*,1989). In addition, when the E2F site was added to the β-globin promoter, transcription could be stimulated in an E1a-dependent fashion; however this experiment must be carefully interpreted as the globin promoter can be activated through the TATA element alone (Kovesdi *et al.*,1987b;Green *et al.*,1983;Lee *et al.*,1991). Nevertheless, induction of E2F appeared to correlate well with activation by E1a whereas the ATF activity appeared to unaffected by viral infection (SivaRaman *et al.*,1986).

Similar studies performed in stem cells suggested E2F was present in EC extracts but not PE extracts (Reichel *et al.*,1987). In addition viral infection of EC cells induced binding to both E2F sites on the promoter although the cellular binding activity present in uninfected cells did not preferentially bind to both sites (Reichel *et al.*,1987;Jansen-Durr *et al.*,1989). This implied that factors in uninfected cells possessed different properties to those in uninfected cells. One explanation for this

could be attributed to the activity of the adenovirus E4 open reading frame 6/7 (orf 6/7) protein. The E4 protein interacts with E2F promoting binding to both E2F sites in the promoter (Hardy and Shenk,1989;Huang and Hearing,1989). Therefore some of the differences in E2F activity observed during adenovirus infection may be partly due to E4. The induction of E2F during infection may also result from post-translational modification which has been shown in cell free extracts (Bagchi *et al.*,1990)

A cellular factor in uninfected EC cells has been identified which binds to a single E2F site (La Thangue and Rigby, 1987). This factor termed DRTF1 (for differentiation regulated transcription factor 1), when assayed via gel retardation resolves as several DNA binding complexes which were named DRTF1**a**,**b** and **c** (La Thangue *et al.*,1990). DRTF1**b**/**c** is abundant in EC cells but reduced in PE cells correlating with the transcriptional regulation of the E2a promoter and the presence of the cellular E1a-like activity (La Thangue and Rigby,1987;La Thangue *et al.*,1990) In contrast to DRTF1**b**/**c**, DRTF1**a** remains relatively constant during differentiation. Purification of the **b**/**c** complex by DNA affinity chromatography produced several polypeptides which activate transcription *in vitro* suggesting DRTF1**b**/**c** is a positively-acting transcription factor (Shivji and La Thangue,1991;Girling *et al.*,1993). The DNA binding specificity of DRTF1**a** is similar to DRTF1**b**/**c** and is sensitive to both detergents and phosphatase treatment however the transcriptional properties of DRTF1**a** remain to be established (La Thangue *et al.*,1990;Shivji and La Thangue,1990). It is possible that DRTF1**a** is a related factor which is appealing since many polypeptides are produced after purification. Alternatively, DRTF1**a** may

contain a similar DNA binding component complexed to other factors to create a slower migrating complex.

Although both DRTF1 and E2F bind to the same site they were originally identified in different cell systems and the relationship between them was unclear. They are likely to be closely related however and isolation of the appropriate cDNA for each protein would be required to distinguish them further.

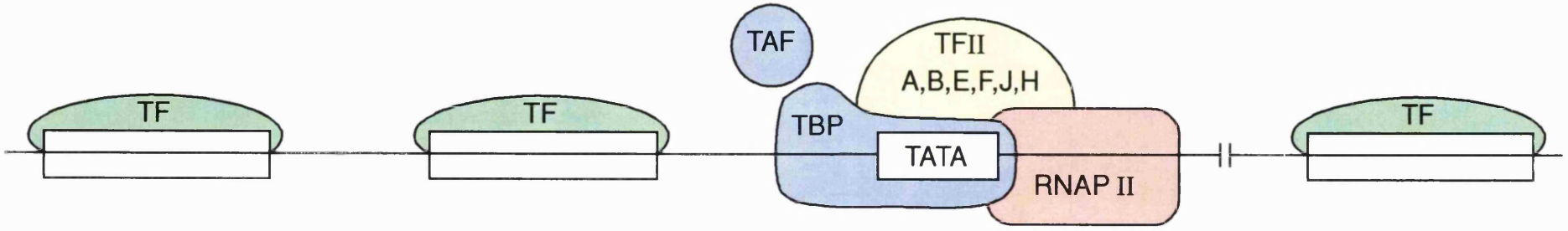
1.8 Aims

The aims of this project were to establish the relationship between DRTF1**a** and DRTF1**b/c** and in particular identify the protein components of each. This would subsequently allow the transcriptional activity of DRTF1**a** to be studied in more detail and the role of this transcription factor in cellular differentiation and cell cycle progression could also be elucidated. DRTF1 is likely to be important in E1a mediated *trans*-activation and may also participate in many of the other biological properties associated with E1a. Therefore the role of DRTF1 complexes in these processes will be investigated further in order to understand how viral proteins modify cellular gene expression and activate viral genes.

Figure 1.1a, *Transcription of RNA polymerase II transcribed genes.* A typical RNA polymerase II transcribed gene is shown. DNA sequences (open boxes) upstream and downstream of the transcription start site (+1) which bind transcription factors (TF) are shown and the position of basal factors (shown in yellow) including the TATA binding protein (TBP) and TAFs are indicated in blue.

Figure 1.1b, *Mechanisms of activating transcription.* Transcription factors contact the basal machinery by looping of the DNA such that these proteins are in close proximity.

a


TAF
TFII
A,B,E,F,J,H
TF
TF
TBP
TATA
RNAP II
TF

b

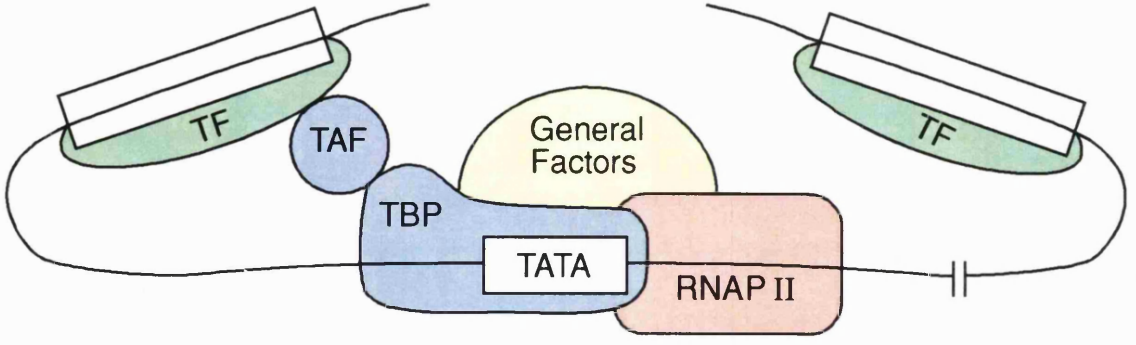

TF
TAF
General
Factors
TF
TBP
TATA
RNAP II

Figure 1.2, *Sequential assembly of basal transcription factors on TATA containing promoters*. The basal factors (TFIIA,IIB,IID,IIE,IIF,IIH and IIJ) required for transcription by RNA polymerase II (RNAP II) are shown and the sequential assembly of these proteins to form a pre-initiation complex is depicted. This diagram only refers to the transcription of TATA containing promoters.

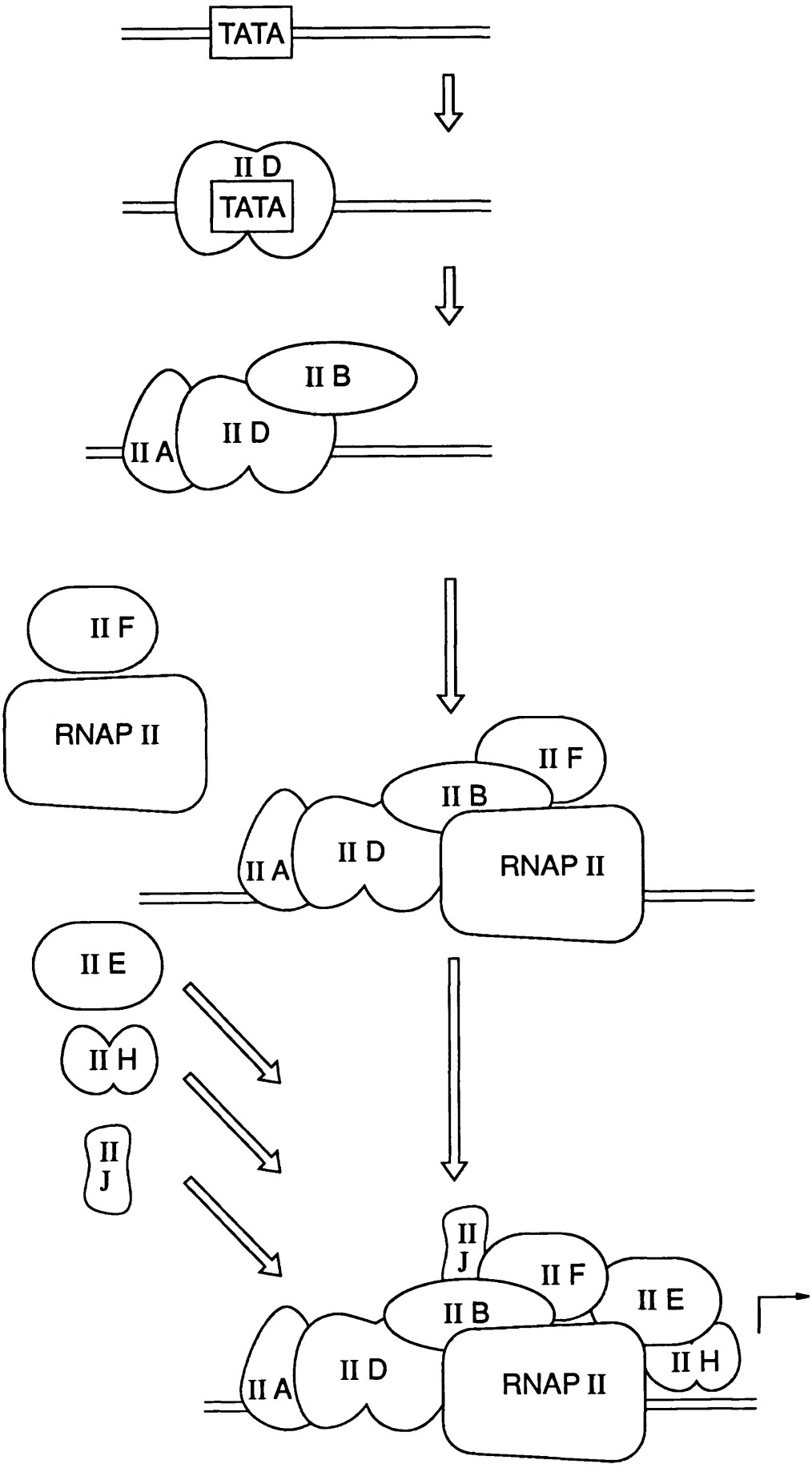

TATA
II D
TATA
II B
II A
II D
II F
RNAP II
II F
II B
II A
II D
RNAP II
II E
II H
II J
II J
II F
II E
II B
II H
II A
II D
RNAP II

Figure 1.3, *Summary of the cell cycle regulatory kinases in mammalian cells and Saccharomyces cerevisiae*. The restriction point (R) and the analogous point in yeast (Start) are shown. In yeast the product of the CDC28 gene (red) functions in G1 and in G2 by binding different groups of cyclins (CLN and CLB). In mammalian cells several cdc2-like kinases and their associated cyclins are also shown in the phases of the cell cycle they are thought to regulate.

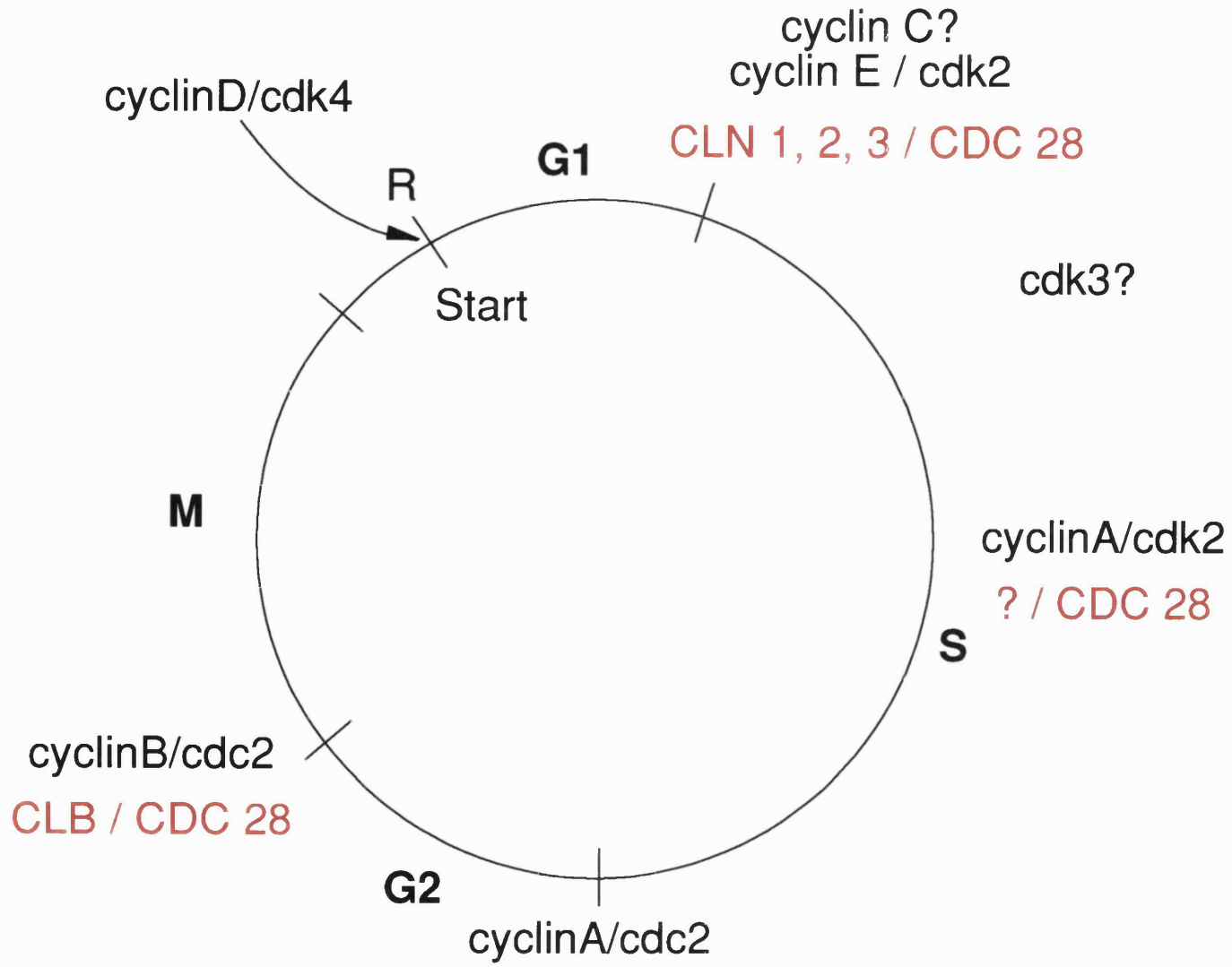
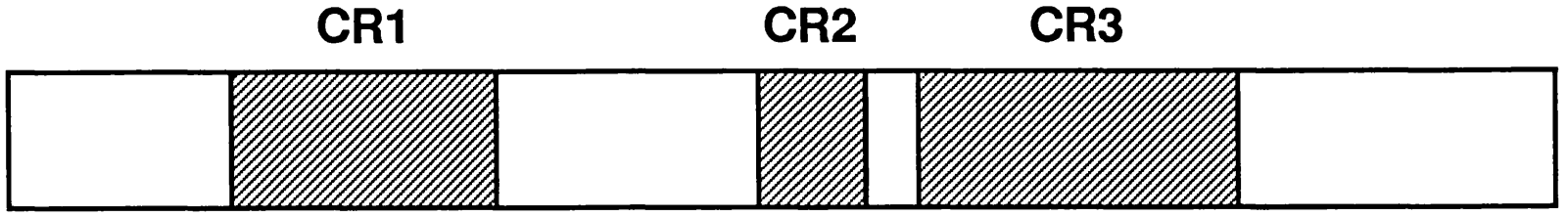

cyclin C?
cyclin E / cdk2
CLN 1, 2, 3 / CDC 28
cyclinD/cdk4
G1
R
Start
cdk3?
M
cyclinA/cdk2
? / CDC 28
S
cyclinB/cdc2
CLB / CDC 28
G2
cyclinA/cdc2

Figure 1.4, *Properties of the adenovirus E1a protein.* The E1a protein is shown with conserved regions shaded (CR1-3). The regions within E1a required for various biological phenomena are indicated and the cellular polypeptides bound by E1a in infected and transformed cells are shown. The sequences within E1a necessary for binding these polypeptides are also indicated.



Cellular Proteins :

p300 Dr1

p130, p107, pRb
cyclinA / cdk2

TBP
Oct 4
ATF2

Biological Activities :

Cellular Transformation
Immortalisation
Induction of DNA Synthesis
Activation of Transcription

Transcriptional
Repression /
Activation

CHAPTER 2

Materials and Methods

2.1 Determination of nucleic acid and protein concentrations

The concentration of nucleic acid solutions was determined with a Uvikon 860 spectrophotometer and quartz cuvettes using the formulae below (Sambrook *et al.*,1989).

1 A260nm=50μg/ml double-stranded DNA

1 A260nm=40μg/ml single-stranded DNA

1 A260nm=40μg/ml RNA

1 A260nm=20μg/ml double-stranded oligonucleotides

Protein concentrations were determined by the method of Bradford (1976) using the Bio-Rad protein concentration assay. Protein solutions were mixed for 10 min in reagent (diluted 1:4 in H_20) and the absorbance measured at 595nm in disposable kartell microcuvettes (Slaughter and Payne). The samples were quantitated with respect to a range of BSA (Sigma) standards to estimate the concentration of test samples.

2.1.1 Standard buffers and enzyme reagents

Plasmid solution I

50mM glucose

25mM Tris-HCl pH 8.0

10mM EDTA

Plasmid solution II

20mM NaOH

1% SDS w/v

Plasmid solution III

5M potassium acetate pH 6.0

1xTE - 10mM Tris-HCl pH 8.0

1mM EDTA

1xTAE- 40mM Tris-acetate

1mM EDTA

pH 8.0

1xTBE- 89mM Tris-borate

89mM boric acid

2mM EDTA

1xL-Broth media (1l)

10g bacto-trptone

5g bacto-yeast extract

10g NaCl

1xAlkaline phosphatase buffer

100mM Tris-HCl (pH9.5)

50mM $MgCl_2$

100mM NaCl

1xLigation buffer

66mM Tris-HCl pH7.6

10mM $MgCl_2$

15mM DTT

0.2mg/ml BSA

1mM ATP

2xSDS protein sample buffer

125mM Tri-HCl (pH6.8)

4% SDS w/v

20% glycerol v/v

0.002% bromophenol blue w/v

5% β-mercaptoethanol v/v

5xSDS gel running buffer

250mM Tris-HCl

2M Glycine

0.5% SDS w/v

5xDNA loading buffer

0.25% w/v bromophenol blue

0.25% w/v xylene cyanol

25% w/v Ficoll (type 400)

10xPCR reaction buffer

100mM Tris pH8.8

15mM $MgCl_2$

500mM KCl

1xImmunoprecipitation buffer

50mM Tris-HCl pH 7.9

125mM NaCl

0.2mM EDTA

1mM DTT

1% NP-40 v/v

1xBandshift reaction buffer

50mM Tris-HCl pH 7.9

6mM $MgCl_2$

40mM $(NH_4)_2SO_4$

0.2mM EDTA

1mM DTT

15% glycerol v/v

1xGST thrombin cleavage buffer

50mM Tris-HCl pH8.0

150mM NaCl

2.5mM $CaCl^2$

0.1% β-mercaptoethanol

1xMicroextraction buffer

20mM Hepes pH 7.8

450mM NaCl

0.2mM EDTA

0.5mM DTT

0.5μg/ml leupeptin

0.5μg/ml protease inhibitor

1μg/ml trypsin inhibitor

40μg/ml bestatin

10xDephosphorylation buffer

500mM Tris-HCl pH8.5

1mM EDTA

1xPBSA

8g/l NaCl

0.2g/l KCl

1.44g/l Na_2HPO_4

0.24g/l KH_2PO_4

10xNick translation buffer

500mM Tris-HCl pH 7.2

100mM $MgSO_4$

1mM DTT

500μg/ml BSA

5xTranscription buffer

200mM Tris pH 7.5

30mM $MgCl_2$

50mM NaCl

10mM spermidine

0.1M column buffer

50mM Tris

6mM $MgCl_2$

40mM $(NH_4)_2SO_4$

2mM EDTA

1mM DTT

15% v/v glycerol

10X restriction enzyme buffers

	Buffer (H)	Buffer (B)	Buffer (A)
NaCl	100mM	100mM	-
Tris-HCl	50mM	10mM	-
$MgCl_2$	-	5mM	-
DTT	-	-	0.5mM
β-mercaptoethanol	-	1mM	-
Potassium acetate	-	-	66mM
Tris acetate	-	-	33mM
Magnesium acetate	-	-	10mM
Final pH	7.5	8.0	7.9

Restriction enzymes used in this study

Enzymes	Buffer	Supplier
AatII	A	Boehringer Mannheim
BamHI	B	Boehringer Mannheim
EcoRI	H	Boehringer Mannheim
EcoRV	B	Boehringer Mannheim
HindIII	B	Boehringer Mannheim
XhoI	H	Boehringer Mannheim

2.2 DNA manipulations:

2.2.1 Extraction and precipitation of DNA

Phenol solution was prepared by equilibrating phenol with 10mM Tris (BDH) and adjusting to 7.5mM with 1M HCl (FSA laboratory supplies). The chloroform solution was made as a mixture of chloroform and isoamyl alcohol 24:1. DNA solutions were first treated with an equal volume of phenol solution vortexed and centrifuged at 13,000(K) rpm for 3 min in a Heraeus sepatech biofuge. The resulting upper aqueous layer was retained and mixed with phenol/chloroform solution (1:1 phenol to chloroform solution) to remove proteins. This was vortexed, centrifuged and the upper layer was mixed with chloroform solution and treated as described above. The resulting aqueous layer was mixed with 0.1 volumes of 3M sodium acetate, pH5.3 (Sigma), 2.5 volumes of 96% ethanol (Hayman Ltd) and precipitated by incubating on solid CO_2 for 25 min. DNA was collected by centrifugation for 25 min at 13K and the pellet was washed in 70% ethanol, air dried for 10 min and resuspended in TE buffer (see section 2.1.1).

2.2.2 DNA oligonucleotides used in experiments

Synthetic oligonucleotides were prepared at the National Institute for Medical Research using an Applied Biosystems automatic synthesiser and supplied heat deprotected. Complementary single stranded oligonucleotides were ethanol precipitated and annealed by heating equimolar quantities to 90^0C in ligase buffer (see section 2.1.1) and cooling overnight to room temperature.

The oligonuleotides used in this study were :

E2F binding site oligonucleotides

71/50, the wild type binding sequence for DRTF1 was derived from the adenovirus (Ad5) E2a promoter from -70 to -50 (La Thangue *et al.*,1990)

5'-GATCTAGTTTTCGCGCTTAAATTTGA-3'

3'-ATCAAAAGCGCGAATTTAAACTCTAG-5'

60*, as above but with a point mutation at position -60

5'-GATCTAGTTTTCGCGATTAAATTTGA-3

3'-ATCAAAAGCGCTAATTTAAACTCTAG-5'

61*, as in 71/50 but with a point mutation at position -61

5'-GATCTAGTTTTCGCTCTTAAATTTGA-3

3'-ATCAAAAGCGAGAATTTAAACTCTAG-5'

60/62, as in 71/50 but with three mutated bases between -60 and -62 (La Thangue *et al.*,1990)

5'-GATCTAGTTTTCGATATTAAATTTGA-3'

3'-ATCAAAAGCTATAATTTAAACTCTAG-5'

63*, as in 71/50 but with a point mutation at position -63 (La Thangue *et al.*,1990)

5'-GATCTAGTTTTCTCGCTTAAATTTGA-3'

3'-ATCAAAAGACGAATTTAAACTCTAG-5'

64*, as in 71/50 but with a point mutation at position -64 (La Thangue *et al.*,1990)

5'-GATCTAGTTTTAGCGCTTAAATTTGA-3'

3'-ATCAAAATCGCGAATTTAAACTCTAG-5'

ATF binding site oligonuleotide

The ATF site oligonucleotide was derived from sequences -58 to -39 of the adenovirus (Ad5) E4 promoter (Gilardi and Perricaudet,1984)

5'-GATCTAACCGTTACGTCATTTTTT-3'

3'-ATTGGCAATGCAGTAAAAAACTAG-5'

p107 oligonucleotides

Fragments of the p107 cDNA were amplified using the following primers:

p107 249-257 contains DNA sequence from amino acid residues 249-257 of p107

5'-CGGGATCCGCAGTCATTACTCCTGTTGCATCAGCC-3'

p107 732-690 contains DNA sequence from amino acid residues 732-690 of p107

5'-GGACAGTGAACTAAAGTGAATTCAAAAACACG-3'

p107 690-732 contains DNA sequence from amino acid residues 690-732 of p107

5'-CGTGTTTTGAATTCACTTTAGTTCACTGTTCC-3'

p107 936-928 contains DNA sequence from amino acid residues 936-928

5'-CGGGATCCTTAATGATTTGCTCTTTCACTGAC-3'

DP-1 oligonucleotides

Fragments of DP-1 were amplified using the following primers:

The 6 sense primer contains DNA sequences from amino acid residues 84-90 of DP-1 (Girling *et al.*,1993).

5'-CGCGGATCCCCCAACACGCATTTTGTG-3

The 15 antisense primer contains DNA sequences from amino acid residues 249-244 of DP-1 (Girling *et al.*,1993).

5'-CGCGGATCCCCTGCGGGCCTGCTG-3'

The 166 antisense primer contains DNA sequences from amino acid residues 160-166 of DP-1.

5'-CGCGGATCCCCGGATGTTCTTCTGGTC-3'

The 146 sense primer contains DNA sequences from amino acid residues 146-153 of DP-1.

5'-CGCGGATCCTTCAGCGCTGCCGACAACCAC-3'

2.2.3 Plasmid DNA constructs used.

E1a plasmid constructs:

E1a13S contains a cDNA for the 13S product of the E1a gene in the Sp65 vector (Pharmacia). E1a12S contains a cDNA for the 12S product of E1a in Sp65. Both

E1a5/3 and E1aCS contain a cDNA for the 13S product lacking CR1 and CR2 respectively in Sp65 and were a gift from N.Jones (Schneider *et al.*,1987).

T antigen construct:

The *HindIII* B fragment of T antigen in Bluescript KS II (Stratagene) was kindly provided by D.Lane (Mole *et al.*,1988).

pRb plasmid constructs:

The GST-Rb contains cDNA sequence from amino acid residue 379 to 928 of pRb in pGEX-2T (Kaelin *et al.*,1991). GST-Rb^{706} and GST-Rb^{Ex22} contain sequences encoding 379-928 with a point mutation changing amino acid 706 or a deletion of exon 22 respectively, these were kindly provided by W.Kaelin and D.Livingston.

p107 plasmid constructs:

The p107 cDNA contains sequences from amino acid residues 189-1068 in Bluescript KS II kindly provided by M.Ewen and D.Livingston (Ewen *et al.*,1991, Zhu *et al.*,1993). GST-p107 contains DNA sequences from p107 encoding amino acid residue 249-936 in pGEX-2T.

cdk2 plasmid construct:

GST-cdk2 contains cDNA sequences for amino acids 1 to 249 of cdk2 in pGEX-2T and was a gift from E.Harlow (Tsai *et al.*,1991).

E2F-1 plasmid constructs:

The E2F-1 cDNA contains the full length sequence from amino acid residues 1-427 in Sp72 and GST-E2F-1 contain sequences for amino acids 89-437 in pGEX-2T, these were kindly provided by D.Livingston and E.Harlow respectively (Kaelin *et al.*1992;Helin *et al.*,1992).

DP-1 plasmid constructs:

The DP-1 cDNA contained the full length sequence encoding amino acid residues 1-410 (Girling *et al.*,1993) in pG4m poly II was provided by M.Zamanian (Webster *et al.*,1989). GST-DP-1^{84-204} contains sequence encoding residues 84 to 204, GST-DP-1^{84-249} contains sequence for 84 to 249, GST-DP-$1^{146-249}$ contains sequence for 146 to 249 and GST-DP-1^{84-166} contains sequences for 84 to 166 all cloned into pGEX-2T.

2.2.4 Diagnostic enzyme digests

Diagnostic digests were performed using 1-10μg of DNA and approximately 5 units (U) of enzyme in the appropriate restriction buffer (section 2.1.1). The reaction volume was kept as small as possible and the volume of enzyme added was not allowed to exceed 10% of the final volume. Reactions were performed at 37^{0}C for 1h after which DNA loading buffer was added (section 2.1.1). The digested sample were electrophorosed though a 0.7-1.5% agarose containing 0.5μg/ml ethidium bromide (Sigma) using a Bio Rad Uniscience mini-gel run at 12V/cm in 1xTBE for 1h. Standard size markers (lambda phage genomic DNA digested with *HindIII*) were run in parallel and the products were examined by ultra violet illumination on a TMP

20 UVP transilluminator and, when necessary the gel was photographed with Polaroid 667 film.

2.2.5 Preparative DNA digests

Usually approximately 100μg of DNA was digested with 2U enzyme/μg DNA in the appropriate buffer. Reactions were incubated for 2-3h at 37^{0}C and when fully digested mixed with DNA loading buffer. Samples were electrophorosed as above through a 1% low melting temperature agarose gel (Sigma) in 1xTBE for 1 hour at 4^{0}C. The desired fragment was excised from the gel using a razor blade and melted in TE buffer by heating to 65^{0}C for 10 min. The molten sample was extracted with warm phenol and twice with phenol/chloroform taking care not to retain any contaminants at the interface. The sample was then chloroform extracted and ethanol precipitated.

2.2.6 Dephosphorylation of DNA

Vector DNA was digested with the appropriate enzymes, extracted, ethanol precipitated and resuspended in H_20. The 5' ends if the vector were then dephosphorylated using calf intestinal alkaline phosphatase (CIP, Boehringer Mannheim) in dephosphorylation buffer (section 2.1.1). Reactions were performed such that 1U of CIP was used per μg of DNA and incubated at 37^{0}C for 45 min. DNA was extracted, precipitated and resuspended in H_2O.

2.2.7 Ligation of DNA

A 10:1 molar ratio of insert:dephosphorylated vector was incubated with 10U of T4 DNA ligase (New England Biolabs) in ligation buffer (section 2.1.1), for blunt end ligations the concentration of ATP was increased to 10mM. Reactions were performed at 14^{0}C overnight and either heat treated to inactivate the ligase or extracted and precipitated.

2.2.8 Preparation of competent bacteria

A 50ml culture of DH5α *E.coli* (Stratagene) bacteria was grown overnight at 37^{0}C and 1ml of this was inoculated into a 250ml of L-Broth. The 250ml culture was incubated at 37^{0}C until the optical density measured at 600nm was 0.9. Cultures were centrifuged at 3K rpm for 5 min at 4^{0}C in a Sorvall RC5C centrifuge. The resulting pellet was resuspended in 50ml of ice cold 100mM $MgCl_2$ to which another 100ml of $CaCl_2$ was added and incubated on ice for 1h. The cells were centrifuged again as above and resuspended in 12.5ml of 85mM $CaCl_2$,15% glycerol, aliquoted and stored at -70^{0}C.

2.2.9 DNA transformation of bacteria

Approximately 50ng of ligated DNA was mixed with 150μl of competent DH5α *E.coli* bacteria and incubated on ice for 30 min. The cells were heat shocked by transferring reactions to 42^{0}C for 2 min. After this 200μl of L-Broth was added before incubating the cells at 37^{0}C for 1h. The cells were subsequently spread plated onto agar plates with 0.1μg/ml ampicillin (Sigma) and incubated overnight at 37^{0}C.

The presence of a correct ligation product was assessed by producing mini-preparations of DNA from individual clones and performing diagnostic restriction enzyme analysis (section 2.2.4).

2.3.1 Mini-preparations of plasmid DNA

Individual colonies or glycerol stocks were used to inoculate 3ml of L-Broth medium containing 0.1μg/ml ampicillin. Cultures were grown at 37^{0}C overnight and 1.5ml of culture was centrifuged at 13K rpm in a microfuge for 1 min. The resulting pellet was resuspended in 250μl of plasmid solution I followed by 250μl of freshly prepared plasmid solution II. The mixture was inverted several times and 250μl of plasmid solution III was added. The mixture was inverted several times again and centrifuged at 13K rpm for 10 min. The supernatant was extracted with phenol/chloroform followed by chloroform extraction and then precipitated using an equal volume of propan-2-ol (BDH). The DNA was immediately centrifuged at 13K rpm for 25 min, air dried for 10 min and resuspended in 50μl of TE. RNA and protein contaminants were removed by adding 70μl of 5M LiCl and incubating on ice for 5 min. The samples were centrifuged at 13K rpm for 10 min and the resulting supernatant ethanol precipitated. Any remaining RNA contaminants were removed by digestion with 2μg of RNAse H (Pharmacia) for 30 min at 37^{0}C. All DNA preparations were assayed by agarose gel electrophoresis prior to use.

2.3.2 Polymerase chain reactions (PCR)

Reactions were all performed using the same reaction conditions in a programmable

thermal controller (Genetic Research Instrumentation Ltd). A typical reaction contained 5ng of plasmid template, 200ng of each oligonucleotide primer 1mg/ml BSA, 10mM of each dNTP and PCR reaction buffer. To this 2U of Taq polymerase (Cetus) was added and overlayed with paraffin oil. Samples were initially heated to 94^{0}C for 2 min and again for 30s then cooled for annealing at 56^{0}C for 1 min. Samples were then heated to 72^{0}C for 1 min and then back to 94^{0}C for 30 seconds. This cycle was repeated 30 times followed by a final incubation at 72^{0}C for 3 min.

2.3.3 Construction of GST-p107

GST-p107 was constructed using a sense oligonucleotide primer for amino acid residues 249-257 and an anti-sense primer covering amino acid residues 732-690 (section 2.2.2) in a PCR, using the p107 cDNA as template (Ewen *et al.*,1991). In a second reaction, a sense primer for amino acid residues 690-732 and an anti-sense primer for amino acid residues 936-928 were used (section 2.2.2). Both PCR products were gel purified, digested with *BamHI* and *EcoRI*, ligated and cloned into the *BamHI* site in pGEX-2T (Pharmacia).

2.3.4 Construction of GST-DP-1 deletions

GST-DP-1$^{84-204}$ was made cloning the *BamHI* fragment of DP-1 (clone 6) into the site of pGEX-2T (Girling *et al.*,1993). GST-DP-1$^{84-249}$ was generated using a sense primer (6 sense) for amino acids 84-89 and an anti-sense primer (15 antisense) for amino acid residues 249-245 (section 2.2.2). GST-DP-1$^{84-166}$ was made using a sense primer, 6 sense and an anti-sense primer (166 antisense) for amino acid residues 160-

166. GST-DP-$1^{146-249}$ was made using a sense primer (146 sense) and an anti-sense primer, 15 anti-sense (section 2.2.2). In all reactions the DP-1^{59-410} (clone B) cDNA was used as template (Girling *et al.*,1993). PCR products were gel purified (section 2.2.5), digested with *BamHI* and ligated into the *BamHI* site of pGEX-2T. GST-DP-1^{59-410} (GST-B) was created by cloning the *EcoRI/XhoI* cDNA fragment of DP-1 (clone B) into the EcoR1 site of pGEX-1N (Pharmacia) blunting only at the 3' end.

2.3.5 Radioactive labelling of oligonucleotides

300ng of double stranded oligonucleotide was incubated with 30μCi of [α-^{32}P]GTP and 5U of Klenow enzyme (Pharmacia) in labelling buffer (section 2.1.1). The reaction was allowed to proceed for 30 min at room temperature and then purified by phenol/chloroform extraction. Labelled oligonucleotides were ethanol precipitated, dried and resuspended in 50μl of H_20.

2.4 Protein assays and manipulations

2.4.1 Heparin Sepharose chromatography

A 30ml heparin Sepharose (Pharmacia) column was packed and equilibrated with 0.1M column buffer (section 2.1.1). Approximately 100ml of whole cell extract was loaded onto the column at a rate of 5ml/h and washed with 12ml of 0.1M column buffer at a rate of 10ml/h. Bound proteins were eluted at 3ml/h with a linear gradient of increasing molarity of KCl generated by a gradient maker. Approximately 5ml fractions were collected during load, wash and elution and assayed for DNA binding

activity using the E2F oligonucleotide 71/50.

2.4.2 Protein extract preparations

Whole cell extracts were prepared as previously described (Manley *et al.*,1980). Whole cell microextracts were prepared from approximately 10^7 cells, as described by Schöler *et al.* (1989) except that sonication was replaced by freeze-thawing three times. Briefly cell pellets were resuspended in extraction buffer (section 2.1.1) and transferred to solid CO_2 until the solution was frozen. This was thawed at 30°C and frozen again and repeated one more time. Samples were then centrifuged for 10 min at 13K in a microfuge at 4°C. The resulting supernatant was aliquoted, assayed for protein concentration and stored at -70°C until required.

2.4.3 Gel retardation assays

Gel shifts were performed essentially as described by La Thangue *et al* (1990) using bandshift reaction buffer (section 2.1.1). Each reaction was made up to a final volume of 20μl containing 2μg of sonicated salmon sperm DNA and either 6μg of whole cell extract or 8μg of microextract. When necessary 300ng of competitor oligonucleotide was added. Where antibodies were included either 8μl of monoclonal supernatant or 1-2μl of crude antiserum was added to reactions (modifications to this general methodology are mentioned in figure legends). For peptide competition, 2nmol of peptide was also added to binding reactions. The reaction components were incubated at 30°C for 10 min prior to addition of labelled probe. Approximately 6ng of oligonucleotide probe was added and the reaction was incubated at 30°C for

another 10 min and loaded on to a non-denaturing 4% polyacrylamide TAE gel (acrylamide: bis-arcylamide; 20:1) at 4^{0}C with buffer (1xTAE) recirculation. Electrophoresis was performed at 500 volts for 2 min and then 150V for 1.5h. The Gels were subsequently vacuum-dried and exposed to X-ray film (Fuji) at -70^{0}C for approximately 12h.

2.4.4 *In vitro* transcription and translation

E1a13S, E1a12S,E1aCS and E1a53 were lineraized with *HindIII*, E2F-1 was linerised with *EcoRV* and were transcribed with Sp6 polymerase (Promega). DP-1 and T antigen were linerarised with *XhoI* and *EcoRI* respectively, and transcribed with T7 polymerase (Promega). Transcription was performed in the presence of 5mM m^7G(5')ppp(5')A sodium (cap), 0.1M DTT, 2.5μg/ul BSA, 10mM rATP,rCTP, rGTP,UTP, RNA guard (Pharmacia) and 40U of RNA polymerase in transcription buffer (section 2.1.1). Reactions were incubated at 37^{0}C for 1.5h and RNA was purified by phenol/chloroform extraction followed by ethanol precipitation. RNA translation was performed using the rabbit reticulocyte lysate (Promega) according to the manufacturers instructions. Approximately 6μl of RNA solution was mixed with 10μl of lysate, 1μl of amino acid mixture 10μM methionine and 1U of RNA guard and incubated for 1h at 30^{0}C. Translation was initially assayed by monitoring ^{35}S-methionine (Amersham) incorporation. For *in vitro* mixing experiments 5μl of *in vitro* translated labelled lysate was mixed with 1μg of each GST fusion protein and made up to 100μl with PBSA containing 1%NP-40. The reaction mixture was incubated at 30^{0}C for 30 min followed by the addition of 30μl of glutathione beads

((Sigma) 1:1 w/v in PBSA). This mixture was rotated for 30 min at room temperature and washed four times with PBSA containing 1%NP-40. Proteins were released by boiling in SDS sample buffer (section 2.1.1) and visualised after SDS-PAGE followed by autoradiography.

2.4.5 Fusion protein production and purification

Fusion proteins were induced and purified essentially as described by Smith and Johnson (1988) with several modifications. Transformed bacteria (*E.coli* DH5α) were inoculated into 50ml of L-Broth with ampicillin (0.1μg/ml) and grown overnight at 37^{0}C. Cultures were subsequently diluted 1/10 with 500mls of fresh L-Broth containing ampicillin (as above) and grown for 1h after which isopropyl-β-D-thiogalactopyranoside (IPTG,Sigma) was added to a final concentration of 0.1mM. Cultures were then transferred to a G25 shaker (New Brunswick Scientific Co. Inc.) and induced overnight at 20^{0}C. Following induction cells were pelleted by centrifugation (6000rpm in a RC5C Sorvall, Dupont), resuspended in PBSA with 200μg of lysozyme (Sigma) and stirred for 10 min at room temperature. Extracts were then centrifuged at 35K rpm for 1h in an L8-70M ultracentrifuge (Beckman) to remove debris and the resulting supernatant incubated with 200μl glutathione agarose beads (1:1 w/v in PBSA) for 10 min at 4^{0}C. Beads were pelleted and washed three times using PBSA containing 0.1% Tween 20 v/v. Fusion proteins were eluted by resuspending the beads in 200μl of 50mM Tris-HCl (pH 8.0) containing 5mM reduced glutathione (Sigma) for 2 min. Beads were pelleted and the supernatant collected for analysis by SDS-PAGE and coomasie blue staining (Harlow and

Lane,1988). Fusion proteins were stored at -70°C until required. For enzyme cleavage of GST fusion proteins approximately 100μg of fusion protein was mixed with 50μl of glutathione beads for 30 min, pelleted and resuspended in thrombin cleavage buffer (section 2.1.1). To this 600ng of human thrombin (Sigma) was added and the mixture rotated for 2h at room temperature. Beads were pelleted and the supernatant recovered for subsequent experiments.

2.4.6 Production of E2F-1 polyclonal antibodies

Three peptides derived from the E2F-1 amino acid sequence were made at the National Institute for Medical research and made up as a 5mg/ml solution in PBSA. The peptides made were from amino acid residues 106-117 (pep1 132) 315-323 (pep2 134) and 427-437 (pep3 136) of E2F-1. A 5mg/ml solution of keyhole limpet haemocyanin (KLH) in PBSA was also made and 2ml of KLH solution was added to 2ml of peptide solution gradually mixing in 2ml of 0.2%v/v glutaldehyde in PBSA. The reaction was stirred at room temperature for 2h and 1M glycine in PBSA (pH 7.2) was added to a final concentration of 200mM. The reaction was stirred for another hour and the final solution dialysed overnight. Half sandy lop rabbits were immunized regularly with the 0.5ml of peptide solutions by the biological services division and test bleeds provided. The presence of anti-E2F-1 antibodies was determined by the ability of crude serum to recognise the GST-E2F-1 fusion protein in western blots or gel retardations.

2.4.7 Antibodies Reagents

XZ55 and C36 are monoclonal antibodies which react with distinct epitope in the human pRb protein kindly provided by Q. Hu and E. Harlow (Whyte *et al.*,1988;Hu *et al.*,1991). The cyclin A polyclonal antisera was made against amino acid residues 77-432 of the bovine cyclin A protein and was kindly provided by T. Hunt. The cdk2 polyclonal sera was made against the full length human cdk2 protein (Tsai *et al.*,1991) and SD9 is a p107 monoclonal antibody which reacts with the p107 protein kindly provided by E. Harlow (S.Dembski and N.Dyson unpublished observations). The SQ41 is a monoclonal antibody which reacts with amino acid residues 369-437 of E2F-1 and the E2F polyclonal antibody was made during this study against a peptide for amino acids 427-437 (Kaelin *et al.*,1992).

Summary of Antibodies used:

Antibody	Isotype	Epitope	Reference
XZ55	IgG1	400-600	Hu *et al.*,1991
C36	IgG1	300-380	Whyte *et al.*,1988
IF8	IgG1	pRb	Bartek *et al.*,1990
cyclin A	polyclonal	77-432	Unpublished
cdk2	polyclonal	cdk2	Tsai *et al.*,1991
SD9	IgG1	p107	Unpublished
SQ41	IgG1	369-437	Kaelin *et al.*,1992
DP-1 A	polyclonal	3-15	Girling *et al.*,1993
DP-1 18	polyclonal	156-168	Girling *et al.*,1993

E2F-1 (136)	polyclonal	427-437	section 2.4.7
IG4	IgG1	HSV 155Kd	La Thangue *et al.*,1984

2.4.8 Immunoprecipitations

For immunoprecipitations with anti-pRb and anti-p107 monoclonal antibodies, 100μg of JM whole cell extract was mixed with 100μl of monoclonal supernatant and 200μl of immunoprecipitation buffer and rotated for 1h at 4^0C. Approximately 40μl of protein G beads ((Sigma) 1:1 w/v in PBSA) were added and the mixture was rotated for a further hour. Beads were washed four times with 400μl of immunoprecipitation buffer and resuspended in PBSA with 1% deoxycholate (DOC) and 1.5% NP-40 for 2 min and the eluted activity was assayed for DNA binding via gel retardation (section 2.4.3). For immunoprecipitations with anti-DP-1 polyclonal antisera (αA), about 1mg of HeLa whole cell extract was incubated with 50μl of antisera with either 20nmol of DP-1 peptide(A) or c-jun peptide 1 and 200μl of immunoprecipiation buffer. Incubation, washing and detergent elution was performed as described above.

2.4.9 Immunoblotting

Protein samples were run alongside protein molecular weight markers (Sigma) on a 10% SDS-PAGE mini-gel (Bio-Rad Mini Protean II) prepared according to the manufactures instructions. Polypeptides were transferred onto 0.45μm pore nitrocellulose membrane using a Bio-Rad mini protein Trans-Blotter according to the

manufacturers instructions. Transferred proteins were visualised by staining the membrane with a 5% Ponceau-S solution (Sigma) and destained with PBSA. The nitrocellulose membrane was blocked in PBSA with 10% Marvel for 1h at room temperature, washed with PBSA and incubated with primary antibody diluted 1/200 in 5% BSA overnight at 4^0C. Blots were washed three times for 10 mins with PBSA 0.1%NP-40 and incubated with secondary antibody either anti-rabbit alkaline phosphatase conjugated (Promega) diluted 1/7000 in 5% BSA or anti-mouse alkaline phosphatase conjugated (Boehringer) diluted 1/1000. This was incubated for 2h at room temperature after which the blots were washed three times with PBA 0.1% NP-40 as above and incubated with substrate in reaction buffer (section 2.1.1). Reactions were terminated with the addition of 0.1% acetic acid.

2.5.1 Cell culture and serum stimulation

F9 EC cells (Bernstine *et al.*,1973) were cultured in Dulbecco's Modified Eagle's medium (DMEM) containing 10% foetal calf serum (Imperial Laboratories Europe), 4mM L-glutamine (GIBCO), 100 units/ml penicillin and 10mg/ml streptomycin (GIBCO) at 37^0C in a 5% CO_2 atmosphere. Cells were passaged every 2-3 days inoculating at a density of approximately $4x10^4$ cells/ml.

NIH 3T3 cells were also cultured in DMEM as described above. For serum stimulation experiments $1x10^6$ cells were plated into DMEM containing 0.5-0.1% foetal calf serum (FCS) and cultured for 36h. Each culture was washed once and subsequently cultured in DMEM containing 10% FCS and cells were harvested at the appropriate times (every 4 hours) to give a time course. For analysis by

immunofluorescence (section 2.5.2) sterile glass coverslips were introduced during the passage of cells and treated as above with the exception that coverslips were removed for staining instead of harvesting. Cultures were assessed for DNA synthesis using a cell proliferation kit (Amersham). Cultures were incubated prior to treatment with DMEM containing 10% FCS in media with bromodeoxyridine (BrDu) diluted 1 in 1000 (Amersham) according to the manufacturers recommendations for 12h.

JM cells were derived from a T-cell leukaemia (Schneider *et al.*,1977) and cultured in RPMI (Imperial Laboratories Europe) containing 10% FCS with glutamine and antibiotic as described above. Cells were passaged every 2-3 days inoculating at a density of $3x10^4$.

2.5.2 Immunofluoresence

Coverslips were removed from culture dishes and washed with PBSA three times. For cells incubated with anti-DP-1 α18 (Girling *et al.*,1993) a fixative of acetone:methanol (1:1) was added for 3 min washed three times with PBSA and then incubated in PBSA with 0.1% Triton X-100 for a further 5 min. Coverslips were washed twice with PBSA and once with PBSA containing 1% FCS. Primary antibody was diluted 1/100 in PBSA containing 1% FCS with either 2nmol of DP-1 peptide 18 or c-jun peptide 1 per μl of antisera used. Antibody reactions were incubated for 30-40 min washed twice with PBSA and three times with PBSA containing 1%FCS. Secondary antibody, fluorescene conjugated anti-rabbit (EuroPath Ltd.) was diluted 1/100 in PBSA containing 10% FCS and incubated for 30 min. Coverslips were washed three times with PBSA wet mounted on slides and stored in the dark at 4^0C.

For cultures labelled with BrDu a fixative containing 90% ethanol 5% acetic acid and 5% water was used for 30 min. Coverslips were washed as above and incubated with primary antibody for 1h according to the manufactures recommendations. Coverslips were washed as described above and incubated with secondary antibody, rhodamine conjugated anti-mouse (EuroPath Ltd.) and processed as above. The percentage of cells in S-phase was assessed by counting 300 cells scoring for those which showed a clear nuclear stain from the BrDu antibody.

CHAPTER 3

DRTF1/E2F forms DNA binding complexes which are modulated by the adenovirus E1a protein

3.0 Introduction

The cellular transcription factor, DRTF1 was originally identified in murine F9 EC cells using the adenovirus E2a promoter (La Thangue and Rigby, 1987). DRTF1, which binds to a single E2F site, resolves as several DNA binding complexes when assayed by gel retardation, termed DRTF1 **a**,**b** and **c**, **a** being the slowest migrating complex and **c** the fastest (La Thangue and Rigby,1987;La Thangue *et al.*,1990). In contrast E2F (E2 factor) was identified using the E2a promoter in human HeLa cells and was believed to require both E2F sites on the promoter and be induced during adenovirus infection (Kovesdi *et al.*,1986a;Yee *et al.*,1987). Therefore, during the initial characterisation of these factors the relationship between the two was unclear. It was possible that they were closely related factors or the same factor which responds differently to adenovirus infection and cellular differentiation. The results presented in a later chapter of this thesis suggest that both DRTF1 and E2F contain common DNA binding subunits and consequently, for simplicity, I will refer to all DNA binding complexes which form on the E2F site in cell extract as DRTF1/E2F.

In EC cell extracts the **b/c** complex is abundant whereas the **a** complex is present in low levels. However, as these cells differentiate to form PE cells the DNA binding

activity of the **b/c** complex is down-regulated (La Thangue *et al.*,1990;Partridge and La Thangue, 1991). This correlates with the activity the E2a promoter which is transcribed efficiently *in vitro* and *in vivo* in EC cells, but not in PE cells (Imperiale *et al.*,1984;La Thangue and Rigby,1987;La Thangue *et al.*,1990). Purification of the **b/c** complex from F9 EC extracts yielded several polypeptides which activated E2a specific transcription *in vitro*, suggesting the **b/c** form was a positively acting binding site-dependent transcription factor (Shivji and La Thangue,1991;Girling *et al.*,1993).

When this study was initiated some of the properties of the **b/c** complex had been established, although the nature of the **a** complex was less well understood. It was possible that the **a** complex represented a related protein or alternatively, was the same protein either modified differently or complexed to other proteins to form a slower migrating DNA binding complex. The DNA binding activity of the **a** complex had been shown to be affected by both phosphatase treatment and detergents, but also possessed the same DNA binding specificity of the **b/c** complex. Therefore they were likely to be closely related (La Thangue *et al.*,1990;Shivji and La Thangue,1991). The aim of the study presented in this chapter was to investigate the properties of the DRTF1/E2F **a** complex in more detail and establish its relationship to the **b/c** form.

3.1 DRTF1/E2F DNA binding complexes vary between different cell types

To study the **a** complex in more detail, DNA binding complexes were examined in lymphocytic cells as previous observations indicated that the **a** complex was

abundant in mouse thymus extracts (Partridge and La Thangue,1991). Microextracts were made from the human JM, T-cell leukaemic line (Schneider *et al.*,1977) and examined by gel retardation using the E2F binding site (Figure 3.0). JM extracts contained a number of specific DNA binding complexes which were competed by the wild-type E2F site but not a mutant site (Figure 3.1a, compare lanes 7,8 and 9). In contrast to EC extracts the **a** complex was abundant in JM extracts whereas the **b/c** complex was less abundant (Figure 3.1a, compare 5 and 9). In order to rule out the possibility that this was an artefact of extract preparation the properties of the ATF site DNA binding activity ECRE2 (Tassios and La Thangue,1991) was also assayed. Although the abundance of ECRE2 varied between the different cell types its mobility was unaffected suggesting that the differences in DRTF1/E2F complexes is a property of the cell type (Figure 3.1a, lanes 6,10 and 14). Interestingly, the **a** complex in JM extracts was composed of two different complexes of slightly different mobility when compared to the **a** complex in EC extracts (Figure 3.1b, compare 5 and 9). Adult mouse thymus extracts also contained abundant **a** complex DNA binding activity as shown previously (Partridge and La Thangue,1991,Figure 3.1a, compare lanes 11-13).

The DNA binding specificity of DRTF1/E2F complexes in JM and EC extracts was established by gel retardation using a panel of mutant oligonucleotides derived from the E2a promoter (summarised in Figure 3.0, La Thangue *et al.*,1990). The DNA binding specificity of EC and JM DRTF1/E2F complexes was similar (Figure 3.1b, compare lanes 2-7 with 8-13) and in good agreement with previously published results using EC extracts a result again suggesting that they are closely related (La Thangue

et al.,1990).

3.2 The DRTF1/E2F **a** complex is modulated by the adenovirus E1a protein

During adenovirus infection the E1a protein is required for *trans*-activation of the E2a promoter through the E2F site (Berk *et al.*,1979;Imperiale *et al.*,1984). To assess whether DRTF1/E2F complexes were affected by the E1a protein, *in vitro* transcribed and translated 13S E1a protein (Figure 3.2a, and Figure 3.2b) was added to JM extracts and assayed by gel retardation. Lysate containing the E1a protein caused the disruption of the **a** complex and an increase in binding activity of the **b/c** form, whereas the lysate alone had no effect (Figure 3.2c, compare lanes 1 with 2, also 3 with 4). This result was specific for DRTF1/E2F complexes since the ECRE2 complex was unaffected by either the lysate alone or lysate containing E1a (Figure 3.2c, lanes 5 and 6). The DRTF1/E2F **a** complex also was specifically disrupted by E1a in EC extracts (Figure 3.2c, compare lanes 7 and 8), suggesting a common mechanism of action in both cell types.

To establish which regions within E1a were responsible for this effect a number of cDNAs encoding proteins which lacked different regions of E1a were transcribed and translated *in vitro* (summarised in Figure 3.2a). All constructs produced the appropriate polypeptide when assessed by the incorporation of ^{35}S-methionine (Figure 3.2b, lanes 1 to 4). The 12S E1a protein lacking conserved region 3 (CR3) disrupted the **a** complex in a similar manner to the 13S product indicating that CR3 is not required for this effect (Figure 3.2d, compare lanes 5 and 6 with 11 and 12).

However, deletions of either CR1 or CR2 severely compromised the ability of E1a to dissociate DRTF1/E2F complexes (Figure 3.2d, compare lanes 5 and 6 with 7-10). Thus, disruption of DRTF1/E2F requires both CR1 and CR2 but not CR3.

3.3 The **a** complex is modulated by SV40 large T antigen

Both SV40 (Simian Virus 40) large T antigen and E1a bind a number of similar cellular polypeptides through regions which show amino acid sequence similarity (see Figure 3.3b, for comparison between E1a and other viral oncoproteins). Therefore I investigated whether T antigen could disrupt the **a** complex in gel retardation assays. T antigen was transcribed and translated *in vitro* as previously described (section 3.2) and added to EC extracts. T antigen disrupted the **a** complex in a similar fashion to E1a whereas the lysate alone had no effect (Figure 3.3a compare lanes 3 and 4 with 5,6,1 and 2). Thus, like E1a, T antigen is also able to modulate DRTF1/E2F complexes suggesting these viral oncoproteins have evolved a common mechanism.

3.4 Discussion

In this study I made several important observations. Firstly, DRTF1/E2F complexes are influenced by the cell type. Thus, in contrast to EC extracts, JM extracts contained predominantly DRTF1/E2F **a** with less of the **b/c** complex. Secondly, the DRTF1/E2F **a** complex was sensitive to both the E1a protein and SV40 large T antigen. Since the **b/c** complex was apparent in the presence of these proteins

it suggested that DRTF1/E2F **a** is composed of the **b/c** complex.

3.4.1 DRTF1/E2F complexes vary in different cell types

The reasons for an abundance of the DRTF1/E2F **a** complex in JM extracts are not clear. It is however unlikely to be a property of leukaemic cell lines since complexes formed in mouse thymus extracts were of a similar nature. In addition Partridge and La Thangue (1991) have shown that DRTF1/E2F complexes vary during mouse embryonic development, supporting the notion that DRTF1/E2F complexes are likely to be influenced by the properties of different cell types. It is possible that different forms of DRTF1/E2F have different transcription properties or transcribe specific target genes. Both the **a** and **b/c** complexes were also shown to contain a similar size DNA binding polypeptide, suggesting they may use the same DNA binding subunit, although one can not exclude that two related factors of a similar molecular weight were present (Partridge and La Thangue,1991). Furthermore, one can not conclude that a DNA binding activity defined using the same oligonucleotide site in different cell types is necessarily the same protein until a molecular characterisation has been performed, since many transcription factors can bind to the same DNA sequence. This is exemplified by the jun, fos and ATF family of transcription factors which can bind a similar DNA sequence (reviewed in Karin and Smeal,1991). In some cases dimerisation between the jun and ATF transcription factors can lead to an altered DNA binding specificity, which may be used to distinguish between these transcription factors (Hai and Curran,1991). The DNA specificity of JM complexes

appeared to be the same as EC complexes supporting the idea that they are closely related or the same factor. It was debatable whether further mutational analysis would be more informative and thus, the DNA binding complexes in JM cells were assumed be DRTF1/E2F.

3.4.2 DRTF1/E2F complexes are modulated by a viral oncoprotein

The adenovirus E1a immediate early protein is able to *trans*-activate the adenovirus E2a promoter and is also known to affect transcription of a number of cellular genes, such as the c-myc and β-tubulin genes (Berk *et al.*,1979;Imperiale *et al.*,1984; Thalmeier *et al.*,1989;Stein and Ziff,1987). In the E2a and c-myc promoters the E2F site has been shown to be important for activation by E1a (Imperiale *et al.*,1984;Thalmeier *et al.*,1989). E1a was found to disrupt the **a** complex specifically in JM extracts resulting in increased binding of the **b/c** complex, a form which is known to activate transcription *in vitro* (Shivji and La Thangue,1991). E1a also disrupted the **a** complex in EC extracts, suggesting this effect is not restricted to JM cells. One explanation for the ability of E1a to activate the E2a promoter could be by increasing the levels of the **b/c** complex from the DRTF1/E2F **a** complex, a model which therefore predicts that the **a** complex has a lower transcriptional activity than **b/c**. Although this is the most obvious interpretation (summarised in Figure 3.4), it does not exclude that E1a activates transcription of the E2a promoter through indirect mechanisms.

During adenovirus infection E1a is required for the *trans*-activation of the

early genes E2a, E3 and E4 (Berk *et al.*,1979;Nevins,1981). In EC cells E1a is not required for *trans*-activation of the E2a promoter which has been taken as evidence for a cellular "E1a-like" activity which is able to substitute for the viral protein in stem cells (Imperiale *et al.*,1984;Jansen-Durr *et al.*,1989). Since EC cell extracts already contain abundance of the **b/c** complex, one explanation for why E1a is not required for *trans*-activation in this cell type is that **b/c** is already present in sufficiently high levels. Therefore, the E1a-like activity may modulate DRTF1/E2F complexes to maintain high levels of the **b/c** complex (summarised in Figure 3.4). In PE cells, the DNA binding activity of the **b/c** complex is down-regulated whereas the **a** complex is relatively unaffected (La Thangue *et al.*,1990). Therefore again, one might imagine in PE cells E1a would be required to produce more of the **b/c** complex. This simplistic model is only likely to be valid for the activation of genes containing E2F sites; the activation of the E3 and E4 genes may occur by different mechanisms, perhaps involving other transcription factors, such as ATF-2 or TBP (Lui and Green,1991;Lee *et al.*,1991).

Disruption of DRTF1/E2F is likely to have another outcome with respect to activation of the E2a promoter which relates to the product of the adenovirus E4 gene. The E4 open reading frame 6/7 (orf6/7) protein can interact with DRTF1/E2F, promoting binding to both E2F sites on the E2a promoter (Huang and Hearing,1989;Reichel *et al.*,1989). This results in a greater activation of transcription since the DRTF1/E2F-E4 complex binds DNA more stably than DRTF1/E2F alone (Huang and Hearing,1989;Neill *et al.*,1990). E4 only interacts with the **b/c** form of DRTF1/E2F and the disruption of heteromeric DRTF1/E2F complexes by E1a is

required for this interaction to occur (Bagchi *et al.*,1990). Therefore, the release of DRTF1/E2F not only produces more transcriptionally active protein but also enables an interaction with the E4 protein (summarised in Figure 3.4).

The E2F sites on the adenovirus E2a promoter are present as an inverted repeat which has not been observed on cellular promoters and the precise positioning and orientation of the sites is essential for activation by E4 (Hardy and Shenk,1989;Neill *et al.*,1990). Thus, the ability of E4 to target DRTF1/E2F is thought to allow selective activation of viral genes rather than cellular ones (summarised in Figure 3.4). Again, because EC cells already contain an abundance of the **b/c** complex, E4 could directly target this factor in the absence of E1a. Therefore, two viral proteins encoded by adenovirus, E1a and E4, have evolved to specifically modulate this cellular factor suggesting DRTF1/E2F plays an important role in adenovirus infection.

3.4.3 DRTF1/E2F may mediate virally induced cellular transformation

E1a contains three conserved regions (CR) which are common between different serotypes of adenovirus. The 12S protein lacks CR3, a region known to be a general activator of transcription (Lille and Green,1989;Moran and Mathews,1987). In this study CR3 was not required for dissociation of DRTF1/E2F complexes however, deletion of either CR1 or CR2 reduced the ability of E1a to disrupt the **a** complex efficiently suggesting both regions are important for this effect. E1a is thought to be oncogenic requiring both CR1 and CR2 for the transformation tissue cultured cells. Therefore disruption of DRTF1/E2F may also play a role in these biological processes

(Whyte *et al.*,1989). It is possible that the oncogenic effects of E1a could be achieved by activating growth promoting genes such as c-myc which are activated through the E2F site (Thalmeier *et al.*,1989). The promoters of other growth regulatory genes such as the N-myc and EGFR (epidermal growth factor receptor) genes also contain E2F binding sites and if these sites are important for the transcriptional regulation, DRTF1/E2F is likely to mediate E1a-induced cellular transformation (Mudryj *et al.*,1990).

The mechanism by which E1a disrupted DRTF1/E2F could be direct, by removing components of the complex or alternatively E1a could activate kinases in the extract to produce this effect. However since E1a binds to a number of cellular proteins a likely explanation for these affects is that E1a sequesters one or more of these proteins from the **a** complex. The regions in E1a required to disrupt DRTF1/E2F are necessary for binding to several cellular proteins some of which have been identified and include, the tumour suppressor retinoblastoma gene product (pRb), pRb-related p107 protein, cyclin A and a cyclin-dependant kinase (Whyte *et al.*,1988;Pines and Hunter,1990;Tsai *et al.*,1991;Ewen *et al.*,1991). Therefore these proteins may be components of the DRTF1/E2F **a** complex.

The SV40 large T antigen and the human papilloma E7 proteins contain regions with sequence similarity to CR1 and CR2 of E1a, therefore as might be predicted both T and E7 are able to transform cells and bind some of the same polypeptides sequestered by E1a (Figure 3.3b, DeCaprio *et al.*,1988;Dyson *et al.*,1989). In this study, T antigen was shown to be able to disrupt DRTF1/E2F complexes and similar results have been obtained for E7 (Phelps *et al.*,1991;Morris *et al.*,1993). This is

consistent with previous observations showing that the E2a promoter could be *trans*-activated by both T antigen and E7. Therefore, activation of the E2a promoter by T antigen and E7, as with E1a, may be by generating more of the transcriptionally active **b/c** complex. Thus, these different viral oncoproteins have evolved a common mechanism to regulate the activity of this transcription factor. Since these viral oncoproteins sequester a number of cellular polypeptides which are also bound by E1a, a common mechanism involving DRTF1/E2F may link the oncogenic effects of E1a, T antigen and E7 to the initiation of transcription.

Figure 3.0, *Summary of the E2F binding site oligonucleotides derived from the adenovirus (Ad5) E2a promoter.* Binding sites for transcription factors are indicated (shaded boxes) on the promoter with respect to the transcriptional start site at +1. Arrows above the E2F sites indicate the orientation of each site. Note that all E2F site oligonucleotides are derived from the distal E2F site only and mutated bases being indicated in bold. The ATF site is derived from the adenovirus (Ad5) E4 promoter.

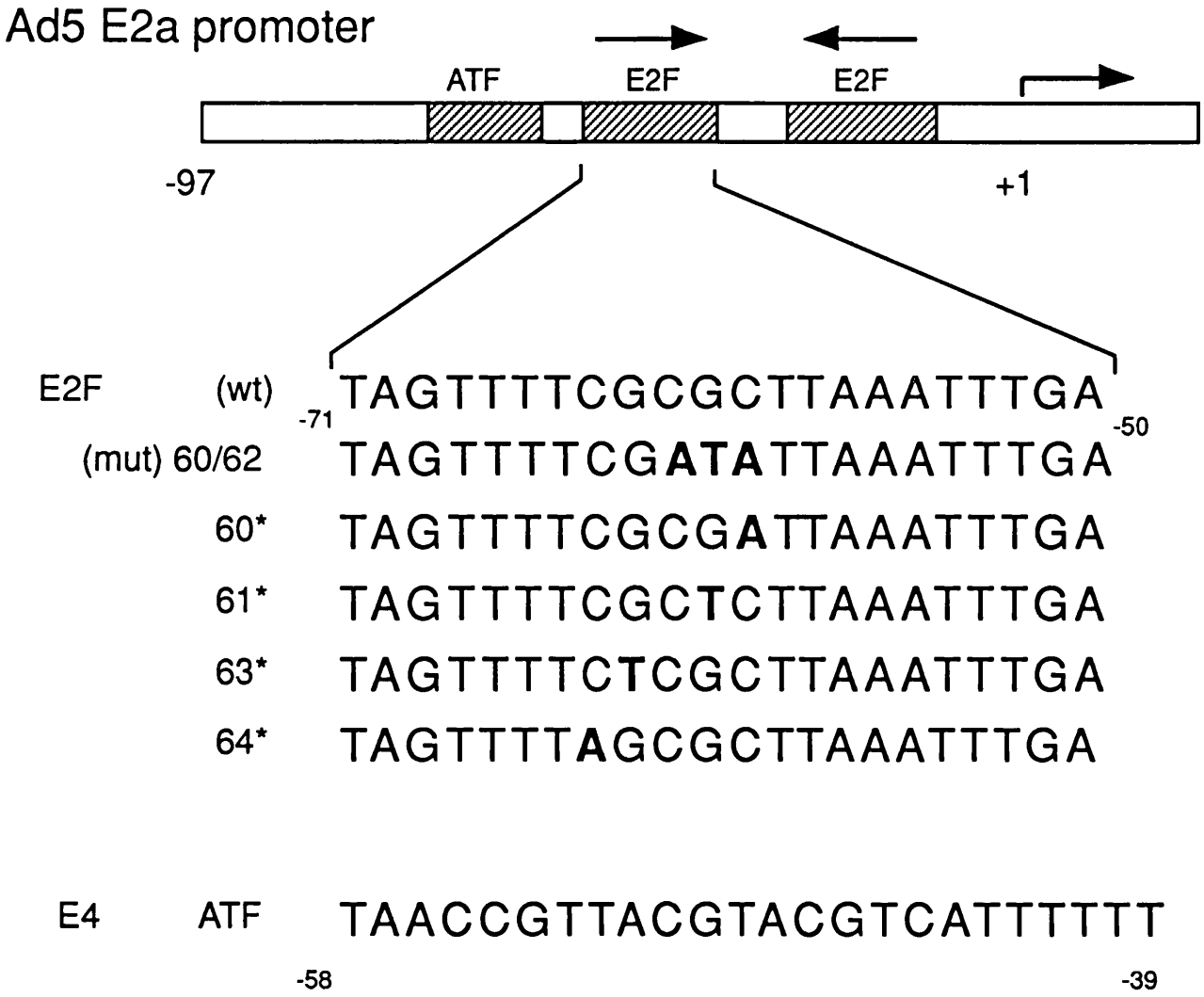

Ad5 E2a promoter
ATF
E2F
E2F
-97
+1
E2F (wt) -71 TAGTTTTCGCGCTTAAATTTGA -50
(mut) 60/62 TAGTTTTCGATATTAAATTTGA
60* TAGTTTTCGCGATTAAATTTGA
61* TAGTTTTCGCTCTTAAATTTGA
63* TAGTTTTCTCGCTTAAATTTGA
64* TAGTTTTAGCGCTTAAATTTGA
E4 ATF TAACCGTTACGTACGTCATTTTTT
-58
-39

Figure 3.1a, *DRTF1/E2F complexes vary in different cell types*. Gel retardations performed with EC (lanes 3-6), JM (lanes 7-10) and mouse thymus (lanes 11-14) microextracts using the E2F binding site (lanes 1,3-5,7-9 and 11-13) or ATF site (lanes 2,6,10 and 14) as probe. Where indicated 300ng (50X molar excess) of wild-type (wt) or mutant (mut, 60/62) competitor was also added. Specific complexes are labelled (**a** and **b/c**) and non-specific binding is shown by *.

Figure 3.1b, *DRTF1/E2F complexes in JM and EC extracts possess a similar DNA binding specificity*. Gel retardations with EC (lanes 2-7) or JM (lanes 8-13) microextracts using the E2F site as probe. Where indicated 300ng of each competitor was added.

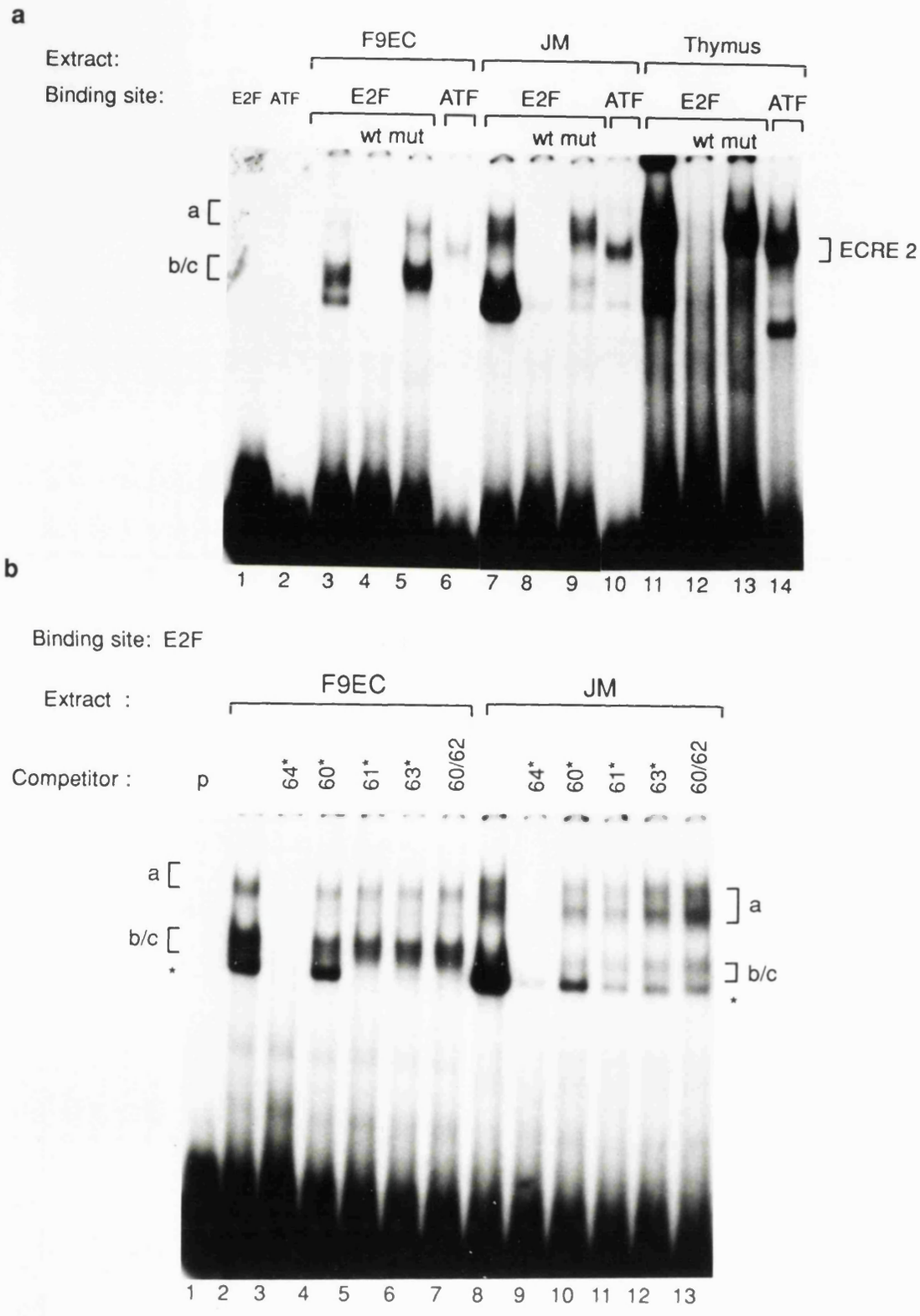
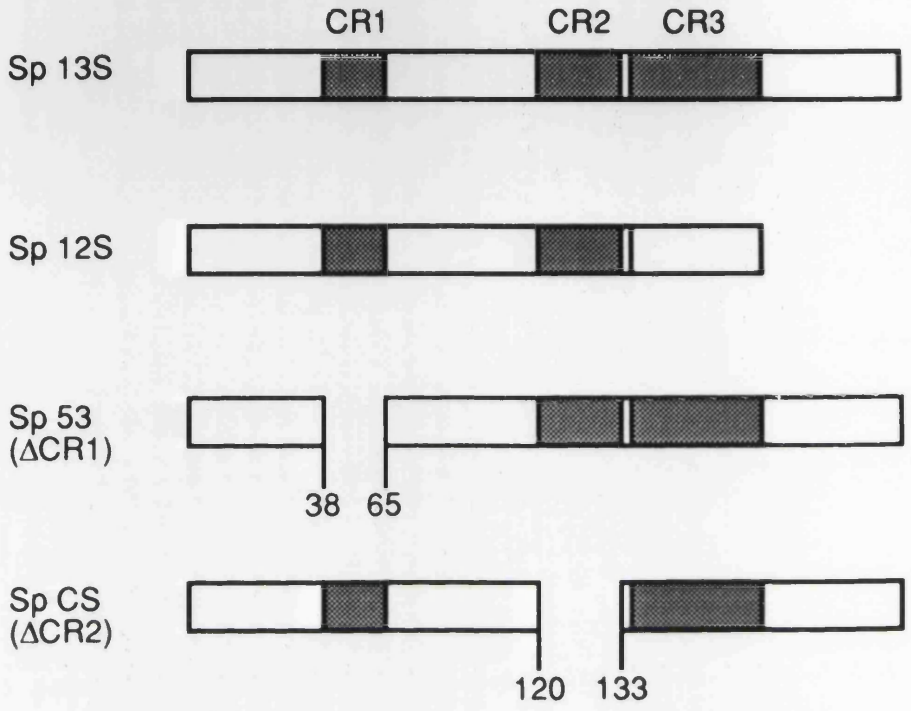

a
Extract:
F9EC
JM
Thymus
Binding site:
E2F ATF
E2F
ATF
E2F
ATF
E2F
ATF
wt mut
wt mut
wt mut
a
b/c
ECRE 2
1 2 3 4 5 6 7 8 9 10 11 12 13 14
b
Binding site: E2F
Extract :
F9EC
JM
Competitor :
p
64*
60*
61*
63*
60/62
64*
60*
61*
63*
60/62
a
b/c
*
a
b/c
*
1 2 3 4 5 6 7 8 9 10 11 12 13

Figure 3.2a, *Summary of E1a constructs used for in vitro transcription and translation.* The conserved regions (CR) of E1a are shown by shaded boxes and the amino acid residues deleted in mutant constructs are indicated.

Figure 3.2b, *Translation of E1a polypeptides.* Each construct was transcribed and RNA was translated *in vitro* with ^{35}S-methionine, E1a polypeptides are indicated as IVT E1a (lanes 1-4).

a


CR1
CR2
CR3
Sp 13S
Sp 12S
Sp 53
(ΔCR1)
38
65
Sp CS
(ΔCR2)
120
133

b

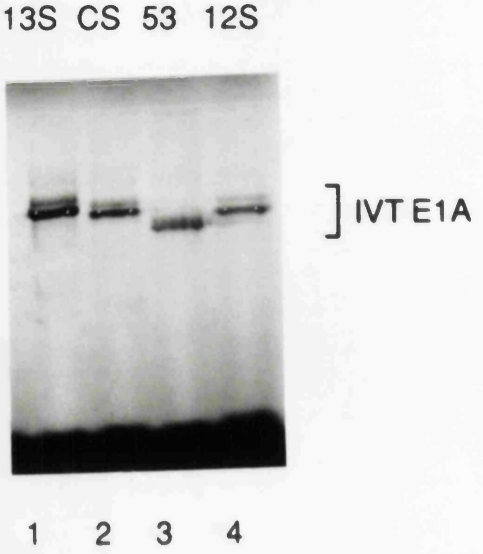
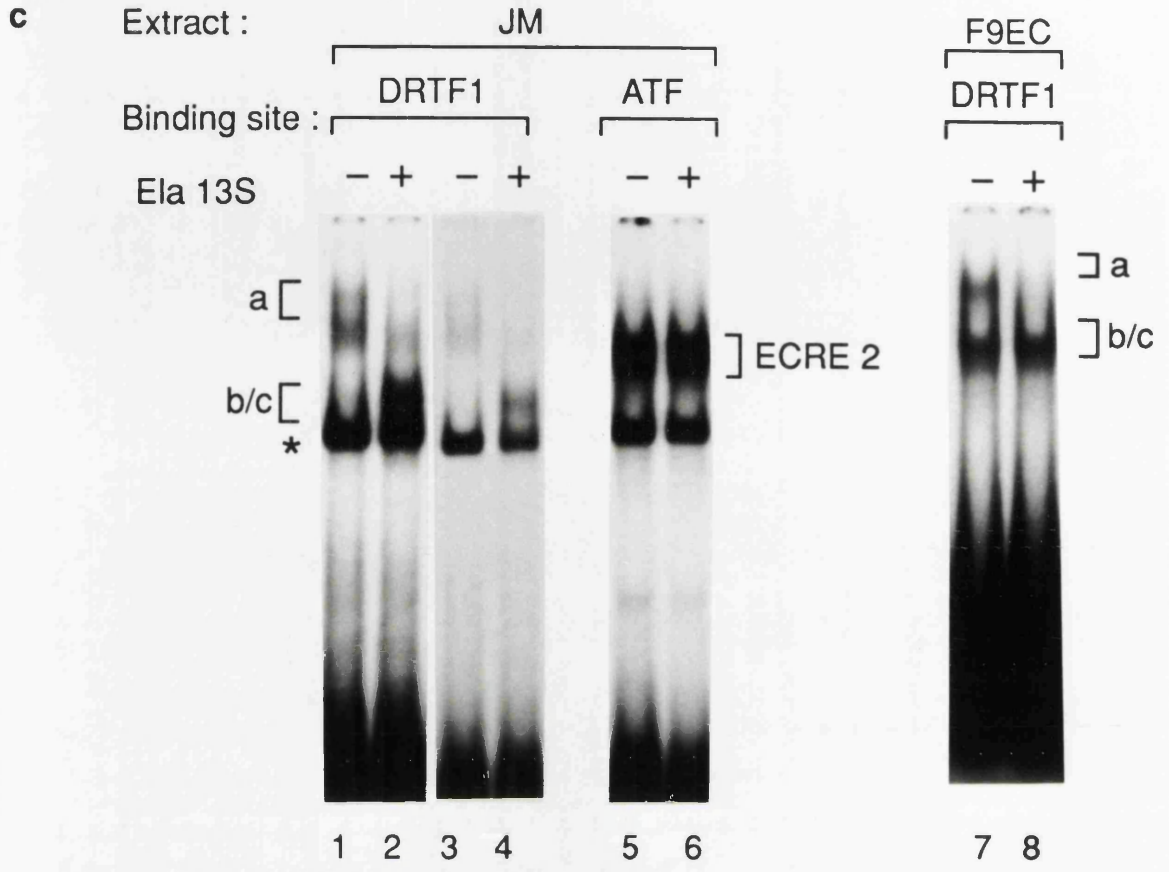

13S
CS
53
12S
IVT E1A
1
2
3
4

Figure 3.2c, *DRTF1/E2F complexes are modulated by the adenovirus E1a protein.* Gel retardations with JM (lanes 1-6) or EC (lanes 7 and 8) whole cell extracts using either the E2F (lanes 1-4,7 and 8) or ATF (lanes 5 and 6) sites as probes. Where indicated either 2μl of lysate alone or lysate containing 13S E1a protein was added to the binding reactions. Note lanes 3 and 4 are a shorter exposure (5 hours) of lanes 1 and 2.

Figure 3.2d *Dissociation of DRTF1/E2F requires CR1 and CR2.* Gel retardations using JM extracts as described above and where indicated either lysate alone (lanes 3 and 4) or lysate containing 13S E1a protein (lanes 5 and 6), CS E1a protein (lanes 7 and 8), 53 E1a protein (lanes 9 and 10) or 12S E1a protein was also added. The probe alone is shown as P and non-specific complexes are shown by *.


c
Extract :
JM
F9EC
Binding site :
DRTF1
ATF
DRTF1
Ela 13S
− + − + − + − +
a
b/c
*
ECRE 2
a
b/c
1 2 3 4 5 6 7 8

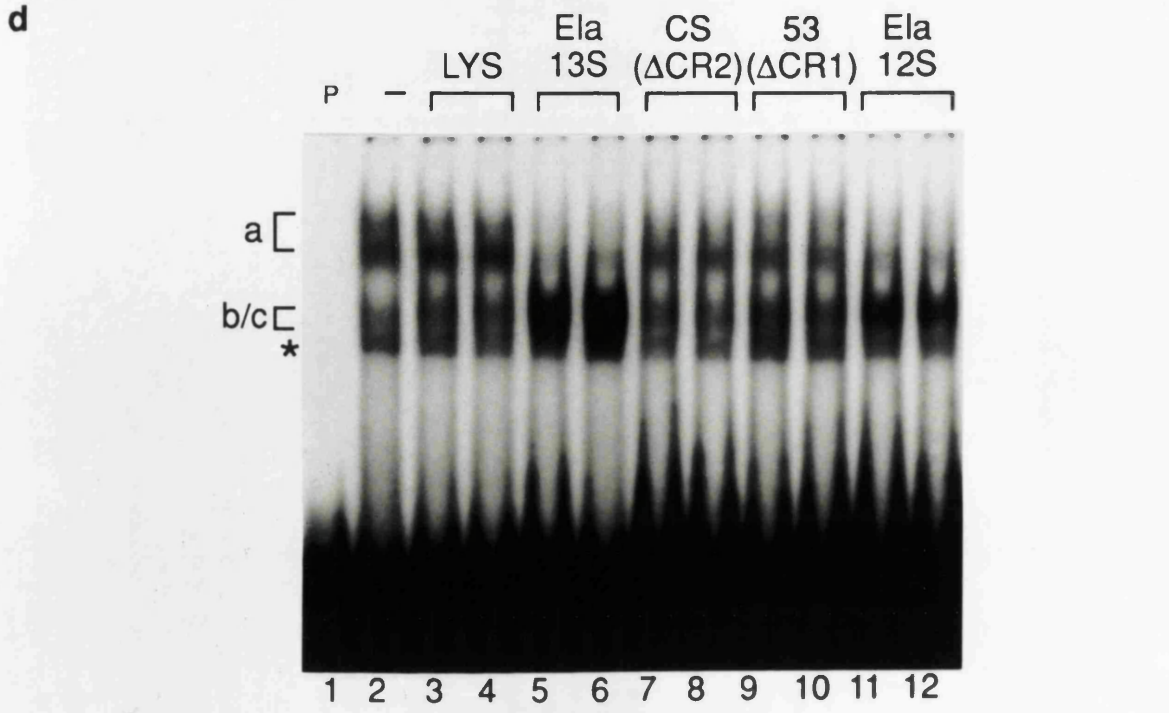
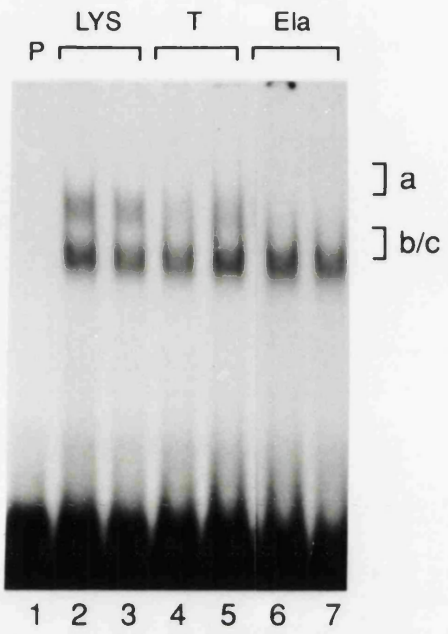

d
P
−
LYS
Ela 13S
CS (ΔCR2)
53 (ΔCR1)
Ela 12S
a
b/c
*
1 2 3 4 5 6 7 8 9 10 11 12

Figure 3.3a *DRTF1/E2F complexes are modulated by the SV40 large T antigen.* Gel retardations were performed by using EC extracts and where indicated 2μl of lysate alone (lanes 2 and 3), lysate containing T antigen (lanes 4 and 5) or lysate containing 13S E1a protein (lanes 6 and 7) was added.

Figure 3.3b, *Summary of the amino acid sequence similarity between adenovirus E1a, SV40 large T antigen and human papilloma virus E7.* The amino acid residues common to adenovirus (Ad5) E1a, T antigen and human papilloma virus (HPV) E7 proteins are boxed and the positions of residues in each protein are indicated.

a


LYS
T
Ela
P
a
b/c
1 2 3 4 5 6 7

b

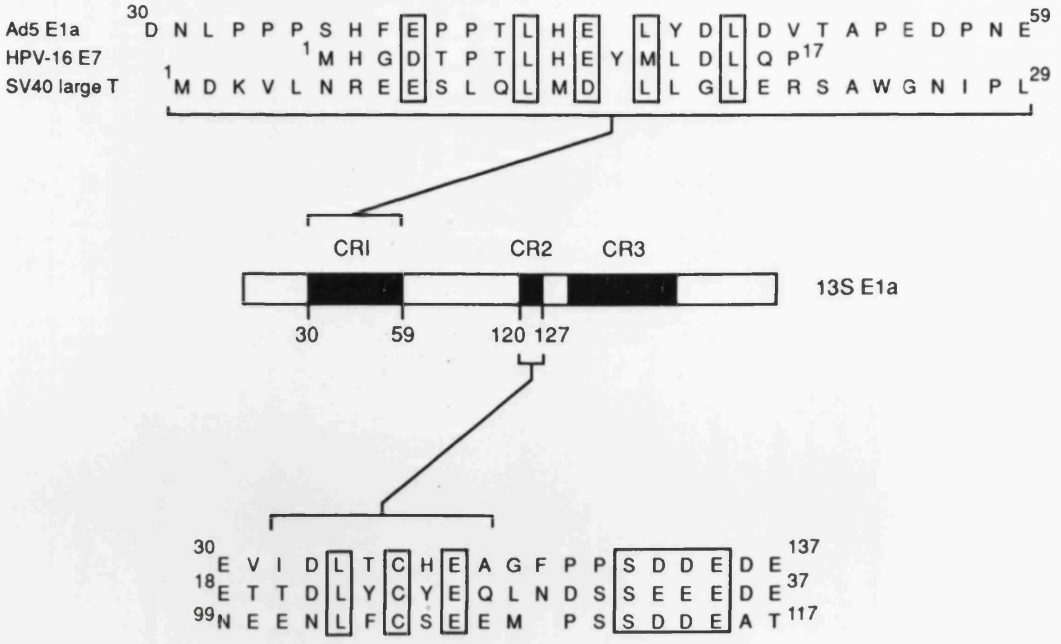
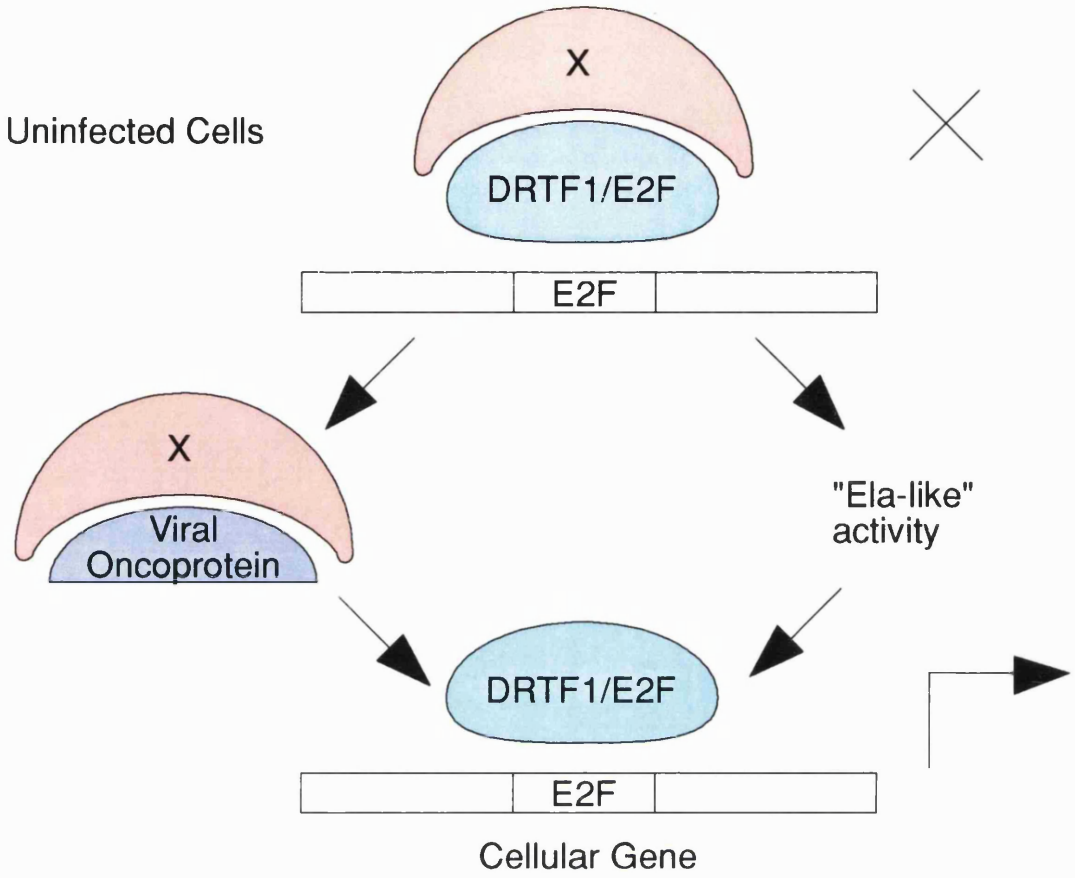
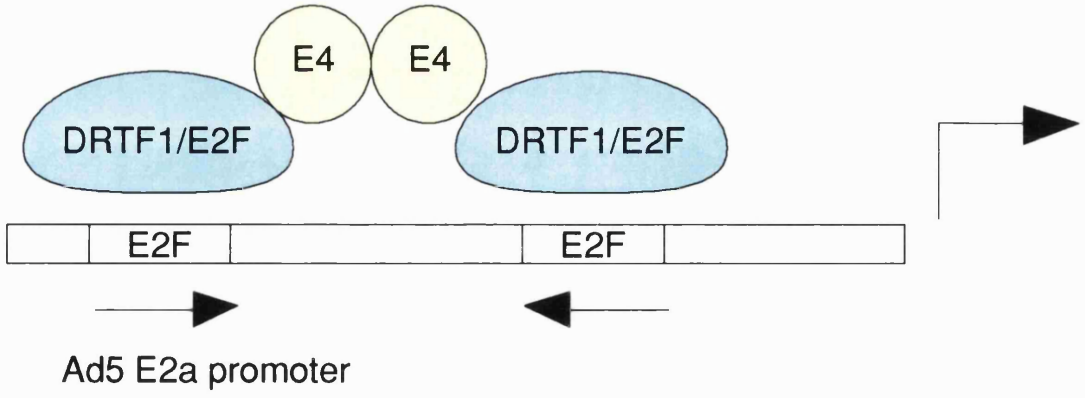

Ad5 E1a
HPV-16 E7
SV40 large T
CRI
CR2
CR3
13S E1a
30
59
120
127

Figure 3.4, *Summary of DRTF1/E2F regulation during adenovirus infection and in embryonal carcinoma cells.* The **b/c** form of DRTF1/E2F is shown in blue and additional proteins which produce the multicomponent complexes are indicated by the red protein X. The effect of viral oncoproteins and the E1a-like activity in EC cells is to cause the appearance of the **b/c** form of DRTF1/E2F which activates transcription of cellular genes. During adenovirus infection the E4 protein (yellow) also interacts with DRTF1/E2F which favours recognition of two appropriately spaced and orientated E2F binding sites such as those in adenovirus E2a promoter.



Adenovirus Infection



CHAPTER 4

DRTF1/E2F binds to the retinoblastoma tumour suppressor gene product

4.0 Introduction

In the last chapter I showed that DRTF1/E2F complexes could be modulated by the adenovirus E1a protein and SV40 large T antigen. The ability of E1a to induce the **b/c** complex at the expense of the **a** complex could arise by a number of mechanisms. E1a may directly or indirectly modify the **a** complex to generate **b/c** or alternatively sequester a component from DRTF1/E2F **a**. The DNA binding activity of the **a** complex has been shown to be sensitive to phosphatase treatment, although it is possible that this may have been an indirect effect rather than due to direct dephosphorylation of the transcription factor (Shivji and La Thangue, 1991). E1a has also been shown to induce DRTF1/E2F DNA binding activity in a phosphorylation-dependent manner in HeLa extracts, although again it is unclear if this was a direct effect (Bagchi *et al.*,1989). However, E1a is associated with a kinase in cell extracts therefore, it is possible that generation of the **b/c** form could be produced through phosphorylation (Tsai *et al.*,1991).

Over-expression of E1a in cells has a variety of the biological effects, such as the induction of DNA synthesis, repression and activation of cellular genes and the transformation and immortalisation of tissue culture cells (Moran and Mathews,1987 summarised in Introduction, Figure 1.3). The conserved regions in the E1a protein

are necessary for these biological effects, for example CR3 contains a transcriptional activation domain which facilitates binding to TBP and other transcription factors, such as ATF-2 or Oct-4 (Lui and Green, 1990;Lee *et al.*,1991;Schöler *et al.*,1991). Furthermore, CR1 and CR2 are necessary for the cell transformation and immortalisation effects of E1a as well as the induction of DNA synthesis (Whyte *et al.*,1989;Moran and Mathews,1987). E1a binds a number of cellular polypeptides in infected and transformed cells which requires both CR1 and CR2, therefore these cellular proteins were thought to be important for mediating the biological effects and have been named according to their size, p300, p130, p107, p105, p60 and p30 (Yee and Branton 1985;Harlow *et al.*,1986;Whyte *et al.*,1989). Several of these have been identified and characterised, the p105 polypeptide was shown to be pRb and the p60 polypeptide was shown to be cyclin A (Whyte *et al.*,1988;Pines and Hunter,1990). Similarly the p30 polypeptide was shown to be the cyclin-dependent kinase $p33^{cdk2}$ and p107 was shown to be a pRb-related protein (Tsai *et al.*,1991;Ewen *et al.*,1991). Since CR1 and CR2 of E1a were also the regions required to disrupt DRTF1/E2F, it was possible that these proteins may be components of the **a** complex. I tested therefore if pRb was a component of DRTF1/E2F.

4.1 The retinoblastoma gene product is a component of the **a** complex

To test if pRb is a component of the **a** complex, monoclonal antibodies which raised against different regions of pRb (Whyte *et al.*,1988;Hu *et al.*,1991) were added to JM whole cell extracts (WCE) or extracts partially purified by heparin Sepharose

(HS) chromatography. The two pRb monoclonals, C36 and XZ55, caused the appearance of a slower migrating complex, referred to as a "supershift", in both WCE and HS extracts which was not apparent with a control monoclonal antibody of the same isotype,IG4 (Figure 4.1a, compare lanes 2 and 3 with 4 to 7 and lanes 8 and 9 with 10 to 13). The interaction of pRb with DRTF1/E2F was specific as another DNA binding activity, ECRE2, was unaffected by either control or anti-pRb antibodies (Figure 4.1b, lanes 2 to 13). It was possible that this effect could result from a cross reaction of the antibodies to DRTF1/E2F. In an attempt to rule out such a possibility, similar experiments were performed using HeLa extracts where the **b/c** form is abundant (La Thangue *et al.*,1990). The **b/c** DNA binding activity in HeLa extracts was unaffected by the control monoclonal antibody, although the pRb monoclonal antibodies produced supershifted complexes which probably resulted from the low levels of the **a** complex present in these extracts (Figure 4.1c, lanes 2 to 7). In contrast the **b/c** complex was unaffected by pRb antibodies implying that these monoclonal antibodies do not cross react with the **b/c** complex in HeLa extracts (Figure 4.1c, compare lanes 2 and 3 with 4 to 7). As in JM extracts, the ECRE2 activity in HeLa was unaffected by either control or pRb monoclonal antibodies (Figure 4.1d, lanes 2-7). The combined conclusion from these data suggests that pRb is a component of the DRTF1/E2F **a** complex.

4.2 Naturally-occurring mutant pRb alleles encode proteins which fail to bind DRTF1/E2F

To gain further support for the idea that DRTF1/E2F is a target for pRb, I tested if pRb would bind DRTF1/E2F *in vitro*. For this, pRb was expressed and purified as a fusion protein from bacteria and added to gel retardations with EC extracts (Kaelin *et al.*,1991;Smith and Johnson,1988). Addition of GST-Rb caused the conversion of **b/c** complex to form a slower migrating complex with similar mobility to the **a** complex (Figure 4.2a, compare lanes 2 and 3). Therefore, pRb binds to DRTF1/E2F in crude extracts.

The pRb gene is often mutated in human tumours which certain cases results in the production of proteins believed to have lost their growth suppressing properties. Two naturally occurring mutant pRb alleles isolated from small cell lung carcinomas (SCLC) which contain a point mutation in exon 21 (amino acid 706) or a deletion of exon 22 respectively (summarised in Figure 4.2b), were therefore tested for their ability to bind DRTF1/E2F (Kaye *et al.*,1990;Horowitz *et al.*,1990). Addition of equal amounts of either GST-Rb^{706} or GST-Rb^{Ex22} failed to cause the appearance of the slow migrating DRTF1/E2F **a** form in conditions where the wild-type GST-Rb could (Figure 4.2a, compare lanes 3-5 with 6-8 and lanes 11-13 with 14-16). Thus, the behaviour of these mutant pRb proteins differs from wild-type protein in that they are unable to bind DRTF1/E2F.

4.3 Discussion

In the last chapter I showed the a complex was disrupted by the E1a protein, a possible mechanism being that it sequestered a component from the complex. In this chapter I have established that one of the components of the DRTF1/E2F a complex is pRb, suggesting a role for pRb at the level of transcription.

It is believed that pRb negatively regulates the cell cycle and, therefore it has been described as a tumour suppressor or anti-oncogene. This idea is based on several observations, for example the pRb gene is frequently mutated in a variety of human tumour cells, such as breast carcinomas, prostate and bladder cancers and small cell lung carcinomas (Harbour *et al.*,1988;Lee *et al.*,1987b;1988;Horowitz *et al.*,1990). Re-introduction of the wild-type gene into some of these tumour cells arrests cell cycle progression and in some cases, produces less tumourigenic growths (Huang *et al.*,1988;Bookstein *et al.*,1989). Also injection of pRb into cells causes growth arrest at the G1/S boundary suggesting that the normal role for pRb is to negatively regulate cellular proliferation (Goodrich *et al.*,1991). As E1a binds pRb through regions necessary for cellular transformation it was thought that some of the oncogenic effects of E1a might be produced by inactivating the growth suppressing effects of pRb (Whyte *et al.*,1989). At the time, however, the mechanism of action of pRb in growth control was unknown.

4.3.1 DRTF1/E2F is a cellular target for pRb

The pRb protein was shown to be a component of the DRTF1/E2F **a** complex suggesting that pRb may function at the transcriptional level by directly binding to a transcription factor. In addition, disruption of DRTF1/E2F by E1a could be explained by the sequestration of pRb from the **a** complex. If DRTF1/E2F were to be a molecule through which pRb exerts its biological effects then E1a may overcome these effects by disrupting its association with this transcription factor. Such a model would imply that DRTF1/E2F positively regulates the cell cycle and may play a central role in cell cycle control.

In human tumour cells the pRb gene is frequently mutated and it is generally assumed that the mutant proteins produced are not capable of growth suppression. Two naturally occurring mutant pRb alleles identified in SCLC, which contain a point mutation in exon 21 or a deletion of exon 22 (Kaye *et al.*,1990;Horowitz *et al.*,1990) encode proteins which failed to bind DRTF1/E2F.

Two conclusions can be drawn from this study. Firstly that regions of pRb protein encoded by exons 21 and 22 are required for binding to DRTF1/E2F. Secondly, these regions are also necessary for pRb growth suppression. Therefore the ability to bind DRTF1/E2F correlates with the ability to suppress growth.

4.3.2 pRb as a regulator of cellular transcription

An obvious question is how pRb modulates DRTF1/E2F transcription? This was

addressed by Zamanian and La Thangue (1992) who showed that E2F site specific transcription was repressed by pRb in transient transfection assays. Furthermore, the mutant pRb gene lacking exon 22 which encodes a protein unable to bind DRTF1/E2F was also unable to repress transcription thereby linking the growth suppressing functions of pRb with the transcriptional repression of DRTF1/E2F (Zamanian and La Thangue,1992). Similar results have also been obtained by a number of other groups (Hiebert *et al.*,1992;Dalton,1992;Qin *et al.*,1992;Qian *et al.*,1992;Hiebert,1993). Thus, the DRTF1/E2F **a** complex is likely to be the transcriptionally inactive form and the **b/c** complex the active form.

For transcriptional activation of the E2a promoter a likely model for the mechanism of action could be that E1a first disrupts transcriptionally inactive pRb-DRTF1/E2F complexes to release "free" DRTF1/E2F which subsequently binds the E4 protein. Therefore the model proposed in figure 3.4 can now be modified by replacing the X component of the **a** complex with pRb. The effect of E1a on DRTF1/E2F could obviously contribute to cellular transformation by enabling **b/c** to activate cellular genes required for cell cycle progression (discussed below).

4.3.3 Regulation of the cell cycle by pRb and DRTF1/E2F

What are the cellular target genes regulated by DRTF1/E2F? As already mentioned in chapter 3, a number of growth promoting genes contain E2F binding site in their promoters (Mudryj *et al.*,1990), and more recently a number of cellular genes required for cell progression, such as the dihydrofolate reductase (DHFR),

DNA polymerase α, thymidine kinase, B-myb and cdc2 genes have been shown to contain E2F sites in their promoters (Blake and Azizkhan,1989;Mudryj *et al.*,1990;Pearson *et al.*,1991;Lam and Watson,1993;Dalton,1992). Many of these genes are transcribed in a cell cycle-dependent manner, with highest expression levels appearing in early S-phase or late G1 (Farnham and Schimke, 1985;Stewart *et al.*,1987;Thompson *et al.*,1986). For the DHFR gene a DNA sequence overlapping the E2F site was shown to be important for the cell cycle regulation of the gene, although a second factor, termed HIP1, was characterised in the early studies (Means *et al.*,1992). However, Slansky *et al.* (1993) subsequently showed that the E2F site alone could confer the cell cycle regulated transcription observed for DHFR to a previously unregulated gene. Similarly, in the B-myb gene, a mutation in the E2F site abolished the periodic expression of this gene, again suggesting that DRTF1/E2F is important for cell cycle regulated transcription (Lam and Watson,1993). DRTF1/E2F could therefore be involved in coordinating the expression of genes required for progression through S-phase. One could imagine therefore that pRb mediated growth suppression is mediated through the repression of these cellular genes, thereby depriving the cell of proteins required for cell cycle progression and leading to cell cycle arrest.

The pRb protein is phosphorylated in a cell-cycle dependent manner being, hypophosphorylated in G1 and gradually phosphorylated upto G2/M, after which it is dephosphorylated (Buchkovich *et al.*,1989;DeCaprio *et al.*,1989;Chen *et al.*,1989;Mihara *et al.*,1989). Since injected pRb arrests cells in G1 when hypophosphorylated pRb is evident, it is generally assumed that hypophosphorylated

pRb is the growth suppressing form. The data presented in this chapter are compatible with the idea that DRTF1/E2F can bind to unphosphorylated pRb. This is because GST-Rb, when expressed in bacteria was unlikely to be produced in a physiologically phosphorylated form, although it was still able to bind DRTF1/E2F (Figure 4.2a). This experiment does not exclude however that DRTF1/E2F can also bind phosphorylated pRb or that pRb becomes phosphorylated in the cell extract. However, it is nevertheless tempting to speculate that DRTF1/E2F binds the growth suppressing form of pRb which is likely to cause the transcriptional repression of genes required for progression through S-phase. In such a model, one could imagine that when pRb becomes phosphorylated or sequestered by E1a and T antigen the active form of DRTF1/E2F is released which is able to transcribe genes necessary for cell cycle progression. Such a model could explain both the mechanism of action of pRb as well as the role of viral oncoproteins in cellular transformation.

4.3.4 Cellular pRb binding proteins with similarity to E1a

Several groups have noted that many of the mutant *pRb* alleles identified in human tumour cells encode proteins which also fail to bind E1a and T antigen (Hu *et al.*,1991;Huang *et al.*,1991;Kaelin *et al.*,1991). One explanation for this is that viral oncoproteins have evolved to sequester pRb by mimicking cellular targets. Therefore, in infected cells E1a and T antigen may compete with these cellular proteins for binding to pRb.

Cellular pRb-binding proteins with amino acid sequence similarity to these viral

oncoproteins have been identified, termed RBP1 (pRb binding protein) and RBP2, although their roles in pRb mediated growth suppression are unknown (Defeo-Jones *et al.*,1991).

An association between pRb and DRTF1/E2F has also been shown by a number of other groups (Bagchi *et al.*,1991;Chellappan *et al.*,1991;Chittenden *et al.*,1991). In one of the studies pRb co-purified with an activity, termed E2F-I, which inhibits DRTF1/E2F DNA binding activity (Bagchi *et al.*,1991). Chellappan *et al.* (1991) showed that purified pRb-DRTF1/E2F complexes contained underphosphorylated pRb a conclusion based only on the mobility of the protein and is therefore not conclusive, although this would support the notion that DRTF1/E2F binds underphosphorylated pRb.

In conclusion, DRTF1/E2F is likely to be an important target for pRb suggesting therefore that one of the mechanisms by which pRb regulates cellular proliferation is at the level of transcription.

Figure 4.1a, *The retinoblastoma gene product in a component of the a complex.* Gel retardations with JM whole cell extracts (WCE) or heparin-Sepharose (HS) purified extracts using the E2F binding site as probe. Where indicated 8μl of pRb monoclonal (C36, XZ55) antibody tissue culture supernatant was added (lanes 4-7 and 10-13) or 8μl of a control antibody (IG4) was added (lanes 2,3,8 and 9). Supershifted DRTF1/E2F complexes are indicated by a^s.

Figure 4.1b, as above except the ATF binding site was used as probe.

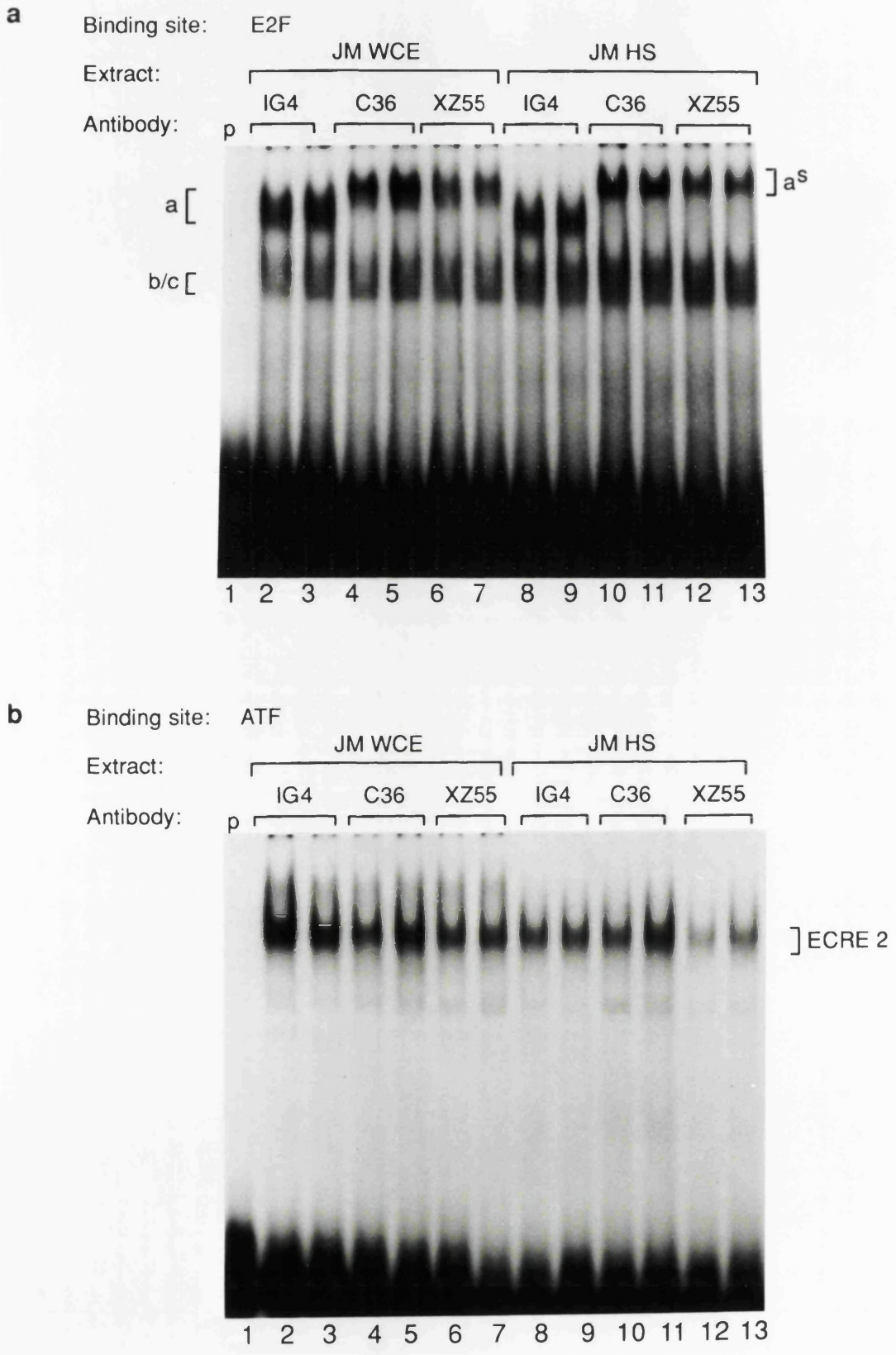

a
Binding site: E2F
Extract:
JM WCE
JM HS
Antibody:
IG4
C36
XZ55
IG4
C36
XZ55
p
a
b/c
a^s
1 2 3 4 5 6 7 8 9 10 11 12 13
b
Binding site: ATF
Extract:
JM WCE
JM HS
Antibody:
IG4
C36
XZ55
IG4
C36
XZ55
p
ECRE 2
1 2 3 4 5 6 7 8 9 10 11 12 13

Figure 4.1c, Gel retardations using HeLa whole cell extracts and the E2F site as probe (lanes 1-7). As in Figure 4.2a pRb monoclonal antibodies (4-7) and control antibody (lanes 2 and 3) were added and supershifted DRTF1/E2F complexes are shown by a^s; note that the **b/c** form of DRTF1/E2F is unaffected by control or pRb antibodies.

Figure 4.1d, as above using the ATF site as probe.

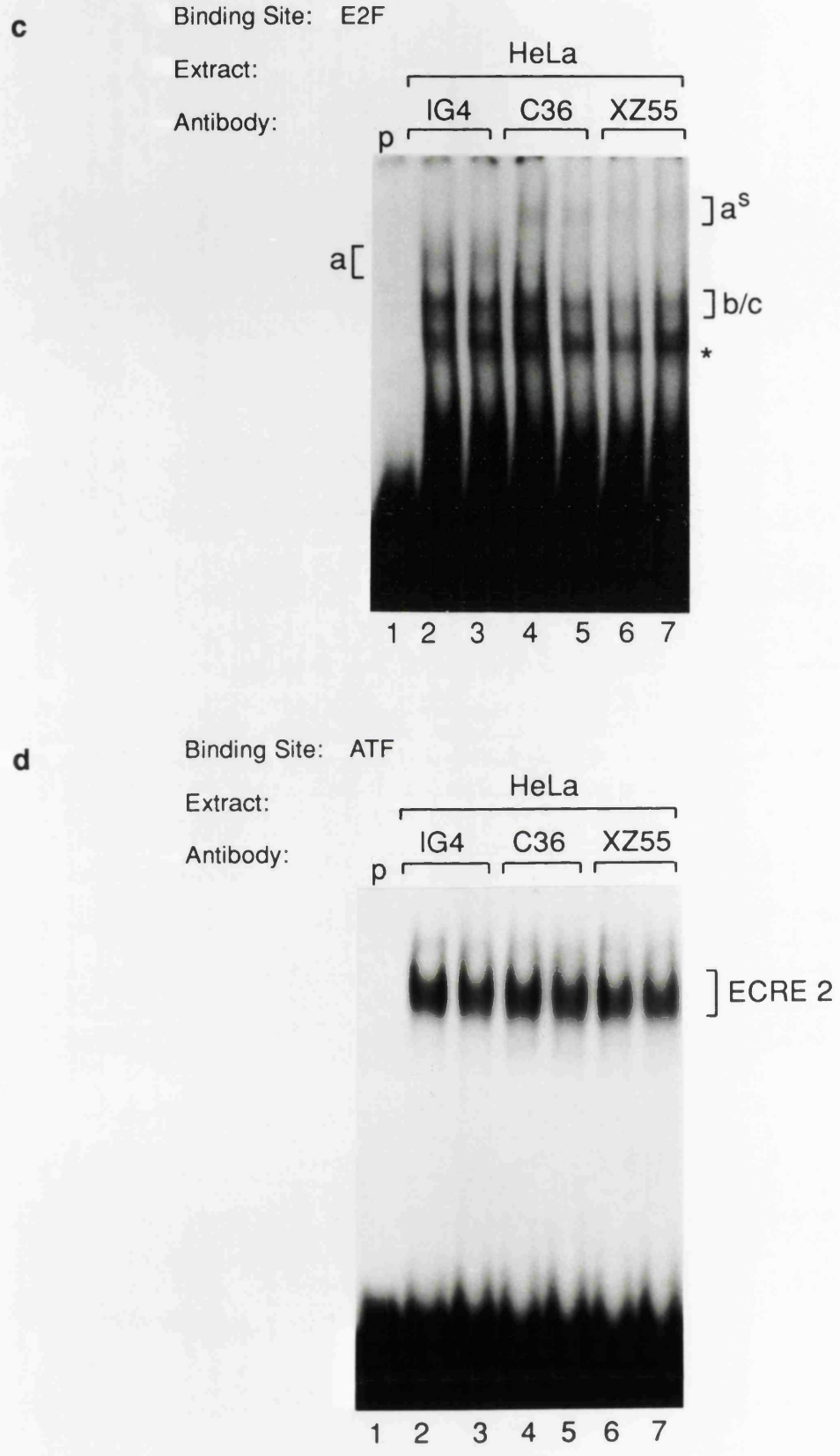
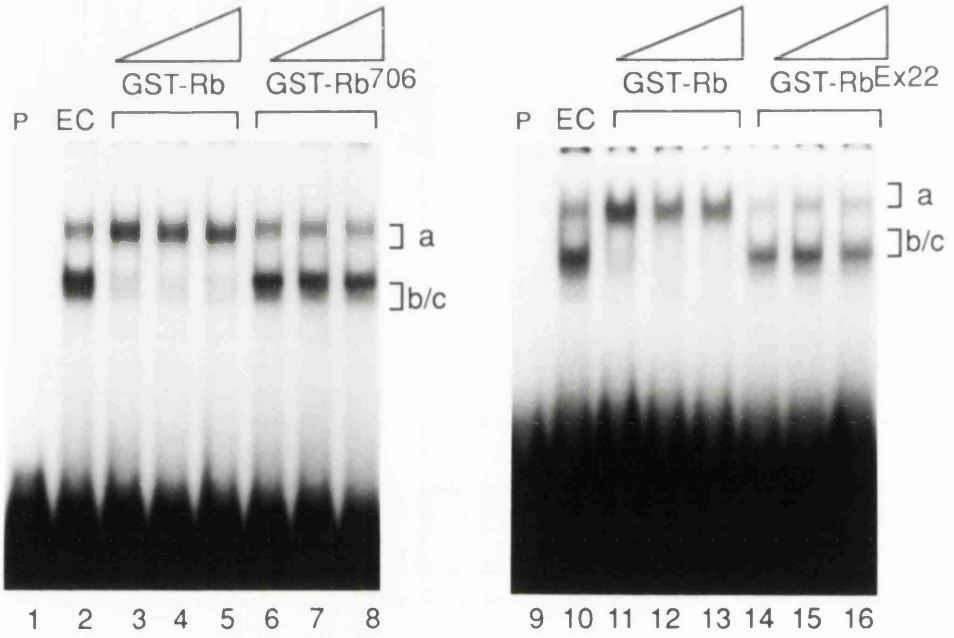

c
Binding Site: E2F
Extract: HeLa
Antibody: IG4 C36 XZ55
p
a[
]a^s
]b/c
*
1 2 3 4 5 6 7
d
Binding Site: ATF
Extract: HeLa
Antibody: IG4 C36 XZ55
p
] ECRE 2
1 2 3 4 5 6 7

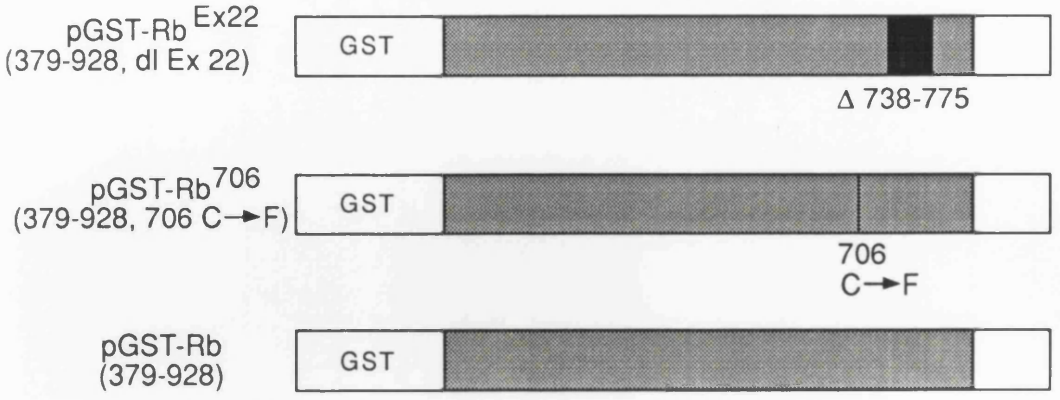
Figure 4.2a, *Naturally occurring mutant pRb alleles encode proteins which fail to bind DRTF1/E2F.* Gel retardations were performed with EC whole cell extracts and where indicated increasing amounts of GST-Rb (lanes 3-5 and 11-13), GST-Rb^{706} (lanes 6-8) or GST-Rb^{Ex22} (lanes 14-16) was added. Approximately 50ng of fusion protein was added in lanes 3,6,11 and 14, 125ng in lanes 4,7,12 and 15 and 450ng in lanes 5,8,13 and 16.

Figure 4.2b, *Summary of GST-Rb constructs.* The positions of mutations in pRb are shown and shaded area indicates the pRb "pocket" domain (amino acid residues 379-792,Kaelin *et al.*,1990).

a


GST-Rb
GST-Rb706
P
EC
a
b/c
1 2 3 4 5 6 7 8
GST-Rb
GST-RbEx22
P
EC
a
b/c
9 10 11 12 13 14 15 16

b


pGST-RbEx22
(379-928, dl Ex 22)
GST
Δ 738-775
pGST-Rb706
(379-928, 706 C→F)
GST
706
C→F
pGST-Rb
(379-928)
GST

CHAPTER 5

The DRTF1/E2F **a** complex is a multicomponent DNA binding complex

5.0 Introduction

The E1a protein sequesters a number of cellular proteins (mentioned in chapter 4) which, like pRb, require CR1 and CR2 for efficient binding (Yee and Branton, 1985;Harlow *et al.*,1986;Whyte *et al.*,1989). Although pRb was shown to be a component of the **a** complex, it was still possible that other proteins could bind to DRTF1/E2F because, for example in JM cell extracts, the **a** complex was composed of several closely migrating complexes with different mobilities which were all dissociated by E1a (chapter 3, Partridge and La Thangue,1991).

One of the proteins sequestered by E1a, cyclin A, is a regulatory subunit for at least two kinase subunits, $p33^{cdk2}$ and $p34^{cdc2}$, which are members of a family of related kinase catalytic subunits (Tsai *et al.*,1991;Meyerson *et al.*,1992). The cyclins and their associated kinases are believed to coordinate cell cycle progression by phosphorylating and modifying the activity of appropriate substrates at the correct time during the cell cycle (Hunt,1989;Sherr,1993).

Cyclin A is sequestered by E1a and recently the E1a-associated p30 polypeptide was shown to be the cdc2-like kinase subunit $p33^{cdk2}$ (Tsai *et al.*,1991). Since cyclin A is a regulatory subunit for $p33^{cdk2}$, the kinase activity associated with E1a is probably derived by binding the cyclin A/cdk2 complex. The biological significance

for E1a binding cdk2/cyclin A is currently unclear, however, since these proteins are important in growth control it is possible their interaction with E1a may contribute to the biological effects of E1a.

The gene encoding the p107 polypeptide has recently been identified and found to encode a protein related to pRb (Ewen *et al.*,1991). The region of similarity between pRb and p107 termed the "pocket" can be divided into two blocks separated by a spacer domain. The pocket defines the minimal sequences in pRb and p107 necessary for binding to E1a and T antigen (Hu *et al.*,1990;Kaelin *et al.*,1990 Ewen *et al.*,1991). Thus, pRb and p107 are referred to as "pocket proteins". It is unclear at present if p107 functions as a tumour suppressor, since the gene has not been reported to be mutated in human tumour cells (Ewen *et al.*,1991), nevertheless, over-expression of p107 in tumour cells can cause cell cycle arrest (Zhu *et al.*,1993).

Another E1a-associated protein, p300, has not been isolated yet, although it appears to require regions in E1a overlapping CR1 but not CR2 distinguishing it from pRb and p107 (Whyte *et al.*,1989). However, p300 is likely to show some structural similarity to pRb as monoclonal antibodies raised against pRb cross react with p300 (Hu *et al.*,1991). p300 is phosphorylated in a cell cycle-dependent manner (Yaciuk and Moran,1991) and has also been shown to bind TBP (Abraham *et al.*,1991). Thus, p300 may play a role during the cell cycle by regulating transcription. Since these proteins were all potential components of the a complex, I tested where possible whether they were components of DRTF1/E2F.

5.1 Cyclin A is a component of DRTF1/E2F complexes

Since cyclin A is sequestered by E1a and therefore a potential component of the **a** complex, a cyclin A polyclonal antiserum raised against the bovine cyclin A protein (PA-CA) was added to JM extracts and its effects assessed by gel retardation. Preimmune serum had no effect on JM complexes, although the immune serum abolished the slowest migrating form of the **a** complex (Figure 5.1a, compare tracks 2 and 3). As the anti-cyclin A reagent was a polyclonal antibody made against a large region of the protein, this effect was unlikely to be due to epitope masking and most likely suggests that only a portion of the **a** complex contains cyclin A.

To further confirm that cyclin A could interact with DRTF1/E2F a cyclin A fusion protein (PA-CA) which is capable of binding a kinase subunit and producing an active kinase (J.Adamczewski, unpublished data), was added to JM extracts. Addition of PA-CA converted the **a** complex into the slower migrating species consistent with the notion that two forms of the **a** complex, cyclin A plus and cyclin A minus, exist (Figure 5.1a, compare lanes 5 and 6 with 7 and 8). Therefore both types of result support the idea that cyclin A is a component of the DRTF1/E2F **a** complex.

Attempts were made to reconstitute the **a** complex from the **b/c** form using the components identified so far. The **b/c** form of DRTF1/E2F, purified by DNA affinity chromatography (Shivji and La Thangue,1991; kindly provided by Rowena Girling) was mixed with pRb and cyclin A fusion proteins and assayed by gel retardation. Addition of PA-CA had no effect on the **b/c** complex (Figure 5.1b, compare 2 and 3 with 4 and 5), whereas addition of GST-Rb reproducibly produced a slower

migrating complex (Figure 5.2b, compare 3 and 4 with 6 and 7). The amount of **a** complex produced in this experiment was small in contrast to the effect seen in EC extracts where addition of GST-Rb converted all of the **b/c** form to the **a** complex (Figure 5.2b, compare lanes 6 and 7 with Figure 4.2a). Addition of both PA-CA and GST-Rb together produced an even slower migrating complex consistent with the idea that both molecules can exist together in the same complex. As a control the effect of heat denatured GST-Rb was also tested. The formation of the slower migrating Rb-DRTF1/E2F complex was not observed (Figure 5.1b, compare 8 and 9 with 10 and 11), indicating that the formation of this complex is dependent on the integrity of GST-Rb.

5.2 The pRb-related protein, p107, complexes with DRTF1/E2F and cyclin A

As discussed earlier, the p107 protein could also be a component of the **a** complex. Therefore EC and JM extracts were supplemented with a monoclonal antibody (SD9) against p107 (S.Dembski and N.Dyson unpublished observations). This antibody supershifted the **a** complexes in both EC and JM extracts whereas the control antibody (IG4) had no effect (Figure 5.2a, compare lanes 2 with 3 and 5 with 6). Indicating that p107, as predicted was also a component of DRTF1/E2F.

Attempts were made to reconstitute the **a** complex using p107 in crude EC extracts where the **b/c** complex is abundant. GST-p107, like GST-Rb was also able to bind DRTF1/E2F to generate more of the **a** complex (Figure 5.2b, compare lane 2 with 3 and 4, and 5 and 6). Since I had shown cyclin A associates with DRTF1/E2F the

effect of adding PA-CA together with pRb and p107 was also investigated. PA-CA had no effect alone (Figure 5.2b, compare 2 with 11 and 12) or when added with GST-Rb (Figure 5.2b, lanes 7 and 8). However, when PA-CA and GST-p107 were added together a slight mobility difference was evident when compared to GST-p107 alone (Figure 5.2b, compare lanes 5 and 6 with 9 and 10), suggesting that both GST-Rb and GST-p107 bind DRTF1/E2F and PA-CA.

5.3 Discussion

Previously I showed that the **a** complex could be disrupted by E1a suggesting that some of the cellular proteins sequestered by E1a were components of DRTF1/E2F. In this chapter I have shown that in addition to pRb, both the cyclin A protein and the pRb-related protein p107, are also components of DRTF1/E2F.

5.3.1 Cyclin A is a component of DRTF1/E2F complexes

Only a proportion of the **a** complex contained cyclin A suggesting that DRTF1/E2F **a** is a heterogeneous mixture of proteins, a result which is consistent with the data presented in chapter 3 (Figure 3.1) where the **a** complex was noted to migrate as a doublet in JM extracts. The slowest migrating complex contained cyclin A whereas the faster did not. However addition of cyclin A converted all of the **a** complex into the slower species indicating that the cyclin A minus form can still bind cyclin A.

That cyclin A was in the **a** complex was both surprising and interesting as cyclins

had not previously been shown to bind transcription factors. Cyclins undergo periodic synthesis and destruction during the cell cycle and are thought to function by binding and regulating a family of related cdc2-like kinases (Hunt,1989;Sherr,1993). These kinases are believed to control cell cycle progression by phosphorylating appropriate substrates and thus regulating their activity at the correct time (Pines and Hunter,1989;Hunt,1989;Sherr,1993). The role of cyclin A in cell cycle control has been supported by a number of observations. For example cyclin A mRNA levels increase gradually from G1 peaking in late S-phase early G2 (Draetta and Beach,1988;Draetta *et al.*,1989;Pines and Hunter,1990). In *Drosophila melanogaster* disruption of the cyclin A gene suggested that it is only necessary for mitosis, although in mammalian cells cyclin A appears to be required for progression through S-phase and mitosis (Lehner and O'Farell,1989;Girard *et al.*,1989;Pagano *et al.*,1991a). Since cyclin A can bind two distinct kinase subunits, $p34^{cdc2}$ and $p33^{cdk2}$, one explanation for these different effects is that each kinase/cyclin A combination controls either S or M phase of the cell cycle. This idea appears to be correct since depletion of $p34^{cdc2}$ and $p33^{cdk2}$ in mammalian and *Xenopus laevis* cells affects progression through mitosis and S-phase respectively (Riabowl *et al.*,1989;Fang and Newport,1991;Tsai *et al.*,1993).

The consequences of cyclin A binding to DRTF1/E2F are not clear although one effect may be similar to the interaction of pRb in that it represses transcriptional activity. Alternatively cyclin A may target a cdc2-like kinase to DRTF1/E2F, resulting in phosphorylation of this transcription factor or a component of the complex. The latter hypothesis is particularly appealing as pRb contains a number of

cdc2 phosphorylation sites which are phosphorylated *in vivo* and can be phosphorylated *in vitro* by cdc2-like kinases (Lin *et al.*,1991b;Hu *et al.*,1991). It is interesting that cyclin A alone had no effect on the b/c complex, although it specifically bound to the a complex suggesting that cyclin A can only bind to DRTF1/E2F indirectly via pocket proteins such as pRb or p107 (discussed below).

Recently several novel cyclins have been isolated referred to as cyclin C,D and E (Koff *et al.*,1991;Lew *et al.*,1991;Matsushime *et al.*,1991;Xiong *et al.*,1991). It has not been possible to assess if they can also bind to DRTF1/E2F due to a lack of reagents. However Lees *et al.* (1992) have shown cyclin E associates with DRTF1/E2F in G1. In contrast to cyclin A, cyclin E is thought to regulate progression through the G1 phase of the cell cycle and hence has been classed as a G1 cyclin (Ohtsubo and Roberts,1993). It is possible that the association of cyclin E with DRTF1/E2F may be necessary for progression through G1 (Koff *et al.*,1991; Lew *et al.*,1991;Ohtsubo and Roberts,1993). Interestingly, pRb mediated growth arrest in SAOS2 cells can be overcome by co-expressing either cyclin A or cyclin E (Hinds *et al.*,1992). This is believed to be mediated through each cyclin activating a kinase subunit and subsequently phosphorylating pRb. Phosphorylation of pRb is thought to inactivate its growth suppressing properties, although neither cyclin appears to stably bind pRb *in vitro* (Hinds *et al.*,1992). If pRb growth suppression is produced through modulation of DRTF1/E2F then the finding of cyclin A and E with DRTF1/E2F is particularly significant. Several reports suggest pRb can be efficiently phosphorylated by D-type cyclins when expressed with cdk4 in insect cells, although complexes with these cyclins and DRTF1/E2F have not been found (Kato *et*

al.,1992;Ewen *et al.*,1993).

In conclusion, several cyclins are believed to modulate phosphorylation of pRb and two of these, cyclins A and E, are also associated with DRTF1/E2F, although their exact function remains to be elucidated.

5.3.2 The pRb-related protein, p107 binds to DRTF1/E2F

The gene for p107 was recently isolated and shown to encode a protein which contains regions of similarity at the amino acid level with pRb (Ewen *et al.*,1991). Given the similarity between pRb and p107 one might expect p107 to have similar effects on the transcriptional activity of DRTF1/E2F. Indeed this is the case as a number of groups have shown that E2F site-dependent transcription can be repressed by p107 in co-transfection experiments (Zamanian and La Thangue,1993;Schwarz *et al.*,1993).

The role of p107 in cell cycle control is unclear, as it has not been reported to be mutated in the tumour cells examined to date (Ewen *et al.*,1991). Nevertheless p107 can suppress growth in certain cells although it is different to suppression by pRb as it is not rescued by co-expressing cyclin A and E (Ewen *et al.*,1991;Zhu *et al.*,1993). The spacer region of p107 is also different to pRb as it is larger and has been shown to stably bind the cyclin A protein (Ewen *et al.*,1992;Faha *et al.*,1992). In this study, it was noted that addition of the p107 and cyclin A fusion proteins to crude EC extracts created a slower migrating complex which was not apparent when using pRb. This would be consistent with the idea that cyclin A binds to p107. Thus, it is

possible that the cyclin A plus and minus forms of DRTF1/E2F probably contain p107, with and without, cyclin A.

A complex between pRb, cyclin A and DRTF1/E2F was only evident when affinity purified DRTF1/E2F was used and may reflect an unstable or unphysiological complex. The latter seems unlikely as others have shown that in some cell types pRb-DRTF1/E2F complexes also contain cyclin A suggesting that these interactions may be cell type specific (Pagano *et al.*,1992b). Furthermore cyclin A is able to rescue pRb-mediated growth arrest implying that both interact *in vivo*. The observation of D-type cyclins specifically binding to pRb implied that these cyclins can stably associate with pRb (Ewen *et al.*1993;Kato *et al.*,1993;Dowdy *et al.*,1993). It is possible the ability of cyclin A to bind pRb in these assays could be artefactual through mimicking cyclin D although this seems unlikely given the observations discussed above. It is likely however that cyclin A only associates with DRTF1/E2F together with pocket proteins such as pRb and p107, although at present it is unclear if cyclin A specifically interacts with p107 or pRb.

5.3.3 Cell cycle regulation of DRTF1/E2F complexes

The interactions of pRb, cyclin A and p107 with DRTF1/E2F are cell cycle regulated (Mudryj *et al.*,1991;Cao *et al.*,1992;Devoto *et al.*,1992;Shirodkar *et al.*,1992). These observations suggested that pRb and p107 DRTF1/E2F complexes occur in different phases of the cell cycle although the interpretation of these experiments is complicated by the problems associated with establishing a fully

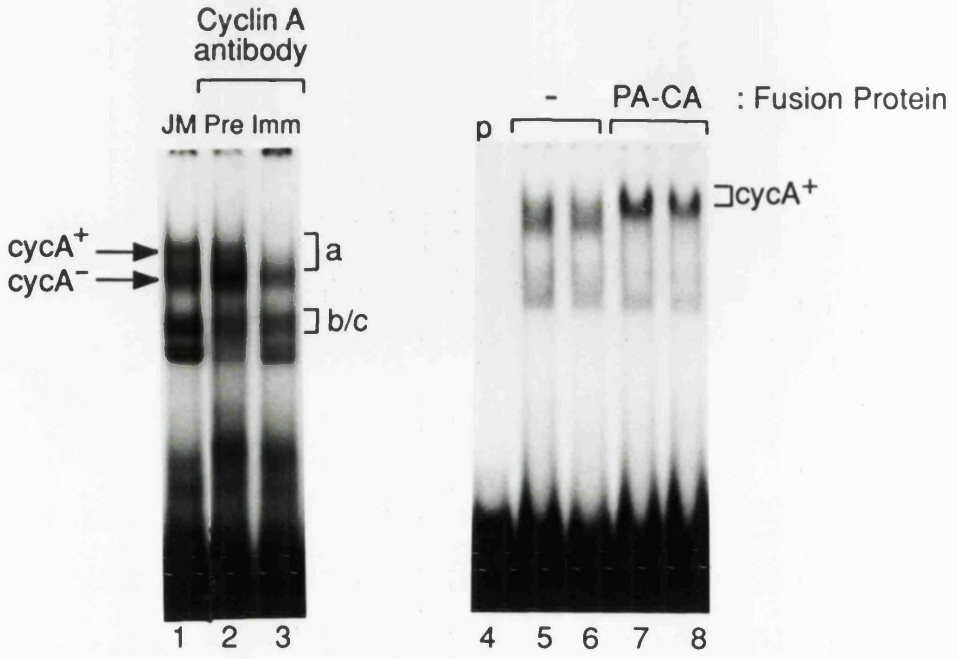
synchronised culture and the use of different cell types. These studies indicate that the pRb containing DRTF1/E2F complexes appear in early G1, persisting into S-phase and then disappearing whereas the DRTF1/E2F complex with p107 appear only in S-phase (Mudryj *et al.*,1991;Devoto *et al.*,1992;Shirodkar *et al.*,1992). In addition, in crude extracts of cells used in these studies, cyclin A was found in p107 complexes with DRTF1/E2F not with pRb. Free uncomplexed DRTF1/E2F appeared at the G1/S transition consistent with the hypothesis that it may regulate genes required for the initiation of DNA synthesis (see also chapter 8). Others have suggested that p107 complexes also exist in G0/G1 (Lees *et al.*,1992;Schwarz *et al.*,1992). For example Lees *et al.* (1992) showed that in G1, DRTF1/E2F complexes with p107 also contained cyclin E, whereas the later S-phase p107 complexes contained cyclin A. Thus, two different cyclins may interact with DRTF1/E2F via the p107 protein.

To conclude, in this chapter two more E1a-associated proteins; p107 and cyclin A were shown to be components of DRTF1/E2F. Although the significance of cyclin A in the DRTF1/E2F complex has yet to be established, it is likely to be involved in coupling the activity of DRTF1/E2F with the cell cycle apparatus by phosphorylating the complex in a cell cycle-regulated manner. The outcome of these interactions may ensure the correct regulation of DRTF1/E2F during cell cycle progression such that S-phase is initiated at the appropriate time.

Figure 5.1a, *Cyclin A is a component of DRTF1/E2F.* Gel retardations using JM whole cell extracts were supplemented with 1μl of cyclin A immune sera (lane 3) or preimmune sera (lane 2). DRTF1/E2F complexes with and without cyclin A are indicated as cycA+ and cycA- respectively. Approximately 500ng of PA-CA fusion protein was added to JM extracts in lanes 7 and 8. Note the slower DRTF1/E2F complex is labelled cycA+.

Figure 5.1b, *Cyclin A and pRb bind DRTF1/E2F.* Gel retardations were performed with 1μl of affinity purified DRTF1/E2F (lanes 2-11). Where indicated 200ng of GST-Rb (lanes 6-9) and 500ng of PA-CA (lanes 3,4,7,8,10 and 11) was added, and 200ng of heat inactivated (HI) GST-Rb was used in lanes 10 and 11. The arrow indicates a complex formed with DRTF1/E2F and GST-Rb alone.

a


Cyclin A antibody
JM Pre Imm
cycA+
cycA−
a
b/c
1 2 3
- PA-CA : Fusion Protein
p
cycA+
4 5 6 7 8

b

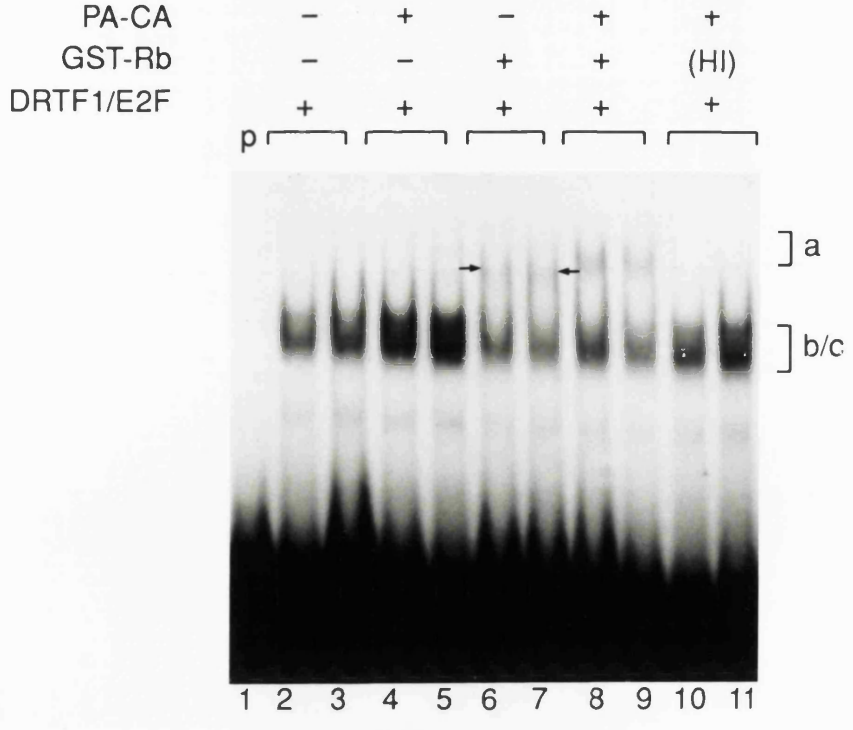
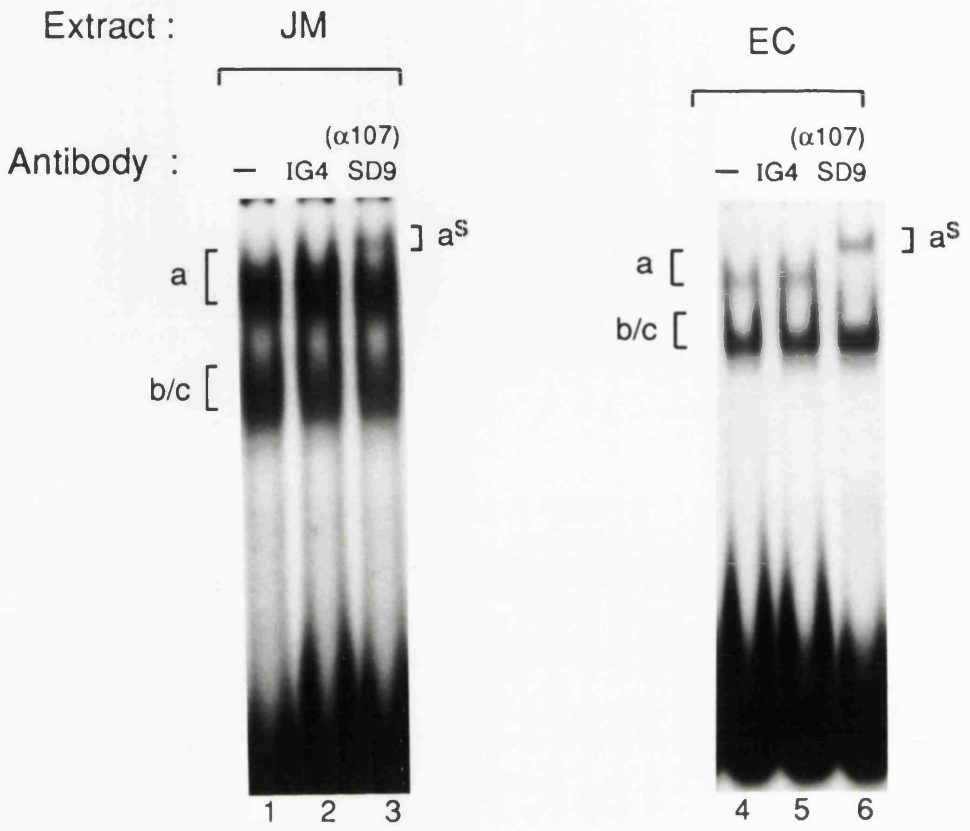

PA-CA − + − + +
GST-Rb − − + + (HI)
DRTF1/E2F + + + + +
p
a
b/c
1 2 3 4 5 6 7 8 9 10 11

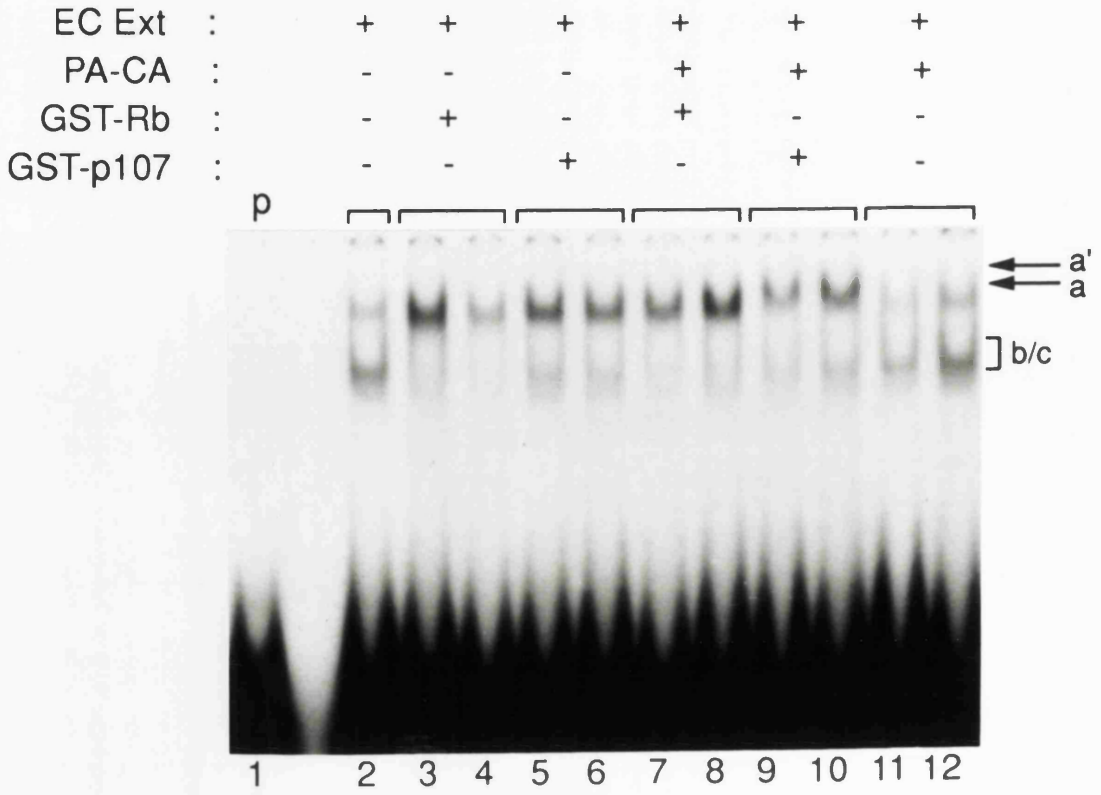
Figure 5.2a, *The pRb-related protein, p107, is a component of the DRTF1/E2F a complex.* Gel retardations with EC and JM whole cell extracts were supplemented with 3μl of p107 monoclonal (SD9) supernatant (lanes 3 and 6) or a control supernatant (IG4) of the same isotype (lanes 2 and 5). Supershifted DRTF1/E2F complexes are indicated as a^s.

Figure 5.2b, *Cyclin A binds DRTF1/E2F with p107 and pRb.* Gel retardation with EC extracts, where indicated 100ng of GST-Rb (lanes 3,4,7 and 8), 100ng of GST-p107 (lanes 5,6,9 and 10) or 500ng of PA-CA (lanes 7-12) was added. The slower migrating complexes are indicated by the arrows. Note that the GST-p107 and PA-CA complex is indicated as a' (lanes 9 and 10) and is distinct from the **a** complex.

a


Extract :
JM
EC
Antibody :
(α107)
— IG4 SD9
(α107)
— IG4 SD9
a
b/c
a^s
a
b/c
a^s
1 2 3
4 5 6

b


EC Ext : + + + + + +
PA-CA : - - - + + +
GST-Rb : - + - + - -
GST-p107 : - - + - + -
p
a'
a
b/c
1 2 3 4 5 6 7 8 9 10 11 12

CHAPTER 6

The cdc2-like kinase subunit $p33^{cdk2}$ can be recruited to DRTF1/E2F by cyclin A

6.0 Introduction

In the last chapter I showed that cyclin A is a component of the a complex. Cyclins comprise a growing family of proteins of which seven classes have been identified named A to F (reviewed in Sherr,1993). The mRNA for cyclins A and B is present late in the cell cycle (Pines and Hunter,1989;1990), whereas others such as cyclins E and C are expressed in G1 (Koff *et al.*,1991;Lew *et al.*,1991). In contrast, the mRNA levels of the D-type cyclins may be constant throughout the cell cycle responding to growth factor stimulation (Matsushime *et al.*,1991;Sewing *et al.*,1993).

Unlike the situation in yeast, the mammalian cdc2 kinase comprises a large family of related cdc2-like proteins with amino acid sequence similarity (Meyerson *et al.*,1992;Sherr,1993). To date five kinase subunits have been classified as cyclin-dependent kinases, cdk1 to 5, as they bind and are regulated by cyclins. In addition, several other proteins defined by PCR based strategies may function as cdks, although their biological activities are unknown (Meyerson *et al.*,1992). In some cases, the kinase subunit can bind more than one cyclin partner and it is believed that each cyclin-kinase combination is required for progression through different phases of the cell cycle (Fang and Newport,1991;Girard *et al.*,1991).

In addition to cyclin A, the G1 cyclin, cyclin E has also been demonstrated in the

p107-DRTF1/E2F complex (Lees *et al.*,1992). Cyclin A binds to at least two kinase subunits, $p34^{cdc2}$ and $p33^{cdk2}$, whereas cyclin E has only been shown so far to bind $p33^{cdk2}$ (Draetta and Beach,1988;Draetta *et al.*,1989;Tsai *et al.*,1991;Koff *et al.*,1992). It was possible that the cyclins behaved in a similar fashion to pRb and p107 in repressing transcription through binding DRTF1/E2F. Alternatively, the interaction of cyclins with DRTF1/E2F may be necessary to recruit a cyclin-dependent kinase to the complex. If this were the case, then it would argue that the cyclins function in part by dictating substrate specificity to the appropriate kinase subunit. As cyclins are not known to function independently of their kinase subunit, I was interested in determining if a cyclin-dependent kinase was associated with DRTF1/E2F and furthermore, whether it could be targeted to the complex through cyclin A.

6.1 Cyclin A recruits the cdc2-like kinase $p33^{cdk2}$ to the **a** complex

Since the PA-CA fusion protein efficiently complexed with DRTF1/E2F *in vitro* I asked if it could recruit the catalytic subunit $p33^{cdk2}$ to the DNA binding complex. For this I used a GST-cdk2 fusion protein (Tsai *et al.*,1991) which binds the cyclin A protein in crude extracts and possess a kinase activity (J. Adamczewski, unpublished observations). The addition of PA-CA to JM extracts caused the appearance of a slower migrating DRTF1/E2F **a** complex, whereas the addition of GST-cdk2 alone had no effect on DRTF1/E2F complexes (Figure 6.1, compare lanes 2 and 3 with 4 and 5, and 6 and 7). However, addition of both PA-CA and GST-cdk2

together resulted in the formation of an even slower migrating complex which was presumably due to the presence of both PA-CA and GST-cdk2 with DRTF1/E2F (Figure 6.1, lanes 8 and 9). Therefore, GST-cdk2 can only associate with DRTF1/E2F after the assembly of PA-CA. The complex also contained pRb as it could be further "supershifted" by the pRb monoclonal antibody C36 and not a control antibody of the same isotype, IG4 (Figure 6.1, compare lanes 10 and 11 with 12 and 13). These results argue that cyclin A is able direct $p33^{cdk2}$ to DRTF1/E2F complexes containing pRb, thus supporting the idea that cyclins may dictate substrate specificity to their associated kinase subunits. The presence of endogenous cdk2 protein in DRTF1/E2F complexes was also confirmed by using a cdk2 polyclonal antibody which caused a "supershift", whereas the preimmune serum had no effect (Figure 6.1, compare 18 and 19).

6.2 Cyclin A recruits $p33^{cdk2}$ to DRTF1/E2F complexes containing p107 or pRb

The ability of PA-CA to recruit GST-cdk2 was also investigated in EC extracts where the **b/c** form of DRTF1/E2F is abundant and therefore the **a** complex had to be created by adding GST-Rb or GST-p107 fusion protein (chapter 5). The proteins used in these experiments are summarised (Figure 6.2c). In contrast to JM extracts, the addition of cyclin A alone had no obvious effect on DRTF1/E2F complexes and similarly, the addition of GST-cdk2 alone also had no effect (Figure 6.2a, compare lanes 2 and 3 with 4,5,8 and 9). However, when both were added together a slower migrating complex was apparent (Figure 6.2a, lanes 10 and 11) with similar mobility

to that observed in JM extracts. The abundance of this complex was much lower than in JM extracts, possibly reflecting the low levels of the **a** complex in EC extracts. This implied that the cyclin A/cdk2 complex can only be produced with the **a** form of DRTF1/E2F, that is, via pocket proteins such as p107 or pRb. In order to test this idea, GST-Rb was added to EC extracts together with PA-CA and GST-cdk2. As before, GST-Rb converted the **b/c** form into the DRTF1/E2F **a** complex (Figure 6.2a, lanes 6 and 7). Addition of either PA-CA or GST-cdk2 alone had no obvious effect on the DRTF1/E2F complex (Figure 6.2a, compare 6 and 7 with 12,13,14 and 15). However, when both were added together with GST-Rb a slower migrating complex was again formed (Figure 6.2a, compare 9 and 10 with 16 and 17). Consistent with presence of more **a** complex was the greater abundance of this slower migrating complex supporting the notion that cyclin A and cdk2 bind to the DRTF1/E2F **a** complex.

Similar experiments were performed using GST-p107 in EC extracts and again addition of either GST-p107 or GST-Rb converted the **b/c** complex into the **a** form whereas PA-CA alone had no effect (Figure 6.2b, compare lanes 2 and 3 with 4 to 9). When PA-CA and GST-cdk2 were added together with GST-p107 or GST-Rb the characteristic slower migrating complex was again evident (Figure 6.2b, compare 12 and 13 with 14 to 17). Thus, in this experimental system GST-cdk2 is able to associate with DRTF1/E2F complexes in a p107 or pRb-dependent manner.

6.3 Cyclin A recruits $p33^{cdk2}$ but not $p34^{cdc2}$ to DRTF1/E2F

Cyclin A is known to bind to at least two different kinases, $p34^{cdc2}$ and $p33^{cdk2}$. Attempts to produce a biologically active mammalian $p34^{cdc2}$ protein have so far been unsuccessful whereas a GST-*Xenopus* cdc2 (GST-Xcdc2) possess cyclin binding and kinase activities (R.Y.C.Poon, unpublished observations). Therefore, the activity of the *Xenopus* cdc2 fusion protein was compared to the human GST-cdk2. When PA-CA and GST-cdk2 were added to extracts the characteristic slower migrating complex previously observed was evident (Figure 6.3, lanes 10 and 11). However, when PA-CA and GST-Xcdc2 were added together a similar complex was not formed (Figure 6.3, compare 10 and 11 with 12 and 13). Therefore, in this *in vitro* assay PA-CA distinguishes between cdc2 and cdk2 in its ability to target the kinase subunit to DRTF1/E2F. This is also consistent with the observation that $p33^{cdk2}$ but not $p34^{cdc2}$ is in DRTF1/E2F complexes detected in crude extracts (Shirodkar *et al.*,1992; Chellappan *et al.*,1992). One can not rule out however that the differences seen are attributed to using the *Xenopus* cdc2 protein instead of the mammalian equivalent or due to different requirements for post-translational modification. The former seems unlikely as the *Xenopus* pRb gene has been isolated and is highly homologous to the mammalian genes (Destrée *et al.*,1993), and *Xenopus* extracts contain a DRTF1/E2F binding activity which can associate with pRb and p107 fusion proteins (R.Girling, unpublished observations).

6.4 Discussion

In the last chapter I demonstrated that several molecules bound DRTF1/E2F to form the **a** complex. In this chapter I established that the kinase subunit $p33^{cdk2}$ is capable of interacting with DRTF1/E2F, and further that it is dependent on the presence of cyclin A.

Although it had been assumed that cdc2-like kinases may modulate the activity of transcription factors, such interactions have not been previously detected in DNA binding complexes. Therefore DRTF1/E2F may be an important substrate for these kinases which is an interesting possibility given the cell cycle regulation of DRTF1/E2F complexes and the genes regulated by it (discussed in chapter 5 and 8).

6.4.1 Cyclin A targets $p33^{cdk2}$ to DRTF1/E2F complexes

In the *in vitro* assay employed cyclin A was able to direct $p33^{cdk2}$ to DRTF1/E2F but only formed complexes with the **a** form, suggesting that pocket proteins provide the interface necessary for this interaction. This idea would be consistent with the ability of cyclin A to bind p107 and form a complex with $p33^{cdk2}$ (Ewen *et al.*,1992;Faha *et al.*,1992). Furthermore, Pepper *et al.* (1993) have shown that cyclin A and $p33^{cdk2}$ bind cooperatively to p107, suggesting p107 may be important intermediate for binding cyclin A/$p33^{cdk2}$ in DRTF1/E2F.

Experiments in this chapter have shown cyclin A was also able to direct $p33^{cdk2}$ to complexes containing pRb, in agreement with earlier observations suggesting that

cyclin A could bind pRb with affinity purified DRTF1/E2F (see chapter 5). Although an *in vitro* system was employed to detect the pRb, cyclin A and $p33^{cdk2}$ complex it is nevertheless likely to be physiologically relevant because several observations suggest that cyclin A and pRb interact *in vivo*. For example, in some cell lines pRb-DRTF1/E2F complexes containing cyclin A have been detected (Pagano *et al.*, 1992b). Also pRb-mediated growth suppression can be overcome by co-expressing cyclin A (Hinds *et al.*,1992 discussed in chapter 5).

It is possible that other cyclins and cdks interact with DRTF1/E2F. However, similar experiment could not be performed using the recently isolated cyclins C,D and E or cdk 3,4 and 5 due to a lack of suitable reagents. One might imagine cyclin E would also be able to target a kinase to DRTF1/E2F as cyclin E has been shown to bind DRTF1/E2F in the G1 phase of the cell cycle. Cyclin E also binds $p33^{cdk2}$ subunit in G1 (Koff *et al.*,1992), although it is currently unknown if $p33^{cdk2}$ associates with cyclin E and DRTF1/E2F.

6.4.2 DRTF1/E2F: a specific substrate for the $p33^{cdk2}$ kinase subunit?

Since $p33^{cdk2}$ could only be targeted to DRTF1/E2F after the incorporation of cyclin A it is likely that the cyclin moiety determines substrate specificity. Thus, cdc2-like kinases need not be substrate-specific but rather locate to specific substrates by virtue of their interaction with cyclin subunits. Cyclin A was able to direct $p33^{cdk2}$ to DRTF1/E2F complexes containing pRb or p107, indicating $p33^{cdk2}$ did not distinguish between the interfaces provided by these two pocket proteins. However, cyclin A was

unable to direct the mitotic kinase $p34^{cdc2}$ to DRTF1/E2F which is known to be able to interact with cyclin A *in vivo* (Draetta and Beach,1988;Pines and Hunter,1990), suggesting some kinase specificity exists in these *in vitro* conditions. It is particularly interesting that $p33^{cdk2}$, but not $p34^{cdc2}$, interacts with DRTF1/E2F since each is thought regulate to a different phase of the cell cycle. For example, depletion of $p33^{cdk2}$ prevents progression through S-phase whereas $p34^{cdc2}$ is necessary for mitosis (Riabowol *et al.*,1989;Fang and Newport,1991;Tsai *et al.*,1993). Therefore progression through S-phase may be influenced by cdk2 regulating the activity DRTF1/E2F and hence genes important for DNA synthesis.

How does $p33^{cdk2}$ regulate DRTF1/E2F? The presence of $p33^{cdk2}$ in DRTF1/E2F complexes may have a number of consequences. For example the active kinase may phosphorylate DRTF1/E2F or another component of the complex. The pRb protein is a candidate substrate as it contains several cdc2 phosphorylation sites which are phosphorylated *in vivo* (Lin *et al.*,1991b;Lees *et al.*,1991;Hu *et al.*,1992). Cyclin A may therefore recruit $p33^{cdk2}$ to phosphorylate pRb which may in turn,regulate the interaction of pRb with DRTF1/E2F. This would subsequently influence the regulation of transcription (summarised in Figure 6.4). It has been shown that expression of pRb in insect cells together with cyclin D and cdk4 results in efficient phosphorylation of pRb, which precludes binding to DRTF1/E2F, although the relevance of this observation *in vivo* remains to be established (Ewen *et al.*,1993;Kato *et al.*,1993). It is also possible that the cyclin A/$p33^{cdk2}$ kinase phosphorylates p107 particularly since p107 can be phosphorylated by cyclin A/$p33^{cdk2}$ *in vitro* on similar sites phosphorylated *in vivo* (Peeper *et al.*,1993). An alternative model is that $p33^{cdk2}$

mediated phosphorylation changes the transcriptional competence of DRTF1/E2F, perhaps by modifying the transcription activation domain or altering DNA binding specificity. In summary, although cyclin A can recruit a kinase subunit to DRTF1/E2F the consequences of this interaction are unresolved.

6.4.3 A model for co-ordinating cell cycle events with transcription

Since cyclin E regulates progression through G1 (Koff *et al.*,1992;Ohtsubo and Roberts,1993), whereas cyclin A is necessary for S-phase and mitosis (Girard *et al.*,1991;Pagano *et al.*,1992a) it is possible that some of these effects may again be mediated by modulating the activity of DRTF1/E2F (summarised in Figure 6.4). Therefore, one might imagine that like cyclin A ,cyclin E targets a kinase to DRTF1/E2F which influences progression through G1. Since two distinct cyclins which regulate different phases of the cell cycle target DRTF1/E2F it is probable that DRTF1/E2F plays an important role in cell cycle control by allowing cdks to integrate their activities with the transcription apparatus. In particular, DRTF1/E2F may coordinate cell cycle events with the initiation of transcription by directly linking growth regulatory proteins such as pRb, p107 cyclins and cyclin-dependent kinases with DRTF1/E2F (summarised in Figure 6.4).

Figure 6.1, *Cyclin A recruits $p33^{cdk2}$ to DRTF1/E2F.* Gel retardation performed using JM whole cell extracts. Where indicated 500ng of PA-CA, (lanes 4,5,8,9,10,11,12 and 13) or 200ng of GST-cdk2 (lanes 6,7,8,9,10,11,12 and 13) was added. In addition either 8μl of anti pRb monoclonal antibody, C36 (lanes 12,13,16 and 17) or 8μl of control antibody, IG4 (lanes 9,10,14 and 15) was also added. The cyclin A/cdk2 complex is indicated by ▲ and the complex supershifted by the pRb antibody is shown by •. Supershifts generated by the pRb antibody in the absence of PA-CA and GST-cdk2 are shown by a^s. Either 1μl of cdk2 antiserum (lane 18) or preimmune serum (lane 19) was also added to JM extracts, the supershifted DRTF1/E2F complex being indicated by the arrow.

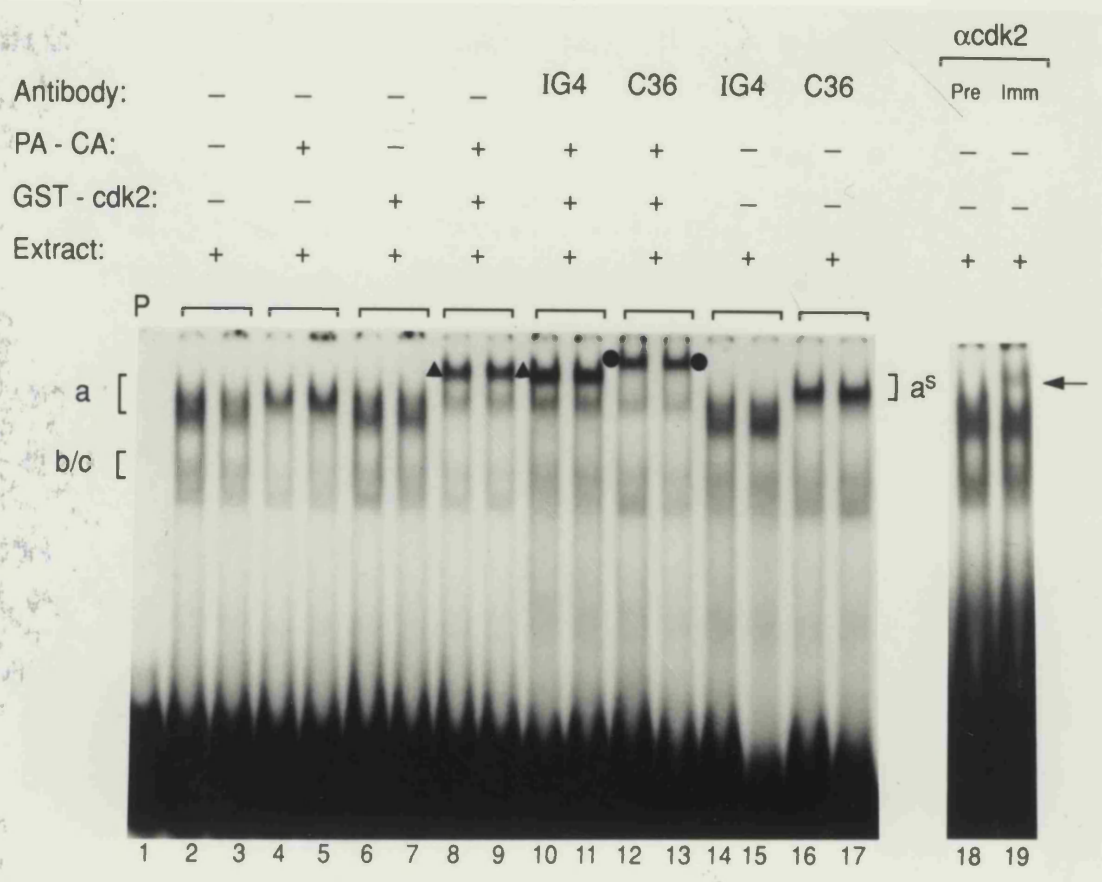

αcdk2
Antibody: – – – – IG4 C36 IG4 C36
Pre Imm
PA - CA: – + – + + + – – – –
GST - cdk2: – – + + + + – – – –
Extract: + + + + + + + + + +
P
a [
b/c [
] a^s
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

Figure 6.2a, *Cyclin A recruits p33^{cdk2} to DRTF1/E2F complexes containing pRb.* Gel retardations were performed with EC extracts. Where indicated 500ng of PA-CA (lanes 4,5,10,11,14,15,16 and 17),200ng of GST-Rb (lanes 6,7,12,13,14,15,16 and 17) and 200ng of GST-cdk2 (lanes 8,9,10,11,12,13,16 and 17) was added. The DRTF1/E2F complex with pRb, cyclin A and cdk2 is indicated by ▲.

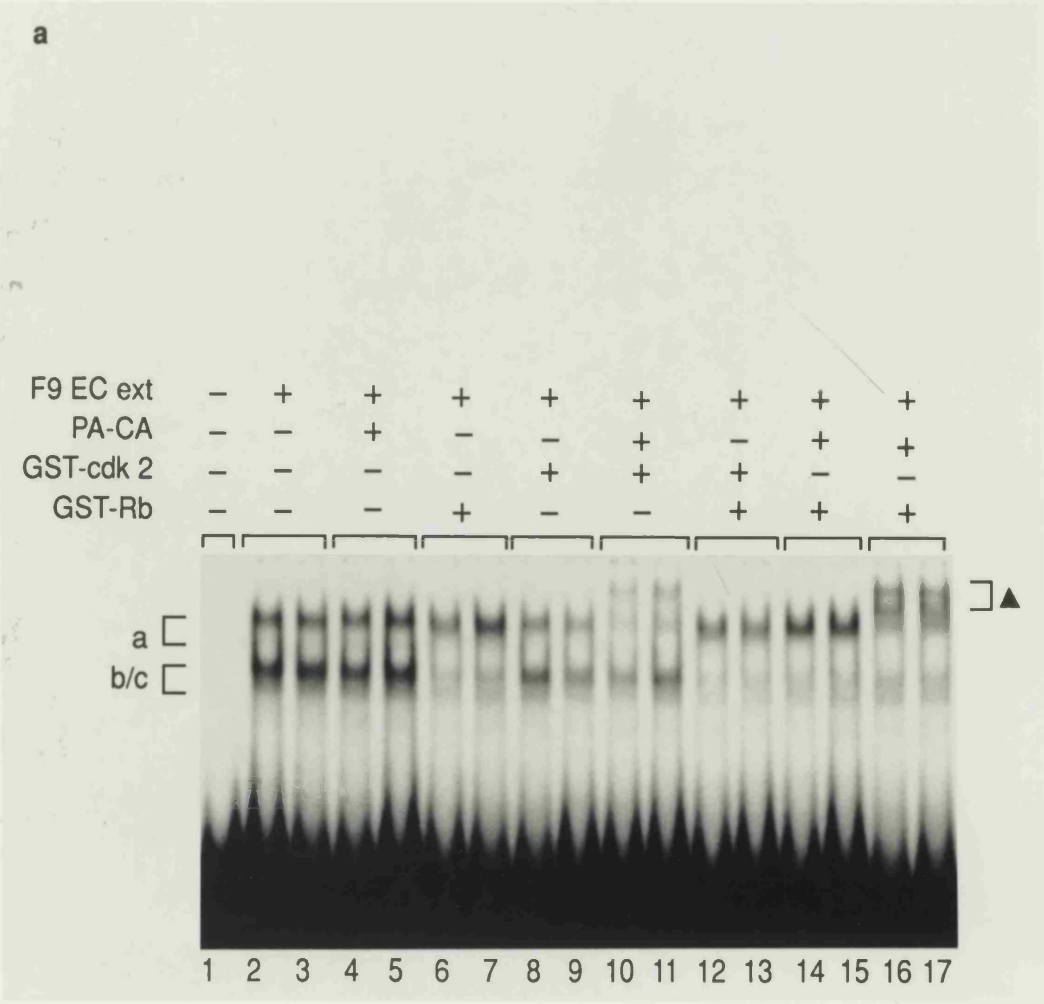
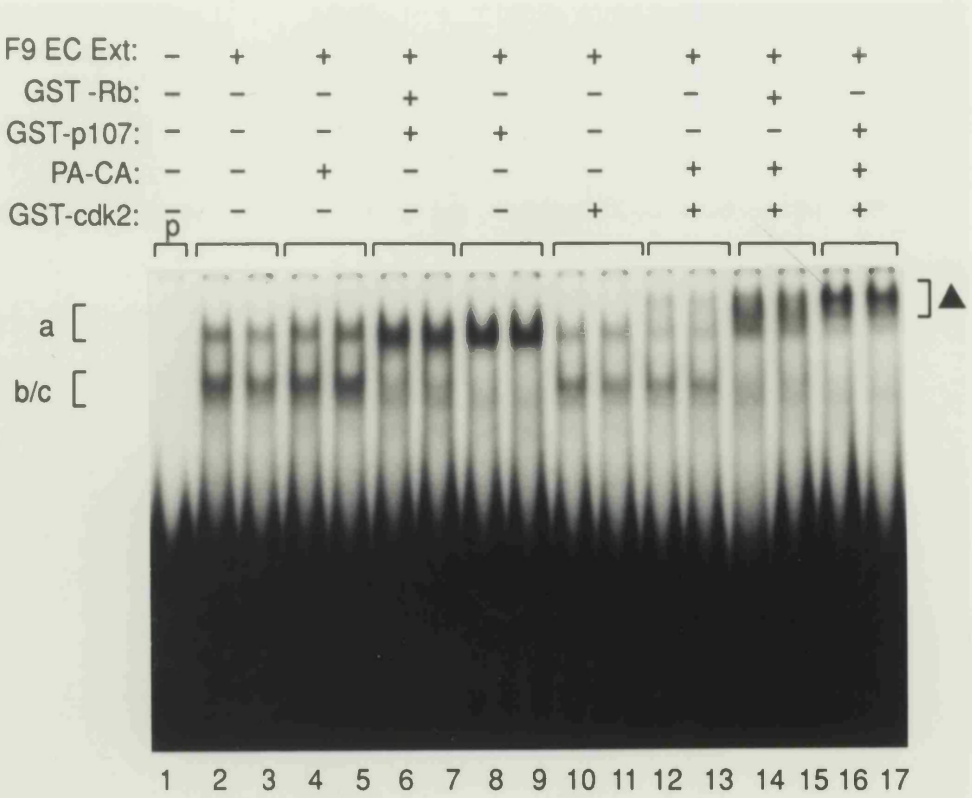

a
F9 EC ext
PA-CA
GST-cdk 2
GST-Rb
a
b/c
▲
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

Figure 6.2b, *Cyclin A directs $p33^{cdk2}$ to complexes containing pRb or p107.* Gel retardations with EC extracts. Where indicated 500ng of PA-CA (lanes 3,4,12,13,14,15,16 and 17), 200ng GST-Rb (lanes 6,7,14 and 15), GST-p107 (lanes 8,9,16 and 17) and 200ng of GST-cdk2 (lanes 10-17) was supplemented to the binding reactions. The DRTF1/E2F complex with cdk2, cyclin A and a pocket protein is indicated by ▲.

Figure 6.2c, *Summary of fusion proteins.* The fusion proteins used in these experiments are depicted showing amino acid residues.

b


F9 EC Ext: – + + + + + + + +
GST -Rb: – – – + – – – + –
GST-p107: – – – + + – – – +
PA-CA: – – + – – – + + +
GST-cdk2: – – – – – + + + +
p
a
b/c
▲
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

c

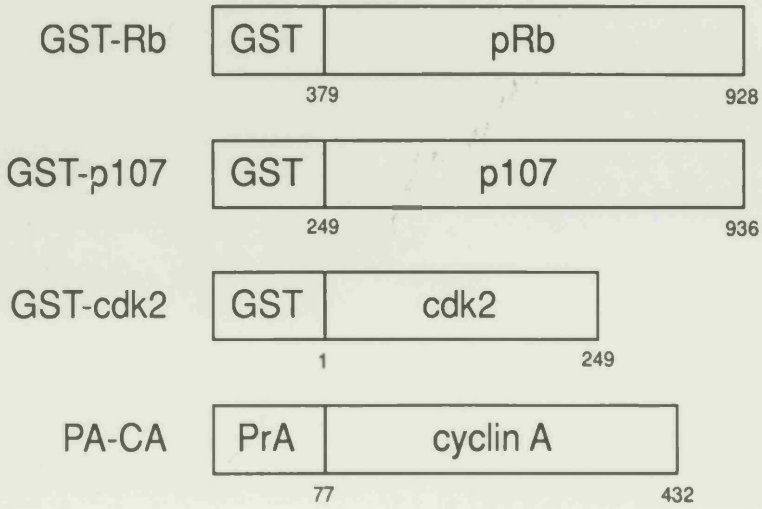

GST-Rb
GST
pRb
379
928
GST-p107
GST
p107
249
936
GST-cdk2
GST
cdk2
1
249
PA-CA
PrA
cyclin A
77
432

Figure 6.3, *Cyclin A recruits p33^{cdk2} but not p34^{cdc2} to DRTF1/E2F.* Gel retardations were performed with JM extracts and where indicated 200ng of GST-cdk2 (lanes 6,7,10 and 11), 200ng of GST-Xcdc2 (lanes 8,9,12 and 13) and 500ng of PA-CA (lanes 4,5,10,11,12 and 13) was added. The cyclin A, cdk2 DRTF1/E2F complex is indicated by ▲, note a similar complex is not formed with Xcdc2.

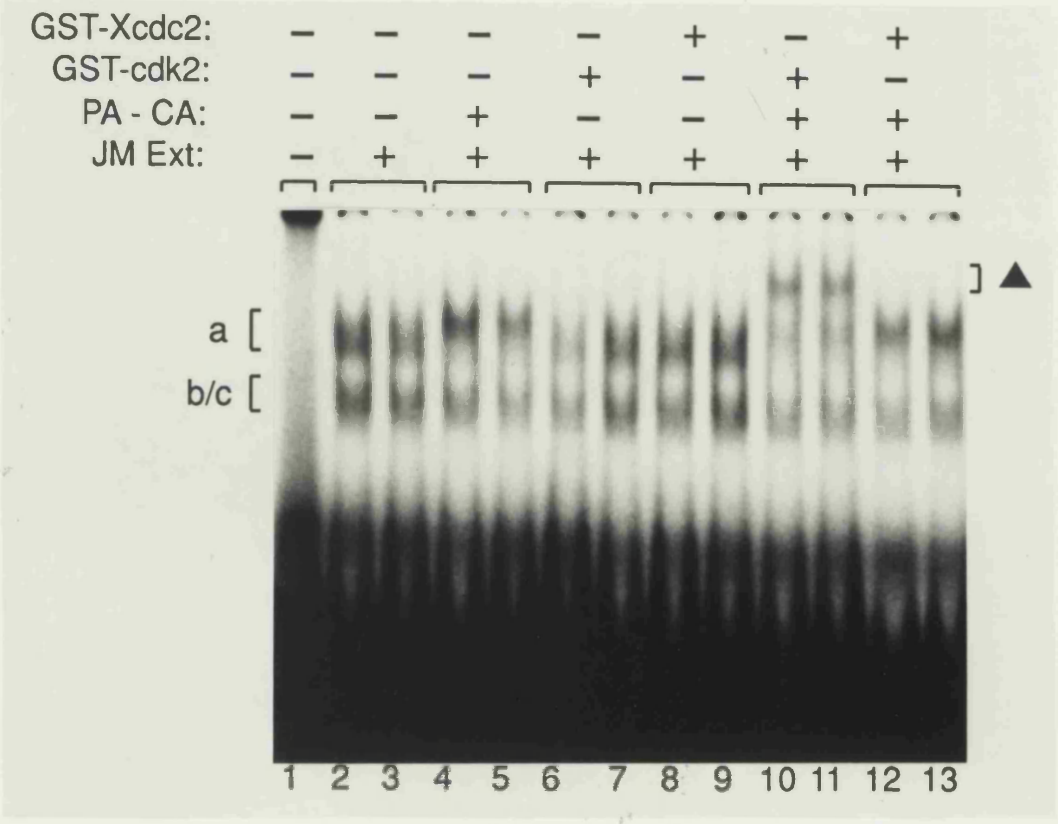
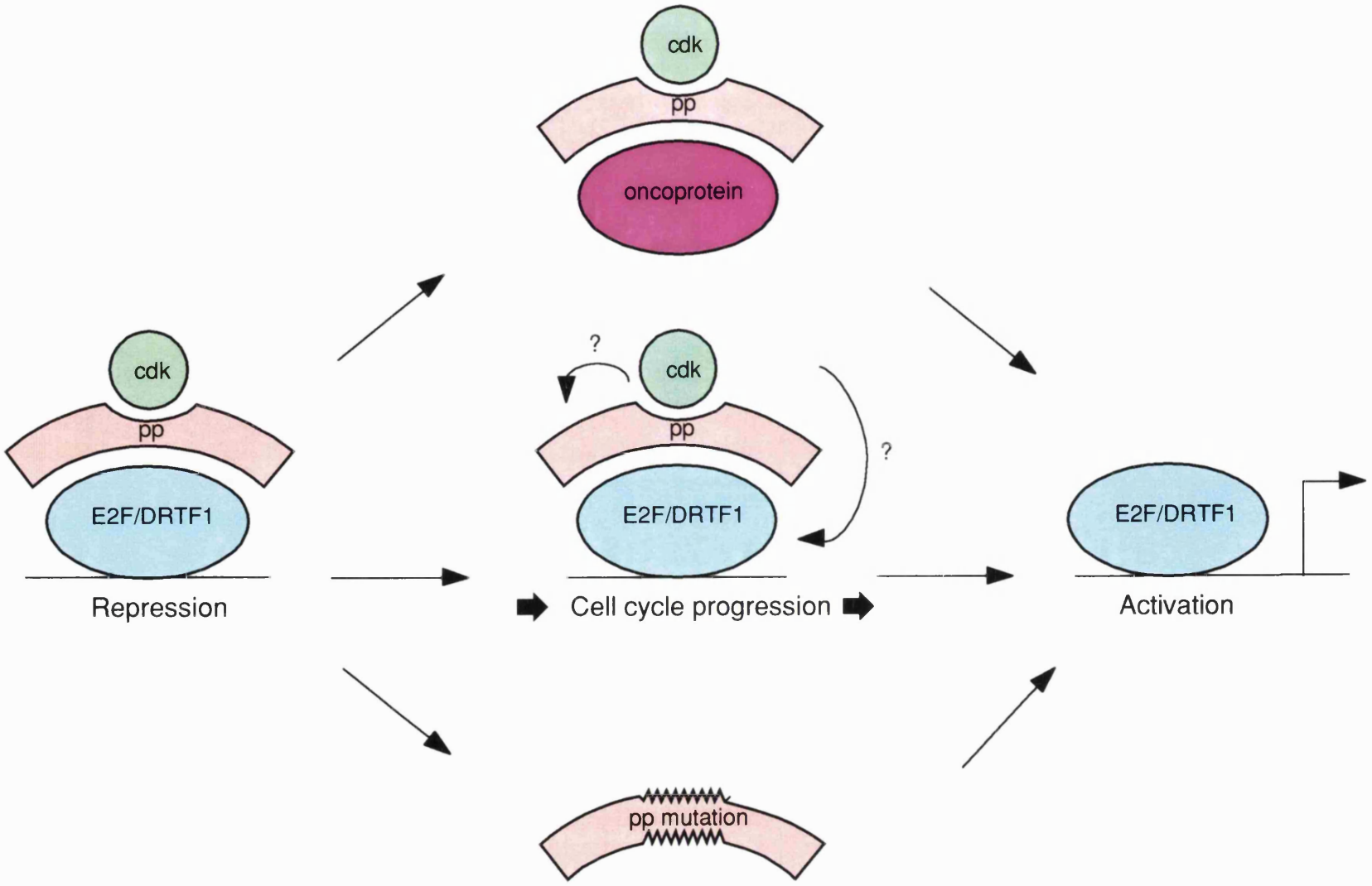

GST-Xcdc2: – – – – + – +
GST-cdk2: – – – + – + –
PA - CA: – – + – – + +
JM Ext: – + + + + + +
a [
b/c [
] ▲
1 2 3 4 5 6 7 8 9 10 11 12 13

Figure 6.4, *Co-ordinating the cell cycle machinery with the initiation of transcription.* DRTF1/E2F (shown in blue) forms heteromeric DNA binding complexes with pocket proteins (pp, shown in red) and cyclin-dependent kinases (cdk, shown in green). Viral oncoproteins (purple) sequester pocket proteins to generate transcriptionally active DRTF1/E2F which may also arise when pocket proteins are mutated in tumour cells. During cell cycle progression active DRTF1/E2F is generated in a cell cycle regulated manner which may arise through phosphorylation of pocket proteins or DRTF1/E2F by cdks (depicted by arrows).


cdk
pp
oncoprotein
cdk
pp
E2F/DRTF1
Repression
?
cdk
pp
E2F/DRTF1
?
Cell cycle progression
E2F/DRTF1
Activation
pp mutation

CHAPTER 7

Characterisation of DP-1, an integral component of DRTF1/E2F DNA binding complexes

7.0 Introduction

In the earlier chapters I established some of the properties and identified components of DRTF1/E2F. However, to further characterise this transcription factor it was necessary to isolate and molecularly characterise the components DRTF1/E2F. Attempts to purify the **b/c** form of DRTF1/E2F by DNA affinity chromatography using the E2F binding site yielded several polypeptides (Shivji and La Thangue,1991;Girling *et al.*,1993). This suggested that either the transcription factor was a multicomponent DNA binding domain or that several related activities recognised the E2F binding site.

Whilst this study was in progress, two distinct DNA binding proteins which recognise the E2F site were isolated, termed E2F-1 and DP-1. E2F-1 was isolated through its ability to bind to pRb and hence contains an pRb binding domain localised to a C-terminal region (Kaelin *et al.*,1992;Helin *et al.*,1992;Shan *et al.*,1992). Furthermore, E2F-1 contains a DNA binding domain near the N-terminus which allows sequence-specific binding to the E2F site and activates transcription of E2F site reporter genes through a domain overlapping the pRb-binding region (Kaelin *et al.*,1992;Helin *et al.*,1992). Therefore E2F-1 possessed many of the properties

expected for a component of E2F/DRTF1.

When antibodies raised against E2F-1 were used in a gel retardation assay only a proportion DRTF1/E2F complexes were affected suggesting the existence of other E2F-like transcription factors (Kaelin *et al.*,1992). This was confirmed when our laboratory isolated DP-1 (for DRTF Protein 1) which was also shown to be capable of binding in a sequence-specific manner to the E2F site (Girling *et al.*,1993). In addition, it was noticed that a small region of amino acid sequence similarity exists between E2F-1 and DP-1 which may imply that these proteins comprise a family of related proteins (La Thangue and Taylor,1993). In contrast to E2F-1, antibodies against DP-1 abolished all DRTF1/E2F DNA binding complexes in EC extracts indicating it is a frequent component of this transcription factor (Girling *et al.*,1993). In this chapter I investigated the properties of DP-1 since I was interested to determine if for example DP-1 was able to bind pRb or p107 and whether it occurs in the same or distinct DNA binding complexes which contain E2F-1.

7.1 DP-1 is a common component of all DRTF1/E2F complexes in EC and HeLa extracts

To test if DP-1 was a component of DRTF1/E2F complexes in EC, HeLa and JM cells I assayed the effect of DP-1 antiserum on the DNA binding complexes which formed on the E2F site using extracts from each cell type. All the DRTF1/E2F complexes in EC extracts were specifically abolished by the DP-1 antisera (αA) since the effect was competed by the peptide used to raise the antibody (A) but not an unrelated c-jun peptide (Figure 7.1a, compare lane 1 and 2). A similar effect was also

apparent in Hela extracts, although JM DRTF1/E2F complexes were unaffected by this DP-1 anti-sera (Figure 7.1a, compare lanes 3 with 4 and 5 with 6).

It was of interest to know if DP-1 associates with either pRb or p107 and I tested therefore if DP-1 co-precipitated with pRb and p107. For this experiment JM extracts were immunoprecipitated with pRb (IF8) or p107 (SD9) monoclonal antibodies and the immunoprecipitates treated with detergent, a treatment known to release DRTF1/E2F DNA binding activity. Both pRb or p107 detergent eluates contained DRTF1/E2F binding activity although the abundance of each was different (Figure 7.1b, lanes 1 and 4). Both activities also contained DP-1 as the DNA binding activity was significantly reduced by an anti-DP-1 antibody (α18) an effect competed by the cognate peptide (18) but not the control peptide (Figure 7.1b, compare lanes 2 with 3 and 5 with 6). Therefore, both pRb and p107 associated DNA binding activities contain DP-1 suggesting that DP-1 has distinct properties to E2F-1 which is only a component of only a proportion of DRTF1/E2F complexes (Kaelin *et al.*,1992).

EC, HeLa and JM extracts were immunoblotted and probed with the peptide purified DP-1 antibody (kindly provided by Troels Sorensen) to assess the nature of DP-1 polypeptides recognised by this antibody. EC extracts contained three distinct polypeptides, p65 and a doublet of p55 termed p55U (upper) and p55L (lower) (Figure 7.1c, lane 2). HeLa and JM extracts also contained polypeptides, p65 and p55U, although p55L was not observed (Figure 7.1c, compare lane 1 with 2 and 3). It is likely that all three polypeptides are forms of DP-1 as the antisera used was peptide affinity purified and other antibodies made against different regions of DP-1 recognise similar polypeptides. However one can not exclude at present that these are

cross reacting polypeptides or the products of closely related genes.

7.2 DP-1 and E2F-1 interact in crude extracts

To assess if DP-1 and E2F-1 co-precipitate *in vivo*, HeLa whole cell extracts were immunoprecipitated with the anti-DP-1 antibody in the presence of either a control peptide (1) or DP-1 peptide (A). Immune complexes were subsequently treated with detergent and assayed for DNA binding activity on the E2F site. The eluted material from DP-1 immunoprecipitates contained DRTF1/E2F binding activity which was not present when the DP-1 peptide was included in the reaction indicating this was specific for DP-1 (Figure 7.2a, compare lanes 5 and 6 with 8 and 9). Furthermore, eluted DRTF1/E2F binding activity contained the E2F-1 protein as immunoblotting the immunoprecipitates with an anti-E2F-1 monoclonal antibody, SQ41 (Kaelin *et al.*,1992) revealed a specific polypeptide with the molecular weight expected of E2F-1 (Figure 7.2b, lane 4). Likewise, the presence of E2F-1 was dependent on the anti-DP-1 DNA binding activity since it was not present in immunoprecipitates performed with the DP-1 peptide (Figure 7.2b, compare lanes 3 and 4). As a positive control for SQ41 a GST-E2F-1 fusion protein was also immunoblotted alongside which as expected also reacted with the E2F-1 antibody (Figure 7.2b, lane 2). Thus, E2F-1 and DP-1 exist in the same protein complex in HeLa cell extracts.

7.3 DP-1 and E2F-1 bind preferentially to the DNA as heterodimers

Many transcription factors are capable of binding DNA as heterodimers and I therefore investigated whether DP-1 and E2F-1 were able to heterodimerise. Thus, both DP-1 and E2F-1 were tested for binding to the E2F site or E2a promoter either separately or together. As a fusion protein GST-DP-1 bound to the E2F site poorly compared to GST-E2F-1 (Figure 7.3a, compare lanes 2 and 3). However addition of both GST-DP-1 and GST-E2F-1 together resulted in synergistic binding activity greater than that for either homodimer suggesting that both proteins interact synergistically (Figure 7.3a, compare lanes 2 and 3 with 4). To confirm that both DP-1 and E2F-1 were present in this complex, antibodies to E2F-1 and DP-1 were added to the DNA binding reaction. The anti-E2F-1 antibody was able to "supershift" the GST-E2F-1 homodimer and also the potential heterodimeric DNA binding activity (Figure 7.3a, compare lanes 3 and 4 with 5 and 6). Similarly, anti-DP-1 antibodies (α18) reduced binding of the heterodimer in a specific manner since it was competed by the homologous peptide but not a control peptide (Figure 7.3a, compare lanes 7 and 8). Thus both E2F-1 and DP-1 are components of the induced DNA binding activity suggesting they can bind DNA as heterodimer, although one can not rule out the possibility that higher order complexes such as tetramers are produced. For simplicity the DP-1/E2F complex will be referred to as a heterodimer this being the most likely explanation.

The ability of DP-1 and E2F-1 to interact was also tested using the E2a promoter from which the E2F site (used in Figure 7.3a) was derived. As before, GST-E2F-1

bound more efficiently than GST-DP-1 although the DNA binding activity of the DP-1 homodimer was also evident (Figure 7.3b, lanes 2 and 3). Again, the E2F-1/DP-1 heterodimer bound DNA more efficiently than either homodimer (Figure 7.3b, compare 2 and 3 with 4). Since the synergistic binding of GST-E2F-1 and GST-DP-1 was observed with truncated proteins lacking N-terminal sequences it was necessary to establish that the full length proteins behave in a similar manner. As these were not available as fusion proteins full length protein were generated by transcribing and translating each cDNA *in vitro*. The reticulocyte lysate containing DP-1 or E2F-1 alone showed little difference to the endogenous DNA binding activity which was apparent with the lysate alone (Figure 7.3b, compare lanes 5 and 6 with 7). However, when both DP-1 and E2F-1 were translated together, greater DNA binding activity was evident (Figure 7.3b, compare lanes 5 and 6 with 7), in agreement with experiments performed using the fusion proteins. Thus, in conclusion both DP-1 and E2F-1 bind to the E2F site synergistically as a heterodimer. Given the data presented it is very likely that this synergistic interaction occurs *in vivo*.

7.4 Dimerisation between DP-1 and E2F-1 requires a region of sequence similarity

Various deletions of DP-1 were made to determine the regions required to interact with E2F-1 (summarised in figure 7.4b). Each fusion protein was cleaved with thrombin such that a faster migrating heterodimer would be evident when assayed by gel retardation. In this experiment the cleaved DP-1 proteins were unable to bind

DNA (Figure 7.4a, lanes 2-5), although both DP-1^{84-204} and DP-1^{84-249} have been shown to weakly but specifically bind to the E2F site (Girling *et al.*,1993). When either DP-1^{84-204} or DP-1^{84-249} were mixed with GST-E2F-1 a faster migrating DNA binding complex was formed at the expense of the E2F-1 homodimer indicating that the cleaved DP-1 protein had dimerised with E2F-1 (Figure 7.4a, compare lane 6 with 7 and 8). The DNA binding of the GST-E2F-1/DP-1^{84-249} heterodimer was greater than either homodimer alone consistent with previous observations using the GST-DP-1 and GST-E2F-1 fusion proteins (Figure 7.3a). However, whereas the E2F-1/DP-1^{84-204} heterodimer was visible the binding activity was far less than with GST-E2F-1/DP-1^{84-249} (Figure 7.4a, compare lanes 7 and 8). This result indicates therefore that the region of DP-1 between amino acid residues 204 and 249 contributes to the increased DNA binding activity. Further deletion of this region from either the N-terminus (DP-$1^{146-249}$) or C-terminus (DP-1^{84-166}) yielded proteins which failed to form a DNA binding heterodimer (Figure 7.4a, lanes 9 and 10). Both DP-1^{84-166} and DP-$1^{146-249}$ greatly reduced E2F-1 binding which may be due to a non-specific effect of adding protein or perhaps because non-DNA binding heterodimers were created.

The DNA binding specificity of the E2F-1/DP-1 heterodimer was compared to the E2F-1 homodimer using the E2F oligonucleotides mentioned in chapter 3. The specificity of the heterodimer was very similar to that of E2F-1 alone and the DRTF1/E2F activity present in EC extracts (Figure 7.4c, compare lanes 2-6 with 7-11 and 12-16). Therefore, the DP-1/E2F-1 heterodimer and E2F-1 homodimers have similar DNA binding specificities.

To characterise the interaction between E2F-1 and DP-1, *in vitro* translated E2F-1

was mixed with the GST-DP-1 fusion proteins and purified with glutathione agarose. In this assay only the DP-1 deletions containing an interaction domain should bind to E2F-1. E2F-1 bound to both DP-1^{84-204} and DP-1^{84-249} but not GST alone, consistent with their ability to form DNA binding heterodimers (Figure 7.4d, compare lanes 2,3 and 6). DP-$1^{146-249}$ also bound E2F-1 whereas DP-1^{84-166} failed to do so (Figure 7.4d, compare lanes 5 and 4). DP-$1^{146-249}$ therefore contains a domain that allows it to interact with E2F-1 but lacks amino acid sequences necessary to form a DNA binding heterodimer.

7.5 DP-1 binds pRb in non-pocket-dependent manner

The pRb-binding properties of DP-1 were investigated further by mixing *in vitro* translated DP-1 with GST-Rb fusion proteins an assay routinely used to study interactions between proteins (Rustgi *et al.*1991;Kaelin *et al.*,1991). As a control, binding to GST alone and glutathione agarose beads was also assessed. For the positive control *in vitro* translated E2F-1 and E1a were also tested for binding to GST-Rb as these have previously been shown to bind wild-type but not mutant pRb proteins (Hu *et al.*,1991;Kaelin *et al.*,1992;Helin *et al.*,1992). DP-1 did not bind to GST alone or the glutathione agarose beads (Figure 7.5, lanes 1 and 2) however binding was observed to both GST-Rb and GST-Rb^{706} with equal efficiency (Figure 7.5, lanes 3 and 4). Both E2F-1 and E1a bound only GST-Rb and less efficiently to mutant GST-Rb^{706} (Figure 7.5, compare lanes 5 with 6 and 7 with 8). Therefore, DP-1 binds to pRb in a non pocket-dependent manner.

7.6 Discussion

In this chapter I established several important properties of DP-1. Firstly, that DP-1 was a major component of DRTF1/E2F DNA binding complexes including pRb and p107 containing complexes. Secondly, DP-1 and E2F-1 can form heterodimers which bind synergistically to the E2F site.

7.6.1 DP-1, a common component of DRTF1/E2F complexes

Initial attempts to purify DRTF1/E2F from HeLa cells suggested a single polypeptide of molecular weight approximately 54Kd (Yee *et al.*,1989), although similar studies using EC extracts indicated more than one component (Shivji and La Thangue,1990;Girling *et al.*,1993). The isolation of two different cDNAs encoding components of DRTF1/E2F suggested that either "free" DRTF1/E2F is a multicomponent DNA binding activity or that these proteins form distinct complexes.

DP-1 was a major component of EC and HeLa DNA binding complexes, although JM DRTF1/E2F complexes were unaffected by the DP-1 antibody. One possible explanation is that DP-1 is not a component of JM complexes, perhaps because a related protein is present. Alternatively DP-1 may be present but the epitope recognised by the antibody is masked, thus preventing an effect by the antisera. Antibodies raised against DP-1 specifically recognise several distinct polypeptides suggesting DP-1 may comprise several components. Current evidence indicates that the p65 is a non-DNA binding polypeptide whereas p55 correlates with the DNA

binding activity. The relationship between p55L and p55U is presently under study although it is likely that the difference between p55U and p55L is due to post-translational modification. This idea is appealing as there is currently no evidence for the presence of different RNA transcripts which could explain these three polypeptides (P.T.Tassios, unpublished observations).

DRTF1/E2F DNA binding activity co-precipitated with both pRb and p107. In both cases the DRTF1/E2F binding activity contained DP-1, supporting the idea that DP-1 is a component of the DRTF1/E2F activity which associates with pRb and p107. Similar experiments with E2F-1 indicated that it is only a component of free DRTF1/E2F complexes and pRb-DRTF1/E2F (Kaelin *et al.*,1992). Therefore, both DP-1 and E2F-1 may be present in pRb complexes. It is possible that DP-1 and E2F-1 bind pRb in separate complexes or the same molecular complex.Alternatively pRb may not bind directly to DP-1. The *in vitro* translated DP-1 protein bound pRb and mutant pRb, whereas both E1a and E2F showed a pocket-dependent interaction with pRb. This may be because DP-1 binds to a different region of pRb and thus reflects a weak association which is pocket-independent.

The earlier results presented in this thesis showed that DRTF1/E2F DNA binding activities in EC extracts bound to pRb in a pocket-dependent manner (chapter 4). Thus, one might predict that other proteins are present in crude extracts which confer pocket-dependent binding to DP-1. E2F-1 is a likely candidate as it binds only wild-type pRb and can dimerise with DP-1. Therefore it is possible that DP-1 stabilises pRb binding to E2F-1.

7.5.2 DRTF1/E2F, a heterodimer of DP-1 and E2F-1?

DP-1 and E2F-1 formed heterodimers which had greater affinity for the E2F site than either protein had alone. Similarly, each homodimer bound to the E2a promoter less efficiently than the heterodimer. On the promoter, two complexes were formed in the presence of both proteins possibly reflecting occupation of both E2F sites. The synergistic binding of the heterodimer suggested that heterodimerisation is likely to be a preferred state and may therefore occur *in vivo*. Consistent with this idea, was the observation of a complex containing the both proteins in crude HeLa extracts. Clearly in different cell types this may not be the case since other proteins, for example a protein related to E2F-1 may dimerise in place of DP-1 or E2F-1. Other studies performed in our laboratory have shown that heterodimerisation also leads to increased transcriptional activation through the E2F site (Bandara *et al.*,1993). Thus, DP-1 and E2F-1 also interact synergistically to activate transcription.

Heterodimerisation as a means of regulating transcription is a common scenario (see Introduction section 1.2.2). For example, jun and fos heterodimers bind the AP-1 site more efficiently than either homodimers alone. Similarly, the myc and max protein also bind more efficiently as heterodimers (Blackwood and Eisenman,1991; Prendergast *et al.*,1991). Intriguingly, the GST-DP-1 protein bound DNA poorly when compared to GST-E2F-1, a result which is analogous to both c-myc and c-fos homodimers which bind DNA less efficiently than their partners, max and jun respectively. However this may simply be due to inefficient post-translational modifications due to the expression of fusion proteins in bacteria and thus may have

little physiological relevance. Alternatively, DP-1 may possess an inhibitory domain which precludes DNA binding as a homodimer which has been shown for the E12 transcription factor (Sun and Baltimore,1991). It is possible however that DP-1 binds DNA poorly *in vivo* and E2F-1 may "help" DP-1 to bind DNA.

The E2F-1 protein contains an activation domain which has been determined using the Gal4 DNA binding domain (Kaelin *et al.*,1992). It also binds DNA in a sequence specific manner, so what does DP-1 provide? There are many possibilities, for example, DP-1 may contain a cryptic activation domain which is exposed upon heterodimerisation. Alternatively, E2F-1 may be unstable in cells and "protected" by heterodimerisation. Another possibility is that DP-1 regulates E2F-1 by allowing growth regulatory signals to be imposed on E2F-1 through phosphorylation by cellular kinases. Many other possibilities exist which can be tested in the future.

7.5.2 Dimerisation between DP-1 and E2F-1.

The region of similarity between DP-1 and E2F-1 was necessary for both to interact and interestingly within this region are amino acid residues which are also conserved in a number of yeast cell cycle regulatory proteins (La Thangue and Taylor,1993). Therefore, this region may be a novel dimerisation domain which has been conserved throughout evolution. Heterodimerisation has two possible outcomes; it not only allows greater DNA binding and transcriptional activity but also confers pocket-dependent binding of pRb to the DP-1/E2F-1 complex. This does not mean that DP-1 can not contribute to pRb binding, as DP-1 may facilitate pRb binding to

E2F-1. To test this idea further, the regions within DP-1 required for pRb binding could be identified and characterised in the DP-1/E2F-1 heterodimer.

It is possible that other E2F-like molecules exist which bind to different pocket proteins, such as p107. If this is the case then perhaps during cell cycle progression different heterodimers of E2F-like and DP-1-like proteins exist which perform different tasks. For example, heterodimerisation may change the specificity for DNA thereby allowing different target genes to be regulated. However, the binding specificity of the DP-1/E2F-1 heterodimer was similar to that of the E2F-1 homodimer and the DRTF1/E2F binding activity in EC extracts. Therefore the mutations in the binding site employed this study did not distinguish between homodimers and heterodimers. Further mutational analysis may be more informative or PCR binding site amplification could be used to isolate the DNA binding sites preferred by homo and heterodimers. Alternatively, there may be no difference in binding specificity but one partner may be limiting to ensure that transcription is regulated by different signals at the appropriate time in the cell cycle.

In conclusion DP-1 is frequent component of DRTF1/E2F complexes and one physiological form is likely to be a heterodimer of DP-1 and E2F-1. In addition, DP-1 binds pRb but in a pocket-independent manner. Therefore dimerisation may influence pRb binding as well as allowing synergistic binding to the E2F site.

Figure 7.1a, *DP-1 is a major component of DRTF1/E2F complexes.* Gel retardation were performed with EC (lanes 1 and 2), HeLa (lanes 3 and 4) and JM (lanes 5 and 6) whole cell extracts. Where indicated 3μl of DP-1 antisera (αA) was added with either 2nmols of peptide 1 (lanes 1,3 and 5) or peptide A (lanes 2,4 and 6).

Figure 7.1b, *DP-1 is associated with p107 and pRb DRTF1/E2F complexes.* DRTF1/E2F DNA binding activities from p107 (SD9) or pRb (IF8) following immunoprecipitations from JM extracts and detergent treatment. Approximately 2μl of each reaction was assayed by gel retardation using the E2F site as probe (lanes 1 to 6). Where indicated 3μl of DP-1 antisera (α18) was added to binding reactions with either 2nmol peptide 18 (lanes 3 and 6) or peptide 1 (lanes 2 and 5). Note the DNA binding activity in lanes 2 and 5 are reduced implying DP-1 is present.

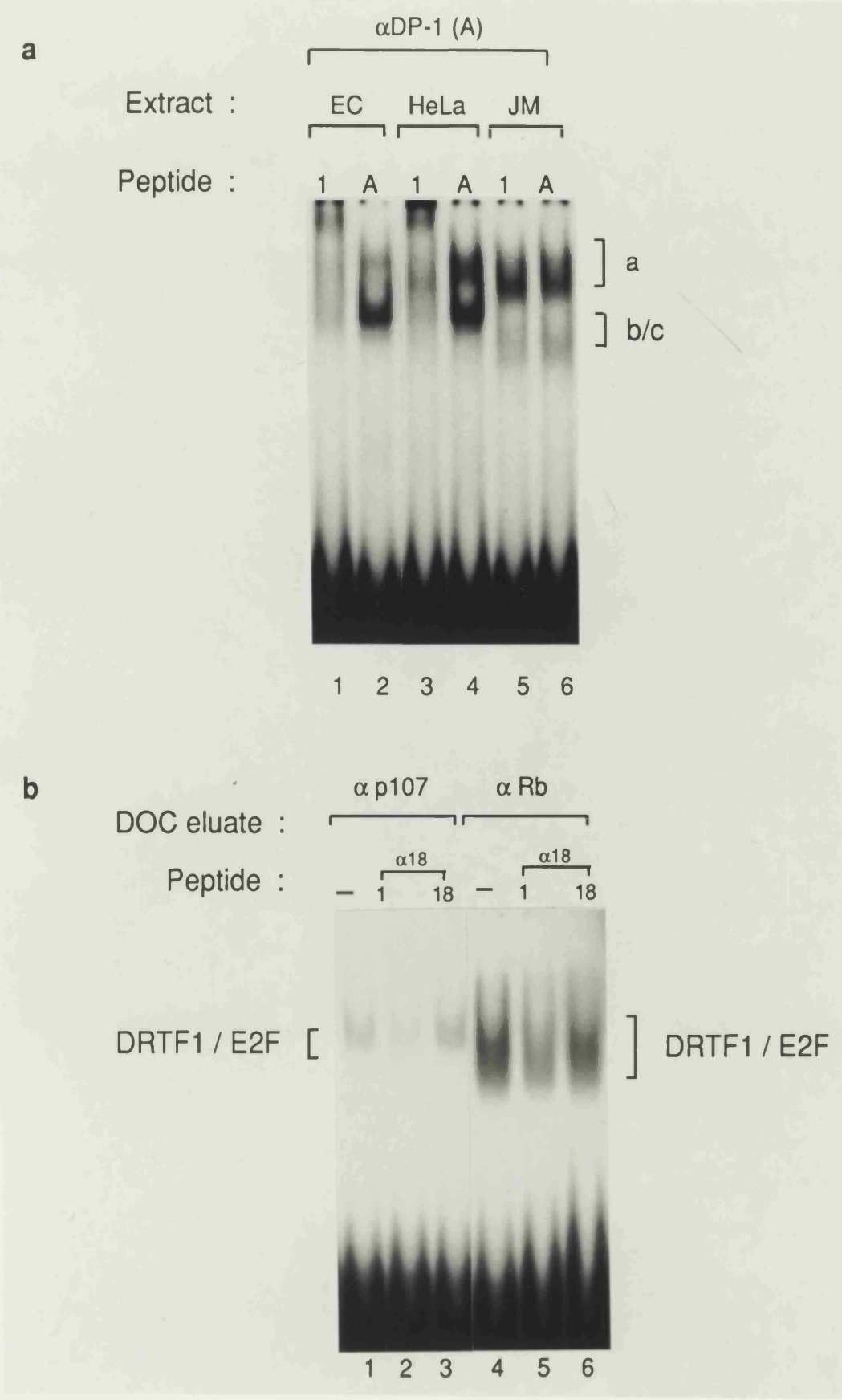

a
αDP-1 (A)
Extract :
EC
HeLa
JM
Peptide :
1 A 1 A 1 A
a
b/c
1 2 3 4 5 6
b
α p107
α Rb
DOC eluate :
Peptide :
α18
– 1 18 – 1 18
DRTF1 / E2F
DRTF1 / E2F
1 2 3 4 5 6

Figure 7.1c, *DP-1 antibodies recognise similar polypeptides in different cell types.* Approximately 40 μg of EC (lane 1), HeLa (lane 2) and JM (lane 3) whole cell extract was western blotted and probed with affinity purified DP-1 antibody (αA). Specific bands of approximately 65,000D (p65), 55,000D (p55U) and 53,000D (p55L) are indicated. The migration and size of molecular weight markers run in parallel are also shown.

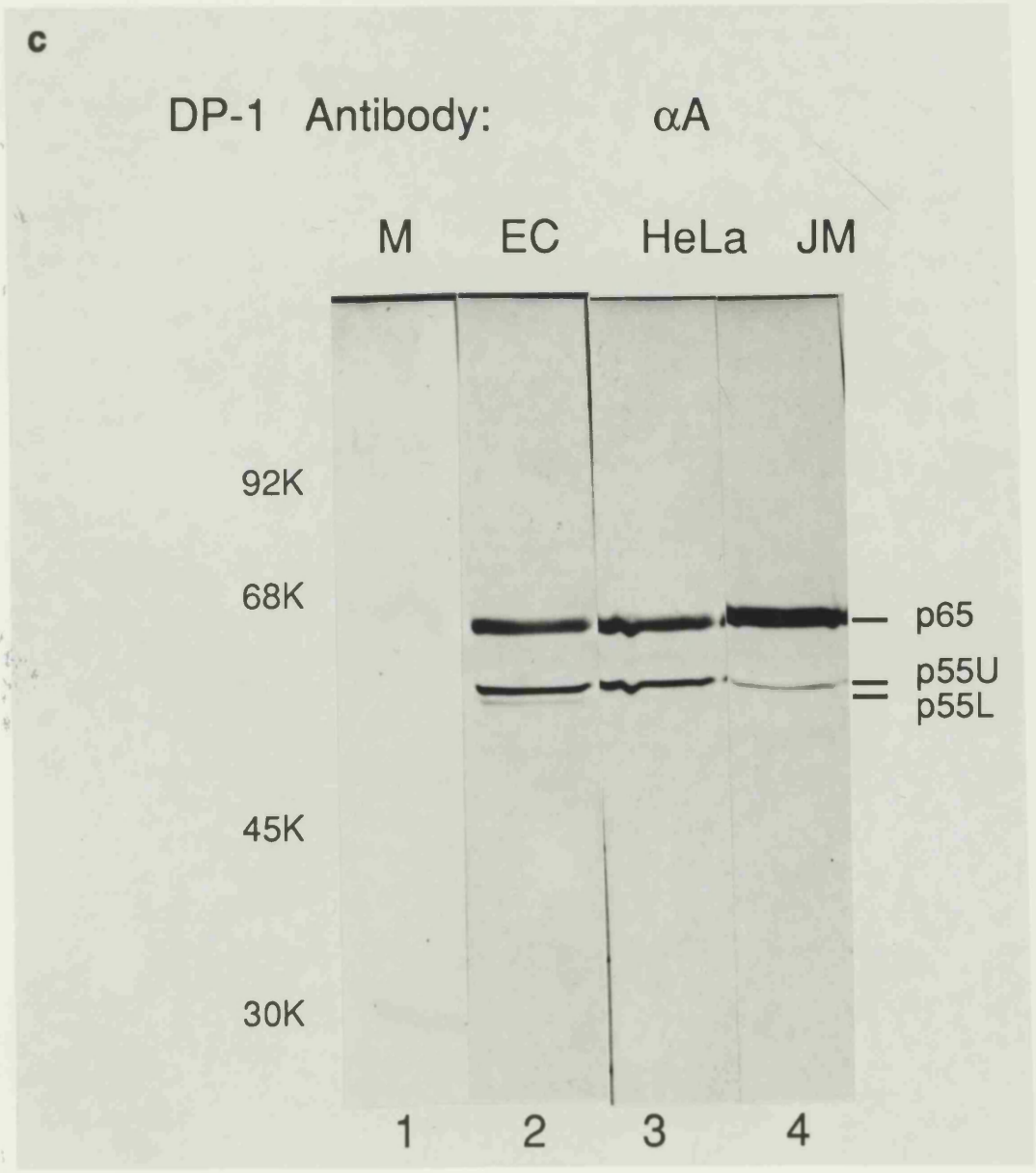

c
DP-1 Antibody: αA
M
EC
HeLa
JM
92K
68K
45K
30K
p65
p55U
p55L
1
2
3
4

Figure 7.2a *DP-1 and E2F-1 co-precipitate in HeLa extracts.* DRTF1/E2F DNA binding activity was assayed following immunoprecipition of HeLa whole cell extracts and detergent release. Immunoprecipitations were performed with DP-1 antisera (αA) in the presence of either 20 nmol peptide A (lanes 2-5) or peptide 1 (lanes 6-9). The binding activity remaining in the extracts after immunoprecipitation is shown by S (lanes 2 and 6) and control washes without DOC detergent are indicated as C (lanes 3 and 7). The DNA binding activity following DOC treatment are shown as W1 and W2 (lanes 4,5,8 and 9) representing two successive washes; note only lanes 8 and 9 contain E2F site DNA binding activity.

Figure 7.2b, W1 and W2 from each immunoprecipitation (with peptide 1 or A) was pooled, blotted alongside GST-E2F-1 (200ng) and probed with the E2F-1 monoclonal antibody, SQ41.

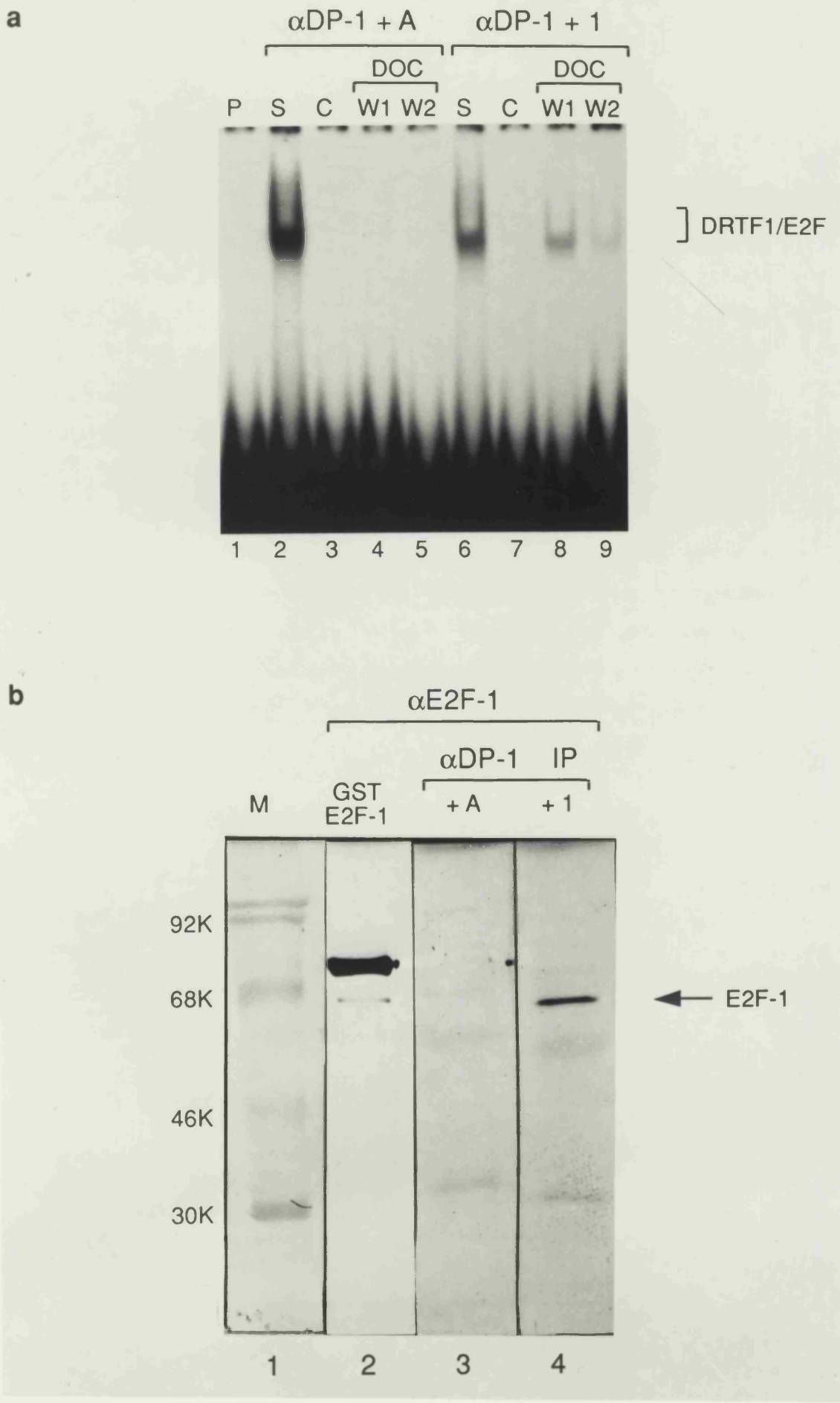

a
αDP-1 + A
αDP-1 + 1
DOC
DOC
P S C W1 W2 S C W1 W2
DRTF1/E2F
1 2 3 4 5 6 7 8 9
b
αE2F-1
αDP-1 IP
M
GST E2F-1
+ A
+ 1
92K
68K
46K
30K
E2F-1
1 2 3 4

Figure 7.3a, *DP-1 and E2F-1 bind the E2F site synergistically as heterodimers.* Gel retardations using the E2F site with either 50ng of GST-DP-1 (lanes 2,4,6,7 and 8) or 25ng of GST-E2F-1 (lanes 3-8). Where indicated either 2μl of E2F-1 antisera (αE2F-1 136) was added (lanes 5 and 6) or 3μl of DP-1 antisera (α18) was added (lanes 7 and 8). With the DP-1 antiserum 2nmol of peptide 18 (lane 8) or peptide 1 (lane 7) was also added. Complexes supershifted by the E2F-1 antibody are indicated as a[s]. The DP-1 antiserum reduced DNA binding activity (compare lane 7 to 8).
Figure 7.3b, Gel retardations were performed using the E2a promoter as probe with 50ng of GST-DP-1 (lanes 2 and 4) or 25ng of GST-E2F-1 (lanes 3 and 4). *In vitro* translated DP-1 and E2F-1 were also assayed for binding to the E2a promoter. Where indicated 1μl of lysate containing DP-1 (lane 5), lysate containing E2F-1 (lane 6) or lysate containing DP-1 and E2F-1 (lane 7) was assayed. Note the lysate alone was also tested (lanes 8) and contained an endogenous E2F site binding activity although this was less than the binding activity produced by DP-1 and E2F-1 together.

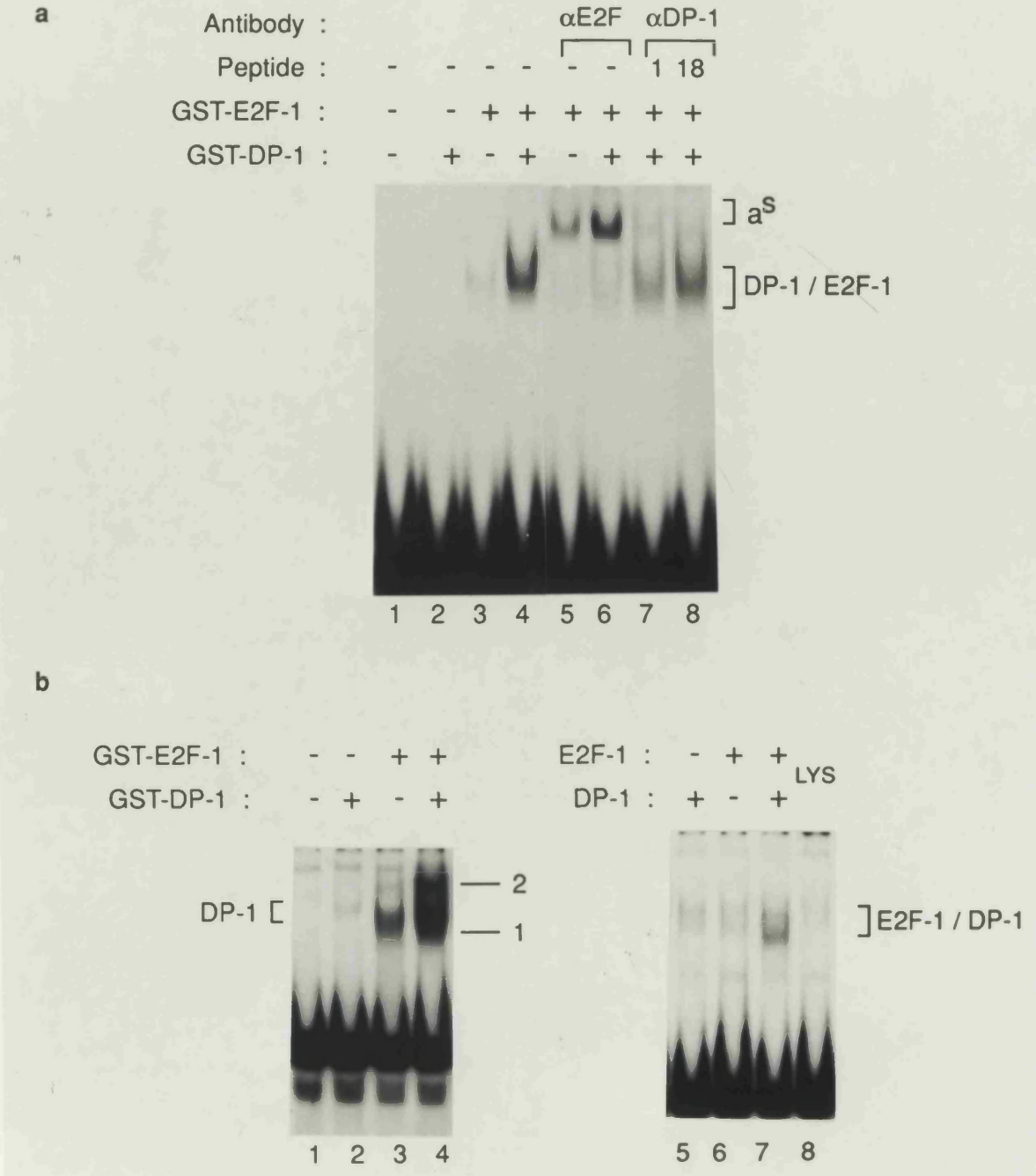

a
Antibody :
αE2F
αDP-1
Peptide : - - - - - - 1 18
GST-E2F-1 : - - + + + + + +
GST-DP-1 : - + - + - + + +
] aS
] DP-1 / E2F-1
1 2 3 4 5 6 7 8
b
GST-E2F-1 : - - + +
GST-DP-1 : - + - +
E2F-1 : - + +
DP-1 : + - +
LYS
DP-1 [
— 2
— 1
] E2F-1 / DP-1
1 2 3 4
5 6 7 8

Figure 7.4a, *Dimerisation between DP-1 and E2F-1 requires a region of similarity.* GST-DP-1 derivatives were cleaved from GST and assessed for DNA binding and the ability to dimerise with E2F-1. Gel retardations were performed using the E2F site as probe. Where indicated 150ng of DP-1^{84-249} (lane 2 and 7), DP-1^{84-204} (lanes 3 and 8), DP-1^{84-166} (lanes 4 and 9) or DP-$1^{146-249}$ (lanes 5 and 10) was added together with 50ng of GST-E2F-1 (lanes 6 to 10). The E2F-1 homodimer and E2F-1/DP-1 heterodimers are indicated. Note that the mutant DP-1 derivative produced a faster migrating complex.

Figure 7.4b, *Summary of DP-1 deletions.* The wild-type DP-1 sequence is shown and deletions are also shown. The shaded domain indicates the region of similarity between DP-1 and E2F-1 (Girling *et al.*,1993).

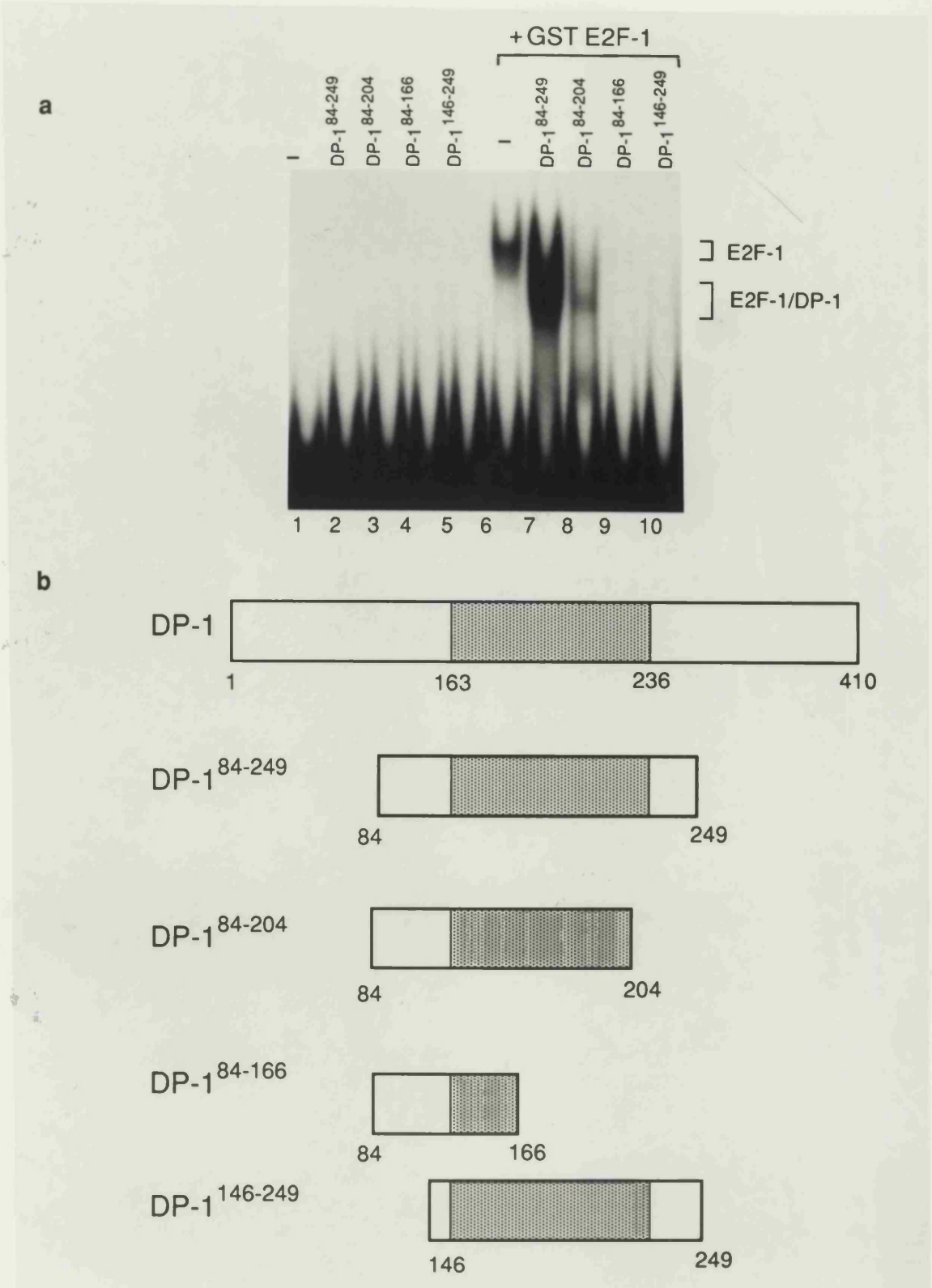
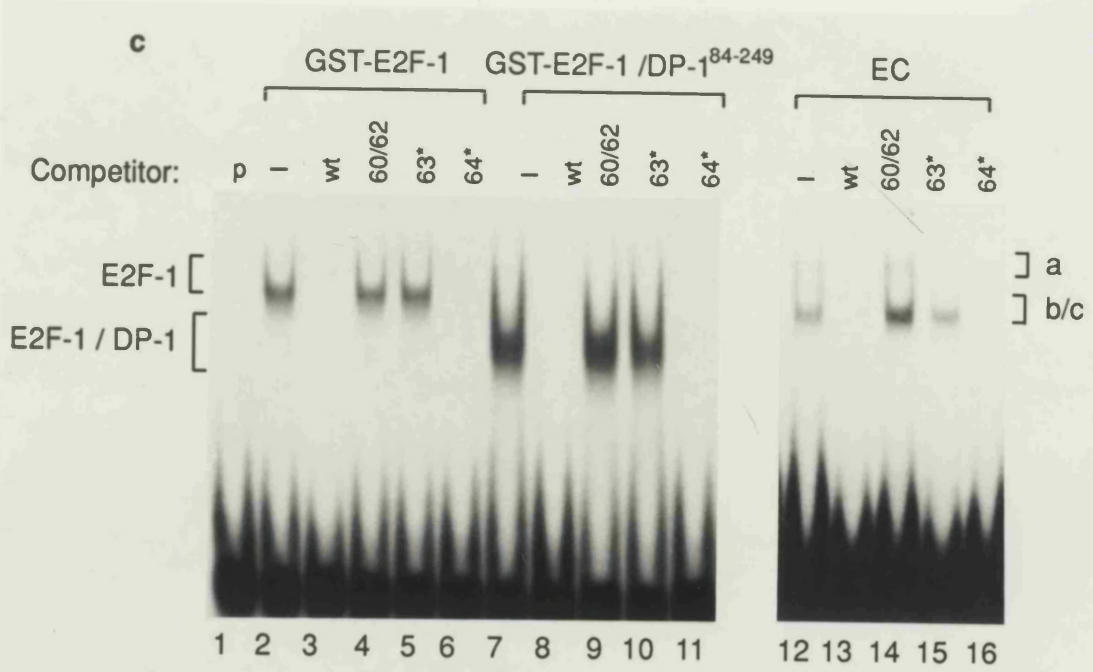

a
+GST E2F-1
–
DP-1 84-249
DP-1 84-204
DP-1 84-166
DP-1 146-249
–
DP-1 84-249
DP-1 84-204
DP-1 84-166
DP-1 146-249
E2F-1
E2F-1/DP-1
1 2 3 4 5 6 7 8 9 10
b
DP-1
1
163
236
410
DP-1 84-249
84
249
DP-1 84-204
84
204
DP-1 84-166
84
166
DP-1 146-249
146
249

Figure 7.4c, *The E2F-1/DP-1 heterodimer possess a similar DNA binding specificity to DRTF1/E2F.* Gel retardations using the E2F site with either 50ng of GST-E2F-1 (lanes 2-11), 150ng of DP-1^{84-249} (lanes 7-11) or 6μg of EC whole cell extract (lanes 12-16). Where indicated 300ng of competing oligonucleotide was added.

Figure 7.4d E2F-1 was translated *in vitro* and radiolabelled using ^{35}S-methionine. Approximately 5μl of lysate was mixed with 1μg of GST-DP-1 fusion protein or GST alone, washed and purified using glutathione agarose. E2F-1 bound to GST-DP-1^{84-249} (lane 3), GST-DP-$1^{146-249}$ (lane 5) and GST-DP-1^{84-204} (lane 6) but not GST-DP-1^{84-166} (lane 4) or GST alone (lane 2). *In vitro* translated E2F-1 alone is also shown (lane 1).


c
GST-E2F-1
GST-E2F-1 /DP-1$^{84-249}$
EC
Competitor:
p
–
wt
60/62
63*
64*
E2F-1
E2F-1 / DP-1
a
b/c
1 2 3 4 5 6 7 8 9 10 11
12 13 14 15 16

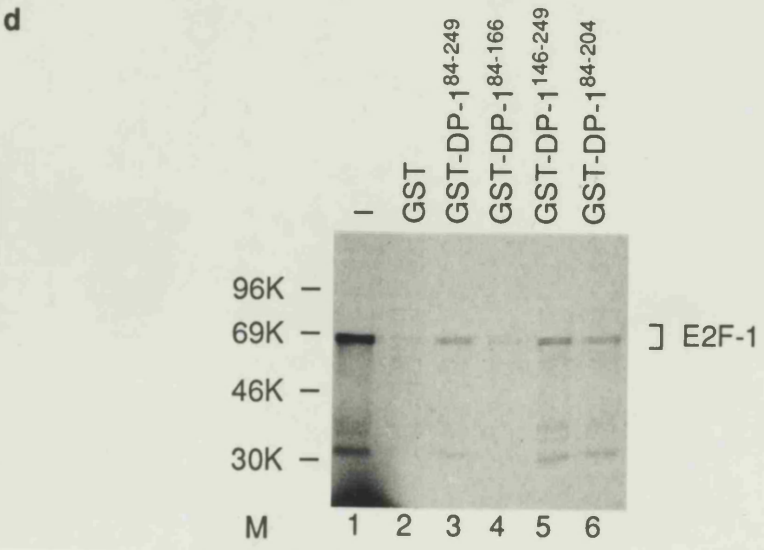
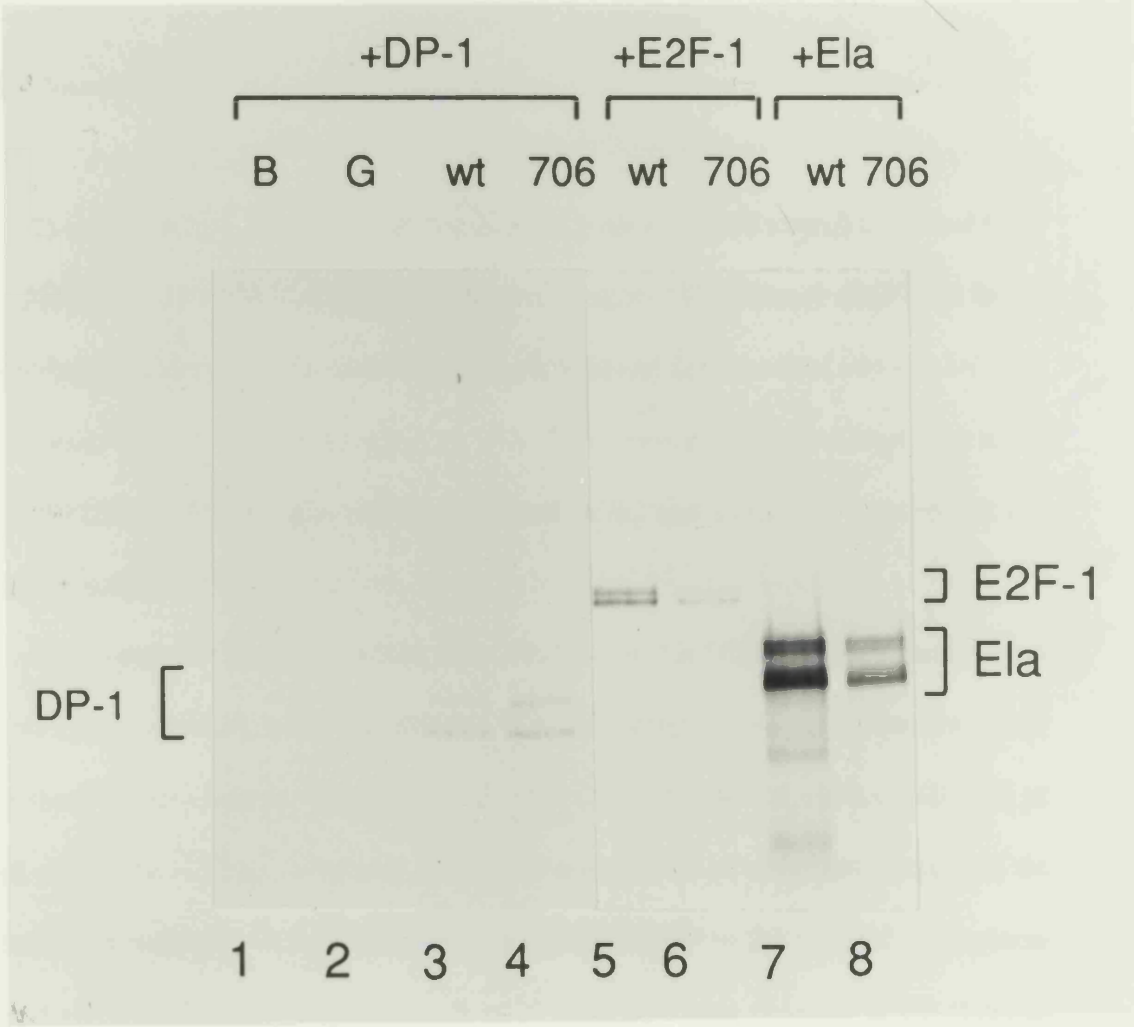

d
–
GST
GST-DP-1$^{84-249}$
GST-DP-1$^{84-166}$
GST-DP-1$^{146-249}$
GST-DP-1$^{84-204}$
96K
69K
46K
30K
E2F-1
M
1 2 3 4 5 6

Figure 7.5, *DP-1 binds pRb in a non-pocket-dependent manner.* DP-1, E2F-1 and E1a were translated *in vitro* together with ^{35}S-methionine. Approximately 1μg of GST-Rb (wt) or GST-Rb^{706} (706) was mixed with 5μl of each lysate, washed and purified using glutathione agarose. Lysate containing DP-1 was also mixed with the glutathione agarose beads (B) alone (lane 1) or 1μg of GST (G) and processed in parallel. DP-1 bound to both GST-Rb and GST-Rb^{706} but not to the agarose beads or GST (lanes 1-4). Both E1a and E2F-1 bound GST-Rb more efficiently than GST-Rb^{706} (lanes 5-8).


+DP-1
+E2F-1
+Ela
B
G
wt
706
wt
706
wt
706
E2F-1
Ela
DP-1
1
2
3
4
5
6
7
8

CHAPTER 8

DP-1, a cell cycle regulated component of DRTF1/E2F

8.0 Introduction

Two distinct components of DRTF1/E2F have been isolated termed DP-1 and E2F-1 (Girling *et al.*,1993;Kaelin *et al.*,1992;Helin *et al.*,1992;Shan *et al.*,1992). In the previous chapter I characterised these two proteins and demonstrated that DP-1/E2F-1 heterodimers were likely to occur *in vivo*. DP-1 was a common component of all DRTF1/E2F DNA binding activities defined in EC and HeLa cell extracts but not others such as JM extracts.

Experiments using synchronised cells have shown that DRTF1/E2F complexes are cell cycle regulated with free DRTF1/E2F appearing at the G1/S boundary and persisting into S-phase (Mudryj *et al.*,1991;Shirodkar *et al.*,1992;Chellappan *et al.*,1992). In addition complexes, with p107 and pRb occur at different stages of the cell cycle with pRb complexes occurring predominantly in G1 and p107 complexes in S-phase, although complexes with p107 have also been detected in G0 (Lees *et al.*,1992;Schwarz *et al.*,1992). In peripheral T-cells and serum stimulated NIH 3T3 cells E2F-1 mRNA is induced at the G1/S boundary and persists into S-phase, which correlates with the appearance of free DRTF1/E2F DNA binding activity (Kaelin *et al.*,1992;Slansky *et al.*,1993). Both in synchronised and asynchronous T-cell extracts, experiments performed using antibodies made against the E2F-1 protein suggest it is

a component of pRb containing DRTF1/E2F complexes and some of the free DRTF1/E2F complexes (Chittenden *et al.*,1993). Since DRTF1/E2F binding activity is present before E2F-1 message is detected it is likely that these DRTF1/E2F DNA binding complexes may contain other E2F-like molecules, such as DP-1.

I was interested therefore in determining which complexes during the cell cycle progression contain DP-1.

8.1 DP-1 is a component of all DRTF1/E2F DNA binding complexes which occur during the cell cycle

NIH 3T3 cells were serum stimulated as these cells have been used frequently in the past for similar experiments (Greenberg and Ziff,1984;Müller *et al.*,1984). In addition the cell cycle regulation of DRTF1/E2F complexes have been studied previously in this cell type (Mudryj *et al.*,1991). Following serum stimulation cultures were harvested every 4 hours to prepare micro-extracts for analysis by gel retardation. In parallel cultures the number of cells in S-phase was monitored by bromodeoxyuridine (BrDu) incorporation. At the early stages of the cell cycle (G0-4 hours) most DRTF1/E2F complexes consisted of the **a** form (Figure 8.1a, lane 2 and 3) which I have previously been shown to contain p107, pRb, cyclins and cyclin-dependent kinases. As cells progressed through the cell cycle, the percentage of BrDu positive cells increased indicating that DNA synthesis was occurring (Figure 8.1a). As cells entered S-phase the **b/c** form was induced in gel retardation assays consistent with previously published cell synchrony experiments performed in both NIH 3T3

cells and other cell types (Mudryj *et al.*,1991;Chellappan *et al.*,1992;Shirodkar *et al*,1992).

The presence of DP-1 was assessed by adding DP-1 antisera (αA) to DNA binding assays at each of the time points. DP-1 was a component of all the DRTF1/E2F DNA binding complexes which could be resolved during cell cycle progression, as the DP-1 antibody abolished all complexes at each time point, an effect which was competed by the homologous peptide (Figure 8.1b, compare lanes 2-9 with 10-18). Therefore, DP-1 appears to be a component of all DRTF1/E2F complexes during cell cycle progression, in contrast to E2F-1.

8.2 Nuclear distribution of DP-1 throughout the cell cycle

The cellular distribution of DP-1 was assessed during the cell cycle progression in 3T3 cells. Nuclear staining was evident in serum starved cells which appeared diffuse and concentrated in speckles (Figure 8.2, a). This staining was specific since it was competed by the homologous DP-1 peptide 18 but not peptide 1 (Figure 8.2, b). Furthermore, it was unlikely to be an artefact of fixation as similar staining patterns were seen in SAOS2 cells using a variety of different fixation techniques (La Thangue, unpublished observations). As NIH 3T3 cells progressed into S-phase, the DP-1 nuclear stain increased although the quality and quantity of speckles were unaffected. Therefore, the increase in nuclear staining correlates with the induction of free DRTF1/E2F DNA binding activity (Figure 8.2, compare panel a with e and g).

The regulation of the DP-1 polypeptides were also studied. Samples from each time point were western blotted and probed with either the DP-1 αA antibody or DP-1 α18 antibody. As previously mentioned DP-1 αA recognised three polypeptides ,p65.p55U and p55L (chapter 7). Both the p65 and p55U polypeptides were unchanged during the cell cycle.In contrast the p55L polypeptide was absent in G0 and induced between 8-12 hours (Figure 8.3a, compare lane 1 and 4). The DP-1 α18 antiserum recognises p55L and a related p68 polypeptide, although it is possible that this is a cross reactive protein or related gene product (N.Burden and T.Sorensen, unpublished observations). As before p55L was induced at the beginning of S-phase correlating with appearance of free DRTF1/E2F (Figure 8.3b, compare lanes 1 and 4). Therefore, DP-1 antisera define several distinct polypeptides of which p55L is cell cycle regulated.

8.3 Discussion

In this chapter I have shown that DP-1 is a component of all DRTF1/E2F DNA binding complexes which occur during cell cycle progression. In addition the polypeptides recognised by anti-DP-1 antibodies suggest they are differentially regulated during the cell cycle.

8.3.1 DP-1 is a major component of cell cycle regulated DRTF1/E2F complexes

DP-1 is a major component of DRTF1/E2F complexes in EC and HeLa extracts and in this chapter was found to be a major component of the DNA binding

complexes which occur during the cell cycle progression in NIH 3T3 cells. Therefore DP-1 has distinct properties to E2F-1 which is present in a portion of DRTF1/E2F complexes (Kaelin *et al.*,1992). The presence of E2F-1 in this cell system could not be determined as attempts to generate antibodies which affect DNA binding complexes in crude extracts have been unsuccessful. However, since E2F-1 has been shown in a subset of DNA binding complexes during the cell cycle progression of T-cells (Kaelin *et al.*,1992;Chittenden *et al.*,1993), it is possible that this occurs during the cell cycle progression in other cells. It could be, for example that E2F-1 is present in the uncomplexed DRTF1/E2F DNA binding activity induced at 8 hours and in addition perhaps those complexes containing pRb. Furthermore, since E2F-1 mRNA appears at the G1/S boundary (Kaelin *et al.*,1992;Slansky *et al.*,1993), DRTF1/E2F complexes which occur early in the cell cycle are unlikely to contain the E2F-1 protein. These complexes did however contain DP-1 suggesting that either DP-1 forms homodimers at this stage of the cell cycle, or perhaps heterodimerises with other E2F-1-like molecules. As cells progress through the cell cycle it is possible that newly synthesised E2F-1 protein would heterodimerise with DP-1 to activate transcription.

What of the other complexes seen during cell cycle progression? The p107-DRTF1/E2F complex appears in S-phase, and again DP-1 was shown to be a component of all S-phase complexes. This is also consistent with the data presented in chapter 7 which indicated that the DRTF1/E2F binding activity from pRb and p107 immunoprecipitates contained DP-1. Since the p107 complexes are unlikely to contain the E2F-1 protein another E2F-like molecule may exist which confers p107 binding

properties and may dimerise with DP-1 in S-phase.

Recently a several E2F-1-like proteins termed E2F-2 and E2F-3 have been isolated and characterised (Lees *et al.*,1993;Ivey-Hoyle *et al.*,1993). Both E2F-2 and E2F-3 specifically bind to the E2F site and also interact with pRb but not p107. The presence of these related proteins in DRTF1/E2F complexes has not been established and it is possible that they are redundant copies of E2F-1 although this remains to be established.

8.3.1 Intracellular distribution of DP-1

DP-1 was shown to be a nuclear protein. The pattern of staining was diffuse throughout the nucleus and also concentrated in "speckles" evenly distributed. As NIH 3T3 cells progressed though the cell cycle, the diffuse nuclear stain increased whereas the speckles remained unchanged. This increase in nuclear staining correlated with the appearance of free DRTF1/E2F observed in the gel retardation assays. The nature of the speckles is unclear although they are reminiscent of DNA replication centres or replication factories (Hozak *et al.*,1993). This idea seems unlikely as the speckles are constant in all cells throughout the cell cycle, even before S-phase can be detected. Attempts to determine whether these co-localised with components of the DNA replication machinery have been unsuccessful (N.B. La Thangue, unpublished observations). The speckled structures are also not only a property of NIH 3T3 cell or due to fixation conditions as SAOS2 cells have similar structures visible using a variety of different conditions for fixation (N.B. La Thangue, unpublished

observations). It is possible that DP-1 is concentrated in a subnuclear compartment for a specific purpose or it may reflect a cross reaction of the antibody to these nuclear locations. The latter seems unlikely since this antibody was purified on a peptide column, although this is not conclusive.

8.3.2 Cell cycle regulation of DP-1

The αA DP-1 sera recognises three polypeptides p65,p55U and p55L, of which the p55 polypeptide correlated with DNA binding activity during DNA affinity purification of DRTF1/E2F (T.Sorensen, unpublished observations). As cells progressed through the cell cycle the p65 polypeptides was unaffected. In contrast the p55L polypeptide was cell cycle regulated being induced in S-phase. This correlated with the appearance of free DRTF1/E2F binding activity and thus, p55L may correspond to a specific form of DP-1 which is under cell cycle control. The DP-1 α18 antibody recognised the p55L polypeptide and a p68 polypeptide. Thus this antibody recognises the induced form of DP-1. Since this was also used for immunofluorescence the increase in nuclear stain observed during cell cycle progression is probably due to p55L. Therefore, it is possible that the speckled staining which remains constant during the cell cycle is due to recognition of p68. At present the nature of p68 is unclear, it is not recognised by other DP-1 antisera although may be a related gene product.

Since p55L is induced at approximately the same time as the **b/c** form of DRTF1/E2F during cell cycle progression it may contribute to the DNA binding

activity induced in S-phase. Similarly the p55U polypeptide may contribute to complexes with pocket proteins as these complexes are present in all phases of the cell cycle. A clearer way of addressing this question would be to immunoprecipitate pRb/p107 complexes and determine if p55U or p55L is selectively retained. The p55L polypeptide was absent from both HeLa and JM extracts but present in EC extracts suggesting that DP-1 may be regulated differently in different cell types.

The difference between p55U and p55L could be due to differential splicing or post-translational modification. The latter is more appealing as there is no evidence for different DP-1 transcripts in NIH 3T3 cells which could account for these polypeptides, however a more informative assay such as RNase protection experiment would need to be performed (P.T.Tassios, unpublished observations). It is possible that p55L is a form of DP-1 that specifically dimerises with E2F-1, an idea which is compatible with the finding that E2F-1 is induced at the G1/S boundary when p55L is induced. Therefore one possible outcome of modifying DP-1 could be to regulate its dimerisation properties. In this model, dimerisation could be influenced by growth regulatory signals which ultimately converge on and modify DP-1. Similar parallels may be drawn with the SRF and Elk-1 transcription factors as these can form a ternary complex on SRE (Shaw *et al.*,1989). When cells are stimulated with growth factors, Elk-1 becomes phosphorylated which in turn stimulates ternary complex formation (Gille *et al.*,1992). To confirm if a specific form of DP-1 binds E2F-1, one could again immunoprecipitate E2F-1 containing complexes to see if the p55L dimerises preferentially.

In conclusion, DP-1 is a common component of all DRTF1/E2F DNA binding

complexes during the cell cycle of NIH 3T3 cells. As cells progress through the cell cycle however, the properties of the DP-1 protein are modulated which may influence its dimerisation properties (summarised in Figure 8.4). This may allow DP-1 to change the pocket protein with which it interacts thus, allowing different growth regulatory signals to modulate transcription through modulating pRb or p107 (summarised in Figure 8.4). For example, pRb may allow regulation in G1 and p107 in S-phase. Alternatively pRb or p107 may regulate distinct genes if heterodimers have different DNA binding specificities by bringing different E2F-like proteins to DP-1. Thus, different levels of regulation may be imposed on DRTF1/E2F during cell cycle progression.

Figure 8.1a, *Cell cycle regulation of DRTF1/E2F.* Serum stimulated NIH 3T3 cells were harvested every four hours and microextracts prepared for gel retardation. DNA binding activity was assessed using the E2F site to which approximately 8μg of extract was added. In parallel cultures the percentage of cells which stained with the BrDu antibody was assessed in approximately 300 cells. Note the **b/c** form of DRTF1/E2F is induced as the percentage of BrDu increases (lanes 4 and 5).

Figure 8.1b, *DP-1 is a frequent component of DRTF1/E2F complexes in NIH 3T3 cells.* Gel retardations were performed with synchronised cell extracts (as above) in the presence of 3μl of αA DP-1 antisera (lanes 2-17). Where indicated 2nmol of peptide A (lanes 2-10) or peptide 1 (lanes 11-17) was also added. Note that all DRTF1/E2F complexes at each time point are abolished by the DP-1 antisera.

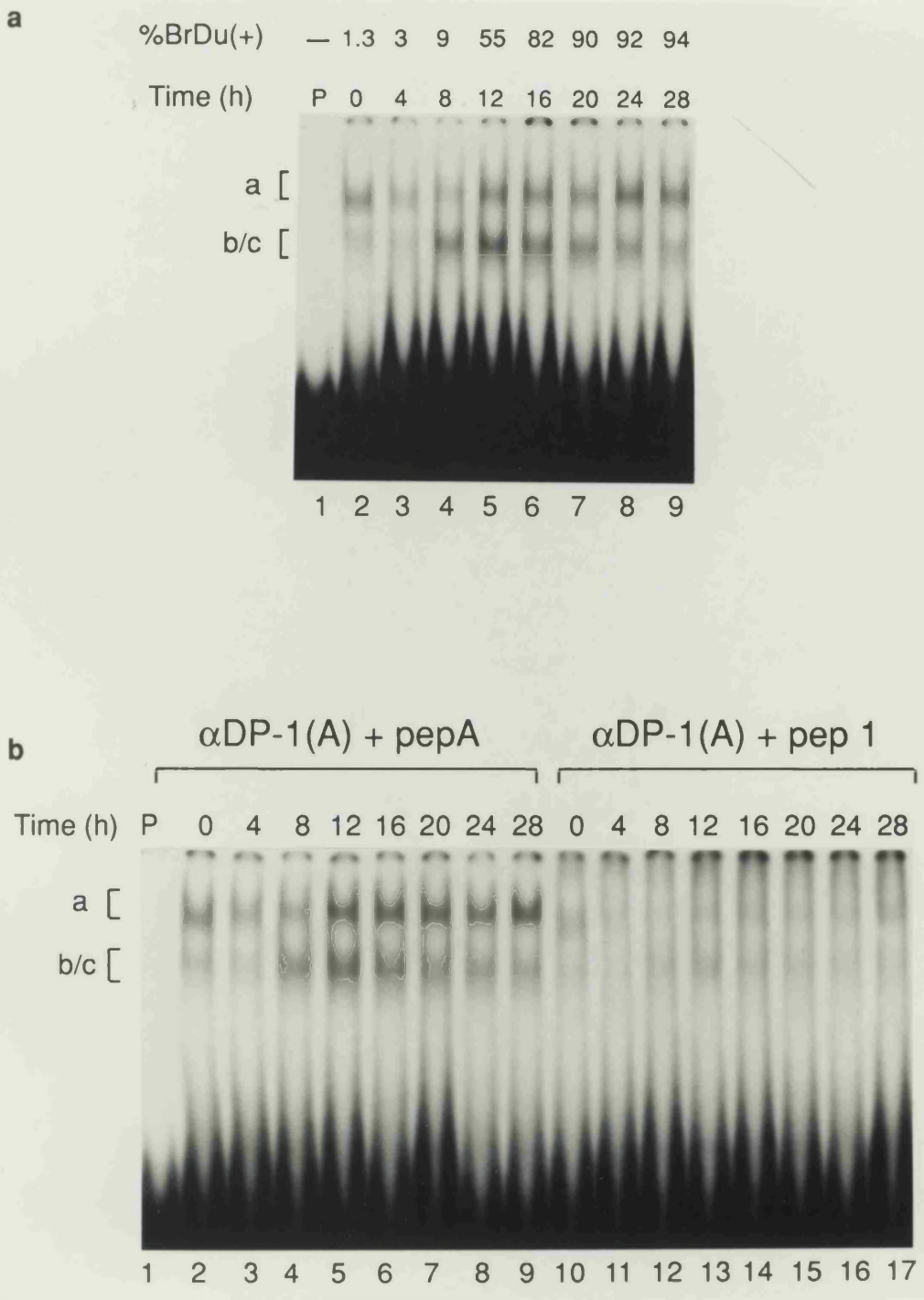
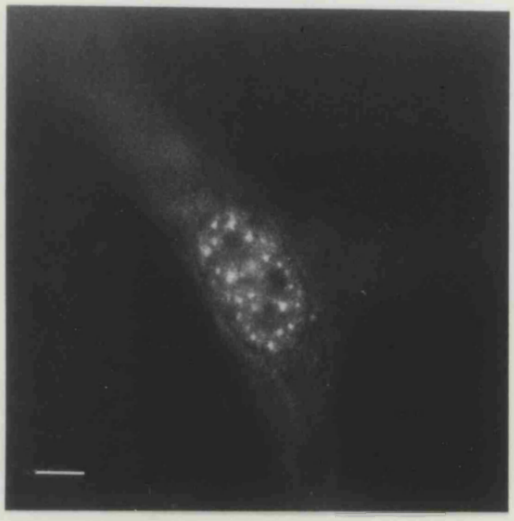

a
%BrDu(+) — 1.3 3 9 55 82 90 92 94
Time (h) P 0 4 8 12 16 20 24 28
a [
b/c [
1 2 3 4 5 6 7 8 9
b
αDP-1(A) + pepA
αDP-1(A) + pep 1
Time (h) P 0 4 8 12 16 20 24 28 0 4 8 12 16 20 24 28
a [
b/c [
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

Figure 8.2, *Nuclear distribution of DP-1 during the cell cycle.* Immnunofluoresence using affinity purified α18 DP-1 antiserum containing 2nmol of peptide 18 (panels b,d,f,h,j,l,m,o) or peptide 1 (panels a,c,e,g,i,k,n,p) per μl of antiserum. In parallel cultures BrDu incorporation was assessed by immunofluoresence using a BrDu monoclonal antibody. The white bar indicates 1μm and specific DP-1 staining at each 4 hour time point following serum stimulation is shown, note the speckled staining pattern (panel a). The percentage of cells staining with the BrDu antibody at each time point is also indicated.

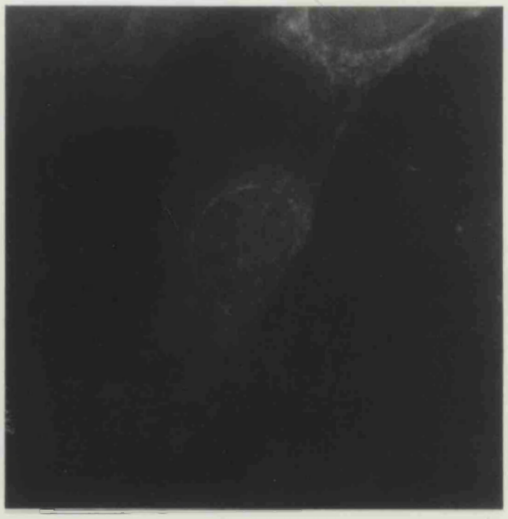
a

αDP-1 + pep1



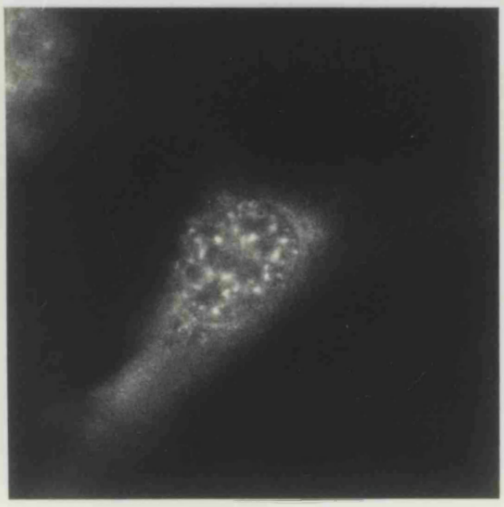
b

αDP-1 + pep18

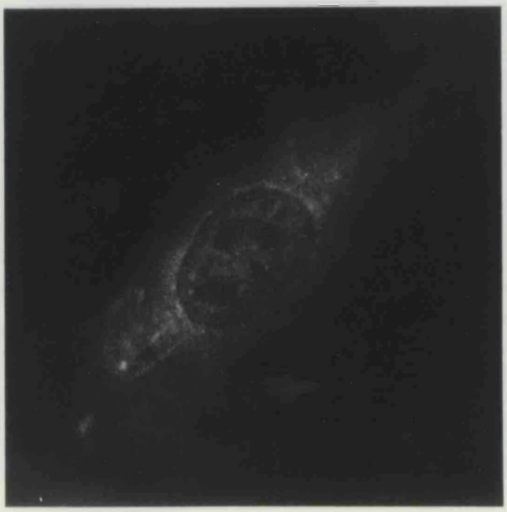


Time: 0h
BrDu (+) 1.3%

c



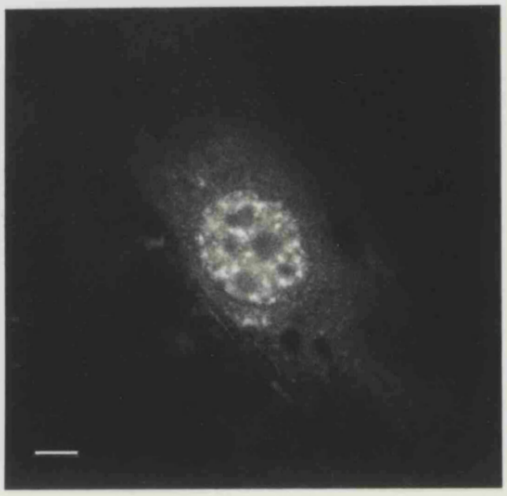
d



Time: 4h
BrDu (+) 3%

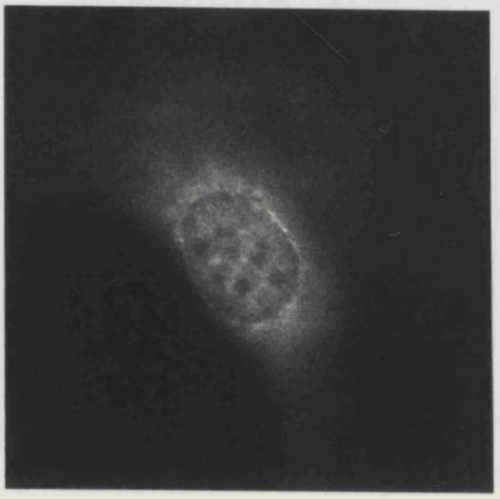
e

αDP-1 + pep1



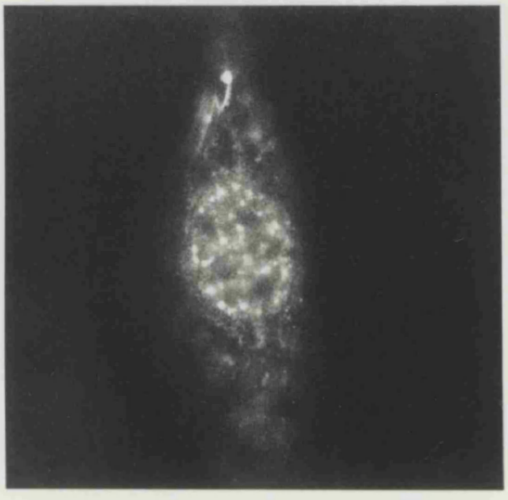
f

αDP-1 + pep18

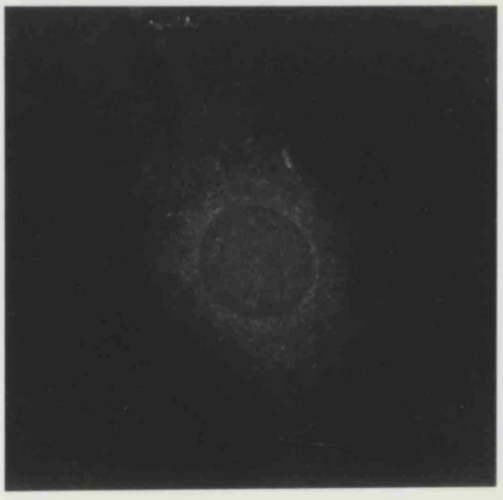


Time: 8h
BrDu (+) 9%

g



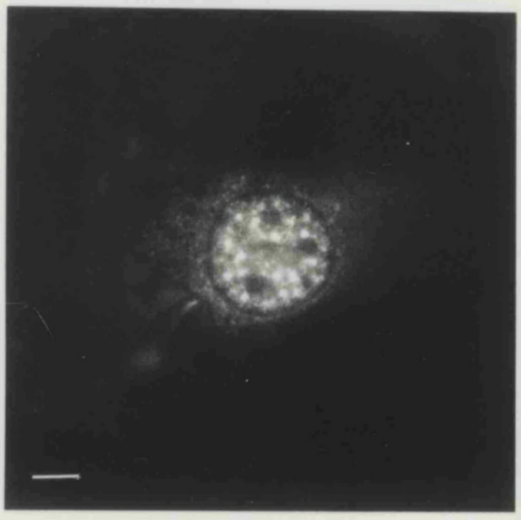
h



Time: 12h
BrDu (+) 55%

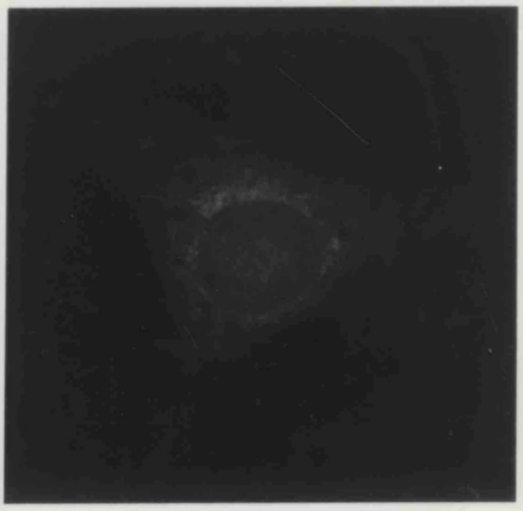
i

αDP-1 + pep1



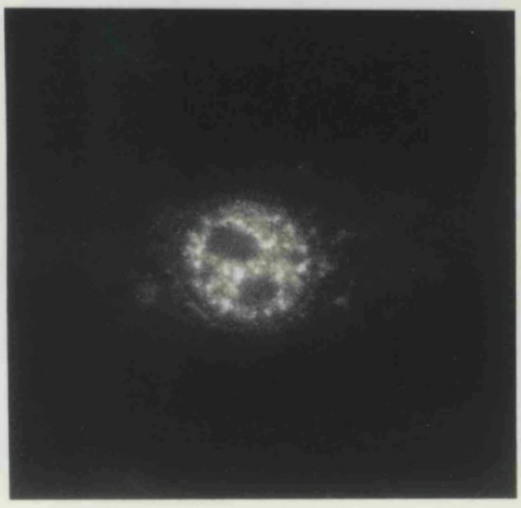
j

αDP-1 + pep18

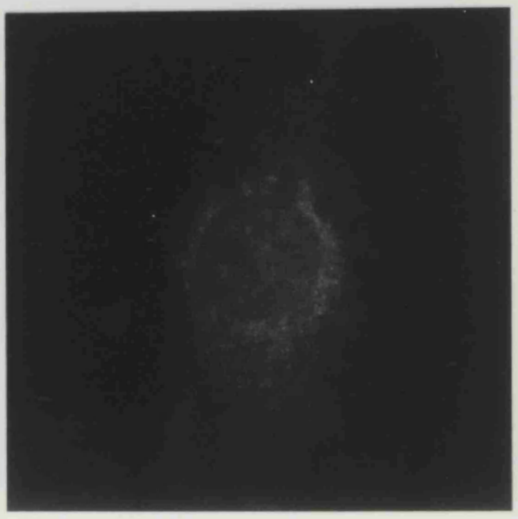


Time: 16h
BrDu (+) 82%

k



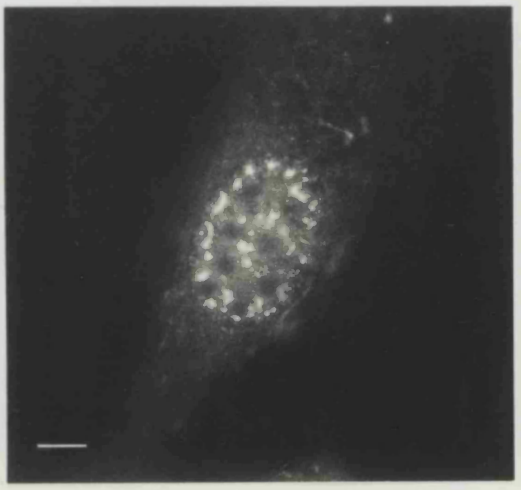
l



Time: 20h
BrDu (+) 90%

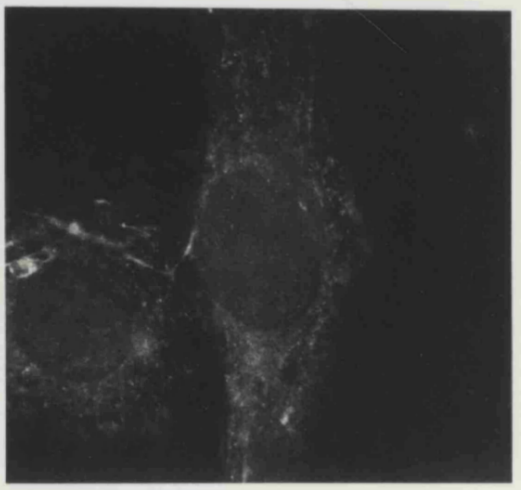
m

αDP-1 + pep1



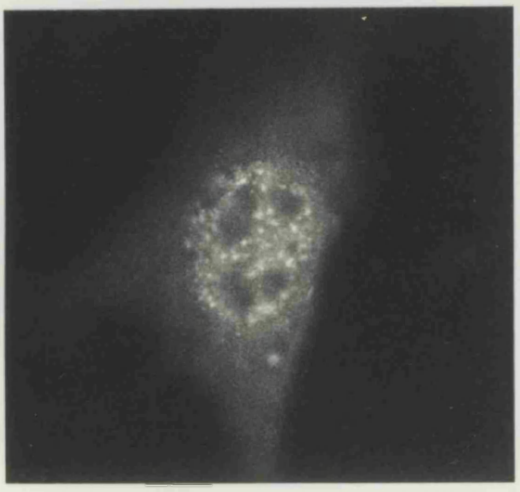
n

αDP-1 + pep18

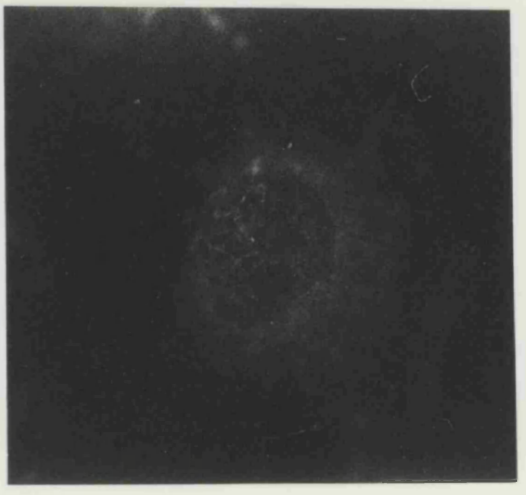


Time: 24h
BrDu (+) 92%

o



p



Time: 28h
BrDu (+) 94%

Figure 8.3a, *DP-1 comprises several distinct polypeptides which are differentially regulated during cell cycle progression.* Approximately 40μg of each microextract was western blotted and probed with affinity purified αA DP-1 antibody. Three polypeptides were revealed of 65,000D (p65), and 55,000D (p55U and p55L). The p65 and p55U polypeptides unchanged at each time point whereas p55L is induced at 4-12h (lanes 4-6).

Figure 8.3b, Approximately 40g of extracts was also blotted and probed with affinity purified α18 DP-1 antiserum which recognises a polypeptide of 68,000D (p68) and 53,000D (p55L). Note again that only the p55L polypeptides is regulated (lanes 4-6).

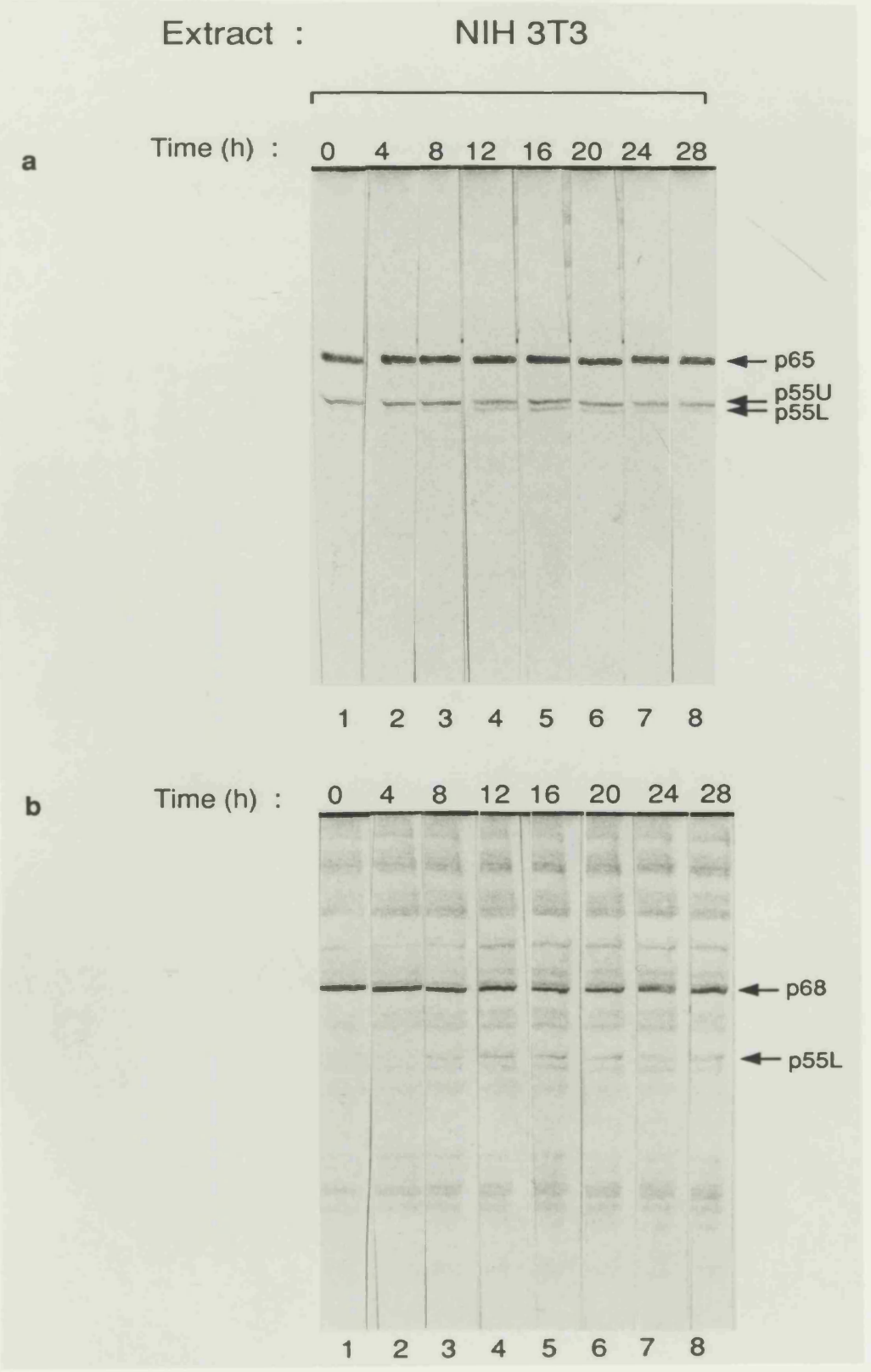
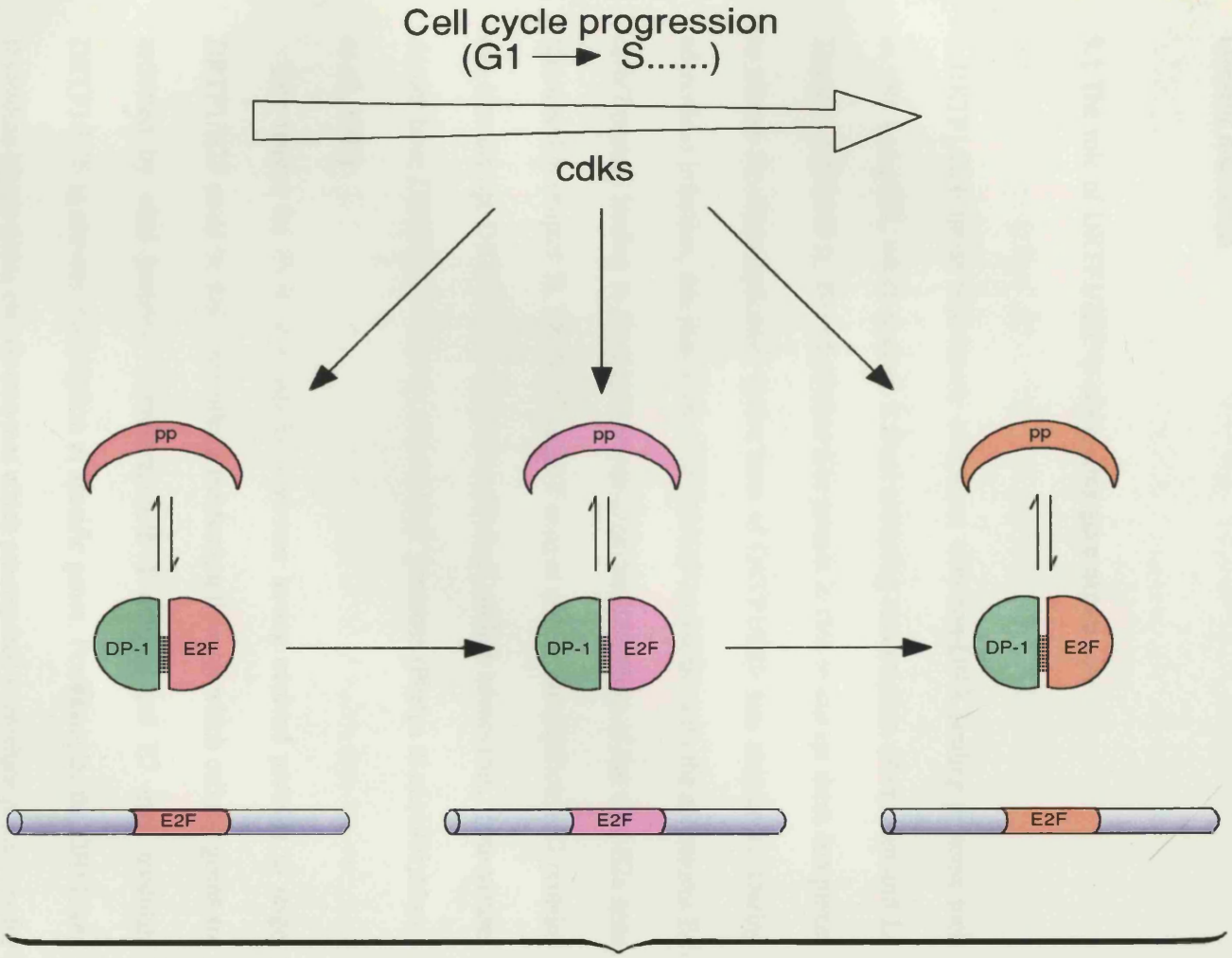

Extract : NIH 3T3
a
Time (h) : 0 4 8 12 16 20 24 28
p65
p55U
p55L
1 2 3 4 5 6 7 8
b
Time (h) : 0 4 8 12 16 20 24 28
p68
p55L
1 2 3 4 5 6 7 8

Figure 8.4, *A model for DRTF1/E2F regulation during cell cycle progression.* DRTF1/E2F binding activity in represented as a heterodimer of DP-1 (shown in green) and different E2F-1-like partners (red,pink and orange). As cells progress through the cell cycle DP-1 is a constant component of DRTF1/E2F but changes its E2F partner. This allows different pocket proteins to bind DRTF1/E2F which may confer different properties to E2F site regulated genes or may allow different growth regulatory signals to be integrated with transcription. This may in turn be mediated by growth regulated kinases such as the cyclin-dependent kinases (cdks) that associate with DRTF1/E2F.


Cell cycle progression
(G1 → S.....)
cdks
pp
pp
pp
DP-1
E2F
DP-1
E2F
DP-1
E2F
E2F
E2F
E2F
E2F site-regulated transcription

CHAPTER 9

General discussion

9.1 The role of DRTF1/E2F in adenovirus gene expression

DRTF1/E2F forms heteromeric complexes with non-DNA binding proteins such as pRb and p107, which prevent it from activating transcription (Zamanian and La Thangue,1992;1993). The adenovirus E1a protein is able to disrupt these complexes to release the transcriptionally active form of DRTF1/E2F (see chapter 3). During adenovirus infection, this then allows DRTF1/E2F to interact with the adenovirus E4 orf6/7 protein leading to the selective transcriptional activation of the viral E2a gene (discussed in chapter 3). The SV40 large T antigen and human papilloma E7 proteins can also disrupt DRTF1/E2F complexes although, unlike adenovirus, these viruses do not have DRTF1/E2F binding sites in their genomes (Phelps *et al.*,1991;Morris *et al.*,1993)

One reason for SV40 and papilloma viruses having evolved proteins to target DRTF1/E2F could be that it provides a mechanism through which cellular genes are activated by viral proteins. Therefore, both T antigen and E7 may modulate DRTF1/E2F to activate transcription of specific genes. For example, the DHFR and thymidine kinase genes encode enzymes which are required to produce precursors for DNA synthesis. These viruses also require many of the same precursors (which are not encoded in their genomes), thus by activating these cellular genes, they ensure

that precursors would be available and non-limiting. These viruses may therefore have evolved a common strategy for activating cellular genes, a process which enables efficient viral replication. The most likely situation where this pathway will be of importance would be during the infection of quiescent cells when the precursors for DNA synthesis are at limiting levels. In contrast other viruses may not modulate the DRTF1/E2F pathway, such as the herpes viruses as they have many of these genes in their genomes.

The E1a,T antigen and E7 proteins are also able to transform and immortalise cells. Again, this could in part be explained by the de-regulation of DRTF1/E2F and the activation of cellular genes required for cell cycle progression. One could imagine that the constitutive expression of these genes would contribute to the continuous proliferation characteristic of tumour cells. To summarise, a number of distinct viral proteins have evolved a common mechanism to modulate DRTF1/E2F in order to regulate cellular genes. Adenovirus has evolved an additional mechanism to utilise DRTF1/E2F through an interaction with the E4 orf6/7 protein. This enables the E2 gene to be specifically activated such that high levels of expression are maintained during a lytic infection.

9.2 The role of pRb in the control of transcription

The product of the pRb gene is believed to be an important regulator of the cell cycle as is it is frequently mutated in human tumour cells and because over expression of pRb in cells causes growth arrest in G1 (discussed in chapter 4). In addition, pRb

is also an essential protein because homozygous transgenic mice lacking the pRb gene are non-viable, dying 12-14 days post coitum (Jacks *et al.*,1992;Lee *et al.*,1992; Clarke *et al.*,1992). As the early stages of murine embryogenesis are not obviously affected by loss pRb in these mice, it is possible that pRb does not regulate cell cycle progression at these stages. Cell cycle control may be fulfilled by an pRb-related gene, such as p107 or alternatively, pRb may be necessary for cells to differentiate (Harlow,1992). Therefore the abnormalities observed in transgenic mice may reflect an inability of cells to differentiate rather than a due to a defect in cell cycle progression.

Since the observations presented in this thesis were described, pRb has become well established as a regulator of transcription and subsequently several other targets have been identified. For example, pRb has been shown to bind to the c-myc protein *in vitro* although the physiological relevance of this is questionable as the these complexes have not been observed in crude extracts (Rustgi *et al.*,1991). A DNA sequence called the retinoblastoma control element (RCE) which was originally identified in the c-fos promoter allows pRb to repress transcription (Robbins *et al.*,1990). A similar sequence has also been identified in the promoters of the c-myc, transforming growth factor β1 and insulin-like growth factor II genes where pRb can both repress and activate transcription through this site depending on the cell type studied (Kim *et al.*,1991;1992a). However, unlike the situation for DRTF1/E2F, pRb has not so far been detected in the complexes which form on the RCE consistent with the idea that pRb regulates these genes indirectly. An RCE was also noted in the promoter of the transforming growth factor β2 gene, which contains a binding site

for the ATF-2 transcription factor (Kim *et al.*,1992b). ATF-2 was shown to bind pRb indirectly in crude extracts an interaction which is believed to activate transcription through the ATF site (Kim *et al.*,1992b).

A direct interaction between the muscle lineage specific transcription factor MyoD and pRb has been shown, and it has been proposed that pRb may activate muscle specific genes through interacting with MyoD (Gu *et al.*,1993). Therefore, one might predict that pRb is necessary for muscle development although mice which lack the pRb gene show no obvious defects in muscle structures (Jacks *et al.*,1992;Lee *et al.*,1992;Clarke *et al.*,1992).

Alternatively pRb may function in a more general manner by influencing basal transcription factors. Regions of amino acid sequence similarity have been observed between pRb and the basal RNAP II factor TFIIB and TBP suggesting that pRb may possess some of the properties of these proteins (Hagemeier *et al.*,1993). Furthermore, it was proposed that pRb may act as an inhibitor of transcription by structurally mimicking basal factors and thereby blocking binding to activating transcription factors. Therefore pRb may be considered as an "anti-adaptor" by binding to activation domains and inhibiting contact to basal factors. Again, these studies were performed *in vitro* and the relevance *in vivo* remains to be established. In conclusion, pRb may interact with a variety of transcription factors to regulate transcription which may mediate its growth suppressing effects.

9.3 pRb and p53: parallel pathways for regulating cellular proliferation?

A number of observations suggest that pRb and p53 have similar properties and may regulate proliferation in an analogous fashion. For example, in normal cells both pRb and p53 behave as tumour suppressors, being able to negatively regulate cell cycle progression through G1 (Goodrich *et al.*,1991;Goodrich and Lee,1992). Furthermore, both pRb and p53 are sequestered by viral oncoproteins; pRb is bound by E1a, T antigen and E7 (Whyte *et al.*,1988;DeCaprio *et al.*,1989;Dyson *et al.*,1989), and p53 by E1b, T antigen and E6 (Lane and Crawford,1979;Werness *et al.*,1982;Sarnow *et al.*,1990). Therefore, these viruses encode proteins which to target two different proteins both of which regulate progression through the G1 phase of the cell cycle.

Interestingly, recent studies suggest that like pRb, p53 also functions as a regulator of transcription. In contrast to pRb which regulates transcription by forming a protein-protein complex with DRTF1/E2F, p53 possess properties of a sequence-specific transcription factor (Fields and Jang,1990;Raycroft *et al.*,1990;Farmer *et al.*,1992). Nevertheless, transcription by p53 can also be regulated through protein-protein interactions as the product of the mdm2 gene can bind p53 and inhibit its transcriptional activity (Momand *et al.*,1992). Therefore, some striking similarities between the mechanisms which regulate DRTF1/E2F and p53 exist: in both cases transcriptional activation is modulated through protein-protein interactions. Furthermore, both pRb and p53 are phosphorylated *in vitro* and *in vivo* by cdks (Lin *et al.*,1991a;Hu *et al.*,1992;Stürzbecher *et al.*,1990;Bischoff *et al.*,1990,), and in

addition both are phosphorylated in a cell cycle dependent manner suggesting that phosphorylation regulates their biological activity (Buchkovich *et al.*,1989;DeCaprio *et al.*,1989;Bischoff *et al.*,1990). Phosphorylation of pRb is thought to disrupt its association with DRTF1/E2F whereas phosphorylation of p53 may control entry into the nucleus (Addison *et al.*,1990). Thus, these cell cycle regulatory kinases may influence the cell cycle by modifying the properties of these transcription factors.

As the viral oncoproteins mentioned above have evolved proteins to target both pRb and p53 it is possible that pRb and p53 function in parallel growth regulatory pathways. It would be advantageous for viruses to inhibit both pathways thereby promoting cellular proliferation. Similarly in tumours cell inactivation of p53 or pRb may contribute to cell transformation.

9.4 DRTF1/E2F, a positive regulator of the cell cycle?

DRTF1/E2F binds two pocket proteins, p107 and pRb. Why have two proteins been conserved to regulate this factor? Although p107 and pRb repress transcription through the E2F site, it is possible that each represses different cellular genes. Alternatively, pRb and p107 may function in distinct regulatory pathways thereby allowing different growth regulatory signals to be relayed to the transcriptional machinery via DRTF1/E2F (chapter 8, summarised in Figure 8.4). Consistent with this idea is the finding that growth suppression by pRb appears to be different from that mediated by p107. Growth arrest by pRb is overcome by expressing cyclin A or cyclin E whereas p107 growth arrest is not (Hinds *et al.*,1992;Zhu *et al.*,1993). It is

possible for example that pRb facilitates progression through G1 and p107 is necessary for the completion of a different phase.

The E1a-associated p300 polypeptides is likely to be structurally related to pRb and p107 as antibodies made against pRb cross react with it (Hu *et al.*,1991). Therefore p300 may be another pocket protein which also regulates DRTF1/E2F. However, other levels of control are possible for p300 since it has been shown to bind TBP and may therefore function as a sequence specific transcription factor (Abraham *et al.*,1993).

Since both pRb and p107 negatively regulate cell cycle progression, it is interesting to speculate that DRTF1/E2F may have a dominant role in regulating cellular proliferation. Some transcription factor genes are mutated in tumour cells and function as oncogenes since they can transform cells (reviewed in Hunter,1990). It is possible that a similar dominant effect on growth may occur if the components of DRTF1/E2F were to be mutated. For instance, mutations in E2F-1 which abolish binding to pRb but leave the activation domain intact, may have the same outcome as mutant pRb proteins which fail to bind DRTF1/E2F. Thus, DRTF1/E2F may be a proto-oncogene with oncogenic counterparts in human tumour cells. Consistent with this notion, over expression of E2F-1 in quiescent cells can promote proliferation (Johnson *et al.*,1993).

9.5 DRTF1/E2F, a family of related transcription factors

DRTF1 and E2F were originally defined as DNA binding activities which form on

the adenovirus E2a promoter and at the time their relationship was unclear (La Thangue and Rigby,1987;Kovesdi *et al.*,1986a). However, during the course of this study it was realised they were likely to be closely related as both activities have been shown to interact with the same molecules. One of the important conclusions from this study was the finding that DRTF1/E2F is a heterodimer of DP-1 and E2F-1. Recent studies have indicated that both DP-1 and E2F-1 are members of families of proteins sharing a conserved DNA binding and dimerisation domain. Several related cDNAs have been isolated recently by searching for homology with E2F-1 and have been termed E2F-2 and E2F-3 (Lees *et al.*,1993;Ivey-Hoyle *et al.*,1993). Both E2F-2 and E2F-3 bind pRb but not p107 although it is not clear if they are also components of DRTF1/E2F DNA binding complexes. It seems likely that a p107-binding E2F-like molecule will also exists.

E2F-1 shares greater amino acid sequence similarity with E2F-2 and E2F-3 than with DP-1, suggesting that DP-1 is more distantly related (Girling *et al.*,1993;Lees *et al.*,1993;Ivey-Hoyle *et al.*,1993). It is possible that E2F-like and DP-like proteins will comprise two families of related gene products. In support of this idea, our laboratory has recently isolated proteins closely related to DP-1 (R.Girling, pers. comm.). If, like DP-1 and E2F-1, these related proteins are able to cross dimerise then it is possible that in different biological situations many possible combinations of DP-1- and E2F-1-like complexes may exist.

It seems probable that the activities referred to as DRTF1/E2F are the result of different heterodimers of DP-1-like and E2F-1-like proteins which recognise the E2F binding site. Therefore, distinct populations of heterodimers may bind to the E2F site

in EC and HeLa cells. Alternatively, heterodimerisation may be regulated in adenovirus infected cells. These ideas can be tested with the appropriate reagents to against the DP-1 and E2F-1 family of proteins.

9.6 DRTF1/E2F, an evolutionary conserved regulator of the cell cycle?

In this thesis I have suggested that DRTF1/E2F is an important co-ordinator of cell cycle progression. If this were the case then one might also expect DRTF1/E2F and the molecules associated with it to be conserved in other organisms. The cyclins and cyclin-dependent kinases have been identified in yeast, plants, amphibians and mammals, consistent with the idea that these are essential components of the cell cycle machinery (Nurse and Thuriaux,1980;Lee and Nurse,1987;Dunphy *et al.*,1988;Hata *et al.*,1991;Hemerly *et al.*,1992;Hirayama *et al.*,1992). In our laboratory the *Xenopus* and *Drosophila* homologues of DP-1 have been isolated indicating that this pathway has been conserved during evolution (R.Girling and X.Feng Hao unpublished observations). At present it is not clear, although likely, that these DP-1 homologues will possess the same properties as the murine gene product.

In the yeast *S.cerevisiae,* a number of genes necessary for DNA synthesis are co-ordinately expressed during cell cycle progression (reviewed in Johnson and Lowndes,1992). A DNA sequence important for the transcriptional regulation of these genes has been identified, ACGCGT, which is also the recognition site for the restriction enzyme *MluI* and hence has been named the MCB (*MluI* cell cycle box. Interestingly, this site resembles the E2F binding site, TTTCGCGC ,suggesting that

a transcription factor related to DRTF1/E2F may be necessary for cell cycle progression in yeast. A cell cycle regulated DNA binding activity that recognises the MCB has been identified termed DSC1 (DNA synthesis control), and comprises several components (Lowndes *et al.*,1991). The DNA binding subunit MBP1 (*Mlul* binding protein) was recently isolated and deletion of this gene in yeast causes de-regulation of DNA synthesis (Koch *et al.*,1993).

The second transcription factor, Swi6, was also shown to be a component of DSC1 (Lowndes *et al.*,1992a), and it is thought to function in concert with Swi4 in regulating the yeast cell cycle. Interestingly, Swi6 may be a target for the $p34^{cdc2}$ kinase (Johnson and Lowndes,1992). Thus, DSC1 may integrate transcription with the cell cycle machinery in an analogous fashion to DRTF1/E2F. In *S.pombe* a similar multicomponent DNA binding activity was found to comprise the product of the cdc10 gene (Lowndes *et al.*,1992b). The cdc10 protein has a small region of similarity with DP-1 and E2F-1 suggesting they may be distantly related (La Thangue and Taylor,1993). This similarity is present in the dimerisation domain of DP-1 and therefore may reflect a conserved interaction domain. Thus, in yeast, proteins related to DP-1 and E2F-1 may control cell cycle regulated transcription. Pocket proteins however have not been identified in yeast and thus this pathway may be analogous but distinct from the situation in mammalian cells.

9.7 Future prospects

A greater understanding of the functions of DRTF1/E2F will not only be achieved

by characterising DP-1 and E2F-1 but also from studying the properties of pRb and p107. Several pRb-binding E2F-1-like cDNAs have been identified and in our laboratory a DP-1-like cDNA has also been isolated. The biochemical properties of these proteins remain to be established. It would be particularly interesting to determine if they preferentially heterodimerise with other members of the family. If this were the case then it would be necessary to determine if these heterodimers are specific to different biological situations.

In chapter 7 I defined a region in DP-1 necessary for interacting with E2F-1. A more detailed analysis of this region could be performed to precisely identify the dimerisation domain and amino acid residues necessary for the interaction. I also created a mutant DP-1 protein which was capable of dimerising with E2F-1 but was unable to bind DNA ($DP\text{-}1^{146\text{-}249}$; chapter 7). It would be interesting to study this mutant protein further as it may function in a dominant-negative manner by dimerising with E2F-1 and inhibiting DNA binding activity; therefore it is possible that $DP\text{-}1^{146\text{-}249}$ will be able inhibit E2F site-dependent transcription. If this were the case then it may inhibit cell cycle progression.

DP-1 was shown to comprise several distinct polypeptides which were differentially regulated during the cell cycle (chapter 8). Determining the relationship between the polypeptides and establishing how they may be produced would also be very informative. Both DP-1 and E2F-1 are likely to be substrates for growth regulatory kinases and one explanation for the variety of polypeptides recognised by DP-1 antisera could be due to differential phosphorylation. It is possible to test this idea radiolabelling cells with ^{32}P-orthophosphate. One might predict therefore that DP-1

is phosphorylated in a cell cycle-dependent manner.

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Human papillomavirus type 16 E7 regulates E2F and contributes to mitogenic signalling

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We have produced human papillomavirus type 16 E7 protein in a bacterial expression system and examined the mitogenic activity of this protein in Swiss 3T3 cells after scrape loading. The ability of E7 to induce cellular DNA synthesis in quiescent mouse fibroblasts is strongly enhanced by the presence of a single growth factor such as insulin. Although only weakly mitogenic, introduction of E7 alone resulted in the rapid induction of the transcriptionally active form of E2F, which was not enhanced further by the addition of insulin. Mutant E7 proteins defective for RB binding failed to induce the active form of E2F or act synergistically with insulin to stimulate DNA synthesis. The ability of E7 to regulate E2F may therefore be necessary, but is not sufficient, for full induction of DNA synthesis.

Introduction

Specific types of human papillomavirus (HPV) are frequently found in association with anogenital carcinomas, and infection with these HPV types confers a high-risk for the development of malignant disease (zur Hausen, 1991). The most common of these high-risk HPV types, HPV-16 and HPV-18, have also been shown to encode transforming and immortalizing activities in cultured cells (Dürst *et al.*, 1987; Pirisi *et al.*, 1987; Bedell *et al.*, 1989). The major viral oncoproteins are encoded by the E6 and E7 genes and, whilst cooperation between E6 and E7 is necessary for the immortalization of primary human genital epithelial cells (Hawley-Nelson *et al.*, 1989; Münger *et al.*, 1989a; Hudson *et al.*, 1990), E7 alone displays the major transforming activity in rodent cells (Kanda *et al.*, 1988; Phelps *et al.*, 1988; Storey *et al.*, 1988; Vousden *et al.*, 1988).

Mutational analyses of E7 have demonstrated that a number of functions are necessary for efficient transforming or immortalizing activity in rodent cells. Like SV40 large T antigen and adenovirus E1a, E7 can bind to the cellular retinoblastoma (RB) protein, the product of a tumour-suppressor gene, and the regions of sequence similarity between these three viral proteins play an important role in their ability to bind RB (DeCaprio *et al.*, 1988; Münger *et al.*, 1989b; Whyte *et al.*, 1989). Point mutations within this region of E7 abolish, or severely reduce, E7–RB binding in *in vitro* assays and destroy transforming activity (Barbosa *et al.*, 1990). Interference with normal RB function is therefore likely to be an important activity of E7 that may contribute to the development of anogenital neoplasia.

Recent studies have shown that RB can bind to, and inactivate, cell transcription factors such as E2F (Bagchi *et al.*, 1991; Bandara & La Thangue, 1991; Chellappan *et al.*, 1991). E2F is involved in the regulation of transcription of several cell genes necessary for cell cycle progression, such as dihydrofolate reductase (DHFR), *myc*, *myb*, *cdc2* and DNA polymerase α (Mudryj *et al.*, 1990; Pearson *et al.*, 1991; Dalton, 1992). The negative control of cell growth mediated by RB is likely to reflect, at least in part, transcriptional repression of these genes (Dalton, 1992; Hamel *et al.*, 1992; Weintraub *et al.*, 1992). Interestingly, mutations within E7 that abolish RB binding and transforming activity also destroy the ability of E7 to *trans*-activate the E2F-responsive adenovirus E2A promoter (Edmonds & Vousden, 1989; Phelps *et al.*, 1991). This is consistent with a role for E7 in dissociation of inactive RB–E2F to release transcriptionally active E2F, a hypothesis further supported by the observation that HPV-expressing cervical carcinoma cell lines or HPV-16-immortalized human keratinocytes contain a reduced level of RB-associated E2F (Chellappan *et al.*, 1992; Pagano *et al.*, 1992). Although E7 has been shown to dissociate E2F–RB-containing complexes in cell-free systems (Chellappan *et al.*, 1992), alterations in E2F-containing complexes in direct response to E7 expression have not been demonstrated in cells.

Despite the importance of the RB-binding activity, this property alone is not sufficient for the full transforming activity of E7. For example, mutations within E7 that result in the replacement of two serine residues normally phosphorylated by casein kinase II can give rise to a protein that retains the ability to bind RB but has a severely reduced transforming and immortalizing activity (Barbosa *et al.*, 1990; Firzlaff *et al.*, 1991). Furthermore, mutations in the extreme N-terminus of E7 also result in loss of transforming and immortalizing functions in rodent cells without affecting RB binding, although the defect in these mutants is not known (Banks *et al.*, 1990; Watanabe *et al.*, 1990; Phelps *et al.*, 1992).

In this study we have used scrape loading (McNeil *et al.*, 1984) to examine the immediate effects of introducing E7 into quiescent Swiss 3T3 cells. We show that, whilst E7 alone is sufficient to induce the transcriptionally active form of E2F, a co-mitogen such as insulin is required for efficient induction of DNA

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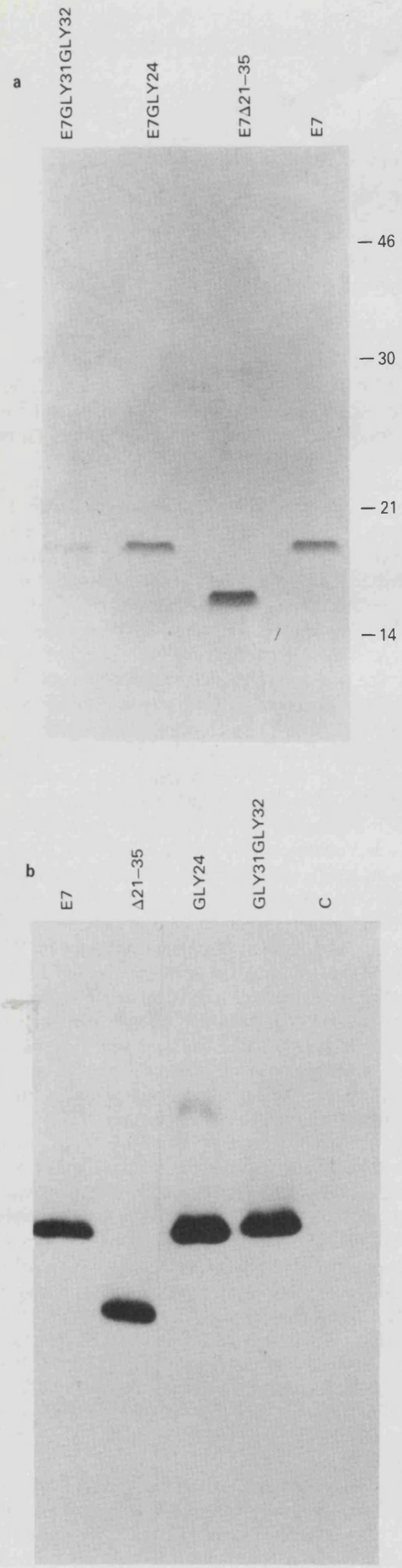

synthesis. Both alterations of E2F complexes and induction of DNA synthesis depend on a functional RB-binding domain of E7. Our results therefore suggest that the ability of E7 to induce active E2F is necessary but not sufficient for mitogenic activity.

Results

Expression of HPV-16 E7 proteins in bacteria

Wild-type HPV-16 E7 protein was expressed as a glutathione *S*-transferase (GST) fusion in pGEX2T. In order to generate unfused E7 protein, an oligonucleotide adaptor encoding a factor X recognition and reconstituting cleavage site and reconsituting the start of E7 was inserted between the BamHI site of pGEX2T and the NsiI site of E7 (see Materials and methods). The introduction of the N-terminal methionine of E7 at the end of the factor X recognition and cleavage site allows the protease to release full-length intact E7. Fusion protein expression was induced as previously described (Smith & Johnson, 1988) and the fusion protein purified over a glutathione–agarose column. The protein was then cleaved with factor X and further purified over a G75 Sephadex column. Fractions containing E7 were identified by SDS–PAGE and silver staining, pooled and concentrated by ultrafiltration. This approach was also used to make additional mutant E7 proteins including a cysteine-to-glycine substitution at amino acid 24 (E7GLY24), serine-to-glycine substitutions in the casein kinase II recognition site at residues 31 and 32 (E7GLY31GLY32) and a deletion of amino acids 21– 35 (E7Δ21–35). Both E7GLY24 and E7Δ21–35 were defective for RB binding, whilst E7GLY31GLY32 retained wild-type RB-binding activity in *in vitro* assays (unpublished observation). SDS–PAGE and Coomassie staining showed that E7, E7GLY24 and E7GLY31 GLY32 migrated with an apparent M_r of 18 000, whilst E7Δ21–35 had an M_r of 16 500 (Figure 1a). In order to confirm that each of the recombinant E7 proteins could be recognized by anti-E7 antibodies, Swiss 3T3 cells were scrape loaded in the presence of 0.5 mg ml^{-1} protein (Morris *et al.*, 1989). Cell extracts were made 3 h after scraping, and wild-type and mutant E7 proteins were shown by immunoblotting to be recognized by a polyclonal and a monoclonal anti-E7 antibody (Figure 1b).

Mitogenic activity of E7

To assess the mitogenic activity of the recombinant E7 protein we scrape loaded the E7 and mutant proteins into quiescent Swiss 3T3 cells using the technique previously described for introduction of recombinant *ras*

Figure 1 (**a**) E7 and mutant proteins expressed in bacteria. A 0.5-μg aliquot of each protein was analysed by 15% SDS–PAGE and Coomassie staining. E7, E7GLY24 and E7GLY31GLY32 migrated with an apparent M_r of 18 000 and E7Δ21–35 had an M_r of 16 500. (**b**) Monoclonal and polyclonal antibodies recognize recombinant E7 and mutant proteins. Scrape-loaded E7, E7Δ21–35, E7GLY24 or E7GLY31GLY32 was immunoprecipitated with a monoclonal anti-E7 antibody and immunoblotted with a polyclonal anti-E7 antibody E7L1

protein into these cells (Lloyd *et al.*, 1989; Morris *et al.*, 1989). As shown in Figure 2, cells scrape loaded with E7 protein alone (0.25–1.5 mg ml^{-1}) show only a small four- to eightfold induction of DNA synthesis (as measured by the increase in [^{3}H]thymidine incorporation into DNA) above background. The DNA response to E7 alone was similar to the stimulation seen following addition of a single growth factor such as insulin (fourfold). However, previous studies have shown that Swiss 3T3 cells require insulin and an additional co-mitogen such as platelet-derived growth factor (PDGF) in order to undergo mitogenesis and initiate DNA synthesis (Pledger *et al.*, 1977; Metcalfe *et al.*, 1985; Rozengurt & Mendoza, 1985). Consistent with this we were able to demonstrate that stimulation of DNA synthesis by E7 was greatly potentiated by the addition of 1 μg ml^{-1} insulin to the medium. The synergy between E7 and insulin consistently resulted in a 100- to 200-fold increase in DNA synthesis (Figure 2), giving a DNA response similar to that seen when cells are treated with PDGF and insulin (data not shown). Cells scraped and allowed to reseal in medium for 10 min prior to the addition of E7 and insulin failed to enter DNA synthesis (data not shown). Analysis of bromodeoxyuridine (BrdU) incorporation into cell nuclei after 25 h showed that E7 plus insulin or PDGF plus insulin stimulated approximately half of the cell population to enter DNA synthesis (43–52%), while fetal calf serum containing a full complement of growth factors stimulated DNA synthesis in almost all the cells (97%). Previous studies have shown that approximately 90% of scraped cells take up protein (Morris *et al.*, 1989), suggesting that not all the cells receiving E7 and insulin undergo DNA synthesis. The mutant E7GLY31GLY32, which lacks casein kinase II phosphorylation sites but retains the ability to bind RB, was also found to be a potent co-mitogen with insulin for the stimulation of DNA synthesis (Figure 2). In contrast, scrape loading of either of the E7 mutants with defects in the RB-binding domain (E7 GLY24, E7Δ21–35) resulted in only a small increase in the DNA response (Figure 2).

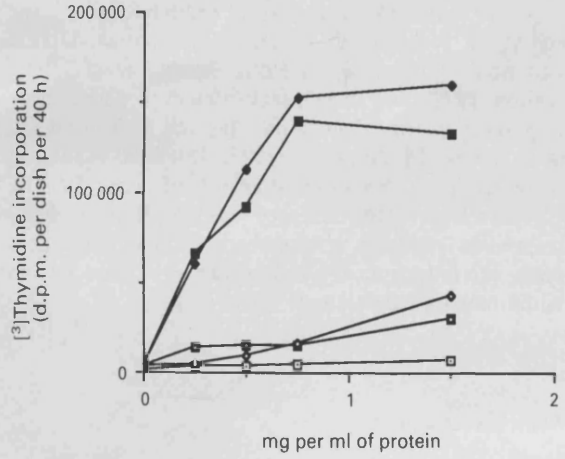

Figure 2 Effect of E7 and mutant proteins on Swiss 3T3 cell DNA synthesis. Quiescent cultures were scraped in the indicated concentrations of E7 or mutant proteins and reseeded in serum-free DMEM with or without 1 μg ml^{-1} insulin. Incorporation of [^{3}H]thymidine into DNA was measured after 40 h. ⊡, E7 alone; ◆, E7 + insulin; □, E7Δ21–35 + insulin; ◇, E7GLY24 + insulin; ■, E7GLY31GLY32 + insulin

Quiescent Swiss 3T3 cells scrape loaded with E7 and treated with insulin underwent the characteristic G_1 lag period prior to the detection of an increase in DNA synthesis at 16 h (Figure 3). This suggests that E7 is able to substitute for the early mitogenic signals provided by an alternative growth factor such as PDGF, but there was no evidence that expression of E7 could induce a more rapid progression into S phase.

E7-induced alterations of E2F complexes

In order to identify an early activity dependent only on E7, we examined the regulation of E2F-containing complexes in quiescent Swiss 3T3 cells following introduction of E7 protein and continued incubation in serum-free medium. Although no effect of E7 on E2F-containing complexes was seen within 45 min of scraping, after 3 h cells receiving E7 showing a marked increase in the b/c form of the E2F complex compared with cells scraped in the absence of E7 protein, and this form of E2F persisted 6 h after scraping (Figure 4). Since the b form of the E2F complex has been shown to be transcriptionally active in other cell types (Mudryj *et al.*, 1991; Shivji & La Thangue, 1991), in contrast to the slower migrating a form, which is probably associated with RB or a related protein (Bandara & La Thangue, 1991), these data are consistent with the ability of E7 to act in quiescent cells to induce transcriptionally active E2F and hence E2F-responsive cell genes. The alteration of E2F complexes does not result from extracellular E7 since extracts from cells scraped in the absence of E7 and left to reseal prior to the addition of E7 showed no increase in the b/c form of E2F compared with control cells scraped in buffer alone (data not shown). The lack of effect of E7 on E2F after 45 min (Figure 4) further indicates that the dissociation observed does not result from an *in vitro* activity of E7 after cell lysis. Addition of insulin to the

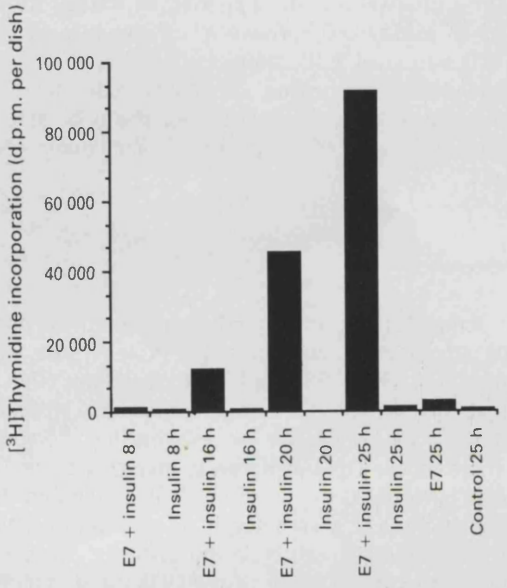

Figure 3 Time course for E7- and insulin-stimulated DNA synthesis. Quiescent cells were scraped with or without 0.5 mg ml^{-1} E7 and samples were treated with 1 μg ml^{-1} insulin where indicated. Incorporation of [^{3}H]thymidine into DNA was measured at the times shown

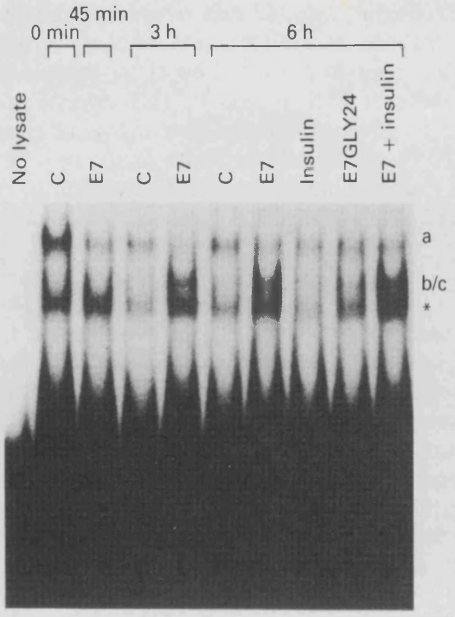

Figure 4 E7 regulates E2F in cells. Swiss 3T3 cells on 90-mm dishes were scraped in 0.5 mg ml^{-1} E7, 0.5 mg ml^{-1} E7GLY24 or buffer alone and treated with 1 μg ml^{-1} insulin where indicated. Scraped cells were transferred to gelatin and fibronectin-coated dishes in serum-free medium. At the times shown reactions were terminated and E2F binding measured by band shift to reveal two specific complexes (a and b/c) and the previously described non-specific band common to all reactions (*) (Partridge & La Thangue, 1991)

scraped cells had no effect on the E2F complexes, and insulin alone was unable to potentiate the effect of E7 (Figure 4). The E7 mutant with reduced RB-binding activity (E7GLY24) showed only a slight increase in the b/c form of the E2F complex (Figure 4), suggesting that the appearance of this complex following the introduction of E7 involves the sequestration of RB or an RB-related protein such as p107 by E7.

These observations are supported by *in vitro* studies in which wild-type E7 protein was shown to dissociate an RB-associated E2F complex to a transcriptionally active form in embryonic carcinoma cells (data not shown). In this system, as in the scraped cells, mutant E7GLY24 had no effect on the E2F-containing complexes.

Discussion

The scrape-loading technique has enabled us to introduce recombinant oncogenic E7 protein into large numbers of Swiss 3T3 cells and therefore offers a unique opportunity to look at immediate signalling events affected by E7. We have shown that the ability of E7 to induce DNA synthesis in quiescent Swiss 3T3 cells is potentiated by the addition of insulin and that E7 functions like a co-mitogen in allowing quiescent cells to re-enter the cell cycle. Interestingly, E7 is also able to act synergistically with PDGF to stimulate a similar DNA response to that seen with insulin and E7 (data not shown). Scrape-loaded E7 alone gave only a weak induction of DNA synthesis under conditions of total serum deprivation, in agreement with previous studies examining the ability of inducible E7 to stimulate DNA synthesis (Sato *et al.*, 1989; Banks *et al.*, 1990). We have confirmed in this study, as in previous reports (Banks *et al.*, 1990), that mutant E7 proteins defective for RB binding also fail to induce a strong mitogenic signal.

Our study indicates that expression of E7 alone is not sufficient for progression from G_0 into S phase. We have been able to show, however, that E7 can independently induce the appearance of the transcriptionally active form of E2F. Mutant E7 protein that is defective for RB binding is also unable to induce appearance of the active E2F complex and fails to stimulate DNA synthesis in cooperation with insulin. This suggests that, like E1a, E7 dissociates an E2F complex containing RB, or an RB-related protein such as p107, thus releasing transcriptionally active E2F. Previous studies in cell-free extracts have shown that E7 can dissociate complexes between E2F and cell factors such as RB and that this activity is dependent on the RB-binding domain of E7 (Chellappan *et al.*, 1992). We have directly demonstrated in cells that the introduction of E7 results in the appearance of the transcriptionally active form of E2F and that this activity is not sufficient for the subsequent induction of DNA synthesis. Interestingly, the E7-stimulated shift in E2F to the faster migrating transcriptionally active complex cannot totally be accounted for by dissociation of the slower migrating a form of the complex. This suggests either that E7 can target an E2F complex that is unable to bind DNA and is not detected in these assays or that E7 induces *de novo* E2F synthesis. The recent cloning of a gene encoding an E2F-like protein will allow further evaluation of the potential interactions with E7 (Helin *et al.*, 1992; Kaelin *et al.*, 1992).

The appearance of transcriptionally active forms of E2F in response to E7 correlates well with previous work on the E7-mediated *trans*-activation of the E2F-responsive adenovirus E2A promoter; E7 mutants that cannot bind RB or alter E2F-containing complexes are also unable to *trans*-activate the E2A promoter (Edmonds & Vousden, 1989; Phelps *et al.*, 1991). It therefore seems likely that E7 will also mediate some transcriptional control over E2F-responsive cell genes that play a role in the control of progress through the cell cycle, such as DNA polymerase α, *myb*, DHFR and *cdc2* (Mudryj *et al.*, 1990; Pearson *et al.*, 1991; Dalton, 1992), and those genes thought to play a more important role in re-entry into the cell cycle from G_0, such as *myc* (Mudryj *et al.*, 1990). It will be of interest to determine whether the induction of active E2F by E7 correlates with increased transcription of E2F-responsive genes or whether expression of these proteins, like induction of DNA synthesis, requires factors additional to induction of active E2F.

Materials and methods

Construction of plasmids encoding GST–E7 fusion proteins

In order to allow expression of HPV-16 E7 proteins with an authentic N-terminus, the 5′ end of the E7 insert in pUCMo E7 (Edmonds & Vousden, 1989) was modified as follows. The parent plasmid was digested with NsiI, and the linear DNA ligated to an excess of a phosphorylated adaptor:

GATCCATCGAAGGTCGTATGCA
GTAGCTTCCAGCAT

The resulting DNA was digested with BamHI and ligated into the BamHI site of pGex 2T (Pharmacia). Clones correctly oriented for expression of GST–E7 were identified by miniprep analysis and the sequence of the Gex adaptor–E7 fusion was confirmed by dideoxy sequencing. The resulting recombinant pGex-E7 plasmid lacks the functional thrombin cleavage site present in pGex2T, but has a reconstructed factor X cleavage motif provided by sequences in the adaptor, in frame with the HPV E7 sequences. Cleavage of the GST–E7 fusion protein with factor X thus generates an intact E7 protein.

For construction of pGex plasmids encoding mutant E7 proteins, the insert from pGex E7 was released by digestion with BamHI and subcloned into M13mp19. Single-strand DNA was isolated by standard procedures, and used for oligonucleotide-directed mutagenesis according to the Amersham version II system. Mutant clones were identified by sequencing and recloned into BamHI-cut pGex 2T to generate in-frame GST–E7 fusions with intact factor X cleavage sites as described above, and reconfirmed by sequencing.

Protein purification

Logarithmically growing cultures of X90 *Escherichia coli* transformed with recombinant Gex E7 plasmids were induced for 4 h with 100 $\mu g\ ml^{-1}$ isopropyl-β-*O*-thiogalactopyranoside (IPTG). Cells were then pelleted, resuspended in 20 ml of ice-cold MTPBS (0.62 $g\ l^{-1}$ sodium dihydrogen phosphate, 2.27 $g\ l^{-1}$ disodium hydrogen phosphate, 8.7 $g\ l^{-1}$ sodium chloride pH 7.0, 10 mM DTT, 1 mM PMSF) and sonicated on ice. Debris was removed by centrifugation, and the supernant loaded onto a 4-ml gluthathione–agarose column and washed with 200 ml of Tris-buffered saline (TBS, 100 mM sodium chloride, 10 mM Tris pH 7.0). The GST–E7 fusion protein coupled to beads was resuspended in 30 ml of TBS, transferred to a 50-ml polypropylene tube and supplemented with 30 μg of factor X (Boehringer) and 60 μl of 0.5 M calcium chloride, and rotated overnight at 4°C. The GST coupled to beads was removed by centrifugation and the supernatant concentrated to 0.5 ml by ultrafiltration under nitrogen pressure. The E7 proteins (except E7GLY31GLY32) were further purified on a G75 Sephadex column in 5% glycerol, 10 mM HEPES pH 7.0 (Imai *et al.*, 1991). Fractions containing E7 were identified by polyacrylamide gel electrophoresis and silver staining, concentrated by ultrafiltration to 3 $mg\ ml^{-1}$ and protein stored under liquid nitrogen.

Scrape loading of protein into cells

Swiss 3T3 cells were seeded at 5×10^4 cells per 30-mm dish or 2×10^5 cells per 90-mm dish (Falcon) in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum. Cultures reached confluence after 3 days and were rinsed once in DMEM and incubated for 16 h in DMEM without serum. Quiescent cells were washed twice in 3 ml of wash solution (120 mM potassium chloride, 30 mM sodium chloride, 0.5 mM magnesium chloride, 10 mM HEPES pH 7.0) and covered with 80 μl of protein per 30-mm dish or 160-μl of protein per 90-mm dish. Cells were scrape loaded and taken up in serum-free DMEM for transfer to gelatin and fibronectin coated dishes as described previously (Morris *et al.*, 1989).

Measurement of DNA synthesis

Cells were labelled with 0.5 $\mu Ci\ ml^{-1}$ [6-^{3}H]thymidine and lysed in 1% SDS for determination of labelled thymidine incorporaiton into trichloroacetic acid-insoluble material. For measurement of bromodeoxyurdine incorporation into nuclei, cells were labelled and treated as described in the Amersham cell proliferation kit.

Generation of polyclonal rabbit anti-E7 antibodies

The purified wild-type E7 protein was used to immunize rabbits in order to produce antiserum specific for the E7 protein. The serum E7L1 produced against the bacterially expressed protein recognized E7 expressed by two HPV-16-positive cervical carcinoma cell lines as efficiently as a previously described rabbit polyclonal antibody raised against an E7 fusion protein (Barbosa *et al.*, 1990) (data not shown).

Immunoprecipitation and Western blotting

For detection of scrape-loaded recombinant E7 proteins, cells were lysed in ELB buffer (250 mM sodium chloride, 5 mM EDTA, 1 mM PMSF, 0.5 mM DTT, 50 mM HEPES, pH 7.0) and then immunoprecipitated with a monoclonal anti-E7 antibody (Triton) and protein A beads as previously described (Edmonds & Vousden, 1989). Samples were anlaysed by 15% SDS–PAGE and transferred overnight onto nitrocellulose. The nitrocellulose blots were blocked with 1% gelatin, probed with the polyclonal anti-E7 antibody (E7LI) and processed by electrochemiluminescence fluorography (Amersham).

Measurement of E2F-binding activity

Preparation of cell extracts and gel retardation was carried out as previously described (Bandara & La Thangue, 1991). The E2F binding site probe contained sequences −71 to −50 taken from the adenovirus E2A promoter and specificity of binding was confirmed by competition with excess unlabelled binding site probe.

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Cyclin A recruits p33^{cdk2} to the cellular transcription factor DRTF1

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Summary

Cyclins are regulatory molecules that undergo periodic accumulation and destruction during each cell cycle. By activating p34^{cdc2} and related kinase subunits they control important events required for normal cell cycle progression. Cyclin A, for example, regulates at least two distinct kinase subunits, the mitotic kinase subunit p34^{cdc2} and related subunit p33^{cdk2}, and is widely believed to be necessary for progression through S phase. However, cyclin A also forms a stable complex with the cellular transcription factor DRTF1 and thus may perform other functions during S phase. DRTF1, in addition, associates with the tumour suppressor retinoblastoma (Rb) gene product and the Rb-related protein p107. We now show, using biologically active fusion proteins, that cyclin A can direct the binding of the cdc2-like kinase subunit, p33^{cdk2}, to complexed DRTF1, containing either Rb or p107, as well as activate its histone H1 kinase activity. Cyclin A cannot, however, direct p34^{cdc2} to the DRTF1 complex and we present evidence suggesting that the stability of the cyclin A-p33^{cdk2} complex is influenced by DRTF1 or an associated protein. Cyclin A, therefore, serves as an activating and targeting subunit of p33^{cdk2}. The ability of cyclin A to activate and recruit p33^{cdk2} to DRTF1 may play an important role in regulating cell cycle progression and moreover defines a mechanism for coupling cell-cycle events to transcriptional initiation.

Key words: cyclin, transcription, retinoblastoma, CDK, tumour suppressor.

Introduction

Cyclins are an evolutionarily conserved group of regulatory molecules that undergo periodic accumulation and destruction during the cell cycle and are required for normal cell cycle progression (Hunt, 1989). They were initially identified in marine invertebrates (Evans et al., 1983), and act by binding to and regulating the activity of the mitotic kinase catalytic subunit p34^{cdc2} or related kinase subunits (Pines and Hunter, 1990a). Cyclins, which have been isolated and characterised from a variety of sources, are grouped into several classes. Cyclins of the B class, which function at mitosis, peak towards M phase and are responsible for activating the mitotic kinase subunit p34^{cdc2} (Draetta et al., 1989; Minshull et al., 1990). Cyclin A, however, accumulates somewhat earlier (Giordano et al., 1989; Minshull et al., 1990; Pines and Hunter, 1990b) and in addition to p34^{cdc2} activates the related kinase subunit p33^{cdk2} (Pines and Hunter, 1990b; Tsai et al., 1991). Other cyclins have recently been defined which are thought to regulate progression through earlier phases of the cell cycle (Matsushime et al., 1991; Xiong et al., 1991; Lew et al., 1991; Koff et al., 1991).

Several reports have suggested that cyclin A regulates progression through S phase. In *Xenopus* egg extracts, p33^{cdk2} or a closely related kinase subunit is required for DNA replication (Fang and Newport, 1991) and is believed to fulfill a similar role in other types of cells (Pines and Hunter, 1991). Moreover, inactivation of cyclin A, either through antibody or antisense approaches, prevents cells from completing S phase (Girard et al., 1991). It is likely, therefore, that cyclin A regulates the kinase activity of p33^{cdk2} which in turn modifies protein substrates that are necessary for cell-cycle progression.

The retinoblastoma (Rb) gene product is a negative regulator of the cell cycle that acts by controlling progression through G_1 (Goodrich et al., 1991). This effect is widely believed to result from the activity of the un- or under-phosphorylated Rb protein, because this form of the protein predominates during G_1 (Buchkovich et al., 1989; DeCaprio et al., 1989). Certain viral oncoproteins, such as adenovirus E1a, SV40 large T antigen and HPV E7 bind the Rb protein (Whyte et al., 1988; DeCaprio et al., 1988; Dyson et al., 1989) and the Rb gene is frequently mutated in tumour cells (Hu et al., 1990; Huang et al., 1990). Both of these processes are thought to inactivate the growth suppressing properties of the Rb protein by preventing it from regulating cellular targets. Moreover, the Rb-related protein, p107,

is also sequestered by adenovirus E1a and SV40 large T antigen (Whyte et al., 1988; DeCaprio et al., 1988) and, although there is no detailed information available yet on its regulation during the cell cycle, is likely to have an important role in cell-cycle control (Ewen et al., 1991).

It is thought that the Rb protein mediates its growth regulating properties by modulating the activity of molecules that regulate progression through the cell cycle, but only recently have potential candidates been identified (Bandara and La Thangue, 1991; Bagchi et al., 1991; Chellapan et al., 1991; Chittenden et al., 1991; Defeo-Jones et al., 1991). A particularly good candidate is the cellular transcription factor DRTF1 which is found in complexes that contain the Rb protein (Bandara and La Thangue, 1991). DRTF1, first defined in F9 embryonal carcinoma (EC) stem cells as a differentiation-regulated transcription factor (La Thangue and Rigby, 1987), is mostly uncomplexed in F9 EC cells, but in other cell-types forms a stable complex with the Rb protein (Bandara and La Thangue, 1991). The Rb-DRTF1 complex is dissociated by E1a, large T antigen and E7 proteins (Bandara and La Thangue, 1991; Bandara and La Thangue, data not shown), which releases the transcriptionally active protein, the form that predominates in stem cells. Moreover, the protein products of all naturally occurring mutant Rb alleles so far studied fail to bind stably to DRTF1 (Bandara et al., 1991; Bandara and La Thangue, data not shown), underscoring the potential importance of DRTF1 in controlling cellular proliferation.

Recent experiments have shown that the Rb protein can specifically repress the transcription of promoters driven by DRTF1, whereas proteins encoded by some naturally-occurring mutant Rb alleles, which fail to bind DRTF1 in vitro, cannot. Furthermore, Rb-mediated transcriptional repression is relieved by the adenovirus E1a protein (Zamanian and La Thangue, 1992). The Rb protein, therefore, represses the transcriptional activity of DRTF1 which thus provides a potential mechanistic explanation for how the Rb protein exerts its growth regulating properties, given that DRTF1 binding sites occur in the promoters of genes that are necessary for cell cycle progression (Blake and Azizkhan, 1989; Pearson et al., 1991).

DRTF1 binds to a similar DNA sequence to that of the HeLa cell transcription factor E2F, and although the exact relationship between these two transcription factors has yet to be established, they are likely to be related because similar interactions have been defined for E2F (Bagchi et al., 1990; Chellappan et al., 1991).

Cyclin A also binds to DRTF1 (Bandara et al., 1991) and previously we have suggested that this allows cell cycle events to be coupled to transcription. We have developed an in vitro assay in which cyclin A efficiently binds to DRTF1 and established that cyclin A can direct the cdc2-like kinase subunit, $p33^{cdk2}$, but not the mitotic kinase subunit, $p34^{cdc2}$, to DRTF1. We suggest that such a kinase will play an important role in regulating the activity of other non DNA-binding proteins, such as the Rb protein and p107, in the DRTF1 transcription factor complex and hence in regulating its transcriptional activity. We also present evidence that the stability of the cyclin A-$p33^{cdk2}$ complex is influenced by DRTF1 or a component within this transcription factor complex.

Results

Cyclin A targets the cdc2-related kinase subunit $p33^{cdk2}$ to DRTF1 complexes that contain either the Rb protein or p107

Cyclin A is part of the DRTF1 transcription factor complex (Bandara et al., 1991). In order to investigate the significance of this interaction we developed an in vitro association assay for cyclin A and DRTF1. Initially, we performed the assay in JM cell extracts where most DRTF1 is bound to the Rb protein (complexed DRTF1 is referred to as DRTF1a) and assessed whether a biologically active cyclin A fusion protein (PA-CA), containing amino acid residues 77 to 432 (Fig.1b), would assemble with JM DRTF1a. Indeed, PA-CA efficiently assembles with DRTF1a, because upon addition of PA-CA to the cell extract the Rb-DRTF1 complex migrates with slower mobility (Fig.1a, compare tracks 2 and 3 with 4 and 5; PA-CA dependent complex indicated by ■). This effect is only apparent with DRTF1a since PA-CA fails to assemble with uncomplexed DRTF1, referred to as DRTF1b (see Fig.2). The binding reaction also requires the integrity of the cyclin box because some derivatives of PA-CA that were mutated in this region fail to assemble with DRTF1a (discussed later). Cyclin A, therefore, contains a domain that enables it to associate with DRTF1a and moreover this association occurs efficiently in vitro.

Since cyclin A regulates the activity of cell cycle kinase subunits, such as $p34^{cdc2}$ (Draetta et al., 1989; Minshull et al., 1990) and $p33^{cdk2}$ (Pines and Hunter, 1990b; Tsai et al., 1991), we reasoned that the cyclin A associated with DRTF1 may provide a targeting function and thus recruit such kinase subunits to a potentially important substrate. We tested this idea directly by determining if an affinity purified fusion protein that contains the entire coding sequence of $p33^{cdk2}$ (amino acid residue 1 to 298; GST-cdk2, Fig.1b) would bind to DRTF1 in JM cell extracts. By itself, GST-cdk2 inefficiently binds to the endogenous DRTF1-Rb complex (Fig.1a, tracks 6 and 7) although in some experiments a weak supershifted cdk2-dependent complex is apparent, presumably caused by a binding reaction with the endogenous cyclin A-DRTF1 complex. Binding is enhanced, however, when additional cyclin A is provided by adding PA-CA at the same time as GST-cdk2, when a novel, slower migrating complex is apparent (Fig.1a, compare tracks 4 and 5 with 8 and 9; indicated by ▲). Thus, GST-cdk2 can assemble into the DRTF1 complex, but does so in a PA-CA-dependent fashion. The association of cdk2 with DRTF1 is thus dependent on cyclin A and therefore cyclin A contains domains that enable it to bind simultaneously to two distinct types of molecule, DRTF1 and cdk2.

The JM DRTF1a complex contains the Rb protein (Fig.1a, compare tracks 14 and 15 with 16 and 17; Rb-supershift indicated by ○). To determine if the cdk2-induced complex also contained the Rb gene product, we tested if the anti-Rb antibody caused a further supershift. Indeed, the cdk2-dependent shift can be further shifted by the Rb monoclonal antibody (Fig.1a, compare tracks 10 and 11 with 12 and 13; cdk2-dependent Rb-supershift indicated by ●). Thus, DRTF1, cyclin A, cdk2 and the Rb gene product can exist in the same molecular complex.

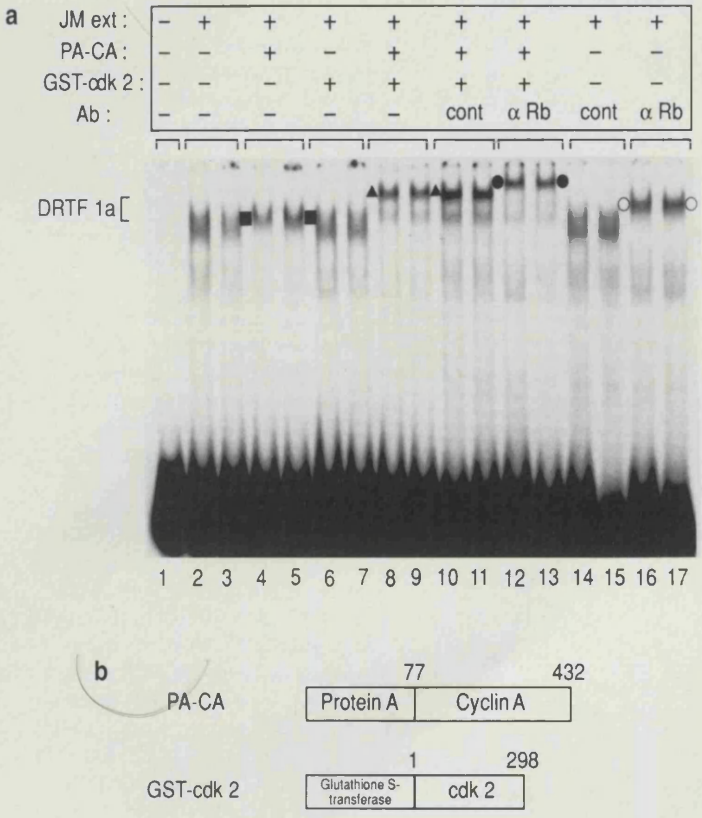

Fig. 1. Cyclin A targets cdk2 to complexed DRTF1. (a) Gel retardation (Shivji and La Thangue, 1991) was performed in a JM whole cell extract (about 2 μg) either before (tracks 2, 3, 14, 15, 16 and 17) or after the addition of PA-CA (about 0.5 μg; tracks 4, 5, 8, 9, 10, 11, 12 and 13) or GST-cdk2 (about 0.5 μg; tracks 6, 7, 8, 9, 10, 11, 12 and 13) in the presence of an anti-Rb, C36 (Whyte et al., 1988), (tracks 12, 13, 16 and 17) or control (tracks 10, 11, 16 or 17) monoclonal antibody; the probe (containing Ad5 E2A promoter sequences from −71 to −50; La Thangue et al, 1990) alone is shown in track 1. DRTF1a is shifted by the anti-Rb monoclonal antibody to ○ (compare tracks 14 and 15 with 16 and 17) and ■ indicates the PA-CA dependent complex (tracks 4 and 5). Note that the cdk-dependent complex, ▲ (tracks 8 and 9), is supershifted to ● by the anti-Rb antibody (compare tracks 10 and 11 with 12 and 13). (b) Structure of fusion proteins used in this experiment. PA-CA was purified as previously described (Bandara et al., 1991). GST-cdk2 contains the entire cdk2 coding sequence (Tsai et al., 1991) fused to glutathione-S-transferase and was purified by affinity chromatography on glutathione Sepharose followed by FPLC on a MonoQ column.

To confirm and extend this result, we tested if cyclin A and cdk2 behaved in a similar fashion in an F9 EC cell extract where most DRTF1 is uncomplexed (Fig.2a, tracks 2 and 3; indicated as DRTF1b). In F9 EC cells, the level of complexed DRTF1 can be increased by adding an Rb fusion protein (containing Rb coding sequence from 379 to 928, GST-Rb) to the cell extract, which efficiently assembles with F9 EC DRTF1b (Bandara et al., 1991 and Fig.2a, compare tracks 2 and 3 with 6 and 7; Rb complex indicated by DRTF1a). When added alone, GST-cdk2 has little effect (Fig.2a, compare tracks 2 and 3 with 10 and 11) and PA-CA a marginal effect (Fig.2a, compare tracks 2 and 3 with 4 and 5). When GST-cdk2 and PA-CA are added together, however, a cdk2-dependent complex with similar mobility to that produced in JM cell extracts is apparent (Fig.2a, compare tracks 2 and 3 with 12 and 13, cdk2-dependent complex indicated by ▲). High levels of uncomplexed DRTF1b still remain, suggesting that GST-cdk2 and PA-CA can only associate with endogenous complexed DRTF1a in F9 EC cell extracts and therefore the amount of cdk2-induced complex reflects the abundance of DRTF1a. This was confirmed by increasing the level of DRTF1a, by adding GST-Rb at the same time as GST-cdk2 and PA-CA when the abundance of the cdk2-dependent complex is enhanced (Fig.2a, compare tracks 12 and 13 with 14 and 15, indicated by ▲); little uncomplexed DRTF1b remains in these conditions. These data indicate that, as in JM extracts, the assembly of $p33^{cdk2}$ with F9 EC DRTF1 is cyclin A-dependent. The combined conclusion of these in vitro assembly assays is thus that the association of GST-cdk2 with DRTF1a is dependent on PA-CA, and that this binding reaction only occurs efficiently with complexed DRTF1a.

The Rb-related protein, p107, which has a degree of similarity with the Rb protein (Ewen et al., 1991), also binds to DRTF1 because a fusion protein containing the p107 coding sequence from amino acid residue 249 to 936 (GST-p107; Fig.2b) efficiently binds to F9 EC DRTF1b (Fig.2a, compare tracks 2 and 3 with 8 and 9). In fact, the characteristics of the p107 and Rb protein binding reactions are very similar indeed both in quality, efficiency of the complex formation and half-life (data not shown).

Since p107 also binds to DRTF1, we assessed whether cyclin A would also bind to the DRTF1-p107 complex and, further, if it is also able to direct $p33^{cdk2}$ to the p107 complex. The p107-induced F9 EC DRTF1a complex is further shifted upon the addition of PA-CA (data not shown), in a similar fashion to the effect that PA-CA has on JM DRTF1a and, once assembled, PA-CA is likewise able to bind GST-cdk2 because the DRTF1-p107 complex is super-shifted upon the addition of GST-cdk2 (Fig.2a, compare 8 and 9 with 16 and 17). Cyclin A can thus bind to and recruit $p33^{cdk2}$ to DRTF1 complexes containing either the Rb gene product or p107.

There are no obvious features of the cdk2-dependent complexes formed with either DRTF1/GST-Rb or GST-p107 that distinguish between them: the mobilities of the complexes are similar (both GST-Rb and GST-p107 cdk2-dependent complexes migrate as a doublet when assayed in F9 EC cell extracts; Fig.3, compare tracks 3 and 4 with 5 and 6) and the stability of these complexes are similar, at least up to 60 min (Fig.3, compare tracks 2 to 6 with 7 to 11, cdk2-dependent complexes indicated by ▲). $p33^{cdk2}$ can therefore, bind via cyclin A to DRTF1 complexes that contain either the Rb protein or p107 and, furthermore, the

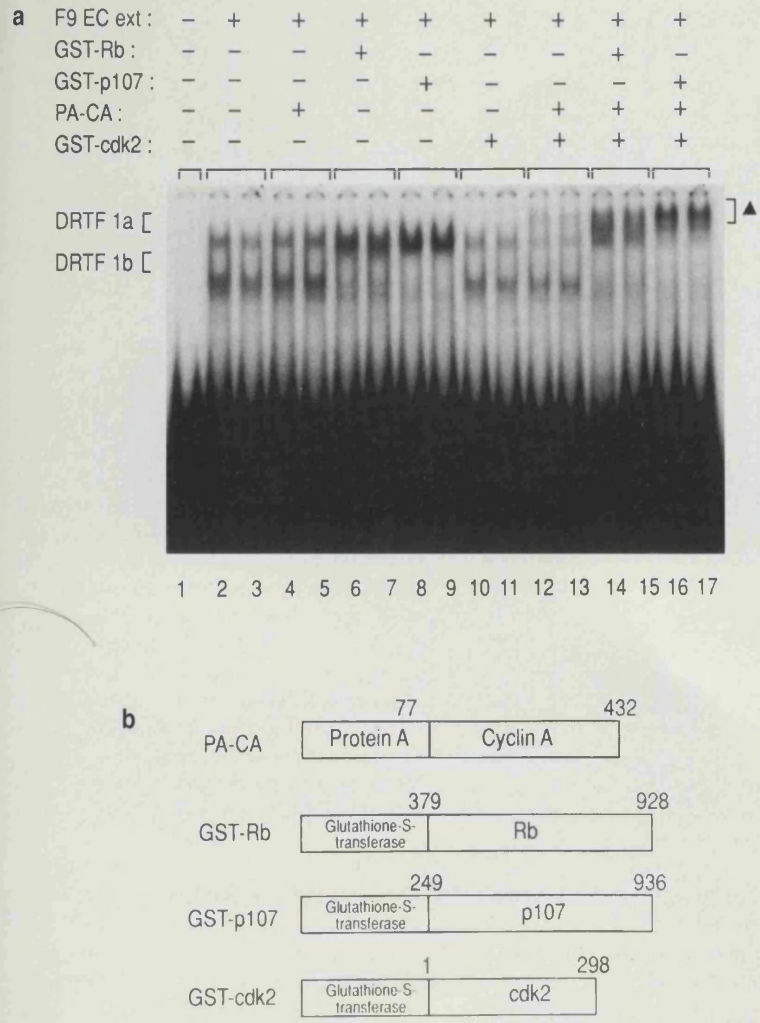

Fig. 2. Cyclin A targets cdk2 to DRTF1 complexes that contain either GST-Rb or GST-p107. (a) Gel retardation was performed in an F9 EC cell extract either before (tracks 2 and 3) or after (about 0.5 μg; tracks 4, 5, 12, 13, 14, 15, 16 and 17) the addition of PA-CA, GST-Rb (about 0.06 μg; tracks 6, 7, 14 and 15), GST-p107 (about 0.02 μg; tracks 8, 9, 16 and 17) or GST-cdk2 (about 0.5 μg; tracks 10, 11, 12, 13, 14, 15, 16 and 17); track 1 shows the probe alone. The cdk2-dependent complex (▲), which migrates as a doublet in the presence of GST-Rb or GST-p107, is apparent in tracks 12 through 17. (b) Structure of fusion proteins used in this experiment. GST-p107 was constructed using a sense oligonucleotide primer for amino acid residues 249-257(5′-CGGGATCCGCAGTC-ATTACTCCTGTTGCATCAGCC) and an anti-sense primer covering amino acid residues 690-732 (5′-GGACAGTGAACTA-AAGTGAATTCAAAAACACG) in a polymerase chain reaction (PCR), using a p107 cDNA (Ewen et al., 1991) as template. In a second PCR reaction, a sense primer for amino acid residues 690-732 (5′-CGTGTTT-TGAATTCACTTTAGTTCACTGTCC) and an antisense primer for amino acid residues 928-936 (5′-CGGGATCCTTAATGATTTG-CTCTTTCACTGAC) were used. Both PCR products were purified, digested with *Bam*HI and *Eco*RI, ligated and cloned into pGEX-2T. GST-p107 and GST-Rb were purified as previously described (Smith and Johnson, 1988).

properties of these binding reactions cannot readily be distinguished in gel retardation assays.

We confirmed that $p33^{cdk2}$ or a closely related subunit is present in the endogenous DRTF1a complexes in F9 EC cell extracts using an antibody raised against the GST-cdk2 fusion protein. The immune but not the preimmune produces a shifted-shift in F9 EC cell extracts (Fig.4, compare tracks 15 and 14); a similar effect is also observed in JM cell extracts (data not shown). We conclude, therefore, that $p33^{cdk2}$ is present in the DRTF1 complex. In this respect, DRTF1 is similar to E2F (Cao et al., 1992; Devoto et al., 1992).

We addressed the specificity of the $p33^{cdk2}$/DRTF1 interaction by asking if the mitotic kinase subunit, $p34^{cdc2}$, could also assemble with DRTF1. To test this, the entire coding sequence of a *Xenopus* $p34^{cdc2}$ (Milarski et al., 1991) was expressed as a fusion protein, GST-Xlcdc2 (Fig.4b), affinity purified and then assayed for any binding activity with DRTF1. It was necessary to use *Xenopus* $p34^{cdc2}$ because repeated attempts to express human $p34^{cdc2}$ as a fusion protein have failed. Although GST-cdk2 is able to assemble efficiently with DRTF1a in a cyclin A-dependent manner (Fig.4a, tracks 4 to 11), GST-Xlcdc2 cannot (Fig.4, compare tracks 10 and 11 with tracks 12 and 13). This lack of effect is not simply caused by a fusion protein that lacked any biological activity because GST-Xlcdc2 is able to bind efficiently to PA-CA in the cell extract to produce H1 kinase activity (R. Y. C. Poon and J. P. A., unpublished data). We therefore conclude that $p33^{cdk2}$ is selectively recruited to DRTF1 by cyclin A.

Complex formation between cyclin A and cdk2 fusion proteins produces a biologically active H1 kinase

Since heterodimerisation of an appropriate cyclin molecule with a compatible catalytic subunit is necessary to produce a biologically active protein kinase, we tested if PA-CA would form a heterodimer with GST-cdk2, and furthermore determined if the heterodimer possessed H1 kinase activity. The ability of PA-CA to bind GST-cdk2 was assessed after incubating both fusion proteins together, either in the presence or absence of JM cell extract, and then harvesting GST-cdk2 (and thus any bound PA-CA) on glutathione beads. These complexes were then denatured and assayed by immunoblotting with an anti-GST antibody which would bind to both GST-cdk2 and PA-CA (through the protein A moiety) and therefore indicate whether GST-cdk2 had formed a complex with PA-CA.

In isolation, the ability of GST-cdk2 to bind PA-CA is slight (Fig.5a, track 16, notice weak reactivity with PA-CA) although, we believe, significant, because when several derivatives of PA-CA in which the cyclin box or the C terminus were mutated (Fig.5c, Δ2, Δ18, Δ24 and ΔC-16) are

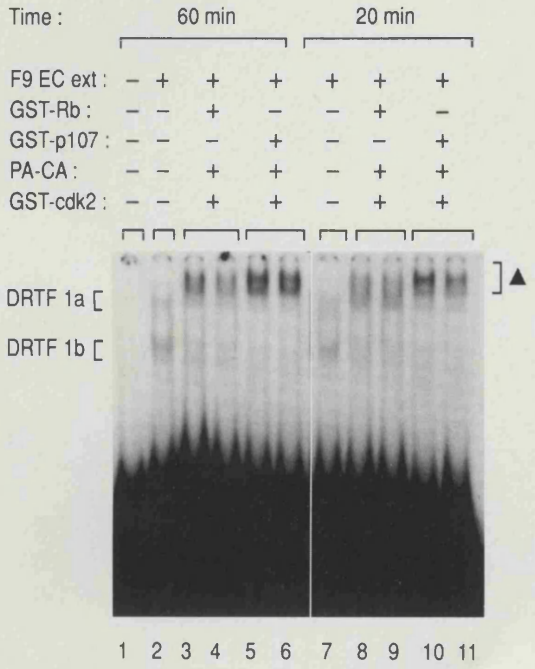

Fig. 3. cdk2-dependent complexes have similar stabilities. Gel retardation was performed in an F9 EC cell extract either alone (tracks 2 and 7) or after the addition of GST-Rb (about 0.06 µg; tracks 3, 4, 8 and 9) or GST-p107 (about 0.02 µg; tracks 5, 6, 10, and 11), together with PA-CA and GST-cdk2 (both at 0.5 µg; tracks 3, 4, 5, 6, 8, 9, 10 and 11), and incubated for either 60 min (tracks 2 to 6) or 20 min (tracks 7 to 11). The cdk2-dependent complex (▲) is indicated. Fusion proteins were as described in Fig. 2.

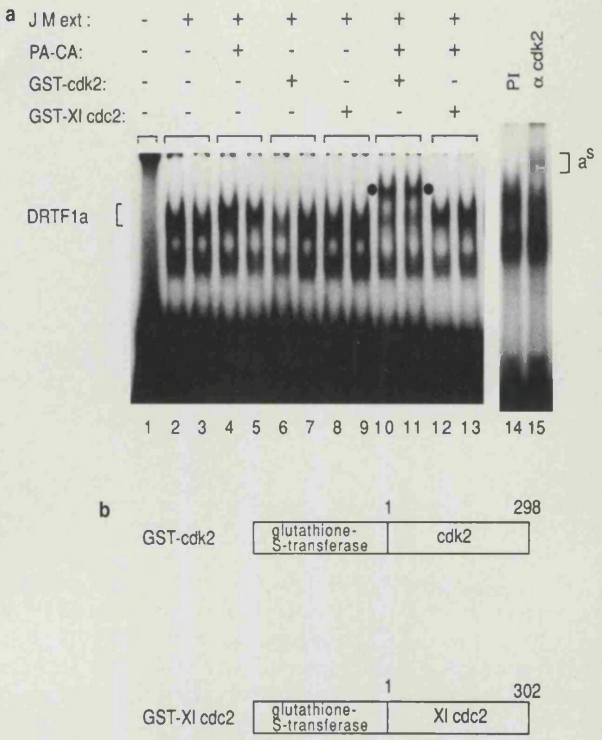

Fig. 4. cdc2 cannot engage with DRTF1. Gel retardation was performed in a JM cell extract either alone (tracks 2 and 3) or after the addition of PA-CA (about 0.5 µg, tracks 4, 5, 10, 11, 12 and 13), GST-cdk2 (about 0.5 µg, tracks 6, 7, 10 and 11) or GST-Xlcdc2 (about 0.2 µg, tracks 8, 9, 12 and 13). GST-Xlcdc2 contains the entire coding sequence of a *Xenopus* cdc2 cDNA (Milarski et al., 1991) in pGEX-2T and was purified as described above. Note that the cdk2-dependent complex, ●, is present only in tracks 10 and 11. For tracks 14 and 15, an F9 EC cell extract was incubated in the presence of either a preimmune (track 14) or anti-GST-cdk2 (track 15) rabbit antiserum. The shifted complex is indicated by a^s.

tested, no detectable binding with PA-CA is apparent (Fig.5a, compare track 16 with 19, 20, 21 and 22). The binding efficiency of PA-CA with GST-cdk2 is, however, enhanced when they are incubated together in the JM cell extract and then assayed (Fig.5a, compare track 9 with 16 and notice the increased amount of PA-CA compared with track 9). This binding requires the integrity of the cyclin box and the C terminus in PA-CA because there is negligible binding when either of these regions are mutated (Fig.5a, compare track 9 through 12 with 15); it is also absolutely dependent on the presence of cdk2, since GST alone or denatured GST-cdk2 do not bind to PA-CA (Fig.5a, compare track 9 with 10 and 11, respectively).

We next determined if heterodimerisation of PA-CA and GST-cdk2 results in H1 kinase activity. Indeed, the affinity purified heterodimer formed in the presence of the JM extract possesses H1 kinase activity (Fig.5b, track 1) which is, as expected, severely compromised when the PA-CA mutants are similarly assayed (Fig.5b, compare track 1 through 4 with 7), and again is dependent on the presence of cdk2 coding sequences (Fig.5b, compare track 1 with tracks 2 and 3). These data show that the PA-CA/GST-cdk2 heterodimer forms a biologically active protein kinase and that an activity in JM cell extracts is necessary for this to occur. Since PA-CA can direct GST-cdk2 to DRTF1 in JM cell extracts, we believe that this would enable heterodimerisation of PA-CA with GST-cdk2 and thus target an active kinase to DRTF1.

The stability of the cyclin A-p33^{cdk2} complex is influenced by DRTF1

The data in the previous section show that PA-CA can bind to GST-cdk2 and that this results in an active H1 kinase. Regions either within the cyclin box or at the C terminus of PA-CA are necessary for this interaction because derivatives in which either region is mutated cannot bind to GST-cdk2 (Fig.5). We wished to determine if these regions were also required for the interaction of PA-CA with DRTF1 and therefore assessed if these mutants assembled with DRTF1a in JM cell extracts. As shown earlier, PA-CA efficiently assembles with DRTF1a, producing a characteristic slower migrating complex (Fig.1, compare tracks 2 and 3 with 4 and 5; PA-CA-dependent complex indicated by ■). Mutant Δ2 is also able to bind to DRTF1 (Fig.6, compare tracks 4 and 5 with 6 and 7; complex indicated by ■) whereas Δ18 and ΔC-16 cannot because there is no effect on the migration of DRTF1a (Fig.6, compare tracks 2 and 3 with 8, 9, 10 and 11); the efficiency of Δ2 binding is, however, lower than the activity of the wild-type sequence. We conclude from these data that the alteration in Δ2 affects the interaction of PA-CA with GST-

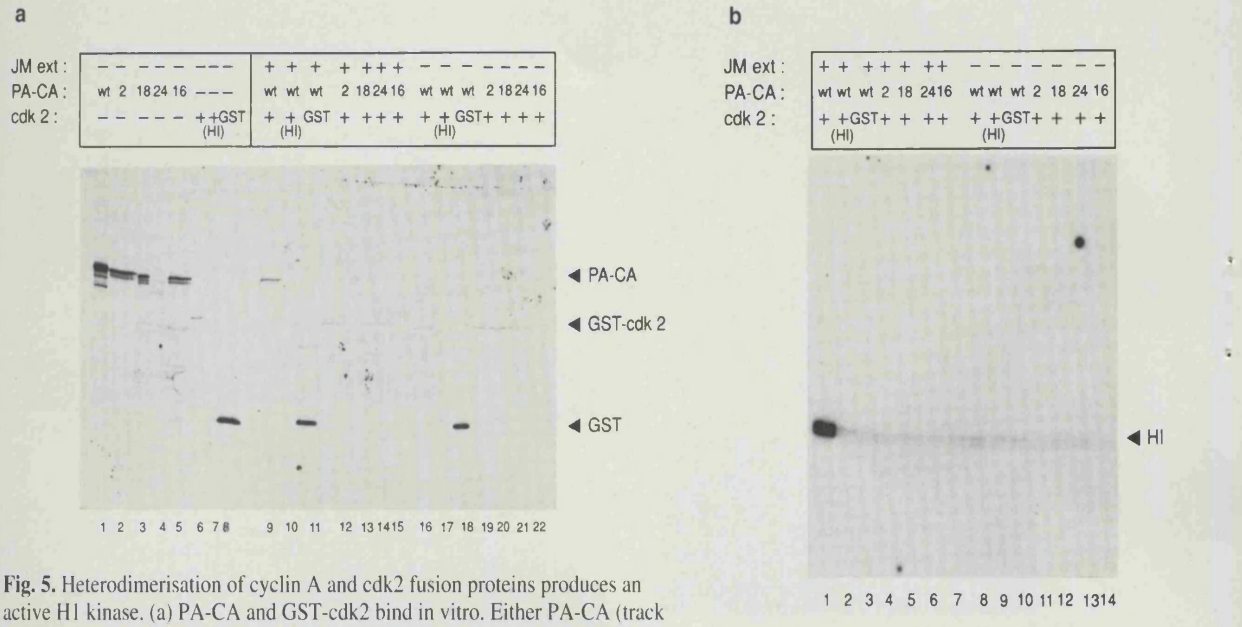

Fig. 5. Heterodimerisation of cyclin A and cdk2 fusion proteins produces an active H1 kinase. (a) PA-CA and GST-cdk2 bind in vitro. Either PA-CA (track 1), PA-CA mutants (Δ2, 18, 24 and C-16; tracks 2 to 5), GST-cdk2 (track 6), heat-denatured GST-cdk2 (track 7) or GST with no fusion (track 8) were assayed after western blotting and probing with an anti-GST antibody. The ability of these fusion proteins to complex in the presence (tracks 9 to 15) or absence (tracks 16 to 22) of the JM extract was assessed for the following combinations: PA-CA and GST-cdk2 (tracks 9 and 16), PA-CA and denatured GST-cdk2 (tracks 10 and 17), PA-CA and GST (tracks 11 and 18), PA-CA ΔAcc2 and GST-cdk2 (tracks 12 and 19), PA-CA Δ18 and GST-cdk2 (tracks 13 and 20), PA-CA Δ24 and GST-cdk2 (tracks 14 and 21) and PA-CA ΔC-16 and GST-cdk2 (tracks 15 and 22). Complexes were affinity purified on glutathione Sepharose, denatured, western blotted and probed with an anti-GST antibody (which binds both to PA-CA and GST). Tracks 1 to 5 show a sample of PA-CA and derivatives loaded directly onto the gel and western blotted. The positions of PA-CA, GST-cdk2 and GST are indicated. (b) Binding of PA-CA to GST-cdk2 produces an H1 kinase. The affinity purified material assayed in Fig.3a, tracks 9 to 22, was assayed in parallel for H1 kinase activity (tracks 1 to 14). The indicated PA-CA fusion proteins (about 300 ng) and GST-cdk2 (about 250 ng) were preincubated either alone or in a JM whole cell extract (about 100 μg) for 15 min at 20°C before the addition of 500 μl of 20 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% NP40 and 1% BSA. Complexes were harvested by incubating with glutathione Sepharose for 2 h and then either assayed for H1 kinase activity (Minshull et al., 1990) or denatured and resolved in a 15% SDS polyacrylamide gel. After transferring onto nitrocellulose, fusion proteins were detected with a polyclonal anti-GST antibody. GST-cdk2 was denatured by heating for 5 min. (c) Description of fusion proteins. PA-CA wt contains the C-terminal 355 amino acid residues of bovine cyclin A, Δ2 has residues 281 and 282 deleted and 283 mutated from serine to threonine, Δ18 lacks residues 273 to 290, Δ24 residues 270 to 293 and ΔC-16 the C-terminal 16 residues; shaded area indicates mutated region.

cdk2 more severely than its ability to interact with DRTF1a because the Δ2 mutant cannot bind to GST-cdk2 (Fig.5) but can bind to DRTF1a.

We next assessed if Δ2 can target GST-cdk2 to DRTF1. When both Δ2 and GST-cdk2 are added to the JM extract, the expected cdk2-dependent shift is produced (Fig.6, compare tracks 12 and 13 with 14 and 15, cdk2-dependent shift indicated by ●). Thus, Δ2 can still direct GST-cdk2 to DRTF1. In contrast, but as expected, both Δ18 or ΔC-16 fail to direct GST-cdk2 to DRTF1 (Fig.6, tracks 16 to 19). We conclude from this experiment that mutant Δ2 can still target GST-cdk2 to DRTF1 even though the ability of these two proteins to interact outside the DRTF1 complex is severely compromised (Fig.5). We suggest, therefore, that the stability of the interaction between GST-cdk2 and mutant Δ2 is influenced by DRTF1 or a component within the transcription factor complex.

Discussion

It is known that cyclins regulate the activity of cell cycle kinases and thus participate in controlling the cell cycle (Hunt, 1989). In this study we have defined a new but widely predicted role for cyclin A by demonstrating that it recruits and thus dictates substrate specificity to the catalytic subunit $p33^{cdk2}$. We believe that such a targeting function is likely to be a general principle that enables cyclin-dependent kinase subunits to locate protein substrates during the cell cycle.

Cyclin A directs $p33^{cdk2}$ to DRTF1

The data obtained from the in vitro association assay in which cyclin A targets $p33^{cdk2}$ to DRTF1 clearly lead us to conclude that cyclin A has at least two binding interfaces: one for DRTF1 and the other for $p33^{cdk2}$. The fact that

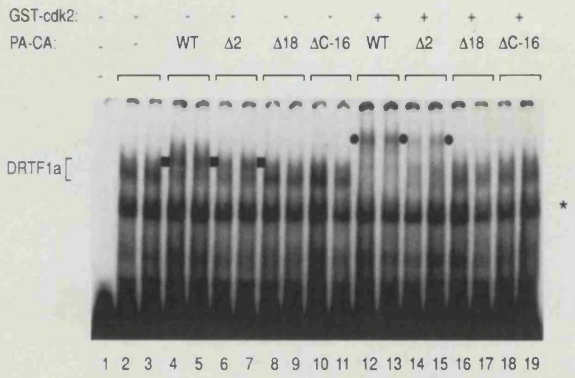

Fig. 6. Evidence that PA-CA directed binding of $p33^{cdk2}$ is influenced by DRTF1. Gel retardation was performed in a JM cell extract either alone (tracks 2 and 3) or after the addition of either wild-type PA-CA (tracks 4, 5, 12 and 13), Δ2 (tracks 6, 7, 14 and 15), Δ18 (tracks 8, 9, 16 and 17) or ΔC-16 (tracks 10, 11, 18 and 19) in the presence (tracks 12 to 19) or absence (tracks 2 to 11) of GST-cdk2. The PA-CA dependent complex is indicated by ■ and note that Δ2 causes the appearence of this complex. Both PA-CA and Δ2 are supershifted upon the addition of GST-cdk2 (indicated by ●). * indicates a non-specific complex.

mutant Δ2, which has amino acid residues 281 and 282 deleted and 283 altered from a serine to a threonine, cannot bind directly to GST-cdk2 (Fig.5a) but still engages with DRTF1a (Fig.6) supports this idea and potentially defines a sequence that distinguishes between these two interaction domains. However, since an increase in the size of the deletion (for example Δ18 which lacks residues 273 to 290) prevents cyclin A from binding to DRTF1, these two binding interfaces may overlap.

Although GST-cdk2 cannot bind directly to Δ2 when it is studied outside the DRTF1 complex it can, nevertheless, be directed and stably bind to the DRTF1 transcription factor complex in an Δ2-dependent fashion (Fig.6). There are at least two possible explanations for this. Perhaps the most likely one is that, in addition to its well characterised binding with cyclin A, $p33^{cdk2}$ interacts with one or more of the components that make up DRTF1 and this serves to stabilise its binding. Alternatively, cyclin A may undergo some modification upon binding to DRTF1 that then enables it to bind more efficiently to $p33^{cdk2}$. Either of these models would also go someway towards explaining the selective targeting function of cyclin A, namely that cyclin A targets $p33^{cdk2}$ but not $p34^{cdc2}$ to DRTF1. We suggest that this selectivity is also established by an additional interaction that occurs between $p33^{cdk2}$ (but not $p34^{cdc2}$) and a domain in DRTF1 which may, of course, be the same domain as that involved in enabling the association of $p33^{cdk2}$ with the Δ2-DRTF1 complex.

If indeed such selective targeting and stable association also occurs for $p34^{cdc2}$ substrates (because of analogous interactions between $p34^{cdc2}$ and appropriate substrates), then it would lead us to make the clear prediction that, although both $p33^{cdk2}$ and $p34^{cdc2}$ can bind to cyclin A, they will, nevertheless, stably associate with different, perhaps overlapping, sets of substrates. This is not an altogether surprising suggestion given that $p33^{cdk2}$ and $p34^{cdc2}$ are thought to regulate progression through two distinct parts of the cell cycle, namely S phase and mitosis respectively (Pines and Hunter, 1991b), roles presumably mediated by modulating different substrates. In conclusion, we believe that these data are consistent with a model in which cyclin A provides a general targeting function for catalytic subunits, such as $p33^{cdk2}$ and $p34^{cdc2}$, that recruits them to a variety of substrates, but that additional interactions between the substrate and catalytic subunit influence whether the catalytic subunit remains stably associated with the substrate.

Cyclin A recruits cdk2 to Rb and p107 complexes

The in vitro assay clearly demonstrates that cyclin A can bind, and recruit $p33^{cdk2}$, to DRTF1 complexes that contain either the Rb protein or p107. This activity is dependent on the presence of Rb or p107, since cyclin A cannot efficiently bind to free DRTF1. However, in cell extracts, cyclin A is found exclusively in E2F complexes that contain p107 (Cao et al., 1992; Shirodkar et al., 1992). It is clear, however, from other studies that a cdc2-like kinase (containing the conserved PSTAIRE amino acid motif) coprecipitates with the Rb protein in anti-Rb immunoprecipitates (Hu et al., 1992). Furthermore, this Rb-associated kinase contains cyclin A (Hu et al., 1992). We believe, therefore, that the Rb-cyclin A-DRTF1 complex generated in our assays is likely to reflect physiological interactions. The difficulty encountered in detecting this complex in cell extracts by gel retardation may reflect, for example, its short half life in vivo. This seems likely because the active kinase (generated by cyclin A plus $p33^{cdk2}$) may phosphorylate the Rb protein, and others have reported that the Rb-E2F complex contains exclusively underphosphorylated Rb protein (Chellappan et al., 1991). Our ability to recreate stable complexes in vitro may thus result from physiological interactions that have been 'frozen' in vitro. This in vitro assay should prove useful in elucidating the role of this kinase in regulating DRTF1.

A mechanism for coupling cell-cycle events to transcription

The ability of cyclin A to direct $p33^{cdk2}$ to DRTF1 is likely to be an important event in controlling cell-cycle progression because some of the genes that are potentially regulated by DRTF1 are necessary for DNA synthesis (Blake and Azizkhan, 1989; Pearson et al., 1991). Moreover, the Rb protein, which acts in G_1 to prevent cells from entering S phase, represses the transcriptional activity of DRTF1 (Zamanian and La Thangue, 1992) and thus could prevent cells from progressing into S phase by limiting transcription of these gene. Since this is likely to be mediated by un- or under-phosphorylated Rb protein (Buchkovich et al., 1989; DeCaprio et al., 1989) we suggest that the ability of a cyclin to direct a kinase to this complex may regulate the activity of the Rb protein within this transcription factor complex. It is possible, therefore, that the transcriptional activation of DRTF1 is necessary for DNA synthesis to occur. Importantly, we do not wish to imply that $p33^{cdk2}$ is the kinase responsible for regulating the transcriptional activity of DRTF1 but merely, based on the data discussed here, that it is a strong possibility.

Moreover, we cannot rule out that the kinase has a different role from what we have suggested, for example, in modulating the activity of other transcription-related molecules. Whatever the targets for this kinase actually are, it is likely that the ability of cyclin A to target and activate such a kinase is an important event in regulating cell-cycle progression.

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Functional synergy between DP-1 and E2F-1 in the cell cycle-regulating transcription factor DRTF1/E2F

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It is widely believed that the cellular transcription factor DRTF1/E2F integrates cell cycle events with the transcription apparatus because during cell cycle progression in mammalian cells it interacts with molecules that are important regulators of cellular proliferation, such as the retinoblastoma tumour suppressor gene product (pRb), p107, cyclins and cyclin-dependent kinases. Thus, pRb, which negatively regulates early cell cycle progression and is frequently mutated in tumour cells, and the Rb-related protein p107, bind to and repress the transcriptional activity of DRTF1/E2F. Viral oncoproteins, such as adenovirus E1a and SV40 large T antigen, overcome such repression by sequestering pRb and p107 and in so doing are likely to activate genes regulated by DRTF1/E2F, such as *cdc2*, c-*myc* and *DHFR*. Two sequence-specific DNA binding proteins, E2F-1 and DP-1, which bind to the E2F site, contain a small region of similarity. The functional relationship between them has, however, been unclear. We report here that DP-1 and E2F-1 exist in a DNA binding complex *in vivo* and that they bind efficiently and preferentially as a heterodimer to the E2F site. Moreover, studies in yeast and *Drosophila* cells indicate that DP-1 and E2F-1 interact synergistically in E2F site-dependent transcriptional activation.

Key words: cell cycle/DNA binding proteins/transcription factors

Introduction

Several lines of evidence suggest that the cellular transcription factor DRTF1/E2F plays an important role in regulating the cell cycle of mammalian cells. For example, DRTF1/E2F DNA binding activity is periodically induced during cell cycle progression, peaking during S phase (Mudryj *et al.*, 1991; Shirodkar *et al.*, 1992), and negatively regulated during cellular differentiation (La Thangue and Rigby, 1987). This binding activity correlates with the transcriptional activity of certain genes that are necessary for cellular proliferation, such as DHFR, DNA polymerase α and p34^{cdc2}, which contain DRTF1/E2F binding sites in their promoters (Blake and Azizkhan, 1989; Dalton, 1992; Means *et al.*, 1992). Furthermore, the retinoblastoma tumour suppressor gene product (pRb), which negatively regulates cell cycle progression from G_1 into S phase and is frequently mutated in tumour cells, binds to DRTF1/E2F (Bandara and La Thangue, 1991; Chellappan *et al.*, 1991). The functional consequence of this interaction is that pRb prevents DRTF1/E2F from activating transcription (Zamanian and La Thangue, 1992). Several other molecules that are implicated in cell cycle control, such as Rb-related p107, cyclins A and E, and p33^{cdk2} also associate with DRTF1/E2F during cell cycle progression (Bandara *et al.*, 1991, 1992; Mudryj *et al.*, 1991; Devoto *et al.*, 1992; Lees *et al.*, 1992). Taken together, these observations suggest that DRTF1/E2F integrates cell cycle events with the transcription apparatus, ensuring that the cell makes the appropriate changes in gene expression at the correct time during cell cycle progression.

Further evidence for the importance of DRTF1/E2F has come from studies on the mechanism of action of viral oncoproteins. Thus, certain oncoproteins, such as adenovirus E1a, SV40 large T antigen and human papilloma virus E7 regulate the activity of DRTF1/E2F by sequestering pRb and the other associated proteins, converting it from a transcriptionally inactive to an active form (Hiebert *et al.*, 1992; Zamanian and La Thangue, 1992, 1993). Because this effect requires regions in these viral oncoproteins previously shown to be necessary for cellular immortalization and transformation (Bandara and La Thangue, 1991; Zamanian and La Thangue, 1992), it is likely that DRTF1/E2F plays an important role in these processes.

Although progress has been made in identifying the cellular proteins that interact with DRTF1/E2F, until recently, relatively little was known about its molecular details. Two distinct polypeptides which are both DNA binding components of DRTF1/E2F have now been molecularly characterized. The first, referred to as E2F-1, was isolated through its ability to bind directly to pRb, which it does through a C-terminal region (Helin *et al.*, 1992; Kaelin *et al.*, 1992). In contrast, DP-1 was defined as a component of DRTF1/E2F DNA binding activity after biochemically purifying DRTF1 from F9 embryonal carcinoma (EC) stem cells, a cell system in which DRTF1/E2F is down-regulated during the differentiation process (La Thangue and Rigby, 1987; La Thangue *et al.*, 1990). cDNAs that encode DP-1 were isolated after obtaining amino acid sequence from affinity purified DP-1 (Girling *et al.*, 1993).

Both E2F-1 and DP-1 contain a region that allows each polypeptide to bind in a sequence-specific fashion as a homodimer to the E2F motif (Helin *et al.*, 1992; Kaelin *et al.*, 1992; Girling *et al.*, 1993). Although the DNA binding domains are not closely related to any previously defined DNA binding structure they are, nevertheless, distantly related to the DNA binding domains in some yeast cell cycle-regulating transcription factors (La Thangue and Taylor, 1993). The functional relationship between DP-1 and E2F-1 has, however, remained unclear. In this study, we show that DP-1 and E2F-1 exist as a complex *in vivo* which

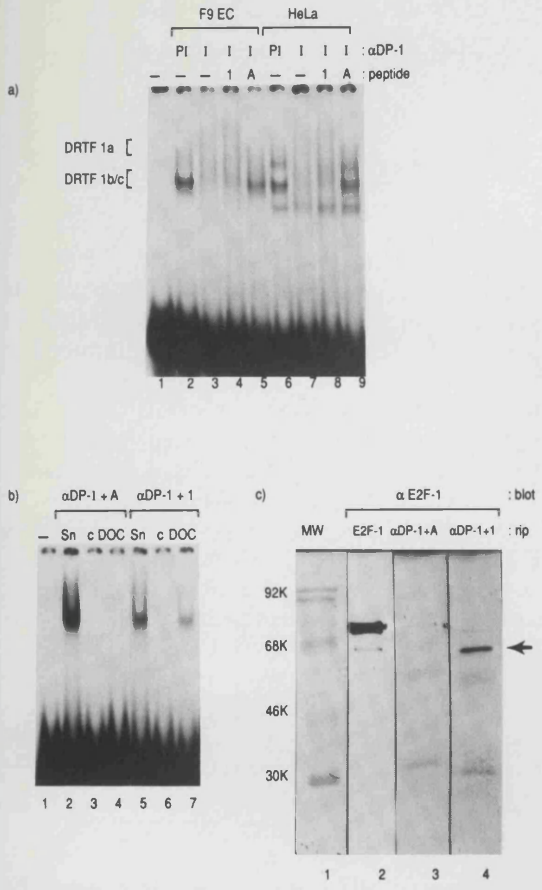

Fig. 1. DP-1 and E2F-1 exist in the same protein complex *in vivo*. (**a**) DP-1 is in DRTF1/E2F DNA binding complexes formed in HeLa cell extracts: gel retardation was performed using F9 EC and HeLa whole cell extracts (in which DRTF1 resolves as three distinct complexes, a, b and c; indicated in figure) with the E2F binding site taken from the adenovirus E2A promoter (nucleotides −71 to −50) in the presence of either preimmune (PI; tracks 2 and 6) or immune (I; tracks 3–5 and 7–9) anti-DP-1 (peptide A) antiserum with the addition of either unrelated peptide 1 (tracks 4 and 8) or peptide A (tracks 5 and 9). In both F9 EC and HeLa cell extracts, all the DRTF1/E2F DNA binding complexes were affected by the anti-DP-1 antibody. (**b**) Anti-DP-1 immunoprecipitates DRTF1/E2F DNA binding activity: immunoprecipitation was performed from HeLa cell extracts with anti-DP-1 in the presence of either homologous peptide A (tracks 2–4) or unrelated peptide 1 (tracks 5–7). The immunoprecipitates were treated with 1% deoxycholate (DOC) and 1.5% NP40, and the detergent-released material assayed for DRTF1/E2F DNA binding activity; the depleted HeLa cell extract is also indicated (Sn; tracks 2 and 5). No DNA binding activity was released in the absence of detergent (indicated by c; tracks 3 and 6). (**c**) Immunoblotting DP-1 immunoprecipitates with anti-E2F-1: anti-DP-1 immunoprecipitates performed in the presence of either peptide A (track 3) or peptide 1 (track 4) were immunoblotted with the anti-E2F-1 monoclonal antibody SQ41; the E2F-1 polypeptide, present in track 4, is indicated by the arrow. As a positive control, ~100 ng of the E2F-1 fusion protein, GST–E2F-1^{89-437}, was immunoblotted in track 2. Track 1 shows standard molecular weights.

recognizes the E2F binding site. Moreover, *in vitro* assays demonstrate that DP-1 and E2F-1 bind efficiently and preferentially as a complex to the E2F site, an interaction which requires the region of similarity between the two proteins. Furthermore, reconstructing DRTF1/E2F in *Drosophila* and yeast cells suggests that DP-1 and E2F-1 interact synergistically in E2F site-dependent transcriptional activation. These data indicate that DP-1 and E2F-1 can functionally interact and that such an interaction is likely to be physiologically relevant in mammalian cells.

Results

DP-1 and E2F-1 exist as a complex in HeLa cells

DP-1 is a component of DRTF1/E2F which is present in murine developmentally regulated and cell cycle regulated DRTF1/E2F complexes (L.R.Bandara, T.S.Sørensen, M.Zamanian and N.B.La Thangue, in preparation) and thus is likely to be a general component of DRTF1/E2F DNA binding activities. Furthermore, DP-1 is the product of a conserved gene since a closely related protein is expressed in amphibians and *Drosophila* (R.Girling and N.B.La Thangue, in preparation; F.-H.Xu and N.B.La Thangue, in preparation). DP-1 thus appears to be a frequent and evolutionarily conserved DNA binding component of DRTF1/E2F. E2F-1, which was isolated through its ability to bind directly to pRb, also interacts in a sequence-specific fashion with the E2F site (Helin *et al.*, 1992; Kaelin *et al.*, 1992). Both proteins contain a small region of similarity that overlaps domains previously shown to be necessary for sequence-specific DNA binding activity (Girling *et al.*, 1993).

We assessed whether DP-1 and E2F-1 exist as a complex in HeLa cell extracts using antibodies that specifically recognize each protein. Initially, we determined by gel retardation whether DP-1 is a component of HeLa cell DRTF1/E2F. Thus, as in F9 embryonal carcinoma (EC) cell extracts, anti-DP-1 peptide antiserum disrupted HeLa cell DRTF1/E2F in a specific fashion since its effects were competed by including in the binding reaction the homologous, but not an unrelated, peptide (Figure 1a, compare tracks 2–5 with 6–9). Anti-DP-1 antiserum was used to immunoprecipitate DRTF1/E2F from HeLa cell extracts, the immunoprecipitate subsequently being released and then immunoblotted with an anti-E2F-1 monoclonal antibody. The DRTF1/E2F DNA binding activity immunoprecipitated by anti-DP-1 (Figure 1b, compare tracks 4 and 7) contained the E2F-1 protein because immunoblotting the immunoprecipitates with an anti-E2F-1 monoclonal antibody revealed a polypeptide with the molecular weight expected for E2F-1 (Figure 1c, track 4, indicated by arrow). The presence of E2F-1 was dependent upon the anti-DP-1 activity since it was not present when the immunoprecipitation was performed in the presence of the homologous peptide (Figure 1c, compare tracks 3 and 4). Thus, DP-1 and E2F-1 exist as a complex in HeLa cell extracts.

DP-1 and E2F-1 interact in vitro in a DNA binding heterodimer

Both DP-1 and E2F-1 contain sequence-specific DNA binding domains, located in similar positions of each protein (between amino acid residues 84 and 204 in DP-1, and 89 and 191 in E2F-1; Girling *et al.*, 1993), which contain a region of similarity that extends outside of the DNA binding domain, to amino acid residue 249 in DP-1. In agreement with previous studies (Helin *et al.*, 1992; Kaelin *et al.*, 1992; Girling *et al.*, 1993) both DP-1 and E2F-1 alone were able to bind to the E2F site, either in the context of the adenovirus E2A promoter (Figure 2a, tracks 2 and 3) or as a single E2F site (which was apparent on increased exposure of Figure 2a, track 6; data not shown). The DNA binding activity of DP-1 was somewhat less than that of E2F-1, the reasons for which are currently unclear. However, when both proteins were

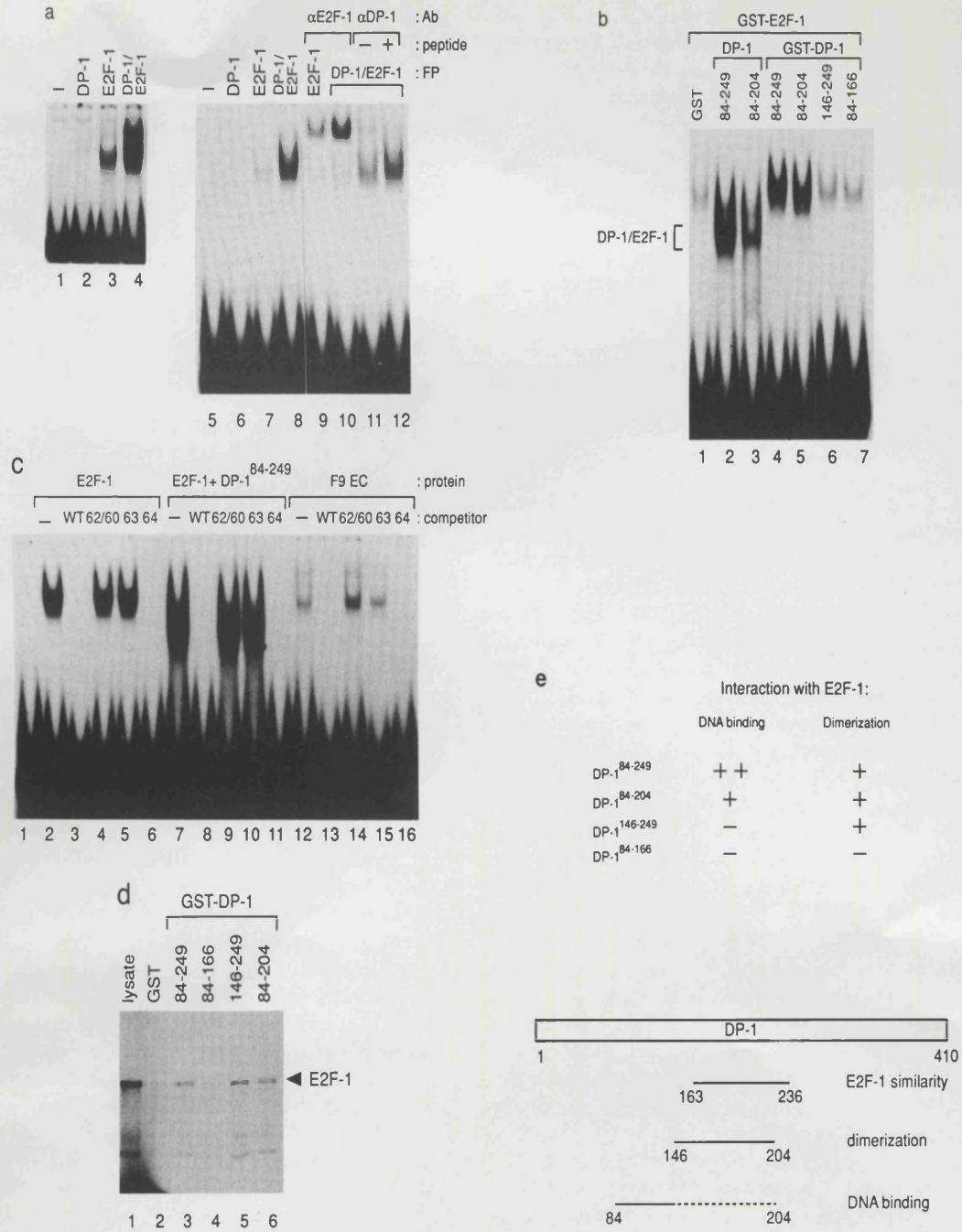

Fig. 2. DP-1 and E2F-1 bind to the E2F site as a complex. (**a**) DP-1 and E2F-1 interact synergistically in DNA binding to the E2F site: GST–DP-1^{59-410} (~25 ng) or GST–E2F-1^{89-437} (~50 ng) were assayed either alone (tracks 2, 3, 6 and 7) or together (tracks 4 and 8) for binding to the adenovirus E2A promoter (tracks 1–4) or the distal E2F site taken from the E2A promoter (tracks 5–8); tracks 1 and 5 show the binding sites alone. Note that a DNA binding complex was apparent in track 6 upon increased exposure (data not shown). The E2F site specificity of the complexes was confirmed by performing the appropriate competition experiments (data not shown). The effect of anti-E2F-1 (tracks 9 and 10) or anti-DP-1 (tracks 11 and 12; anti-peptide 18; Girling *et al.*, 1993) was assessed on GST–E2F-1^{89-437} alone (track 9) or GST–E2F-1^{89-437} and GST–DP-1^{59-410} together (tracks 10, 11 and 12). In addition, the reactions in tracks 11 and 12 contain either an unrelated (track 11) or the homologous (peptide 18; track 12) peptides. (**b**) DP-1 and E2F-1 form DNA binding heteromers: GST–E2F-1^{89-437} (~50 ng) was incubated with a control GST fusion protein (~300 ng; track 1) or DP-1^{84-249} or DP-1^{84-204} (~150 ng, released after cleavage with thrombin; tracks 2 and 3), GST–DP-1^{84-249}, GST–DP-1^{84-204}, GST–DP-$1^{146-249}$ or GST–DP-1^{84-166} (~300 ng, without cleavage; tracks 4, 5, 6 and 7). (**c**) Sequence specificity of the E2F-1^{89-437}/DP-1^{84-249} heteromer: the DNA sequence specificity of complexes formed by either GST–E2F-1^{89-437} (50 ng; tracks 2–6) or GST–E2F-1^{89-437} with DP-1^{84-249} (50 ng and 150 ng respectively; tracks 7–11) was determined by competing with the wild-type or mutated derivatives of the distal E2F site from the adenovirus E2A promoter (~100-fold molar excess of the binding sites indicated). For comparison, a similar experiment is shown in an F9 EC cell extract (tracks 12–16). Both mono- and heteromeric DNA binding complexes had very similar sequence specificities to F9 EC cell DRTF1/E2F. Track 1 shows the probe alone. Details of the competing binding sites are given in Materials and methods. (**d**) DP-1 contains a dimerization domain: the indicated regions of DP-1 were expressed as GST fusion proteins (tracks 3–6) and ~2 μg incubated with 5 μl of a reticulocyte lysate containing translated wild-type E2F-1^{1-437}. GST fusion proteins, or GST protein alone (track 2), were collected with glutathione–agarose beads and bound E2F-1 polypeptide released. Track 1 shows the lysate with the E2F-1 polypeptide. Note that DP-$1^{146-249}$ binds to E2F-1, and that usually between 10 and 20% of the input E2F-1 was specifically retained. (**e**) Summary of the data and molecular properties of DP-1. The C-terminal border of the DNA binding domain, which is known to lie within the region indicated by the broken line, has not been defined.

present in the same binding reaction, increased E2F site DNA binding activity was apparent (Figure 2a, compare tracks 2 and 3 with 4, and 6 and 7 with 8). The DNA binding activity was much greater than that expected from an additive effect of the two DNA binding activities, indicating that together DP-1 and E2F-1 recognize the E2F site synergistically.

The presence of both DP-1 and E2F-1 in the DNA binding complex was confirmed using antisera specific for either protein. An anti-E2F-1 peptide antiserum supershifted the DNA binding complex (Figure 2a, compare track 8 with track 10), whereas the anti-DP-1 peptide antiserum inhibited the DNA binding activity (Figure 2a, compare tracks 11 and 12). However, the effect of the anti-DP-1 antiserum was less dramatic, the reasons for which are unclear, but may be related to the availability of the epitope which, for this antibody, is located close to the DNA binding domain of DP-1 (Girling *et al.*, 1993).

We used this assay to determine the regions in DP-1 which are necessary to produce a DNA binding complex with E2F-1. Thus, various derivatives of DP-1 were expressed as GST fusion proteins, cleaved with thrombin, and then assessed for any interaction with E2F-1. Since these derivatives of DP-1 were truncated versions of the wild-type protein, any of them which was able to interact with E2F-1 to produce functional DNA binding activity should result in a smaller and hence faster migrating DNA binding complex. Moreover, if only one faster migrating complex were apparent, a heterodimer of the two proteins would be the most likely explanation. Indeed, when either DP-1^{84-249} or DP-1^{84-204} were mixed with E2F-1 (GST–E2F-1^{89-437}), a faster migrating DNA binding complex was formed relative to E2F-1 alone (Figure 2b, compare track 1 with 2 and 3) or E2F-1/DP-1 (Figure 2a) indicating that these two derivatives of DP-1 were able to interact with E2F-1 and that they were likely to form a heterodimer. Again, the DNA binding activity of the E2F-1/DP-1^{84-249} complex was greater than that for E2F-1 alone (Figure 2b, compare track 1 with 2 and 3) or DP-1^{84-249} which had low DNA binding activity in the conditions employed in this assay (data not shown) but nevertheless can specifically recognize the E2F site (Girling *et al.*, 1993). The DNA binding activity of the E2F-1/DP-1^{84-204} reaction was less than E2F-1/DP-1^{84-249} indicating that the region of DP-1 between amino acid residues 204 and 249, which shows significant similarity to E2F-1 (Girling *et al.*, 1993), also influences DNA binding activity. The synergistic DNA binding effects of DP-1^{84-249} and DP-1^{84-204} were also apparent when the uncleaved GST fusion proteins were mixed with E2F-1 although, because of their increased size, a faster migrating DNA binding complex did not occur (Figure 2b, compare track 1 with 4 and 5). Further deletion of this region, either from the N- or C-terminus (DP-$1^{146-249}$ and DP-1^{84-166} respectively) yielded derivatives of DP-1 that failed to form a DNA binding complex with E2F-1 either as GST fusion proteins (Figure 2b, compare track 1 with 6 and 7) or after cleavage (data not shown), indicating that DP-1^{84-204} is the minimal region so far defined which is capable of producing a DNA binding complex with E2F-1.

Analysis of the DNA binding specificity of the E2F-1/DP-1^{84-249} complex with a panel of binding sites derived from the adenovirus E2A promoter distal E2F site (La Thangue *et al.*, 1990; Shivji and La Thangue, 1991) indicated that it was very similar to that for E2F-1 alone (Figure 2c, compare tracks 3–6 with 8–11) and, furthermore, the DRTF1/E2F site DNA binding activity defined in F9 EC cell extracts (Figure 2c, compare tracks 13–16).

To characterize further the interaction between DP-1 and E2F-1 we employed an assay in which *in vitro* transcribed and translated E2F-1 polypeptide could bind to DP-1–GST fusion proteins. The ability of E2F-1 to interact with DP-1 was assessed after collecting the GST fusion protein with glutathione–agarose beads and subsequently releasing the bound E2F-1 polypeptide. Both DP-1^{84-249} and DP-1^{84-204} could interact with E2F-1 since the amount of E2F-1 bound to GST–DP-1^{84-249} and GST–DP-1^{84-204} was significantly greater than that bound by the GST beads alone (Figure 2d, compare track 2 with 3 and 6), consistent with their ability to form a DNA binding heteromer (Figure 2b). DP-$1^{146-249}$ also bound to E2F-1 whereas DP-1^{84-166} failed to do so (Figure 2d, compare track 2 with 4 and 5). DP-$1^{146-249}$ therefore contains a domain, which based on the earlier results is likely to be a dimerization domain, that allows it to interact with E2F-1 but lacks sufficient amino acid sequence for the heteromer to bind to DNA. The additional information in DP-1^{84-249} is necessary for the complex to bind to DNA. These data therefore suggest that the region of DP-1 which is similar to E2F-1 (amino acids 163–236) contains a dimerization domain, and that additional N-terminal sequence is necessary for DNA binding activity. A summary of these data is presented in Figure 2e.

DP-1 and E2F-1 interact in yeast cells

To determine if DP-1 and E2F-1 interact directly *in vivo* we adapted a previously described assay system in yeast cells (Fields and Song, 1989) which utilized expression vectors that synthesize two hybrid proteins, one derived from DP-1 and the other from E2F-1. In the first, the DP-1 coding sequence was fused to the DNA binding domain of the bacterial LexA protein, to make pLEX.DP-1 and in the second, pGAD.E2F-1, the E2F-1 coding sequence was fused with the acidic transcriptioal activation domain (AAD) of the yeast Gal4 protein. pLEX.DP-1 failed to activate a reporter construct driven by a LexA binding site, whereas a hybrid protein that contained the *trans*-activation domain taken from the p53 protein could (Figure 3). However, when pLEX.DP-1 and pGAD.E2F-1 were expressed together, the transcriptional activity of the LexA reporter construct was increased considerably (~75-fold) relative to its activity when either pLEX.DP-1 or pGAD.E2F-1 were expressed alone (Figure 3). This result, combined with the earlier studies presented in this paper, strongly suggest that DP-1 and E2F-1 interact directly *in vivo*. Using the same experimental strategy, we have failed to obtain evidence for an interaction between pLEX.DP-1 and pGAD.DP-1 (V.M.Buck, L.H.Johnston and N.B.La Thangue, data not shown).

DP-1 regulates E2F site-dependent transcription in vivo

Increasing the levels of the DP-1 protein in a variety of mammalian cells (for example, F9 EC, SAOS-2 and 3T3) and growth conditions failed to stimulate significantly the transcriptional activity of an E2F site-dependent reporter (data not shown). In order to assess if DP-1 and E2F-1 functionally interact we therefore had to take an alternative approach which involved developing the appropriate assay

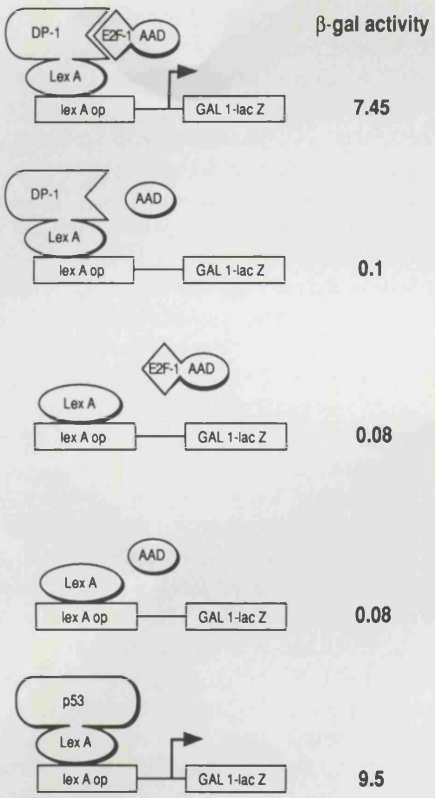

Fig. 3. DP-1 and E2F-1 interact in yeast cells: summary of results. Details of the expression vectors and reporter construct are described in Materials and methods.

in *Drosophila* SL2 cells, a cell system which has been used previously to study the activity of mammalian transcription factors (Courey and Tjian, 1988). These cells were particularly appropriate for this analysis because the endogenous E2F site DNA binding activity is very low when assayed by gel retardation (data not shown). In order to assess the functional interaction of DP-1 and E2F-1, we determined the effects of each protein alone and when expressed together on the transcriptional activity of p3×WT, a reporter construct driven by three E2F sites (Figure 4a; Zamanian and La Thangue, 1991). Thus, E2F-1 was able to activate p3×WT in a dose-dependent fashion (Figure 4b and c, compare lanes 1 and 2) whereas DP-1 failed to do so (Figure 4b and c, compare lanes 3 and 4), results which are similar to the behaviour of E2F-1 and DP-1 in mammalian cells (Helin *et al.*, 1992; Kaelin *et al.*, 1993; and data not shown). However, when DP-1 and E2F-1 were expressed together much greater E2F site-dependent transcriptional activation was apparent relative to either alone (Figure 4b and c, compare lanes 1, 3 and 5). Moreover, this synergistic effect was titratable because increasing the level of DP-1 produced more E2F site-dependent transcription (Figure 4b and c, compare lanes 1, 5 and 6) and specific since co-expression of an unrelated DNA binding, derived from the Gal4 protein, did not produce any significant effects (Figure 4b and c, compare lanes 5 and 6 with 7 and 8). Moreover, similar expreriments performed with p3×MT indicated that this activation was specific for the wild-type E2F site (data not shown). We conclude therefore that DP-1 and E2F-1 functionally interact in E2F site-dependent transcription and that this interaction is synergistic.

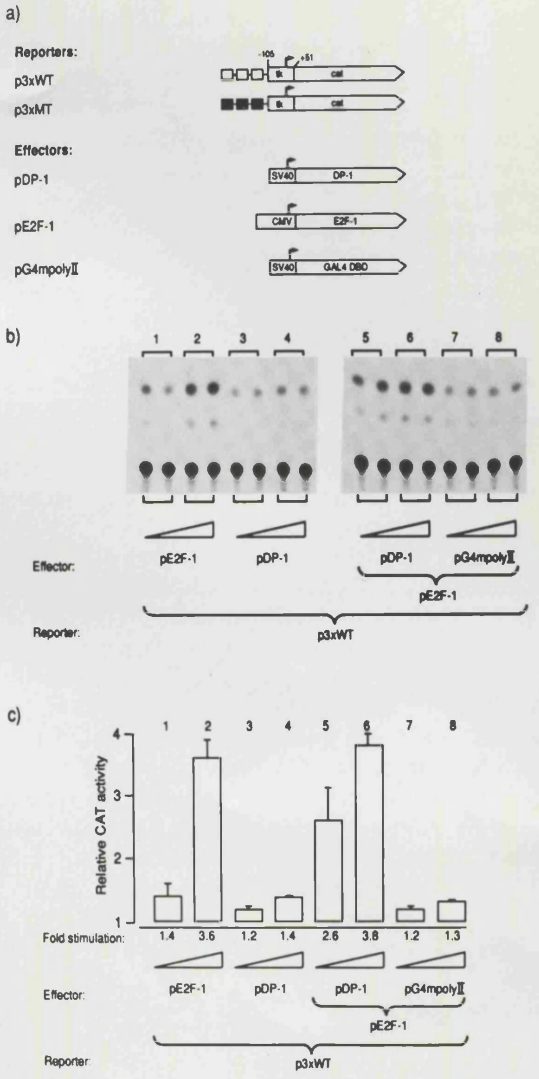

Fig. 4. Functional synergy between DP-1 and E2F-1 in *Drosophila* SL2 cells. (**a**) Summary of constructs: p3×WT and p3×MT have been described previously (Zamanaian and La Thangue, 1992). pDP-1 and pE2F-1 contain full length proteins, and pG4MpolyII the Gal4 DNA binding domain. (**b**) and (**c**) SL2 cells were transfected with p3×WT and the indicated expression vectors. The amounts of expression vector in each treatment were as follows: 50 ng (lanes 1, 5, 6, 7 and 8) or 500 ng (lane 2) for E2F-1, 5 μg (lanes 3 and 5) or 10 μg (lanes 4 and 6) for DP-1, and 3.7 μg (lane 7) or 7.0 μg (lane 8) for pG4Mpoly II. All values are expressed relative to p3×WT alone which was given an arbitrary value of 1.0, and are representative of at least three separate experiments. (b) shows an example of the crude data which is quantitatively represented in (c).

DP-1 and E2F-1 activate E2F site-dependent transcription yeast cells

We next assessed if DP-1 and E2F-1 can functionally interact in E2F site-dependent transcription in yeast cells. For this, we used constructs in which the yeast *cyc1* promoter was driven by E2F binding sites taken from the adenovirus E2A promoter. In p4×WT CYC1, four E2F binding sites drive the *cyc1* promoter (Figure 5a), activating transcription ~12-fold above the activity of p4×MT CYC1 (data not shown). This transcriptional activity could be stimulated further upon introduction of the E2F-1 expression vector, pGAD.E2F-1. Thus, pGAD.E2F-1 increased the transcriptional activity of p4×WT CYC1 ~10-fold, compared with the small effect that the DP-1 expression

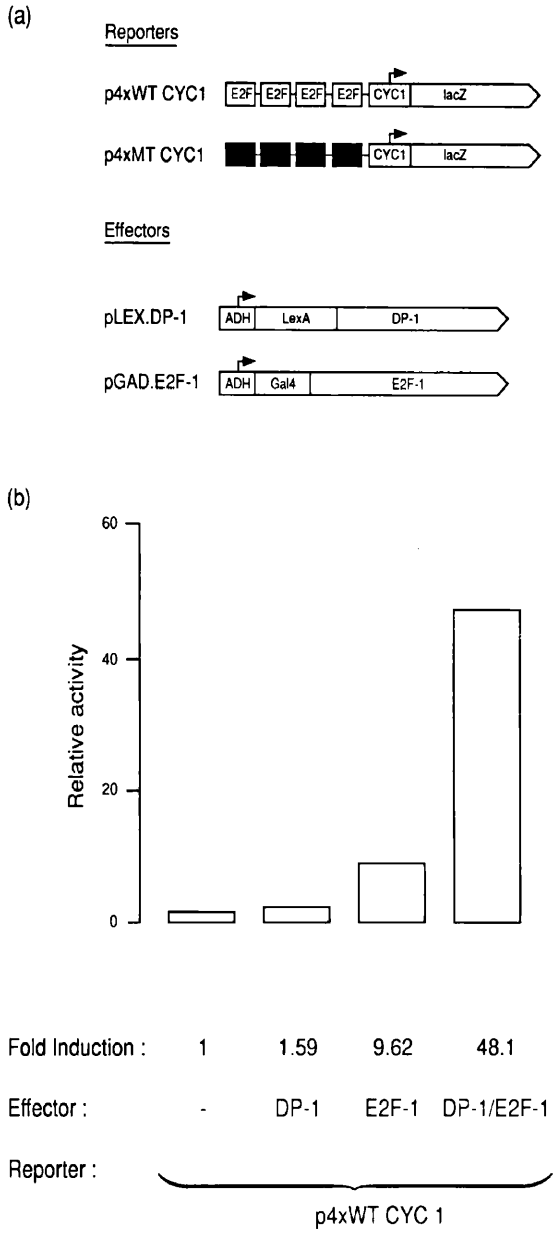

Fig. 5. DP-1 and E2F-1 activate E2F site-dependent transcription in yeast cells. (**a**) Summary of constructs. (**b**) β-galactosidase activity was measured in *S.cerevisiae* strain W3031a carrying p4×WT CYC1 and the indicated effector expression vector. All values are expressed relative to the activity of p4×WT CYC1 which was given an arbitrary value of 1.0 and are representative of at least three separate experiments.

vector, pLEX.DP-1, had on the same reporter construct (Figure 5b). However, when E2F-1 and DP-1 were expressed together, the activity of p4×WT CYC1 was even greater, and usually ~50-fold above basal p4×WT CYC1 activity (Figure 5b); the activity of p4×MT CYC1 was not significantly affected by either the E2F-1 or DP-1 expression vector (data not shown). We conclude that DP-1 and E2F-1 activate E2F site-dependent transcription more efficiently when present together than either does alone, suggesting again that DP-1 and E2F-1 interact synergistically in E2F site-dependent transcriptional activation.

Discussion

DP-1 and E2F-1 interact in mammalian cells

Previous studies have indicated that DP-1 is a universal component of DRTF1/E2F DNA binding activity in F9 EC cells because all the DNA binding complexes that occur on the E2F site are disrupted by anti-DP-1 antibodies (Girling *et al.*, 1993). The same situation exists in HeLa cell extracts where all the DRTF1/E2F DNA binding complexes are affected by anti-DP-1 antibodies (Figure 1a). Based on these observations, and combined with studies performed in other cell types (L.R.Bandara, T.S.Sørensen, M.Zamanian and N.B.La Thangue, in preparation), we believe that DP-1 is a frequent component of transcription factor DRTF1/E2F.

In the light of these observations, we were interested to determine if DP-1 can interact with the other E2F site DNA binding protein, E2F-1 (Helin *et al.*, 1992; Kaelin *et al.*, 1992) and, furthermore, establish whether such an interaction occurs in physiological conditions. Our results indicate that DP-1 and E2F-1 exist as a complex in HeLa cell extracts, and thus imply that at least a proportion of the total DRTF1/E2F DNA binding activity is likely to be a heteromeric complex involving DP-1 and E2F-1. It is unclear, at the moment, just how much of the DRTF1/E2F DNA binding activity is a complex of DP-1 and E2F-1 because our attempts to use anti-E2F-1 antibodies to affect the DNA binding activity in gel retardation assays have been unsuccessful (data not shown). Also, we cannot rule out that other proteins bind to DP-1, in the place of E2F-1. In fact, this would seem a likely possibility because several polypeptides in affinity purified DRTF1/E2F with distinct molecular weights (from 45 to 55 000) are capable of specifically binding to the E2F site (Shivji and La Thangue, 1991; Girling *et al.*, 1993).

A physical interaction between DP-1 and E2F-1 in vitro and in yeast cells

We established that DP-1 and E2F-1 can interact directly by studying their DNA binding properties in gel retardation assays. DP-1 and E2F-1 formed a heteromeric DNA binding complex with exactly the same DNA binding specificity as that possessed by DRTF1/E2F in crude cell extracts (Figure 2 and La Thangue *et al.*, 1990). Moreover, it was apparent that the DNA binding activity of the heteromer was considerably greater than for E2F-1 or DP-1 alone, suggesting that DP-1 and E2F-1 interact synergistically. A molecular analysis of the region in DP-1 which was necessary to form a DNA binding complex with E2F-1 indicated that the region of similarity between the two proteins, together with an additional N-terminal domain, was required (summarized in Figure 2e). The region of similarity allowed DP-1 and E2F-1 to bind to each other and thus is likely to constitute a dimerization domain.

We confirmed these observations in yeast cells using an assay which makes use of the modular organization of transcription factors (Fields and Song, 1989). Thus, DP-1 was fused to the bacterial LexA DNA binding domain and, in a separate molecule, E2F-1 to the acidic transcriptional activation domain of the yeast Gal4 protein. In this assay, a functional activation domain is recruited to the LexA-dependent promoter only if there is a physical interaction between the two hybrid proteins. When the two hybrid proteins were expressed together there was strong activation of the LexA-dependent reporter. Thus, DP-1 and E2F-1 are able to interact physically in yeast cells. Moreover, this result indicates that they are able to do so in the absence of DNA binding since the DNA binding specificity was provided by

LexA and thus took place independently of the E2F binding site.

Transcriptional synergy by DP-1 and E2F-1 in vivo

We addressed the functional consequences of the interaction between DP-1 and E2F-1 for E2F site-dependent transcription in both *Drosophila* and yeast cells. We took this approach because our attemps to activate transcription by introducing wild-type DP-1 into mammalian cells have met with limited success, the reasons for which are unclear but may be related to the levels of endogenous DP-1 protein.

Both types of assay, whether performed in *Drosophila* or yeast cells, indicated that DP-1 and E2F-1 interact synergistically in E2F site-dependent transcription since when both proteins were expressed together transcriptional activation was more efficient than for either protein alone. A likely explanation for such an effect is that the DNA binding activity of the DP-1/E2F-1 heterodimer is more stable than either homodimer and thus transcriptional activation is more efficient. This idea would be entirely consistent with the *in vitro* DNA binding data presented earlier in this study which suggested that DP-1 and E2F-1 interact synergistically. We cannot, however, rule out other potential influences, such as activation of a cryptic transcriptional activation domain in the DP-1/E2F-1 heterodimer and, in fact, recent experiments have suggested that such a possibility is likely to be correct (M.Zamanian and N.B.La Thangue, unpublished data).

In conclusion, we have demonstrated that DP-1 and E2F-1 interact in transcription factor DRTF1/E2F, to produce a DNA binding complex which is the preferred state over either homodimer. Since E2F-1 can bind to pRb (Helin *et al.*, 1992; Kaelin *et al.*, 1992) in such a complex it is likely that E2F-1 will provide an interface recognized by pRb, thus enabling the transcriptional activity of this particular E2F site DNA binding activity to be regulated by pRb. It is possible that other molecules heterodimerize with DP-1, in the place of (and perhaps related to) E2F-1, providing an interface recognized by other proteins which are known to interact with DRTF1/E2F, such as p107 (Zamanian and La Thangue, 1993), thus allowing these molecules also to regulate E2F site-dependent transcription. We suggest therefore that distinct heterodimers recognize the E2F site, with DP-1 as a common component, enabling different molecules, such as pRb and p107, to integrate their biological activities with the transcription apparatus and hence to regulate genes driven by DRTF1/E2F.

Materials and methods

Preparation of cell extracts, gel retardation and immunochemical techniques

Cell extracts were prepared as previously described (La Thangue *et al.*, 1990). Gel retardation in F9 EC and HeLa cell extracts (~6.0 μg) in the presence of anti-DP-1 was performed as previously described (Girling *et al.*, 1993), and immunoprecipitation with anti-DP-1 from HeLa cell extracts was performed by standard procedures. The immunoprecipitates were treated with 1% DOC and 1.5% NP40 and the detergent released material assayed for DRTF1/E2F by gel retardation and the presence of E2F-1 by immunoblotting with the anti-E2F-1 monoclonal antibody SQ41 (Kaelin *et al.*, 1992). The anti-DP-1 antibodies, anti-peptide A and anti-peptide 18, have been previously described (Girling *et al.*, 1993). Rabbit anti-E2F-1 antiserum (antiserum 134) was raised against a peptide which represents E2F-1 amino acid sequence 426–437. The sequences of the binding sites used to assess DNA binding specificity were derived from the adenovirus E2A promoter (−71 to −50) and were as follows: WT; TAGTTTTCGCGCTTAAATTTGA; 62/60, TAGTTTTCGATATTAAATTTGA; 63, TAGTTTCTCGCTTAAATTTGA; 64, TAGTTTTAGCGCTTAAATTTGA. In Figure 2a (tracks 1–4), the adenovirus E2A promoter (−96 to +68) was used; in all other cases, the distal E2F site in the E2A promoter (sequences −71 to −50) was used. About 100-fold excess of competing binding sites were used in the gel retardation assays.

Fusion proteins and in vitro translation

DP-1 and E2F-1 were expressed as, and released from, GST fusion proteins as previously described (Girling *et al.*, 1993). About 100-fold excess of the competing binding sites were used in gel retardation assays, with the binding site taken from the adenovirus E2A promoter (−71 to −50). The wild-type E2F-1 coding sequence was transcribed and further translated using reticulocyte lysate (Promega) and radiolabelled with [^{35}S]methionine. In the dimerization assay (Figure 2d), GST–DP-1 fusion protein was incubated with E2F-1 polypeptide for 30 min at 30°C, collected with glutathione–agarose (Sigma), and washed repeatedly with 0.1% NP40 in PBSA. Bound E2F-1 polypeptide was released by denaturation in SDS sample buffer and resolved in a 10% polyacrylamide gel.

Yeast assays

pBTM116 contains the complete LexA coding sequence (1–202) under the control of the yeast *ADH1* promoter. pLEX.DP-1 carries the coding sequence for DP-1 (from amino acid 59 to the C-terminus) downstream of the LexA coding sequence in pBTM116. pBTM126 carries the wild-type murine p53 coding sequence (amino acids 1–346) downstream of the LexA DNA binding domain. pGAD.L6 is a derivative of pGAD2F (Chien *et al.*, 1991) containing the Gal4 transcription activating domain (from amino acid residue 768 to 881) under the control of yeast *ADH1* promoter. pGAD.E2F-1 contains the entire E2F-1 coding sequence (from amino acid 1 to 437) downstream of the Gal4 activation domain. p4×WT CYC1 and p4×MT CYC1 were derived from pLGΔ178 (Guarente and Mason, 1983). The wild-type E2F site was taken from the −71 to −50 region of the adenovirus E2A promoter and the mutant site was mutated in nucleotides −62 to −60 (La Thangue *et al.*, 1990). For the yeast interaction assay (Figure 3), the indicated expression vectors were transformed into the yeast strain CTY10-5d (*MATa ade2 trp1-901 leu2-3, 112 his3-200 gal4 gal80 URA3::lexAop-lacZ*) which contains an integrated plasmid which carries two copies of a 78 bp oligonucleotide, each copy containing two colE1 operators or four binding sites for LexA dimers upstream of the transcription start site of GAL1–lacZ. For the yeast E2F site-dependent transcription assay (Figure 5), the yeast strain W3031a (*MATa ade 2-100 trp1-1 leu2-3,112 his3-11 ura3*) was used carrying either p4×WT CYC1 or p4×MT CYC1 and was transformed with the indicated expression vectors. β-galactosidase activity of mid-log phase cultures was quantitated as described previously (Johnson *et al.*, 1986). β-galactosidase activity was measured for at least three independent transformants.

Transfection of Drosophila tissue culture cells

Reporter constructs were all derived from pBLcat2 and have been described previously (Zamanian and La Thangue, 1992). Open and solid boxes denote wild-type and mutant E2F binding sites, respectively. pDP-1 encodes a complete DP-1 protein, and pG4mpolyII the Gal4 DNA binding domain (Webster *et al.*, 1989). pE2F-1 has been previously described as pCMV RBAP-1 (Kaelin *et al.*, 1982). Cells were transfected by the calcium phosphate procedure and harvested 40–45 h later and for each transfection, pBluescript KS was included to maintain the final DNA concentration constant. All transfections included an internal control pCMV β-gal. The assay for CAT activity, correction for transfection efficiency and quantitation of TLC plates have been described previously (Zamanian and La Thangue, 1992).

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A new component of the transcription factor DRTF1/E2F

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TRANSCRIPTION factor DRTF1/E2F coordinates events in the cell cycle with transcription by its cyclical interactions with important regulators of cellular proliferation like the retinoblastoma tumour-suppressor gene product (Rb) and the Rb-related protein, p107 (refs 1–8). DRTF1/E2F binding sites occur in the control regions of genes involved in proliferation[9,10], and both Rb and p107 repress the capacity of DRTF1/E2F to activate transcription (refs 11, 12; M. Zamanian and N.B.L.T., manuscript submitted). Mutant Rb proteins isolated from tumour cells are unable to bind DRTF1/E2F (refs 11–13), and certain viral oncoproteins, such as adenovirus E1A, sequester Rb and p107 in order to free active DRTF1/E2F (refs 5, 11, 12, 14, 15). Here we report the isolation of a complementary DNA encoding DRTF1-polypeptide-1 (DP-1), a major sequence-specific binding protein that is present in DRTF1/E2F, including Rb- and p107-associated DRTF1/E2F. The DNA-binding domain of DP-1 contains a region that resembles that of E2F-1 (refs 16, 17), and recognizes the same sequence. DRTF1/E2F thus appears to contain at least two sequence-specific DNA-binding proteins.

E2F was defined as an activity induced in HeLa cells after adenovirus infection[18], whereas DRTF1 was defined as a transcription factor downregulated during the differentiation of F9 embryonal carcinoma (EC) cells[19,20]. As they bind to similar DNA sequences and associate with the same proteins[5,6,8,21–24], they are likely to be closely related. Here we refer to the DNA binding activity in whole cell extracts which recognizes the E2F site as DRTF1/E2F.

DRTF1 was purified from F9 EC whole cell extracts by sequential applications to a DNA-binding-site affinity matrix containing either a wild-type or mutant binding site[25]. The DNA-binding site was taken from a region of the adenovirus E2A promoter (−71 to −50) that contains a high-affinity E2F binding site[20]. We routinely purified a group of polypeptides (Fig. 1*a*, track 2) that could activate transcription *in vitro* in a binding site-dependent fashion[25]. Several polypeptides in the affinity-purified material specifically bound to the wild-type site (Fig. 1*a*, compare tracks 5 to 6; ref. 25). One polypeptide (M_r ~46,000 (46K); Fig. 1*a*, track 2, indicated as p46) was excised and digested with a lysylendopeptidase; ten of the resulting fragments were purified and sequenced (Fig. 1*b*; peptides boxed). We predicted the DNA sequences that encode two of the peptides (Fig. 1*b*, legend) and then used these as primers to amplify a cDNA fragment derived from F9 EC RNA. Clones representing the complete coding sequence of p46 (referred to as DP-1) were finally isolated from an F9 EC cDNA library.

The complete DP-1 coding sequence contains an open reading frame encoding 429 in-frame amino acids that includes eight of the peptides from the p46 sequence (Fig. 1*b*). The cDNA probably includes the initiating methionine as there is an in-frame termination codon immediately upstream and because the sequence flanking and including this methionine codon would be an efficient translation initiation signal[26]. By northern analysis of poly(A)$^+$ F9 EC RNA, the DP-1 transcript was estimated as ~2.8 kilobases (kb) (data not shown); it is expressed in many different cell types and in a wide range of tissues during murine embryogenesis (J.F.P., P. Tassios and N.B.L.T, manuscript in preparation). Homology searches of the Leeds and Swiss protein data bases failed to detect any significant similarity with any other protein. But a small region within the DNA-binding domain of DP-1 has significant similarity to an analogous region in the DNA-binding domain of E2F-1.

To confirm that DP-1 is a component of DRTF1/E2F, peptide antisera were raised against DP-1 (Fig. 2*e*). Immunoblotting affinity-purified DRTF1/E2F with anti-peptide 15 revealed two polypeptides with apparent M_rs 46K and 55K (Fig. 2*a*, track 2) that were recognized by the antiserum (Fig. 2*a*; compare tracks 5 and 6). We confirmed that DP-1 was part of the DRTF1/E2F DNA-binding complex in F9 EC cell extracts from which DRTF1 resolves as complexes DRTF1a, b and c (ref. 25). Addition of anti-peptide A, but not the preimmune serum, causes either a supershift and concomitant reduction in DRTF1a, b and c (Fig. 2*b*, antiserum 3; compare tracks 1 and 2) or abolishes all DRTF1 complexes (Fig. 2*b*, antiserum 4; compare tracks 5 and 6). The effects of both antisera 3 and 4 could be competed by peptide A but not the unrelated peptide 1 (Fig. 2*b*; compare tracks 3, 4 and 7, 8). Extracts from other cell types, including HeLa, 293, PCC3, PCC4, SAOS-2, and several SV40-transformed mouse-cell lines gave similar results (data not shown). We conclude that DP-1 is a component of DRTF1/E2F and that it is present in all DRTF1/E2F DNA-binding complexes in F9 EC cell extracts.

When anti-peptide A was incubated with affinity-purified DRTF1/E2F, a supershift was apparent (Fig. 2*c*; compare tracks 1, 2 and 5, 6; clearest for antiserum 3). The effect was not as marked as in F9 EC cell extracts, probably because some of the DP-1 polypeptide loses its N-terminal region during purification (data not shown). Therefore we used anti-peptide 18 (Fig. 2*e*; antiserum 11), representing sequence from the DNA-binding domain of DP-1, which specifically inhibits the DNA-binding activity of affinity-purified DRTF1/E2F (Fig. 2*c*; compare tracks 11, 12).

We assessed whether DP-1 was present in DRTF1/E2F complexes that form on different E2F binding sites using the class 1 and class 2 consensus E2F binding sites which were defined by binding site selection[27] and occur in the transcriptional control regions of certain cellular genes[9,10,28]. Anti-peptide A abolishes DRTF1/E2F complexes formed on both class 1 and class 2 binding sites (Fig. 2*d*; compare tracks 3, 4 and 7, 8).

To determine whether DP-1 is present in complexes containing Rb, we immunoprecipitated them with an anti-Rb monoclonal antibody[29] from extracts of the human leukaemic cell line JM, which contain high levels of the DRTF1/E2F–Rb complex[5]. DRTF1/E2F DNA-binding activity was released from Rb by detergent, but not from the immunoprecipitate with the control monoclonal antibody A7 (Fig. 3*a*; compare tracks 2 and 3, and respective depleted extracts, tracks 4 and 5). The presence of DP-1 in Rb-associated DRTF1/E2F was tested with anti-peptide A (antiserum 3) and anti-peptide 18 (antiserum 11). Anti-peptide A caused a supershift (Fig. 3*a*, compare tracks 6 and 7; shift indicated by 's'), whereas anti-peptide 18 caused a reduction in DRTF1/E2F DNA-binding activity (Fig. 3*a*; compare tracks 8 and 9). We conclude that DP-1 co-immunoprecipitates with Rb from JM cell extracts and is therefore likely to associate with Rb *in vivo*.

In F9 EC cell extracts, an anti-p107 antibody disrupted DRTF1a (Fig. 3*b*; compare tracks 2 and 3), which, together with our result that the anti-DP-1 peptide antibodies interfered with DRTF1a (Fig. 2*b*), suggested that p107 and DP-1 exist in the same DRTF1/E2F complex. Furthermore, DRTF1/E2F DNA binding activity released from an anti-p107 immunoprecipitation was specifically reduced by anti-peptide 18 (Fig. 3*b*;

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‡ To whom correspondence should be addressed.

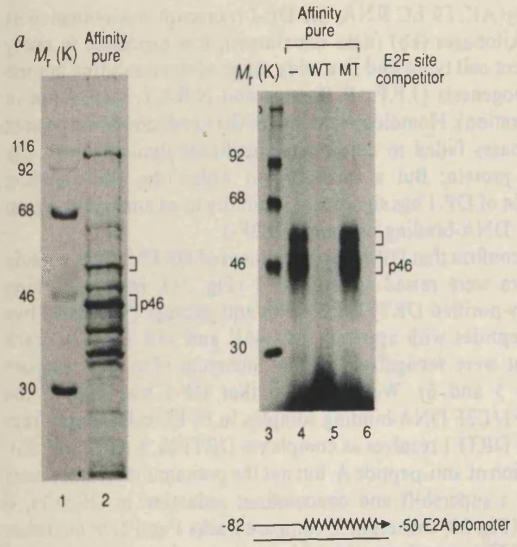
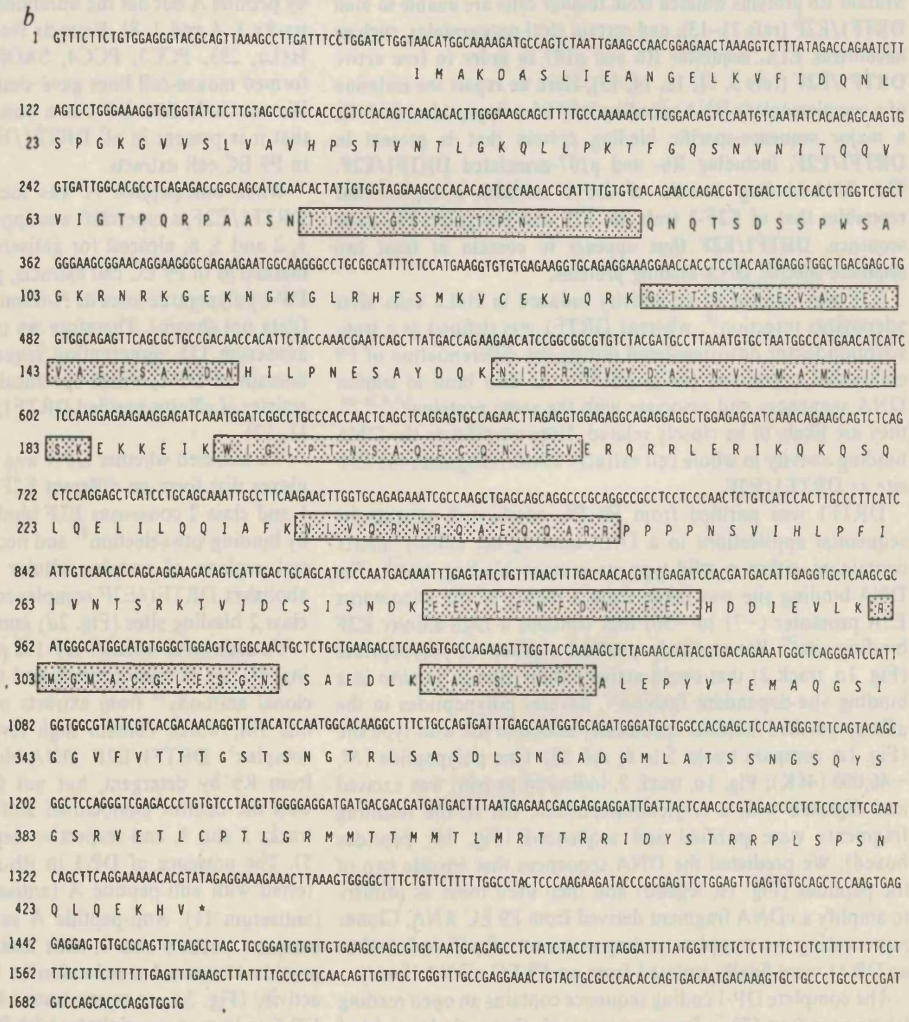


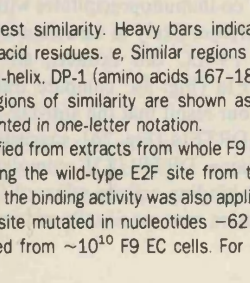
FIG. 1 *a*, Affinity purification of DRTF1/E2F from F9 EC cells. Polypeptides in affinity-purified DRTF1/E2F (about 5 μg from about 5×10^{10} F9 EC cells) were separated by SDS-polyacrylamide gel electrophoresis and stained with Coomassie blue (track 2); track 1 shows M_r standards. DNA-binding polypeptides in affinity-purified DRTF1/E2F were assayed by crosslinking to the adenovirus E2A promoter distal E2A site (binding site details indicated at bottom right) either in the absence (track 4) or presence of competing wild-type (track 5) or mutant (track 6) E2F binding sites; M_r standards shown in track 3. The p46 polypeptide is indicated; upper bracket shows another group of polypeptides which also specifically bind to the E2F site ($M_r \sim 55{,}000$). *b*, Nucleotide sequence of cDNA encoding murine DP-1 and predicted amino-acid sequence. Boxed sequences are peptides obtained from microsequencing of DP-1. Two peptide sequences could not be accounted for from the cDNA sequence. *c*, Comparison of DP-1 and E2F-1. DNA-binding domains in DP-1 and E2F-1 (amino acid 84–204 in DP-1, and 89–191 in E2F-1; ref. 16). A region of significant similarity (amino acid 163–236 in DP-1, 162–226 in E2F-1) includes sequences outside the DNA-binding domain. *d*, Alignment of amino-acid sequences of DP-1 and E2F-1 in regions of highest similarity. Heavy bars indicate identical and light bars similar amino-acid residues. *e*, Similar regions of DP-1 and E2F-1 form an amphipathic α-helix. DP-1 (amino acids 167–183) and E2F-1 (amino acids 166–182) regions of similarity are shown as a helical wheel. Amino acids are represented in one-letter notation.



d

DP-1	84 KGVVSLVAVHPSTVNTLGKQLLPKTFGQSNVNITQQV 123
E2F-1	89 KGVVSLVAVHPSTVNTLGKQLLPKTFGQSNVNITQQV 128
DP-1	124 IGTPQRPAASNLVVGSPPPPNTFISQNQTSDSSPWSA 163
E2F-1	129 IGTPQRPAASNLVVGSPPPPNTFISQNQTSDSSPWSA 168
DP-1	164 KRNRKGEKNGKGLRHFSMKVCEKVQRKGTTSYNEVAEL 203
E2F-1	169 KRNRKGEKNGKGLRHFSMKVCEKVQRKGTTSYNEVAEL 208

e



c, Comparison of DP-1 and E2F-1. DNA-binding domains in DP-1 and E2F-1 (amino acid 84–204 in DP-1, and 89–191 in E2F-1; ref. 16). A region of significant similarity (amino acid 163–236 in DP-1, 162–226 in E2F-1) includes sequences outside the DNA-binding domain. *d*, Alignment of amino-acid sequences of DP-1 and E2F-1 in regions of highest similarity. Heavy bars indicate identical and light bars similar amino-acid residues. *e*, Similar regions of DP-1 and E2F-1 form an amphipathic α-helix. DP-1 (amino acids 167–183) and E2F-1 (amino acids 166–182) regions of similarity are shown as a helical wheel. Amino acids are represented in one-letter notation.

METHODS. DRTF1/E2F was affinity-purified from extracts from whole F9 EC cells[25] using affinity matrices containing the wild-type E2F site from the adenovirus E2A promoter (−71 to −50); the binding activity was also applied to a matrix containing an E2F binding site mutated in nucleotides −62 to −60. About 5.0 μg protein was purified from $\sim 10^{10}$ F9 EC cells. For UV crosslinking of DRTF1/E2F to the E2F site[25], we used ~50 ng of affinity-purified protein. A 100-fold molar excess of either wild-type (−71 to −50) or mutant oligonucleotides was used in competition assays. Affinity-purified DRTF1 was precipitated with TCA, electrophoresed through SDS-polyacrylamide, and p46 electroeluted; p46 was confirmed as pure by electrophoresis and silver staining of eluted material. p46 was digested with a lysylendopeptidase, and peptides purified by HPLC using Aquapore AX-300 and RP-300 in series in 0.1% TFA with a 1% per min acetonitrile gradient. Peak fractions were sequenced by automated Edman degradation in a 477A/120A gas-liquid pulse sequencer (Applied Biosystems). A set of degenerate oligo-

compare track 4 and 5). We conclude therefore that DP-1 can exist in DRTF1/E2F complexes that contain either Rb or p107.

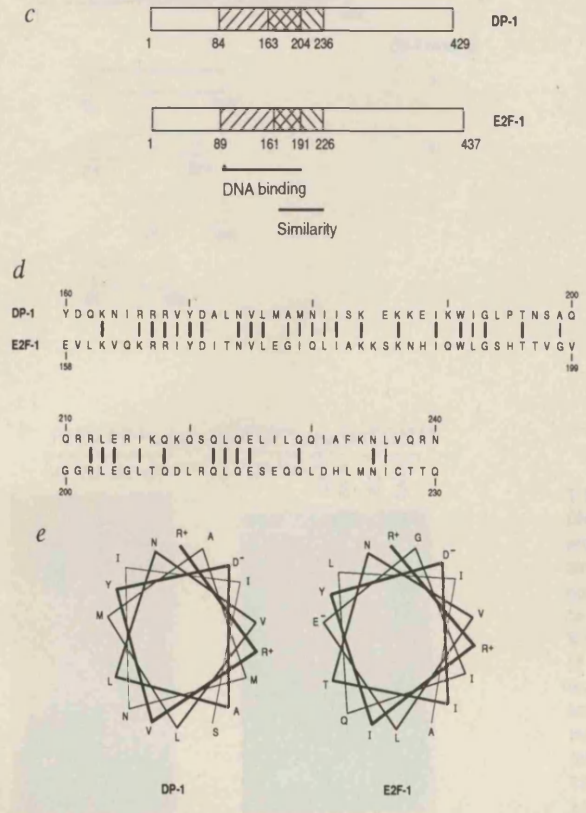
To determine whether DP-1 could bind to the E2F site in a sequence-specific way, regions were expressed as glutathione-*S*-transferase (GST) fusion proteins (Fig. 4*a*), affinity-purified and tested for DNA-binding activity. The smallest region defined that retains sequence-specific DNA-binding activity contains DP-1 protein sequence from amino-acid residue 84 to 204 (DP-1_{84-204}; Fig. 4*b*, compare tracks 4 and 5); in the same conditions, an unrelated sequence-specific transcription factor, GATA-1 (ref. 30), failed to bind to the wild-type E2F site (Fig. 4*b*; compare tracks 8, 9 and 10, 11). Smaller regions of DP-1 (DP-$1_{146-249}$ and DP-1_{84-166}) failed to bind to the E2F site, whereas DP-1_{84-249} retained sequence-specific DNA binding (Fig. 4*a* and *b*; compare track 6 and 7). Thus DP-1 has DNA-binding specificity which is similar to DRTF1/E2F.

We noticed a region (DP-1 residues 160–200) with significant similarity to a sequence in the DNA-binding domain of E2F-1 (Fig. 1*c*), in which 42% is identical and 70% similar (Fig. 1*d*). These regions could include two α-helixes, one of which would be amphipathic (helical wheel in Fig. 1*e*). As DP-1 and E2F-1 bind to the same sequence (this work, and refs 16, 17), this

region of similarity may be involved in recognizing an E2F binding site. This idea is supported by the potential similarity of two other DNA-binding proteins that regulate transcription during the yeast cell cycle. These proteins, encoded by *SW14* and *cdc10*, bind to a DNA sequence that resembles the E2F site[31], and contain a region within their DNA-binding domains with features in common with the DP-1/E2F-1 α-helical region already discussed (Fig. 1*e*, and ref. 32).

Another region of similarity between DP-1 and E2F-1 is apparent outside the DNA-binding domain (DP-1 amino acids 210–240, 41% identity; Fig. 1*d*) which, like the earlier region, could form an amphipathic α-helix. It is possible that DP-1 and E2F-1 interact because DP-1 is present in all DRTF1/E2F complexes, some of which contain E2F-1, rather as in the transcription factor AP-1, in which different proteins interact through related domains[33].

The region of similarity between DP-1 and E2F-1 is likely to be an important part of their DNA-binding domains, but we cannot distinguish whether it is involved in recognizing the E2F sequence *per se* or if it provides a dimerization domain which then allows sequence-specific recognition. We believe, however, that it includes the DNA-recognition domain because of its distant, but significant, similarity with the DNA-binding domains of the yeast cell cycle-regulating proteins SWI4 and cdc10 (ref. 32). □



nucleotide primers were synthesized on the basis of amino-acid sequence in peptides 6 (75–91) and 5 (235–249) as follows: C-terminal regions of peptide 6 (PNTHFV), 5′-CGCGGATCCCC(ACGT)AA(CT)AC(ACGT)CA(CT)-TT(CT)GT-3′; and peptide 5 (AQESQN), antisense strand 5′-CGCGGATCCA-(AG)(AG)TT(CT)TG(ACGT)(CG)(AT)(CT)TC(CT)TG(ACGT)GC-3′; both oligonucleotides included a linker sequence at the 5′ end. Peptide 5 antisense oligonucleotide was used to synthesize cDNA from F9 EC cell RNA which was then used in a PCR with both peptide 6 and 5 primers. Products were subcloned, sequenced and cDNAs derived from DP-1 RNA identified by the presence of peptides 21 and 3, two further peptides obtained by microsequencing peptides derived from p46, which are located between peptides 6 and 5. This cDNA fragment was used to screen several murine cDNA libraries. DP-1 cDNA clones were frequently rearranged and the nucleotide sequence shown is from cDNAs from an F9 EC cell library.

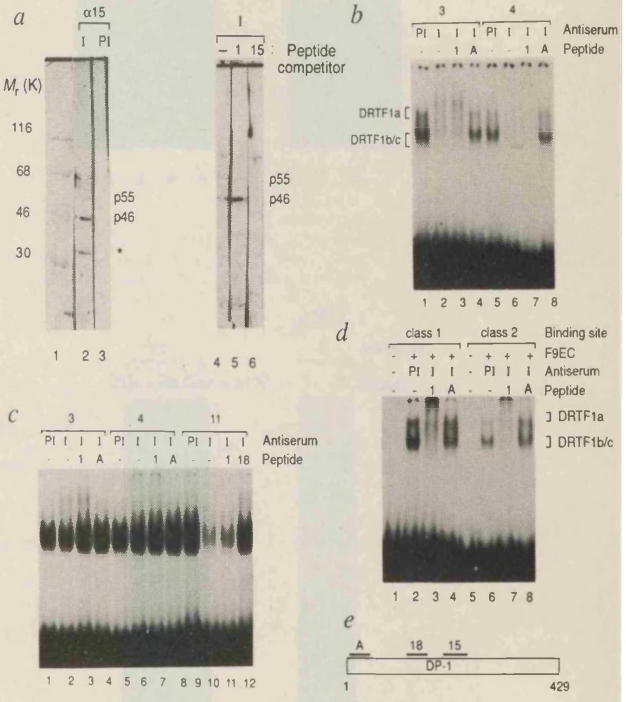
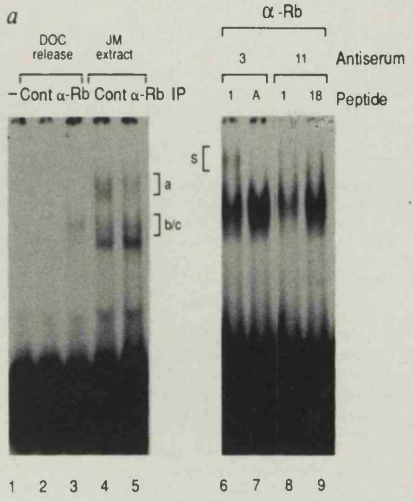


FIG. 2 DP-1 is a DNA binding polypeptide in DRTF1/E2F. *a*, DP-1 is present in affinity-pure DRTF1/E2F. Immunoblot with affinity-purified DRTF1/E2F and anti-peptide 15 (amino acids 235–249). Reactivity with p46 and p55 was only present in the immune (I, track 2) but not preimmune (PI, track 3) serum. This was specific because it was competitively inhibited by peptide 15 but not by an unrelated peptide, peptide 1 (compare tracks 6 and 5). p46 is likely to be a derivative of p55 because anti-peptide A (see below, derived from the N-terminal region of DP-1) only detected p55 (data not shown). M_r standards are shown in track 1, asterisk indicates a nonspecific reaction. *b*, DP-1 is in DRTF1/E2F DNA-binding complexes formed in F9 EC cell extracts. Gel retardation was done in F9 EC whole cell extracts (in which DRTF1/E2F resolves as complexes a, b and c) with an E2F binding site (E2A promoter sequences −71 to −50) in the presence of either preimmune or immune anti-peptide A serum with the addition of either unrelated peptide 1 (tracks 3 and 7) or peptide A (tracks 4 and 8); 3 and 4 represent two different rabbit antisera. *c*, DP-1 is in the affinity-pure DRTF1/E2F DNA-binding complex. Gel retardation was done with affinity-purified DRTF1/E2F together with an E2F binding site (E2A promoter sequences −71 to −50) in the presence of preimmune or immune anti-peptide A (tracks 1–8) or anti-peptide 18 (antiserum 11, tracks 9–12) together with either unrelated peptide 1 (tracks 3, 7 and 11) or the homologous A peptide (tracks 4 and 8) or 18 (track 12). *d*, DP-1 is in DRTF1/E2F DNA binding complexes that form on divergent E2F binding sites. Gel retardation was done in F9 EC whole cell extracts with either a class 1 (tracks 1–4) or class 2 (tracks 5–8) binding site in the presence of either preimmune or immune anti-peptide A (antiserum 4), together with either unrelated peptide 1 (tracks 3 and 7) or peptide A (tracks 4 and 8). *e*, Location of peptides A, 18 and 15 in DP-1. METHODS. Peptides 15, A or 18 were coupled to keyhole limpet haemocyanin and used to immunize rabbits. Generation of antibodies and immunoblotting followed standard procedures. In gel retardation assays ~5.0 ng affinity-purified DRTF1/E2F or ~5.0 μg of F9 EC crude cell extract were assayed with an oligonucleotide containing E2A promoter sequences −71 to −50 in the presence of ~100-fold molar excess of −62/−60 (E2A sequences −71 to −50 mutated in positions −62, −61 and −60 (ref. 20)). In *d*, class 1 (TTTTGGCGGGAAT) or class 2 (ATTTGCGCGGGAAT) binding sites[27] were used. Antisera were added during the preincubation period.



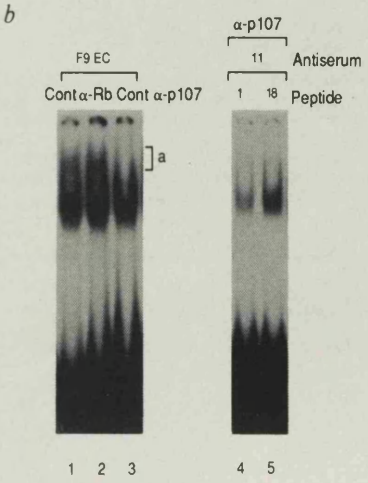
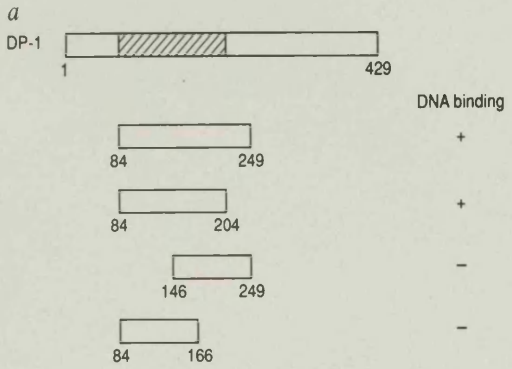


FIG. 3 DP-1 in pRb and p107-DRTF1/E2F complexes. *a*, DP-1 associates with Rb. Immunoprecipitation was from JM cell extracts with either anti-Rb monoclonal antibody IF8 (ref. 29) or a control monoclonal antibody A7. Immunoprecipitates were treated with 1.0% deoxycholate and the released material assayed for DRTF1/E2F activity in gel retardation. DRTF1/E2F binding activity was only detected in the anti-Rb immunoprecipitates (compare tracks 2 to 3); the JM cell extract depleted with anti-Rb had reduced levels of the Rb complex (compare tracks 4 to 5, depleted complex indicated by 'a'). Detergent-released DRTF1/E2F was further assayed for reactivity with anti-peptide A (antiserum 3; tracks 6 and 7) and anti-peptide 18 (antiserum 11; tracks 8 and 9) in the presence of either unrelated peptide 1 (tracks 6 and 8) or the homologous peptides (tracks 7 and 9). Anti-peptide A caused a supershift (indicated by 's' in track 6), whereas anti-peptide 18 interfered with DRTF1/E2F DNA-binding activity (compare tracks 8 and 9). *b*, DP-1 associates with p107; DRTF1/E2F was resolved in an F9 EC cell extract in the presence of either the control monoclonal antibody (A7; track 1), anti Rb (IF8; track 2), or anti-p107 (SD9; track 3); note that anti-p107 reduced level of DRTF1a. In tracks 4 and 5, JM cell extracts were immunoprecipitated with anti-p107 monoclonal antibody. DRTF1/E2F was released as described in *a*, and assayed for reactivity with anti-peptide 18 (antiserum 11) in the presence of either unrelated peptide 1 (track 4) or the homologous peptide 18 (tracks 5). Note that anti-peptide 18 caused a reduction in DRTF1/E2F DNA-binding activity (compare tracks 4 and 5).



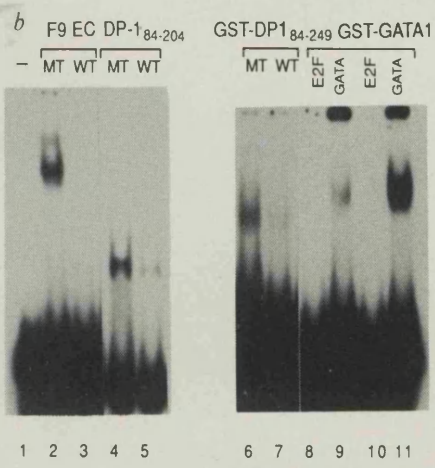


FIG. 4 DP-1 is a sequence-specific DNA binding protein. *a*, Summary of DNA binding properties of DP-1 fusion proteins. The indicated regions of DP-1 were expressed as fusion proteins, affinity-purified and assessed for DNA-binding activity in a gel retardation assay. *b*, DP-1_{84-204} is sufficient for sequence-specific DNA binding to an E2F site. Affinity-purified GST-DP-1_{84-204} was cleaved with thrombin and the released polypeptide assayed (~1.0 μg) for binding to the E2F site in the presence of ~100-fold excess of competing mutant (track 4) or wild-type (track 5) E2F site (the distal E2F site, −71 to −50, from the adenovirus E2A promoter). Competition with the same binding sites in F9 EC cell extracts is shown in tracks 2 and 3; tracks 1 to 5 were taken from the same experiment. GST-DP-1_{84-249} had similar DNA specificity (compare tracks 6 and 7; about 2.5 μg of uncleaved fusion protein in the presence of 100-fold excess of competing mutant or wild-type binding site), whereas DP-$1_{146-249}$ and DP-1_{84-166} failed to bind in these conditions (data not shown). Likewise, GST-GATA 1 (~1.5 μg) failed to bind to the E2F site (tracks 8 and 10) but did to its cognate binding site (tracks 9 and 11) in the presence (tracks 8 and 9) or absence (tracks 10 and 11) of nonspecific salmon sperm DNA (2 μg); tracks 6 to 11 are from the same experiment.

METHODS. Regions of the DP-1 cDNA were amplified in a PCR and subcloned into pGEX-2T (ref. 13). Oligonucleotide primers used in each PCR were as follows; DP-1_{84-249}, 5′-CGCGGATCCCCCAACACGCATTTTGTG-3′ and 5′-CGCGGATCCCCTGCGGGCCTGCTG-3′; DP-1_{84-204}, 5′-CGCGGATCCCCCAACACGCATTTTGTG and CGCGGATCCGTTCTGGCACTCCTGAGC; DP-$1_{146-249}$; 5′-CGCGGATCCTTCAGCGCTGCCGACAACCAC and 5′-CGCGGATCCCCTGCGGGCCTGCTG; DP-1_{84-166}, 5′-CGCGGATCCCCCAACACGCATTTTGTG and CGCGGATCCCCGGATGTTCTTCTGGTC. GST fusion proteins were affinity-purified on glutathione-sepharose and their purity assessed by PAGE (ref. 13). Gel retardation with the distal E2F binding site from the adenovirus E2A promoter (−71 to −50 region) and the DP-1 derivatives was done in the absence of salmon sperm DNA (tracks 4, 5, 6 and 7). Thrombin digestion of GST-DP-1_{84-204} was by resuspending the GST-fusion protein/glutathione-sepharose beads in 50 mM Tris, pH 8.0, 150 mM NaCl, 2.5 mM $CaCl_2$, 0.1% β-mercaptoethanol containing human thrombin at 3 μg ml^{-1} for 2h. The presence of cleaved DP-1_{84-204} in the supernatant was confirmed by immunoblotting. GST-GATA-1 contained a full-length murine GATA-1 coding sequence[30] and was incubated with a synthetic GATA-1 binding site.

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Adenovirus E1a prevents the retinoblastoma gene product from complexing with a cellular transcription factor

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THE transforming proteins of several DNA tumour viruses, including adenovirus E1a and simian virus 40 large T antigen, complex with the retinoblastoma (Rb) tumour-suppressor gene product[1,2]. This requires regions in these viral proteins necessary for transformation and is thought to inactivate the growth-suppressing properties of the Rb protein by disrupting its interaction with cellular targets[3]. Indeed, regions of Rb required to form a complex with E1a and large T antigen are often mutated in transformed cells[4]. The level at which the Rb protein regulates proliferation is unknown, although one possibility is transcription. We have previously characterized a sequence-specific transcription factor, DRTF1, the activity of which is downregulated as embryonal carcinoma stem cells differentiate. DRTF1 is found in several discrete protein complexes (a, b and c) which are of different sizes but have the same DNA specificity[5,6]. We now show that one of these also contains the Rb protein and, further, that the adenovirus E1a protein causes the dissociation of the Rb protein from this complex. This requires conserved regions 1 and 2 of E1a that are known to be required for efficient transformation[7]. These results demonstrate that the Rb protein forms a complex with a DNA-bound transcription factor, and suggests that the Rb protein might act by regulating transcription.

In F9 EC cell extracts the 1b/c form of DRTF1 (from now on referred to as 1b) is more abundant than the 1a form[8]. But as F9 embryonal carcinoma (EC) stem cells differentiate to F9 parietal endoderm-like cells (F9PE), DRTF1b is strongly downregulated, whereas DRTF1a becomes the predominant form (J.F.P. and N.B.L.T., manuscript in preparation). DRTF1a can be dissociated into 1b by a variety of treatments (J.F.P. and N.B.L.T., manuscript in preparation), suggesting that DRTF1a contains the 1b DNA-binding activity and another polypeptide(s). DRTF1 binds to the same motif as the HeLa cell transcription factor E2F, although the relationship between DRTF1 and E2F is unclear.

Two cell lines derived from human leukaemias, JM and H9, were found to contain DRTF1a in abundance, but little 1b (Fig. 1*a*, compare lanes 1, 2 and 3). These activities had the expected DNA-binding specificity[8] but slightly different mobilities to the F9 1a form (Fig. 1*a* and *b*); JM cell extracts were used to characterize further the DRTF1a activity.

The downregulation of DRTF1 during F9 EC cell differentiation correlates with the degree of cellular E1a-like activity[5], originally defined by the ability of F9 EC cells, but not their differentiated derivatives, to allow the expression of an adenovirus deletion mutant which lacks a functional viral E1a gene[9]. Because DRTF1b is abundant in F9 EC cells where there is an endogenous cellular E1a-like activity, we tested whether 1a was modulated by viral E1a. The mobility of DRTF1a in F9 EC, F9 PE and JM cell extracts increased to that of 1b after the addition of adenovirus E1a (Fig. 2*a*). This effect was specific for DRTF1 as E1a did not affect the binding of ECRE2 (ref. 13) to the ATF DNA-binding site (Fig. 2*a*). This could mean that the adenovirus E1a protein sequesters a protein(s) from the 1a complex to produce the 1b complex. That 1b is abundant and 1a scarce in F9 EC cell extracts is consistent with the presence of a cellular E1a-like activity that acts analogously to viral E1a.

The adenovirus E1a gene is differentially spliced to produce mRNAs whose protein products have distinct biological properties[7]. Both the products of the 12S and 13S coding sequence contain two regions, known as conserved regions (CR) 1 and 2 (Fig. 2*c*), which are required for E1a to bind several celluar polypeptides, including the Rb protein[4,10], and for immortalization and cooperation with other oncogenes[7]. Both 13S- (Fig. 2*a* and *b*) and 12S- (Fig. 2*b*) encoded polypeptides dissociate 1a, but two 12S mutants with deletions in either CR1 (Sp53) or CR2 (SpCS) (Fig. 2*c*) failed to cause the dissociation of 1a (Fig. 2*b*), suggesting that both CR1 and CR2 are required for this. As these conserved domains in E1a are also required for binding cellular proteins, we reasoned that E1a may act to sequester a cellular protein from the 1a complex.

A candidate for this cellular protein was the Rb protein, and to test this we assayed a number of different monoclonal antibodies that react with distinct epitopes in the Rb protein (ref. 1; Q. Hu and E. Harlow, manuscript in preparation) for their ability to super-shift the DRTF1a-complex, which would indicate the presence of Rb in the DNA-bound protein complex. Two monoclonal antibodies, C36 and XZ55, which react with different regions of the Rb protein, each produced a super 1a-shift (Fig. 3*a*, lanes 4 to 7, and 10 to 13), confirming that the Rb protein is indeed present in DRFT1a. A control monoclonal

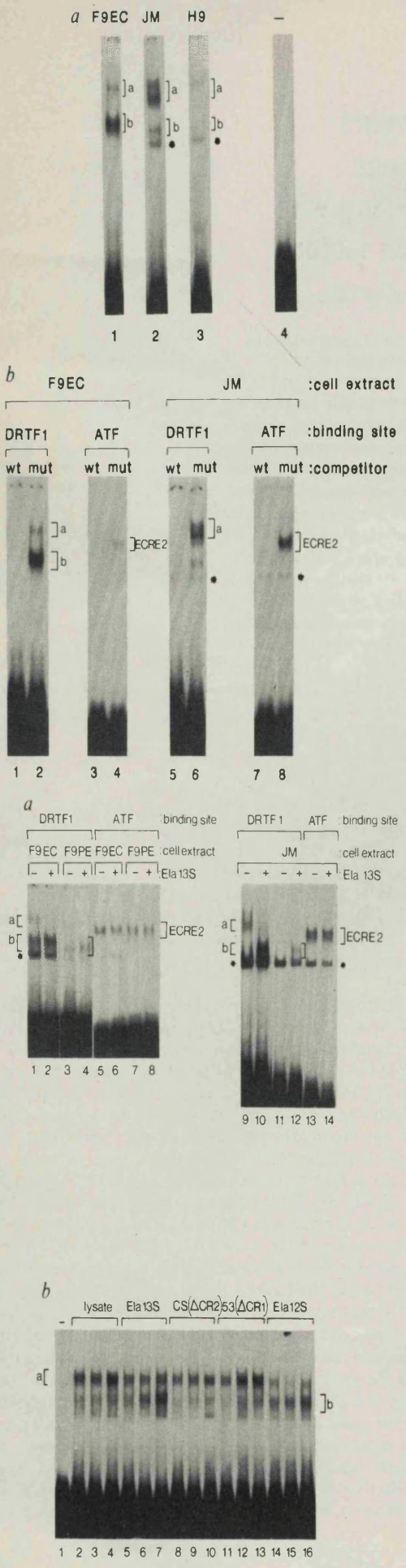

FIG. 1 *a*, Distinct forms of DRTF1 in different cell-types. Complexes formed on a probe containing a DRTF1 site in F9 EC (lane 1), JM (lane 2) and H9 (lane 3) cell extracts; complexes a and b are indicated. Lane 4 shows the probe alone, and the asterisk indicates a non-specific complex. *b*, Sequence specificity of DRTF1 complexes in F9 EC and JM cell extracts. Specificity of the complexes formed on the probe containing the DRTF1-site was determined by competition with oligonucleotides containing either wild-type (lanes 1 and 5) or mutant (oligonucleotide 62/60, lanes 2 and 6) DRTF1 binding sites; DRTF1 complexes are indicated. As a control, a similar assessment of complex specificity was performed with a probe containing an ATF site[8], using either a wild-type (wt; lanes 3 and 7) or mutant (mut; lanes 4 and 8) ATF-site as competitor; the ATF-site binding activity, ECRE2, is indicated.
METHODS. Gel retardation assays were performed as described previously[8] in F9 EC, JM or H9 cell extracts prepared according to standard procedures. The wild-type DRTF1 binding site probe contains sequences from the adenovirus E2A promoter −71 to −50 (ref. 8), and the mutant 62/60 has sequences −62 to −60 mutated[8]. Probe P contains a wild-type ATF binding site taken from the adenovirus E4 promoter[13]; Pm1, is a mutant ATF site, unable to bind ATF (ref. 13).

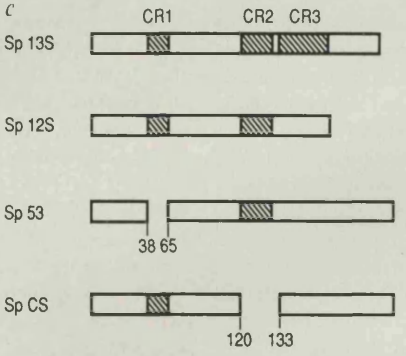

FIG. 2 Adenovirus E1a dissociates DRTF1a. *a*, The 13S E1a coding sequence was transcribed, translated and assessed for its effect on DRTF1 in either F9 EC (lanes 1 and 2), F9 PE (lanes 3 and 4) or JM cell extracts (lanes 9 to 12). The 13S E1a product dissociated the 1a complex in all three cell extracts (lanes 2, 4, 10 and 12) to produce the faster migrating 1b-complex whereas the control lysate containing translated BMV RNA did not (lanes 1, 3, 9 and 11); lanes 11 and 12 show a short exposure of lanes 9 and 10. The E1a 13S product had no effect on the binding activity of ECRE2 to the ATF site in any of the extracts studied (lanes 5 to 8, 13 and 14). *b*, The indicated coding regions of E1a were transcribed, translated and assessed for their ability to dissociate the 1a complex in the JM extract. Both 13S (lanes 5 to 7) and 12S E1a (lanes 14 to 16) dissociated 1a (compare the induction of 1b to the lysate alone; lanes 2 to 4), whereas Sp52 (ΔCR1) and SpCS (ΔCR2) did not (lanes 8 to 13). Each series of lanes shows the results of titrating in equal amounts of lysate, with increasing concentration from left to right. The reticulocyte lysate alone was titrated in lanes 2 to 4. *c*, Summary of coding sequences and deletions. The shaded regions in E1a and derivatives indicate CR1 and CR2.
METHODS. All coding sequences were cloned into Sp65 and transcribed and translated by standard procedures. Translation efficiency was assessed by examining the polypeptides on standard SDS-polyacrylamide gel electrophoresis before they were used in gel retardation experiments. Translated polypeptides were added to the JM cell extract before addition of the binding site; binding site probes are as used in Fig. 1.

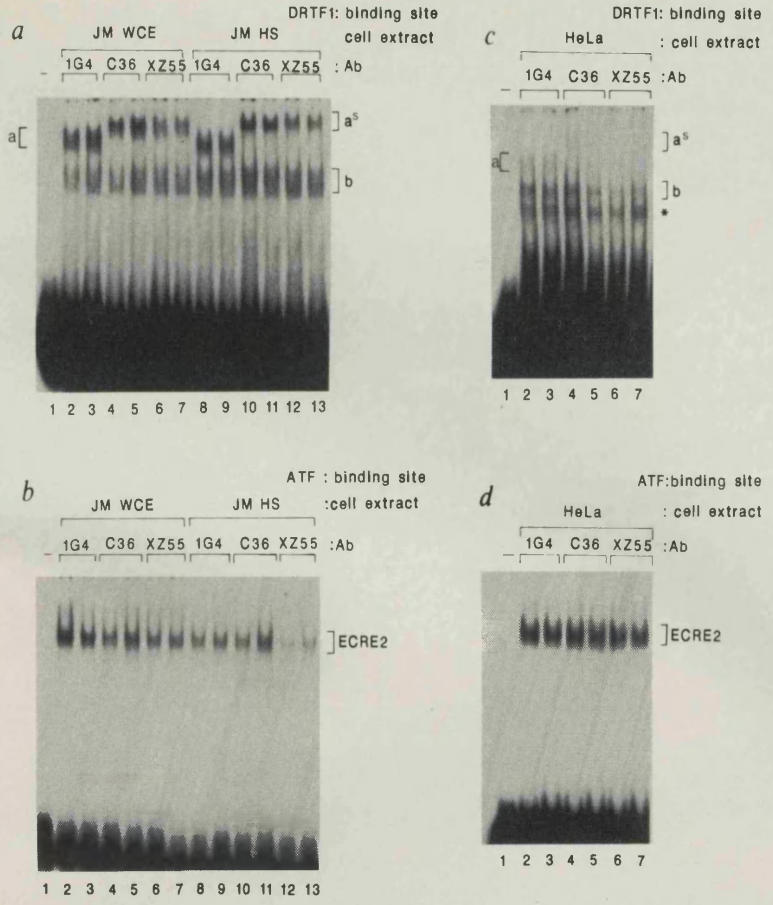

FIG. 3 The retinoblastoma gene product complexes with DRTF1. *a*, Monoclonal antibodies against the Rb protein were added to either JM (*a* and *b*) or HeLa (*c* and *d*) whole cell extracts (WCE) and assessed by gel retardation using either a DRTF1 (*a* and *c*) or an ATF (*b* and *d*) binding site. Shifted-shifts are indicated in JM extracts (*a*) and the much weaker shifted complex in HeLa cell extracts (*c*) as a^s. Note that the 1b complex in HeLa and JM cell extracts is unaffected by the anti-Rb antibodies, as is the ECRE2 complex formed in both cell-types on the probe containing the ATF site. A heparin-Sepharose fraction from JM cell extracts (HS) was used in lanes 8 to 13. METHODS. Monoclonal antibodies C36 and XZ55, which react with different regions of the Rb protein (1; Q. Hu and E. Harlow, manuscript in preparation), were added to the extract and incubated for 10 min before the addition of the appropriate binding site. As a control, a monoclonal antibody, IG4, which reacts with an unrelated antigen[12] but has the same isotype as C36 and XZ55, was used. Binding site probes are as in Fig. 1.

antibody that reacts with an unrelated protein[12] had no effect on the 1a-shift (Fig. 3*a*, lanes 2 and 3, and 8 and 9). Also, the super-shift was specific for DRTF1 because the ATF activity ECRE2 was unaffected by the antibodies (Fig. 3*b*). This clearly establishes that the Rb protein is present in DRTF1a, and strongly argues that E1a acts to sequester Rb from the complex. If this is the case, then DRTF1b should not be affected by the anti-Rb antibodies because this form is generated by E1a (Fig. 2*a*). This prediction was tested in a HeLa cell extract which contains predominantly the DRTF1b form[8] (possibly because HeLa cells express the human papilloma virus HPV-18 E7 protein). As predicted, this complex was unaffected by the anti-Rb antibodies, although low amounts of a shifted-shift were visible with C36 and XZ55 (Fig. 3*c*, lanes 4 to 7), most probably derived from the small amount of 1a in HeLa cell extracts[8]. Similarly, the small amounts of 1b in the JM cell extract was not affected (Fig. 3*a*). We conclude from these studies that the Rb gene product when complexed with DRTF1 produces 1a and that adenovirus E1a acts to disrupt this complex by sequestering the Rb protein.

Although the mechanism by which the Rb protein negatively regulates cellular proliferation is unknown, it is clear that it binds a number of cellular proteins which might be involved, an idea supported by the inability of some naturally occurring Rb mutants to bind these activities[3]. Here we have demonstrated that the Rb protein is complexed to a cellular transcription factor, thereby enabling the Rb protein to localize to DNA. As DRTF1 is a transcriptional activator, it is possible that by complexing with it, the Rb protein regulates this activity. Indeed, the transcriptional activity of affinity-purified DRTF1a is severely compromised when assayed by *in vitro* transcription (data not shown).

This provides a molecular explanation of how E1a, and other viral transforming proteins such as simian virus 40 large T antigen, might in part act: by forming a complex with Rb they inactivate the transcription-repressing properties of the Rb protein and thereby indirectly activate transcription of those genes normally subject to Rb-mediated negative regulation. Similarly, mutations that occur in the Rb gene in a variety of transformed cells are often found to modify the region known to bind E1a[4] and might interfere with the transcription properties of the Rb protein. It will be interesting to establish whether these mutant Rb genes encode proteins that can complex with DRTF1.

Finally, certain stem cells, F9 EC for example, have a cellular E1a-like activity which reduces the level of the DRTF1/Rb complex. Presumably a cellular E1a-like activity functions to regulate the association of the Rb protein with cellular transcription factors such as DRTF1. This activity might be important in regulating the proliferation of stem cells. □

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Cyclin A and the retinoblastoma gene product complex with a common transcription factor

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THE retinoblastoma gene (*Rb*) product is a negative regulator of cellular proliferation[1], an effect that could be mediated in part at the transcriptional level through its ability to complex with the sequence-specific transcription factor DRTF1 (ref. 2). This interaction is modulated by adenovirus E1a, which sequesters the Rb protein[3] and several other cellular proteins[3], including cyclin A (refs 4, 5), a molecule that undergoes cyclical accumulation and destruction during each cell cycle[6,7] and which is required for cell cycle progression[8]. Cyclin A, which also complexes with DRTF1, facilitates the efficient assembly of the Rb protein into the complex. This suggests a role for cyclin A in regulating transcription and defines a transcription factor through which molecules that regulate the cell cycle in a negative fashion, such as Rb, and in a positive fashion, such as cyclin A, interact. Mutant loss-of-function *Rb* alleles, which occur in a variety of tumour cells, also fail to complex with E1a and large T antigen[9,10]. Here we report on a naturally occurring loss-of-function *Rb* allele encoding a protein that fails to complex with DRTF1. This might explain how mutation in the *Rb* gene prevents negative growth control.

DRTF1 is a cellular transcription factor that complexes with the *Rb* gene product in a cell-specific fashion[2]. In JM cells, which are derived from a human T-cell leukaemia, most DRTF1 is complexed to the Rb protein (DRTF1a), whereas in murine F9 embryonal carcinoma (EC) stem cells less Rb is complexed and DRTF1 exists mostly in the dissociated, Rb-free form (DRTF1b). The Rb protein can be sequestered from DRTF1 by adenovirus E1a, an effect that requires E1a conserved regions 1 and 2 (ref. 2), also necessary for the immortalizing activity of E1a (ref. 11). In F9 EC cell extracts DRTF1 DNA-binding activity resolves as three discrete complexes, referred to as a, b and c[12], where DRFT1a migrates more slowly than b and c (from now on collectively referred to as DRTF1b) (Fig. 1, track 2). DRTF1 binds to the same motif as the HeLa cell transcription factor E2F, although the relationship between DRTF1 and E2F is at present unclear.

As DRTF1 complexes with the Rb protein, we tested whether it also complexed with other E1a-associated proteins, several of which have been characterized[3,13]. One of these is cyclin A (refs 4, 5), a member of the family of molecules first defined in marine invertebrates that undergo cyclic accumulation and destruction during each mitosis of the cell-cycle[6,7]. Cyclin A has been implicated in the regulation of mitosis from genetic studies[8] and because of its cyclic accumulation and association with cdc2-related kinases[5].

To investigate whether cyclin A also complexed with DRTF1, we assessed the effect of an anti-cyclin A polyclonal antibody. Anti-cyclin A antibody, but not the preimmune serum, disrupted DRTF1a, causing it to migrate faster, but had no effect on DRTF1b (Fig. 1, compare tracks 3 and 4; indicated by a′), and neither antibody affected the ATF-site DNA-binding activity ECRE2 (Fig. 1, compare tracks 7 and 8). The anti-cyclin A antibody caused a faster migrating DRTF1a complex probably by dislodging cyclin A from the transcription factor complex. Two proteins involved in cell cycle control, the Rb protein and cyclin A, are thus present in DRTF1a and both are targeted and sequestered by viral E1a (refs 3–5).

The low level of DRTF1a and high level of DRTF1b in F9 cells could result from limiting amounts of available Rb protein, cyclin A or both. We tested this by expressing and purifying Rb and cyclin A fusion proteins (Fig. 2*a*), and supplemented F9 cell extracts with each protein before assaying the effect by gel retardation. The Rb fusion protein contained human *Rb* coding sequence from residues 379 and 928, which includes the simian virus 40 large T/E1a binding region[9,10], fused to glutathione *S*-transferase (Fig. 2*a*); the purification strategy yielded an Rb fusion protein, GT-Rb, free from other contaminating polypeptides (Fig. 2*b*, track 4).

GT-Rb efficiently complexed with F9 EC DRTF1b because there was an induction of DRTF1a and a concomitant reduction of DRTF1b on addition of GT-Rb to the F9 EC whole cell extract (Fig. 3; compare track 2 with 3, 4 and 5), suggesting that free and appropriately modified Rb protein is limiting and in part responsible for the low level of DRTF1a in F9 EC cells. This also argues that the underphosphorylated form of the Rb protein can assemble into the complex as GT-Rb was purified from prokaryotic cells and should therefore lack the physiologically relevant phosphorylation provided by eukaryotic cells. We

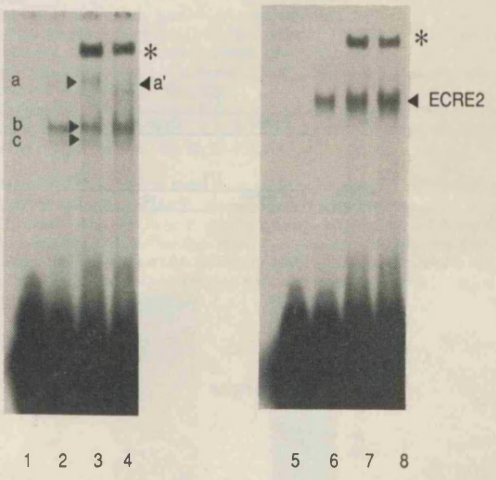

FIG. 1 Cyclin A is complexed to a transcription factor. A rabbit polyclonal antiserum raised against purified bovine cyclin A was added to an F9 EC cell extract and the effect assessed by gel retardation using either a DRTF1 (tracks 1 to 4) or an ATF (tracks 5 to 8) binding site. The F9 EC cell extract alone is shown in tracks 2 and 6, the extract with preimmune rabbit serum in tracks 3 and 7, and with the polyclonal anti-cyclin A in tracks 4 and 8. DRTF1a, b/c and ECRE2 complexes are indicated, a′ indicates the faster mobility complex that appears in the anti-cyclin A-treated track (track 4), and asterisks indicate a nonspecific complex caused by the rabbit antisera (tracks 3, 4, 7 and 8). The probes alone are shown in tracks 1 and 5.

METHODS. Gel retardation was performed as described[20] in F9 EC whole cell extracts prepared according to standard procedures. The DRTF1 and ATF binding-site probes contained sequences −71 to −50 and −82 to −60, respectively, taken from the adenovirus E2A promoter[20]. The rabbit polyclonal anti-bovine cyclin A antiserum was prepared by expressing a partial bovine cyclin A complementary DNA clone (J.P.A. and M. Carrington, unpublished results) as a fusion protein in the T7-inducible open reading frame of pET5b. The cyclin A sequence starts at residue 26 (ref. 5) and extends into the 3′ nontranslated region. After induction of the fusion protein, inclusion bodies were collected, the fusion protein separated by preparative gel electrophoresis and electroeluted from the gel slice. Rabbits were injected with 4 doses of 200 μg of pure protein at 4-week intervals. The antibody recognizes a single polypeptide with the predicted molecular weight for cyclin A in a wide variety of vertebrate extracts (data not shown).

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went on to test whether GT-Rb would associate with affinity-purified DRTF1, which consists predominantly of F9 EC DRTF1b (ref. 12) and contains several polypeptides of relative molecular mass in the range of 50,000 (50K) that bind specifically to the DRTF1 motif, and one of 30K, which binds less efficiently; affinity-purified DRTF1b is a binding site-dependent positively acting transcription factor when its activity is assayed in F9 cell extracts[12]. Although GT-Rb efficiently complexed with DRTF1b in F9 EC crude cell extracts, it failed to do so when added to affinity-purified DRTF1b (Fig. 2*c*, compare tracks 2 and 3 with 6 and 7); but there was a weak interaction between GT-Rb and DRTF1b (Fig. 2*c*, tracks 6 and 7). This suggested that another molecule present in crude extracts but absent from the affinity-purified fraction was required. An obvious candidate for this was cyclin A because it is present with the Rb protein in DRTF1a (Fig. 1, and ref. 2). To establish whether cyclin A has a role in assembling the Rb protein into DRTF1a, a protein A-cyclin A fusion protein (PA-CA) containing the C-terminal 355 residues (residues 77 to 432) of cyclin A (Fig. 2*a*) was expressed and purified to homogeneity (Fig. 2*b*, track 2). PA-CA retains all of the expected mitotic functions of cyclin A so far investigated because it is capable of activating H1 kinase in *Xenopus* egg and HeLa cell extracts (J.P.A. and T.H., manuscript in preparation). Unlike GT-Rb, there was only a marginal effect on DRTF1a when PA-CA was added to crude F9 EC cell extracts (data not shown).

PA-CA did not complex with affinity-purified DRTF1b (Fig. 2*c*, compare tracks 2 and 3 with 4 and 5), again arguing that another component was required for PA-CA to assemble efficiently into DRTF1a. But when both GT-Rb and PA-CA were added together, there was a significant enhancement of DRTF1a (Fig. 2*c*, tracks 8 and 9). This required the activity of both fusion proteins because it did not occur when either of them was denatured (Fig. 2*c*, tracks 10 to 13), nor was it independent of affinity-purified DRTF1b as the fusion proteins alone had no DNA-binding activity (Fig. 2*c*, tracks 14 to 15).

Cyclin A activates H1 kinase through its association with cdc2 or cdc2-related kinases[5]. Although it is unlikely that affinity-pure DRTF1b contained such kinases as the purification involved four applications to a binding-site affinity column, we cannot exclude this, so these kinases might have been involved in assembling GT-Rb and PA-CA into DRTF1a. This possibility was, however, extremely unlikely because GT-Rb, PA-CA and DRTF1b assembled in the absence of ATP and, moreover, the assembly was not affected by including a monoclonal antibody against the conserved PSTAIRE motif in cdc2 and related kinases (data not shown). Cyclin A therefore facilitates the assembly of the Rb protein into DRTF1a, a role which is independent of cdc2 kinase. We believe that cyclin A facilitates the assembly of the Rb protein, rather than the converse, because there was weak binding between GT-Rb and DRTF1b but not between PA-CA and DRTF1b (Fig. 2, compare tracks 4 and 5 with 6 and 7).

The *Rb* gene is frequently mutated in a variety of tumour cells to produce loss-of-function alleles, either through point mutation or deletion[14,15]. These mutants simultaneously lose the ability to complex with E1a and SV40 large T antigen[9,10]. It has therefore been proposed that mutant Rb proteins can no longer

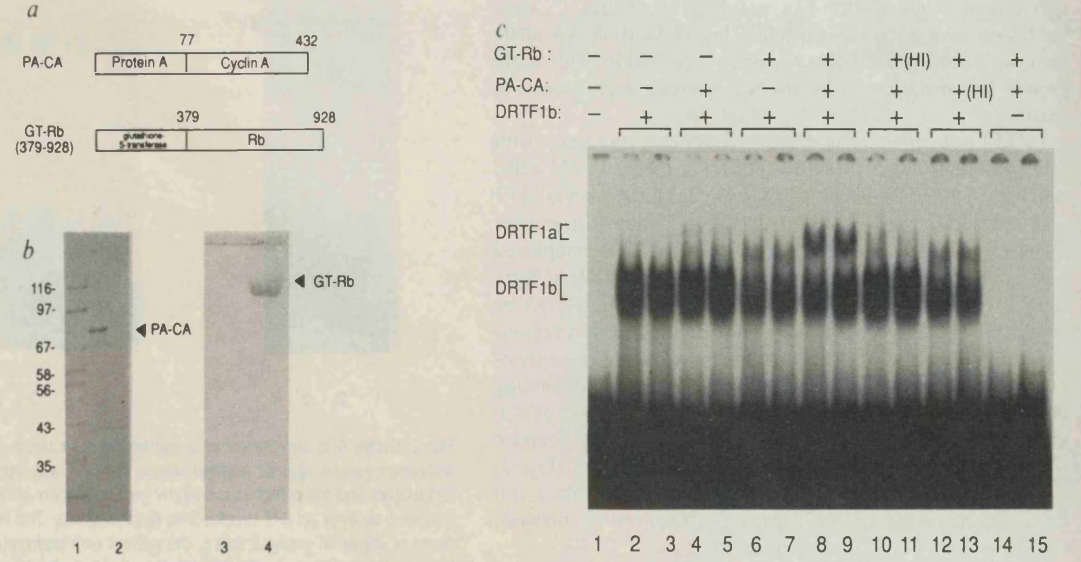

FIG. 2 Cyclin A is necessary for the Rb protein to complex efficiently with DRTF1b. *a*, Diagram of glutathione *S*-transferase-Rb (GT-Rb) and protein A-cyclin A (PA-CA) fusion proteins. pGT-Rb contains human Rb sequence from residues 379 to 938, and pPA-CA, the C-terminal 355 residues of bovine cyclin A from residues 77 to 432. *b*, Affinity-purified PA-CA (track 2) and GT-Rb (track 4) fusion proteins. Track 1 shows molecular mass markers (K) for track 2, and track 3 shows a control eluate from uninduced pGT-Rb-containing bacteria. The relative molecular mass of GT-Rb is ~80K. *c*, Either affinity-purified PA-CA (~1 μg) or GT-Rb (~150 ng) was added alone (tracks 4 and 5, and 6 and 7, respectively) or together (tracks 8 and 9) to affinity-purified F9 EC DRTF1b (~5 ng; shown alone in tracks 2 and 3). Either GT-Rb or PA-CA was denatured by heat-inactivation (HI) and added to native PA-CA (tracks 10 and 11) or GT-Rb (tracks 12 and 13), together with affinity-purified DRTF1b; there was no DNA-binding activity of the fusion proteins alone (tracks 14 and 15). Note that there was very weak binding between GT-Rb and DRTF1b, and that this was enhanced when both fusion proteins were added together (indicated by 1a in tracks 8 and 9).

METHODS. The protein A-cyclin A fusion protein vector (pPA-CA) was constructed by isolating the 1.6-kilobase fragment encoding the C-terminal 355 residues of bovine cyclin A and ligating this in-frame with the protein A gene from pRIT2T (Pharmacia), cloned into pET3a, enabling expression to be regulated from the T7 promoter. This fusion protein comprises the N-terminal 265 residues of protein A followed by the C-terminal 355 residues of cyclin A. After induction of the fusion protein the extract was centrifuged at 60,000 r.p.m. for 15 min, precipitated with ammonium sulphate, dissolved in 20 mM Tris-HCl, pH 8.0, 20 mM NaCl, 1 mM DTT and fractionated over an ACA-34 gel filtration column. Fractions were assayed by SDS-PAGE, the peak fractions pooled and loaded onto a Mono-Q column; protein was eluted with a linear gradient to 1 M NaCl. The pooled peak fractions were dialysed extensively against 10 mM Tris-HCl, pH 8.0, 1 mM DTT. pGT-Rb contains human *Rb* coding sequence (residue 379 to 928) cloned into the glutathione *S*-transferase fusion protein vector pGEX-2T as described[21]. After induction, GT-Rb was purified by glutathione *S*-transferase affinity chromatography and eluted with reduced glutathione[22]. DRTF1b was purified from F9 EC whole cell extracts as before by applying four times to the binding-site matrix[12]. Gel retardation and binding-site probes as in Fig. 1. Fusion proteins were added to affinity-pure DRTF1b and preincubated for 10 min at 30 °C before addition of the binding site.

complex with a cellular protein, which is predicted to have some degree of homology with the Rb-binding domains in E1a and large T antigen and is necessary for the wild-type Rb protein to mediate its growth-suppressing effects. Because DRTF1 is complexed with the wild-type Rb protein *in vivo*[2] and as this complex is destroyed by E1a (ref. 2) and SV40 large T antigen (data not shown), we reasoned that mutation in the *Rb* gene may also compromise the DRTF1-Rb interaction. We tested this using the *in vitro* Rb assembly assay in which GT-Rb, containing wild-type sequence, efficiently complexed with DRTF1b (Fig. 3). The assembly of GT-Rb was compared with GT-Rb^{706}, which contains the same sequence as GT-Rb apart from a single cysteine → phenylalanine substitution at residue 706 (ref. 14). This natural mutation occurred in a small cell lung carcinoma and no longer binds E1a or large T antigen[14]. GT-Rb induced DRTF1a efficiently (Fig. 3, compare track 2 with 3, 4 and 5), whereas there was no effect on addition of GT-Rb^{706} (Fig. 3, compare track 2 with 6, 7 and 8). The single amino-acid change in GT-Rb^{706} therefore produces an Rb protein incapable of complexing with DRTF1.

The wild-type *Rb* gene product is a negative regulator of cellular proliferation, an effect that might be mediated at the transcriptional level through its ability to regulate the activity of transcription factors such as DRTF1 (ref. 2). As the underphosphorylated form of the Rb protein is in DRTF1a, and given that the transcriptional activity of Rb-complexed DRTF1 may be compromised[2], an important mechanism for Rb-mediated growth control might involve the conversion of active transcription factors to their inactive counterparts. A naturally occurring mutation in the Rb gene, which changes a single amino acid in the wild-type sequence[14], encodes an Rb protein that cannot interact with DRTF1, arguing that DRTF1 is important for wild-type Rb to mediate its normal cellular function of negative growth control. Loss-of-function *Rb* alleles also fail to bind to E1a or large T (refs 9, 10, 14). It is likely, therefore, that the normal function of Rb is mediated through a protein that has a region with structural similarity to the Rb-binding domain in E1a and large T. We propose DRTF1 as a candidate for such a molecule.

Both cyclin A and the Rb protein locate to DNA by virtue of an ability to complex with DRTF1, suggesting that cyclin A might have a role in regulating transcription. Cyclin A also functions to assemble the Rb protein efficiently into the transcription factor complex, an effect independent of cdc2 and cdc2-like kinases. It is still possible that dissociation of the complex is dependent on these kinases because the activity of cdc2 is activated when it complexes with cyclin A[5]. In this respect, DRTF1-associated cyclin A may serve two separate functions: first, to guide cdc2 to key substrates, such as the Rb protein in DRTF1, and second, to activate cdc2 kinase once it has located its substrate. It is consistent with such a model that the Rb protein is efficiently phosphorylated by human cdc2 kinase[16]. Given that the underphosphorylated form of the Rb protein complexes with DRTF1, the form thought to mediate its growth regulating properties[17-19], phosphorylation may release the Rb protein from DRTF1a and so enable DRTF1b to activate transcription of target genes. □

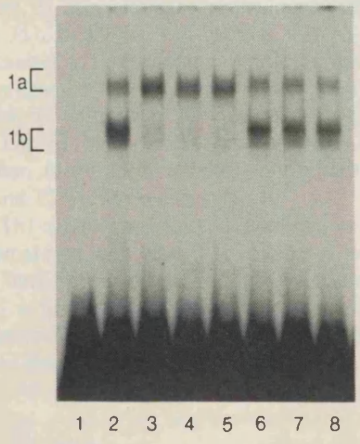

FIG. 3 A naturally occurring mutant Rb protein, Rb^{706}, does not complex with DRTF1. Either affinity-purified GT-Rb (tracks 3, 4 and 5) or GT-Rb^{706} (tracks 6, 7 and 8) fusion proteins were added in increasing amounts (~45 ng, 75 ng and 105 ng, respectively) to an F9 EC cell extract (shown alone in track 2) and the effect assessed using a DRTF1 binding site probe; track 1 shows the probe alone.

METHODS. GT-Rb and GT-Rb^{706} were expressed and purified as described in the legend to Fig. 2. Fusion proteins were preincubated with the extract for 10 min before addition of the binding site probe. Binding site probes are described in Fig. 2 legend.

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