A Thesis entitled

Characterisation of DP-1

Presented by

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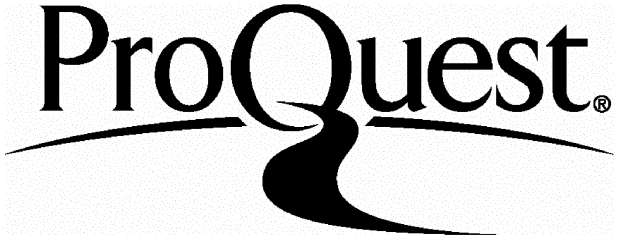

til Mor og Far

Ibegekumene tsores iz gut tsu dertseylin

Abstract

The transcription factor DRTF1/E2F regulates genes required for cell cycle progression and occupies a central role in the control of cellular proliferation by integrating cell cycle machinery with transcription. DRTF1/E2F transcriptional activity is regulated in part by the binding of the tumour suppressor pRb which is mutationally inactivated in a large range of human cancers.

Mutations in the p53 tumour suppressor gene are the most frequently observed genetic alterations in human neoplasia. Recent data suggests that the p53 gene product controls a cell cycle checkpoint responsible for maintaining the integrity of the genome, although the exact mechanism by which this occurs is still unclear.

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The DNA binding activity of DRTF1/E2F is believed to be a heterodimer composed of one of each of the DP- and E2F- polypeptide families. I present evidence that one member, DP-1, can exist in a hypo- or hyper-phosphorylated state *in vivo*, which in turn correlates with altered DRTF1/E2F affinity for its DNA binding site. Data indicates that the different phosphorylation state may affect DP-1's ability to heterodimerise with partners such as E2F-1 and E2F-5, an event essential for high affinity DNA binding and subsequent transcriptional *trans*-activation. These results potentially define a new level of control for DRTF1/E2F in which its DNA binding activity is modulated by cell cycle-regulated phosphorylation events on DP-1.

Also presented is evidence suggesting that one phosphoform of DP-1 can form an *in vivo* complex with p53 and that p53 can repress the DNA binding activity of DP-1/E2F-1 heterodimers. These results contribute to the establishment of DP-1 as a common cellular target in two distinct and independent pathways of growth control mediated through the activities of the pRb and p53 tumour suppressor proteins. The integration of p53 with DP-1 and the consequent regulation of DRTF1/E2F DNA binding activity defines novel potential pathway through which p53 can influence cell cycle progression.

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Declaration

All work presented within was performed entirely by myself and in no way forms part of any other thesis. Any material support by other persons is specifially acknowledged. The work was carried out while I was a graduate student at the National Institute for Medical Research, Mill Hill, London, from October 1992, and for the final year, on the premises of the Institute of Biomedical and Life Science, University of Glasgow. I was under the supervision of Professor Nicholas B. La Thangue (formerly N.I.M.R., now University of Glasgow) and Dr Ben Carritt (University of London).

Troels Seyffart Sørensen

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Publications and Meeting Abstracts

The following publications and meeting abstract were submitted during the course of the work presented in this thesis.

Girling, R., Bandara, L.R., Zamanian, M., Sørensen, T.S., Xu, F.H. & La Thangue, N.B. (1993). DRTF1/E2F Transcription Factor - an Integrator of Cell-Cycle Events with the Transcriptional Apparatus. *Biochemical Society Transactions*, **21,** 939-942.

Sørensen, T.S., Bandara, L.R.,Girling, R. & La Thangue, N.B. (1993). DP-1 : A Constitutively Expressed Gene which Encodes a Conserved and Integral DNA Binding Component of the Cell Cycle-Regulating Transcription Factor DRTF1/E2F. *Meeting Abstract - I.C.R.F. Tumour Virus Meeting, Cambridge.*

Bandara, L.R., Lam, E.W.F., Sørensen, T.S., Zamanian, M., Girling, R. & La Thangue, N.B. (1994). DP-1 - a Cell-Cycle-Regulated and Phosphorylated Component of Transcription Factor DRTF1/E2F which is Functionally Important for Recognition By pRb and the Adenovirus E4-Orf-6/7 Protein. *EMBO J.*, **13,** 3104-3114.

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Abbreviations

All genes in the main text are written in *itallics*.

A	Adenine or peptide A (angel)
a	DRTF1/E2F "a" complex (complexed heterodimer)
α	Anti-(body)
APα–	Epitope affinity purified anti-peptide-
APS	Ammonium persulphate
ATF	Activating Transcription Factor
ATP	Adenosine 5'-triphosphate
b/c	DRTF1/E2F "b/c" complex (non-complexed heterodimer)
BCIP	5-Bromo-4-chloro-3-inodolyl phosphate
BD	TFIID/TFIIB complex
BSA	Bovine Serum Albumin
C	Cytosine or peptide C / Control peptide
CAK	Cyclin Activating Kinase
cAMP	Cyclic adenosine mono-phosphate
CDC-	Cell Division Cycle -
CDI	Cyclin-CDK Inhibitor
CDK-	Cyclin Dependent Kinase -
cDNA	Complementary deoxyribonucleic acid
CIP	Calf Intestinal Phosphatase
cm	Centimetre
cpm	Counts Per Minute
D	Peptide D
DAF	DP-1 Associated Factor
Dal	Daltons
DHFR	Dihydofolate reductase
dH_2O	Distilled water
dm^3	Litre
DMEM	Dulbecco's Modification of Eagles Medium
DMF	Dimethyl formamide
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DOC	Deoxycholate
Dp	Phosphorylated peptide D
DP-	DRTF1 Polypeptide
dplt	F9EC LSL extract depleted with 32.3
DRTF1	Differentiation Regulated Transcription Factor 1
DRTF1/E2F	single E2F-site DNA binding activity
DTT	Dithiothreitol
EC	F9 Embryonal Carcinoma
E.coli	*Eschericha coli*
EDTA	Ethylenediaminetetra-acetic acid
E2F-	E2 factor
ELISA	Enzyme-Linked Immunosorbent Assay
Enh	Enhancer element
FCS	Foetal Calf Serum
F9EC	F9 Embryonal Carcinoma
G	Guanine
g	Gram or Gravities

G0	Quiescence
G1	Gap 1 phase
G2	Gap 2 phase
GST	Glutathione-S-transferase
GTP	Guanosine 5'-triphosphate
H	Heat treated
h	Hour
H_6	Histidine tag (HHHHHH)
HEPES	N-[2-Hydroxethyl]piperazine-N'-[2-ethanesulfonic acid]
HPV	Human Papilloma Virus
IB	Immunoblot
II	TFII (RNA polymerase II transcription factor)
IL-2	Interleukin-2
Inr	Initiator element
IP	Immunoprecipitation
IPTG	Isopropyl-ß-D-thiogalactopyranoside
K	Thousand rpm or kilo
K^+	Positive control
K^-	Negative control
KDal	Kilodalton
LSL	Low Salt Lysis buffer/Extract
lys	Reticulolysate
M	Molar or Mitosis
mA	Milliamp
MAT-1	Menage A Trois -1
MBS	M-Makimidobenzoyl N- hydroxy succinimide
MEB	Micro Extract Buffer
mg	Milligram
µg	Microgram
min	Minute(s)
ml	Millilitre
µl	Microlitre
mM	Millimolar
mm	Milimetre
µM	Micromolar
mRNA	Messenger RNA
n	Nano
NBT	Nitroblue tetrazolium
ng	Nanogram
NIMR	National Institute for Medical Research
nm	Nanometres
NP-40	Nonidet P40
OD	Optical Density
P	Phosphorylation
p	Pico
p55	DP-1 doublet as resolved by immunoblotting
PAGE	Polyacrylamide gel electrophoresis
pRb	Retinoblastoma susceptibility gene product
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline Tween (20)
PC	Positive Cofactor
PCNA	Proliferating Cell Nuclear Antigen
PE	Perietal Endoderm cells
PE(7d)	Seven-day post differention agent-exposure Perietal Endoderm cells
PIC	Protease Inhibitor Cocktail
PKC	Protein Kinase C
p55L	Faster migrating half of DP-1 p55 doublet

p55λ	Forms of p55 generated by artificial dephosphorylation
Pol	RNA polymerase
pRb	Retinoblastoma gene product
p55U	Slower migrating half of DP-1 p55 doublet
Py	Pyrimidine
RB	Retinoblastoma susceptibility gene
re-IP	Re-Immunoprecipitation
RNA	Ribonucleic acid
rpm	Revolutions Per Minute
rRNA	Ribosomal RNA
S	Synthesis phase
SDS	Sodium Dodecyl Sulphate
SRB	Suppressor of RNA polymerase B
SV40	Simian virus 40
T	Thymine
TAF	TBP-associated factor
T.agn	SV40 large T antigen
TATA	TATA box
TBP	TATA binding protein
TF	RNA polymerase transcription factor
Tris	Tris(hydroxymethyl)methylamine
tRNA	Transfer RNA
Tween 20	Polyoxyethylene sorbitan monolaurate
U	Units/uracil or un-(heat) treated
UV	Ultra Violet light
V	Volts
VC	Peptide VC
$^{v}/_{v}$	Volume per Volume
wt	Wild-Type
$^{w}/_{v}$	Weight per Volume

3d/5d/7d	Days post-differentiation agent-exposure perietal endoderm cells
17	Peptide 17
24	Peptide 24
26	Peptide 26
32.3	Monoclonal antibody to peptide D and DP-1

(+/+)	Wild type
(+/-)	Heterozygous mutant
(-/-)	Homozygous mutant

Single Letter Amino Acid Code

Alanine	A
Cysteine	C
Aspartic acid	D
Glutamic acid	E
Phenylalanine	F
Glycine	G
Histidine	H
Isoleucine	I
Lysine	K
Leucine	L
Methionine	M
Asparagine	N
Proline	P
Glutamine	Q
Arginine	R
Serine	S
Threonine	T
Tryptophan	W
Valine	V
Tyrosine	Y

Chapter 1

Introduction

RNA polymerase II transcription

Specific mRNA synthesis mirrors the progressive phases of the cell cycle as key protein products are required for the mechanical and regulative tasks of cellular replication. Regulation of transcription is likely to be a crucial component in how the cell cycle exerts its control over the proliferative machinery. It is believed that basal transcription components are regulated by gene specific activating or repressive transcription factors whose activity in turn can be influenced by second messenger cascades or the periodicity of cell cycle factors.

Basal transcription

Eukaryotic cells contain three RNA polymerases (Pol I, II and III), each primarily responsible for the transcription of one of the three broad classes of RNA polymers : Ribosomal, messenger and transfer respectively. Pol II is of particular interest because it will target genes whose protein products are associated with cellular proliferation.

Basal transcription factors

Early preparations of Pol II were incapable of initiating transcription selectively at promoters *in vitro* (Chambon, 1975) suggesting that components in addition to the polymerase are involved in selective initiation. Many factors have been found to associate with Pol II on promoter DNA and painstaking work has narrowed these down to the components required to permit the polymerase a low level of unregulated transcription *in vitro* (Conaway & Conaway, 1993 ; Zawel & Reinberg, 1993 ; Buratowski, 1994 ; Maldonado & Reinberg, 1995 ; Zawel & Reinberg, 1995a). For Pol II these are : TFIIB, TFIID, TFIIE, TFIIF, TFIIH and TFIIJ, and are referred to as the general or basal transcription factors, and the sequential assembly of these factors on core promoter elements forms the basis of the transcriptional pre-initiation complex (above references and see below). More

factors have been characterised and are either required for regulated transcription (e.g. TFIIA - see below) while others may have augmentative or gene-specific roles (e.g. TFIIG, TFII-I - see below).

Enough basal transcription factor components have been cloned to make it possible to reconstitute basal transcription *in vitro* and has permitted mutational analysis which has produced a great deal of information on the functions of individual factors (Kornberg *et al.*, 1994). Five basal transcription factors related to those found in humans : a, b, d, e and g, have been identified in fission yeast (Kornberg *et al.*, 1994). The polypeptide components of Pol II and most of the polypeptide components of these basal transcription factors are well conserved amongst eukaryotes from yeast to man (Koleske & Young, 1995) and consequently yeast provides a powerful tool with which to probe this system, not just as an easily genetically manipulated eukaryote, but also as a provider of the true substrate of RNA polymerases : Cellular chromatin, as opposed to the "naked" DNA employed in *in vitro* assays. The RNA polymerase and the set of basal transcription factor complexes are common for all the genes of a particular RNA class, and contain many individual polypeptide factors which are common to all RNA polymerases (Tjian & Maniatis, 1994 ; Zawel & Reinberg, 1995a).

Core promoter elements

The promoters of Pol II transcribed genes contain core promoter elements which are necessary and sufficient DNA binding sites for specific recognition, assembly and low-level activity by basal transcription factors *in vitro* (Conaway & Conaway, 1993). The best characterised core element is the TATA box (consensus sequence : TATAAA : Davidson *et al.*, 1983), and is generally found at around position -30 to -25 relative to the transcription initiation start site (Zawel & Reinberg, 1993). The less well characterised Initiator elements (Inr - consensus : $PyPyA_{+1}NT/\underline{A}PyPy$: Kaufmann *et al.*, 1996) are found at, or overlapping, the start site itself (transcription is often initiated at the Adenine). Our understanding of basal complex assembly and regulation on TATA-containing promoters is more defined than that on promoters which contain only an Initiator. A large number of class II genes contain promoters that lack any recognisable TATA element, most of these are "housekeeping" genes, that is, genes active in all cells (Zawel & Reinberg, 1993). Though these promoters are not as strong as TATA-box containing promoters they can modulate accurate transcription initiation and are believed to utilise the same basal transcription factors as the TATA element (Zawel & Reinberg, 1993 ; Kaufmann *et al.*, 1996 - see below).

TFIID

The TFIID complex contains the TATA element binding polypeptide, the TATA-Binding Protein (TBP), which targets TFIID, and consequentially the other basal transcription factors, to the TATA transcriptional-initiation box (Zawel & Reinberg, 1995). TBP is bound by a range of TBP-Associated Factors (TAFs) which together make up the TFIID complex (Zawel & Reinberg, 1995). TAFs are thought to mediate the action of gene-specific regulatory transcription factors (Sauer *et al.*, 1995 and see below).

TFIID binding to the TATA element appears to be an initial rate limiting event which promotes the assembly of the transcriptional pre-initiation complex (Sauer *et al.*, 1995 and see below). New evidence concerning the structure of TFIID has shed some light on how this might be achieved. This involves speculation as to how such a complex gains access to specific sites in the densely packed eukaryotic chromosome.

Each human cell contains over a meter of DNA which must be intensively packaged if it is to be fitted into a nucleus with a diameter of a few micrometers. The basic packaging unit is an octamer formed from two molecules each of the histones H2A, H2B, H3 and H4 called a nucleosome (Wolffe & Pruss, 1996). Each of the four core histones has a very similar C-terminal domain structure that directs the formation of specific heterodimers between the histones and also determines the path of the DNA wrapped round the nucleosome (Wolffe & Pruss, 1996). 147 base pairs of DNA are wound in two-and-a-half turns round the hetero-octamer and is "sealed" in place by histone H1. A linear array of such nucleosomes is wound into a fibre 30nm in diameter, which is itself coiled further to form chromosomes (Wolffe & Pruss, 1996).

Chromatin, not purified DNA, is the physiological substrate of the pre-initiation complex. Biochemical and genetic evidence suggests that chromatin structure plays an active role in transcription (Struhl, 1996). TBP alone binds extremely poorly to TATA elements in chromatin templates and consequential nucleosomal repression appears to affect all genes (Felsenfeld, 1992). It seems that the chromatin structure presents an inherent transcription repressive activity which has to be overcome. New results indicate that TAF structures, within the transcription factor TFIID, closely resemble those of the histones, raising the possibility that the DNA at the promoter is bent or wound around TFIID in a similar way as at the histone octamer and may provide a means of access into the chromatin for the TFIID complex (Hoffmann *et al.*, 1996 ; Xie *et al.*, 1996).

The amino-terminal sequences of two *Drosophila melanogaster* TFIID components, dTAF$_{II}$63 and dTAF$_{II}$62, distantly resemble those of histones H3 and H4 respectively (Kokubo *et al.*, 1994). The structure of a complex containing

dTAF$_{II}$63 and dTAF$_{II}$62 (Xie *et al.*, 1996) suggested that, both in crystals and in solution, the two subunits form a tight heterotetramer closely resembling the $[H3/H4]_2$ heterotetrameric half of the histone octamer (Arents *et al.*, 1991). Additionally it has been reported that the sequence of the human hTAF$_{II}$20, distantly resembles that of H2B (Hoffmann *et al.*, 1996). H2B which can interact strongly with itself and with histones H3 and H4, was demonstrated also to complex with the human equivalents of dTAF$_{II}$42 and dTAF$_{II}$62, hTAF$_{II}$31 and hTAF$_{II}$80 (Hoffmann *et al.*, 1996). Together these results suggest that TFIID may contain a histone octamer-like sub-structure composed of two dimers of hTAF$_{II}$20 attached to a tetramer of hTAF$_{II}$31 and hTAF$_{II}$80 (Hoffmann *et al.*, 1996). This structure would be consistent with observed stoichiometry of TFIID (Hoffmann *et al.*, 1996).

Proteins with similar structure can have very different functions, but the TAF$_{II}$s in question retain the positively charged amino acids which their histone counterparts employ to contact the DNA in the nucleosome (Arents & Moudrianakis, 1993) suggesting that DNA may in some way be wrapped around parts of TFIID in the same way that it is wrapped around the nucleosome. Furthermore, TFIID can compete with nucleosomes for DNA binding and confer a similar pattern of protection against cleavage by nucleases (Sawadogo & Roeder, 1985 ; Nakajima *et al.*, 1988) while, conversely, core promoter packaging by histone octamers prevents TFIID or TBP binding to the TATA element and effectively represses transcription (Owen-Hughes & Workman, 1994). Histone-like DNA interactions and bending may contribute to the stability of the transcription initiation complex and could account for the functional significance of downstream regions on weak TATA-containing (Nakatani *et al.*, 1990) or TATA-less (Martinez *et al.*, 1995) promoters. Retention of TFIID components on transcriptionally inactive mitotic chromosomes (Hoffmann *et al.*, 1996) is indicative of a nucleosome-like stability of the TFIID promoter complex within physiological chromatin and may suggest how the transcriptional apparatus gains access within the chromatin structure (discussed further below).

Assembly of the pre-initiation complex on TATA promoters

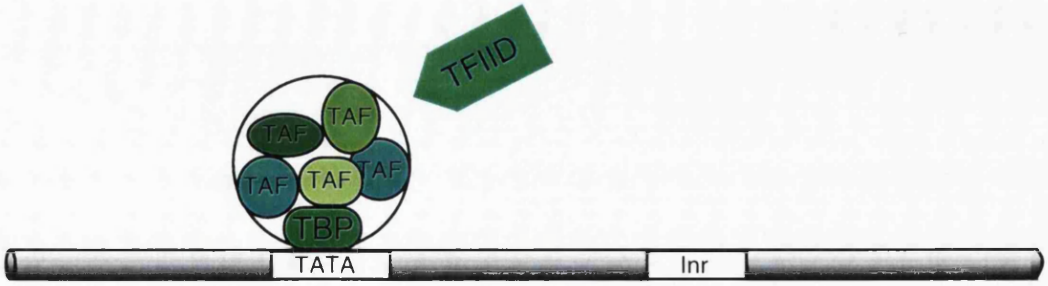
The binding of the TFIID to the TATA element is the first step in the formation of a transcription-competent complex and provides the site for assembly of the other general transcription factors and Pol II (Zawel & Reinberg, 1995 and figure 1.1). The pre-initiation complex consists of over 40 polypeptides with a total molecular mass in excess of 2 million Daltons, nearly the size of a prokaryotic ribosome (Zawel & Reinberg, 1993). Studies employing DNA footprinting, template competition studies, gel retardation analysis and kinetic assays have suggested that the basal transcription factors and Pol II can assemble in an ordered pathway on a

Figure 1.1

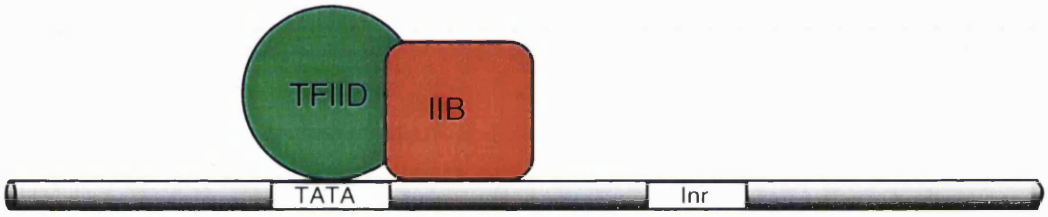
Assembly of the RNA polymerase II transcription initiation complex at TATA containing promoters.

(i)TFIID is composed of multiple TAFs and TBP (different shades of green). The sequential assembly of the transcription initiation complex is commenced by TFIID binding to the DNA (grey bar) TATA motif upstream of the transcriptional initiation point at the Initiator (Inr) site. (ii) This is in turn bound by TFIIB (IIB - orange square), (iii) RNA polymerase II (Pol II - yellow oval), TFIIF (IIF - magenta circle) , (iv) TFIIJ (IIJ - blue oval), TFIIE (IIE - cream) and TFIIH (IIH - pink).

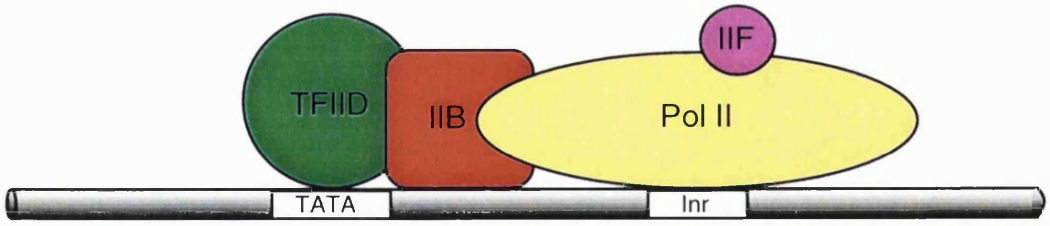
i)


TFIID
TAF
TAF
TAF
TAF
TAF
TBP
TATA
Inr

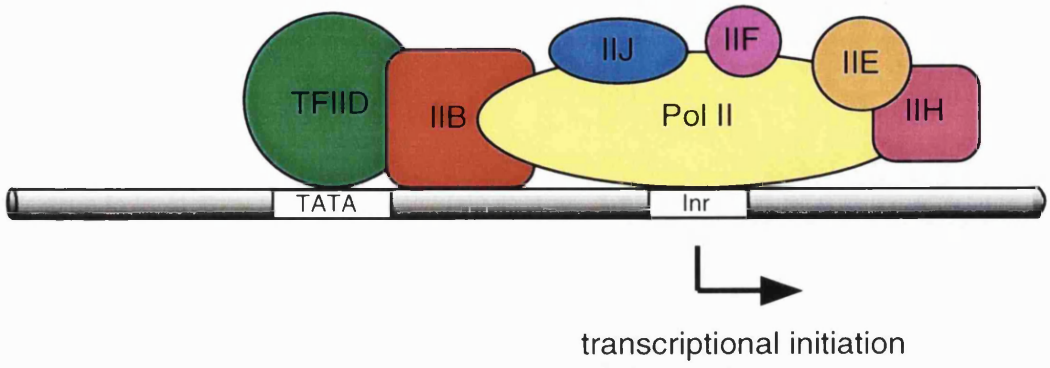
ii)


TFIID
IIB
TATA
Inr

iii)


IIF
TFIID
IIB
Pol II
TATA
Inr

iv)


IIJ
IIF
IIE
TFIID
IIB
Pol II
IIH
TATA
Inr
transcriptional initiation

promoter to form an initiation complex *in vitro* prior to formation of the first phosphodiester bond in the nascent mRNA transcript (Flores *et al.*, 1992 ; Zawel & Reinberg, 1993 ; Ma *et al.*, 1993 ; Buratowski, 1994 ; Goodrich & Tjian, 1994 ; Zawel & Reinberg, 1995a).

TFIID binds to the TATA box first and is then bound by TFIIB creating a TFIIB-TFIID-DNA initiation-platform (the BD complex) that is recognised by Pol II, possibly aided by and attached to TFIIF, which binds at the same time (Tschochner *et al.*, 1992 ; Ha *et al.*, 1993). TFIIB is believed to play a pivotal role in transcription initiation by serving as a bridge between the promoter-bound TBP (the TFIID complex) and Pol II (Tschochner *et al.*, 1992 ; Ha *et al.*, 1993). The interaction between TFIIB and Pol II seems to be critical in determining the transcription start site since mutations in the yeast gene encoding TFIIB (*SUA7*) shifts the start site of several yeast genes *in vivo* (Pinto *et al.*, 1992) and mutations in the largest subunit of Pol II have been shown to affect initiation in a similar manner (Berroteran *et al.*, 1994). Taking advantage of different initiation sites in the *S. cerevisiae* and *S. pombe* transcription systems, subunit interchange between the two reconstituted systems of fractionated components, found that the Pol II/TFIIB complex will determine the start site (Li *et al.*, 1994) .

The extension of the complex beyond the TFIID/B/F-Pol II stage does not seem to increase the size of the DNA footprint, indicating that the following interactions are predominately made with the previous factors as the sole DNA-associating platform (Zawel & Reinberg, 1993). TFIIE binds followed by TFIIH, probably in a co-operative manner, and finally TFIIJ binds completing the assembly of transcription competent pre-initiation complex (Goodrich & Tjian, 1994 ; Zawel & Reinberg, 1995). TFIIA can add to the complex at any time after TBP binding as its interaction is directly and exclusively with TFIID, it is not required for basal transcription using TBP and highly purified factors (Zawel & Reinberg, 1993) but has been reported to be required for activated transcription (Maldonado & Reinberg, 1995 and see below).

TFIIH is the only general transcription factor known to contain enzymatic activities in that it participates in nucleotide excision repair (Drapkin *et al.*, 1994 ; Mu *et al.*, 1995) and contains ATPase, kinase and helicase activity (Maldonado & Reinberg, 1995 ; Drapkin *et al.*, 1994 ; Drapkin & Reinberg, 1994). Possession of helicase activity has led to speculation that TFIIH might travel with Pol II during elongation (Zawel *et al.*, 1995 and see below). Interestingly, the kinase activity has been identified as the cyclin-dependent kinase MO15/CDK7 with its regulatory partner, cyclin H (Roy *et al.*, 1994 ; Serizawa *et al.*, 1995). Together they will specifically phosphorylate the carboxy-terminal domain of Pol II which may have an inhibitory effect on its DNA binding activity (Roy *et al.*, 1994 ; Serizawa *et al.*,

1995). The Cyclin H/CDK7 complex is otherwise known as CAK, for CDK-Activating Kinase, and phosphorylates and activates CDC2, CDK2 and CDK4 (Fisher & Morgan, 1994 and see Cell Cycle section). The CAK-connection represents a potentially influential pathway for the cell cycle cascades to crosstalk with the fundamental components of transcription (Maldonado & Reinberg, 1995).

Holoenzyme model

The notion of stepwise assembly of the transcriptional initiation complex has been challenged by observations made in the yeast system in which transcriptionally active Pol II holoenzyme complexes has been reported (Koleske & Young, 1995).

The discovery of budding yeast Pol II holoenzyme containing Pol II, TFIIF, TFIIB, TFIIH and SRB proteins (Suppressor of RNA polymerase B - see below) suggests an alternative model for pre-initiation-complex formation (Koleske & Young, 1994). The holoenzyme is highly stable in the absence of DNA and is capable of efficient selective initiation when supplemented with TBP and TFIIE (Koleske & Young, 1994). These results support a model in which the assembled holoenzyme is recruited to promoters at which TFIID is already bound. Purified mammalian Pol II, TFIIF, TFIIB and TFIIH can also form a complex independently of the DNA template *in vitro* (Serizawa *et al.*, 1993) and a holoenzyme complex has also been observed in human cells (Chao *et al.*, 1996). If this model is correct then two of the major regulatory steps in transcription initiation are formation of a TFIID-promoter complex and association of the holoenzyme with this "landing pad" (Koleske & Young, 1995).

Additional polypeptides, SRB proteins, known to have a role in transcriptional initiation *in vivo*, stimulate both basal and activated transcription (Koleske & Young, 1995). They were discovered through genetic methods designed to reveal genes whose products are involved in transcriptional activation. Each is tightly associated with the Pol II holoenzyme in both yeast and mammalian cells *in vivo* (Koleske & Young, 1995 ; Halle & Meisterernst, 1996).

Assembly of the pre-initiation complex at Inr promoters

Mutational analysis of the terminal *deoxynucleotidyltransferase* (*TdT*) promoter demonstrated that around 17 nucleotides surrounding the transcription start site contain the information necessary to direct transcriptional initiation independently and was termed the Initiator (Inr - Smale & Baltimore, 1989). Many Inr sites have been analysed and fall into different families but generally this element appears to be present in many promoters regardless of the presence of the TATA element or not

(Zawel & Reinberg, 1993). An Inr can be defined as a core promoter element as it is capable of determining the location of the start site in a promoter that lacks a TATA box and has the ability to enhance the strength of a promoter that contains a TATA box if this is located at a correct relative spacing (25-30 base pairs upstream from the Initiator) : The $PyPyA_{+1}NT/APyPy$ sequence can impart both of these characteristics (Javahery et al., 1994 ; Kaufmann *et al.*, 1996). Despite an increasingly extensive knowledge of the proteins required for TATA-mediated transcription, the requirements for initiation from promoters that lack a TATA box remain poorly defined.

In vitro reconstitution experiments using TATA-less promoters indicate that transcription from these sites requires all the general transcription factors (including TFIID) that have been associated with binding to the TATA element (Zawel & Reinberg, 1993 ; Pugh & Tjian 1991 ; Martinez *et al.* 1994 ; Kaufmann *et al.* 1996). The recognition of both the Inr and the TATA by similar complexes suggests that the two elements direct transcriptional initiation through similar mechanisms.

There are several theories as to how the pre-initiation complex is assembled on the Initiator. Pol II is thought to be able to direct the formation of the pre-initiation complex by recognising and binding weakly to the Inr element (Carcamo *et al.*, 1991 ; Aso *et al.*, 1994 ; **figure 1.2**). The Pol II-Inr complex then provides a platform for the assembly of the general transcription factors, possibly in the same manner as on TATA sites (Zawel & Reinberg, 1993). Complex assembly stabilises the interaction with the Initiator although addition of TATA containing oligonucleotides will compete off the Inr-complexes (Carcamo *et al.*, 1991) indicating that this latter site is always preferred. It is possible that the newly discovered histone-like containing structure of TFIID (see above) may help explain how it could stabilise the weak interaction between Pol II and the Initiator.

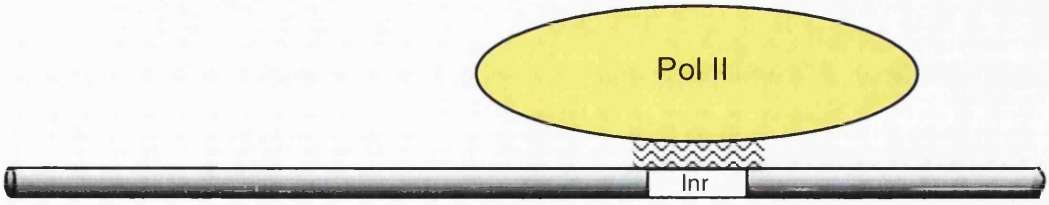
Studies from several laboratories have shown that the TFIID complex is required for the efficient activity of Inr elements (Smale *et al.*, 1990 ; Pugh & Tjian, 1991 ; Kaufmann & Smale, 1994) but furthermore highly purified *Drosophila* and human TFIID complexes bind to consensus Inr elements with the Inr/TFIID interaction apparently dependent on the precise nucleotides needed for Inr function (Kaufmann & Smale, 1994 ; Purnell *et al.*, 1994). The mechanistic role of such an interaction in the assembly of the pre-initiation complex however remains undefined. A recently identified factor, CIF (Cofactor of Initiator Function), stimulates Inr activity in reactions containing TFIID, but not TBP, and may thus be associated with transcriptional activation processes (Kaufmann *et al.*, 1996). CIF contains multiple components one of which appears to be the mammalian homologue of *Drosophila* $TAF_{II}150$ (Kaufmann *et al.*, 1996). CIF is not needed for TATA mediated transcription and may thus illustrate structural differences between pre-initiation

Figure 1.2

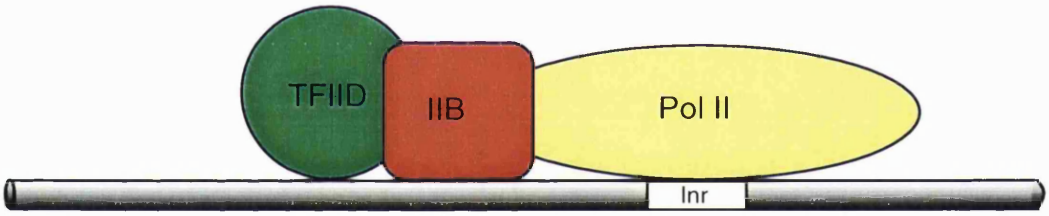
Assembly of the RNA polymerase II transcription initiation complex at TATA-less promoters.

(**i**) The sequential assembly of the transcription initiation complex at TATA-less promoters is possibly commenced by RNA polymerase II (Pol II - yellow oval) which associates weakly with the transcriptional initiation point at the Initiator (Inr) motif on the DNA template (grey bar). (**ii**) This interaction is stabilised by the complexing of TFIID (green circle) bridged by TFIIB (IIB - orange square). (**iii**) The assembly is then thought to be follow in the same manner as on TATA-containing promoters (see figure 1.1) : TFIIF (IIF - magenta circle) , TFIIJ (IIJ - blue oval), TFIIE (IIE - cream) and TFIIH (IIH - pink).

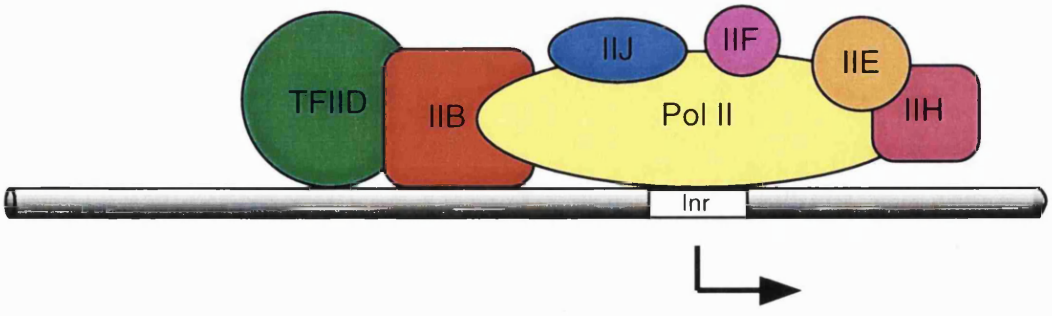
i)



ii)



iii)



transcriptional initiation

complexes at the two core promoters and may explain why some activating transcription factors appear to prefer Initiators over TATA complexes or *vice versa* (see below). For example, the glutamine-rich activation domain of Sp1 stimulates transcription from Inr-containing core promoters much more strongly than from core promoters that lack an Inr element (Emami *et al.*, 1995).

Pre-initiation complex assembly at the Initiator has also implicated TFII-I which binds to Inr sequences in some promoters and appears to directly recruit TBP to an upstream TATA box (Roy *et al.*, 1993). Another protein, YY1, binds with high affinity to Inr elements containing a CCAT core sequence and directs transcriptional initiation in the absence of TFIID or TBP (Usheva & Shenk, 1994)

There are various lines of evidence that TATA and Inr elements function co-operatively when present simultaneously to ensure specific initiation. Double mutations of TATA and Inr sites are transcriptionally non-functional which is not the case when either element is mutated (Concino *et al.*, 1984). When the TATA box is replaced with a random sequence, transcription levels are greatly decreased *in vivo* and *in vitro,* but accurate initiation is maintained (Smale & Baltimore, 1989). Since the position of the Inr and the transcriptional start point relative to the TATA box is relatively constant, it has been suggested that the TFIID/TFIIB complex at the TATA box actually stabilises the otherwise weak Pol II interaction with the Inr at the start site (Zawel & Reinberg, 1993).

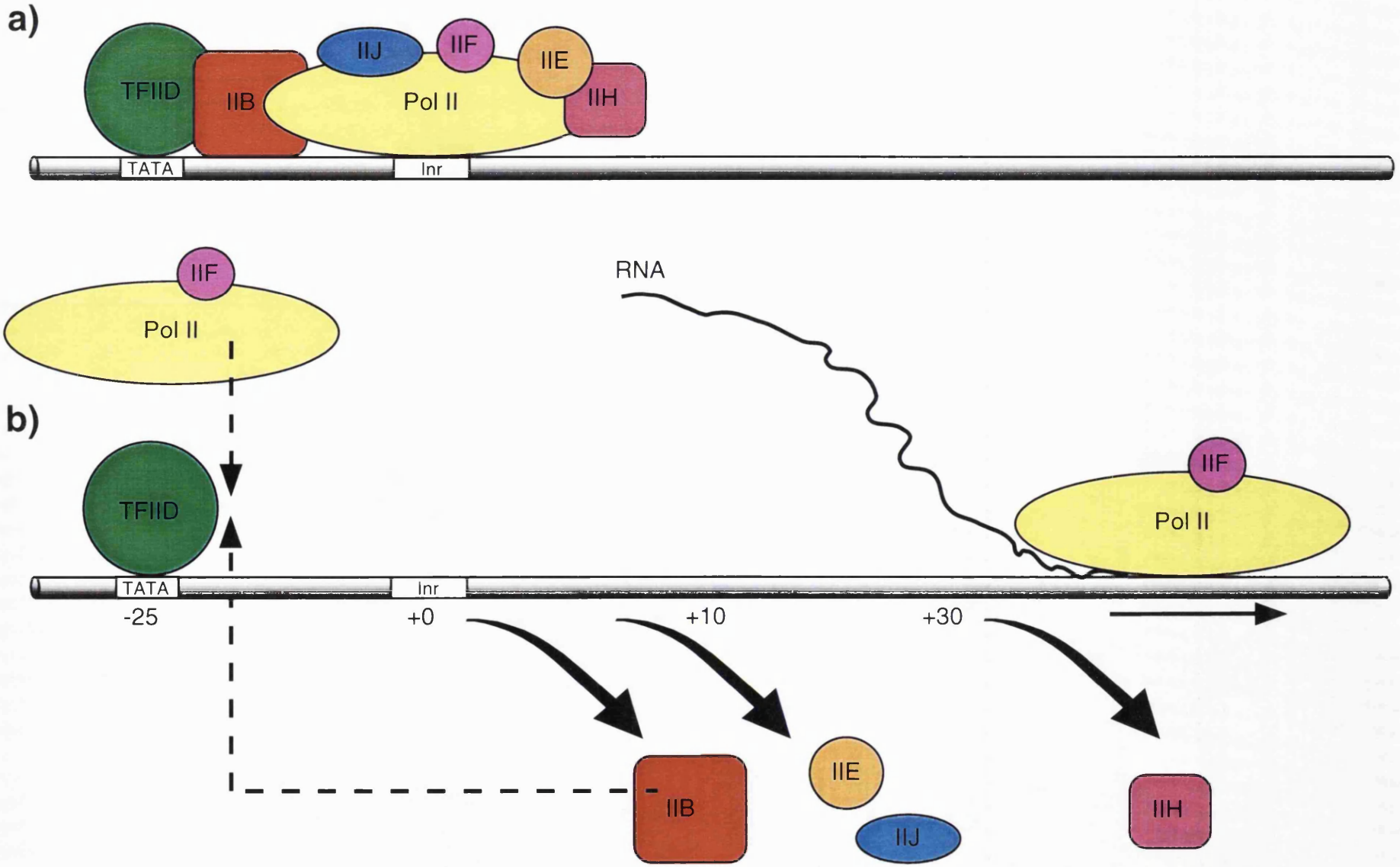
Elongation

The disassembly of the pre-initiation complex during the transition from the initiation to elongation phases of transcription, appears to be as specific a series of event as that of the assembly (**figure 1.3**). In the presence of nucleotide tri-phosphates, maturation of the pre-initiation complex assembly process is followed by strand separation and the formation of an open complex. This event requires the hydrolysis of ATP and is concomitant with the phosphorylation of the large subunit of Pol II (Bunick *et al.*, 1982 ; Jiang & Gralla, 1995). During *in vitro* elongation, Pol II initiates transcription and is released from the grasp of TFIID which appears to remain at the core promoter, thus supporting rapid re-initiation of transcription (Zawel & Reinberg, 1993 ; Zawel *et al.*, 1995b). While TFIID remains promoter-bound, TFIIB, TFIIE, TFIIF and TFIIH are released, a process thought to be modulated by TFIIE and TFIIH (Zawel *et al.*, 1995b). Upon release, TFIIB re-associates with TFIID, thus reforming the Pol II docking site : The DB complex (Zawel *et al.*, 1995b). TFIIE is released before formation of the tenth phosphodiester bond while TFIIH release occurs after +30. TFIIF is unique in that it is the only basal factor detected in the Pol II elongation complex (Zawel *et al.*, 1995b).

Figure 1.3
Elongation of the RNA polymerase II transcription initiation complex.

a. The fully assembled minimal pre-initiation complex (see figure 1.1) composed of RNA polymerase II (Pol II - yellow oval), TFIID (green circle), TFIIB (IIB - orange square), TFIIF (IIF - magenta circle) , TFIIJ (IIJ - blue oval), TFIIE (IIE - cream circle) and TFIIH (IIH - pink square), bound to the TATA and Initiator (Inr) motifs on the DNA template (grey bar).

b. Elongation/transcriptional initiation of the preinitiation complex involves the unbinding from TFIID which remains bound to the TATA box. TFIIB is released as mRNA synthesis begins and reassembles with TFIID to promote the assembly of a new RNA polymerase II/TFIIF complex. The other basal transcription factors are sequentially shed with TFIIH as the last one after the +30 point. TFIIF is unique amongst the basal transcription factors in that it appears to remain bound to Pol II throughout mRNA polymerisation.


a)
TFIID
IIB
IIJ
IIF
Pol II
IIE
IIH
TATA
Inr
IIF
Pol II
RNA
b)
TFIID
TATA
Inr
IIF
Pol II
-25
+0
+10
+30
IIB
IIE
IIJ
IIH

Regulated transcription

Transcriptional activation of eukaryotic genes involves the regulated assembly of multiprotein complexes on gene-specific enhancers and promoters. The central players in this process are sequence-specific regulator transcription factors that prompt the assembly of the transcription pre-initiation complex at the start site of mRNA synthesis, and provide the gene with individual regulatory cues for expression. Regulating transcription factors are thought to provide a medium through which the basal transcriptional complex can respond to the demands of cellular regulatory systems such as the cell cycle.

Regulatory promoter elements

Eukaryotic promoter elements and the macromolecular entities that bind them can be placed in three distinct classes : The Initiator, TATA box and enhancer elements containing : Pol II/holoenzyme, TBP and associated proteins, and activator proteins respectively (Ptashne, 1992).

Core promoter elements : The Initiator and the TATA box, are thought to provide very little of the specific regulatory information which is conferred to genes by additional *cis*-regulatory elements : The enhancer elements. Proximal enhancer elements are required for the correct initiation and regulation of transcription and are found close to the transcription start site and the core promoters. Distal enhancer elements are generally found very far upstream from the core promoter but sometimes also in introns or downstream of the genes (Ptashne, 1992 ; Struhl, 1996). Distal enhancer elements however generally refer to sequences which activate transcription from many kilobases up or downstream of the start site but which still rely on proximal enhancer elements. A great diversity of specific DNA binding proteins are responsible for the specific regulatory potential of promoters and enhancers. The regulation of these factors is a key target in the regulation of gene expression (Ptashne, 1992 ; Struhl, 1996).

Regulatory transcription factors

Regulatory transcription factors and enhancers are composed of modular components. A typical transcription factor contains a specific DNA binding domain, a multimerisation domain that allows the formation of homo- or heteromultimers, and a transcriptional activation domain (Tjian & Maniatis, 1994). These domains can be combined in a modular fashion to generate novel and fully functional transcription

factors. Many transcription factors are grouped together in families because they contain regions of significant amino acid similarity, often corresponding to the binding of similar DNA sequences (Tjian & Maniatis, 1994).

The activation domains of sequence-specific transcription factors are believed to interact directly or indirectly with components of the basal transcription apparatus. The overall potency of a transcription factor is determined by a number of factors : The affinity for its site on the DNA, the strength of any subunit interactions necessary to assemble a functional activator, as well as the strength of the interaction between an activation domain and its "target" in the basal transcription complex (Tjian & Maniatis, 1994).

Activator transcription factors have been loosely classified into groups based on the properties of their activation domains. Yeast activator Gal4 and herpes simplex virion protein VP16 are thought of as acidic activators because their activation domains are rich in aspartate and glutamate residues, whereas the activation domains of Sp1 and CTF are rich in glutamine and proline residues respectively (Zawel & Reinberg, 1993). Thus far, the structural relationships and mechanisms of specificity of these different activation domains remain obscure, especially since not all activation domains of a given class appear to interact with the same target (Gill *et al.*, 1994).

The regulation of the activities of these transcriptional activators is in turn the target of regulation. Transcription factors that heterodimerise can for example be controlled by the limiting availability of one partner. This is likely to be the case for the oncoprotein c-Myc which binds DNA poorly as a homodimer but more efficiently as a heterodimer with c-Max (Blackwood & Eisenman, 1991). c-Max homodimers can bind DNA but in contrast to c-Myc do not possess an activation domain and hence causes repression of transcription by blocking c-Myc DNA binding (Kato *et al.*, 1992). Hence the regulation of the proportional concentrations of these two factors can affect expression of their target genes. Also, c-Fos homodimers do not bind DNA whereas c-Jun homodimers can do (Sassone-Corsi *et al.*, 1988). However c-Jun/c-Fos heterodimers bind more efficiently than either homodimer to activate transcription of AP-1 target genes (Sassone-Corsi *et al.*, 1988). In this way, the abundance of c-Fos can regulate the DNA affinity of c-Jun.

Another form of post-translational regulation of transcription factors is by the phosphorylation of key functional regions. Effects of phosphorylation on transcription factors can be both stimulatory and repressive and have been documented to target DNA binding, nuclear translocation and *trans*-activation/repression domains, with both activating and repressive effects on these functions (Hunter & Karin, 1992 ; Karin, 1994). For example, SWI5 is phosphorylated at a basic nuclear localisation

signal which impedes this function (Moll *et al.*, 1991). c-Jun is phosphorylated with resultant loss of DNA binding (Boyle *et al.*, 1991) and CREB mediates transcriptional activation of cAMP-inducible genes upon phosphorylation by protein kinase A on its activation domain (Gonzalez *et al.*, 1991).

TFIID and transcriptional activation

Transcription in eukaryotic genes is regulated by interactions between the large pre-initiation complex assembled at the transcription start site and activators and repressors bound to DNA up to tens of thousands of base pairs away (Ptashne & Gann, 1990 ; Gill & Tjian, 1992). All transcriptional activators that have been tested thus far require the TFIID complex for activation *in vitro* (Tjian & Maniatis, 1994). *In vitro* transcription experiments strongly implicate TAFs as being specifically involved in the response to activators (Tjian & Maniatis, 1994). Thus stimulation of *in vitro* transcription by sequence specific transcription factors can be detected with partially purified TFIID but not with purified or recombinant TBP alone (Pugh & Tjian, 1990 ; Dynlacht *et al.*, 1991). TFIID and TBP support comparable levels of "basal" transcription but only TFIID can respond to activators in *in vitro* transcription assays (Pugh & Tjian, 1990 ; Dynlacht *et al.*, 1991).

The additionally required factors are found in the form of at least eight TBP-associated factors (in humans : TAF_{II} : 250, 150, 110, 80, 60, 40, 30α, 30β) that with TBP form a stable and active TFIID complex, and so it is proposed that TAF_{II}s may serve as a functional link between transcription factor activation domains and the basal transcription apparatus (Zawel, 1993 ; Zawel & Reinberg, 1995). One function of activators appears to be the stabilisation of TBP/promoter complexes since artificial recruitment of TBP by physical connection to a promoter-bound protein, by-passes the need for an activation domain (Struhl, 1995) suggesting that the role of TAFs could be to provide a bridge between activators and TBP.

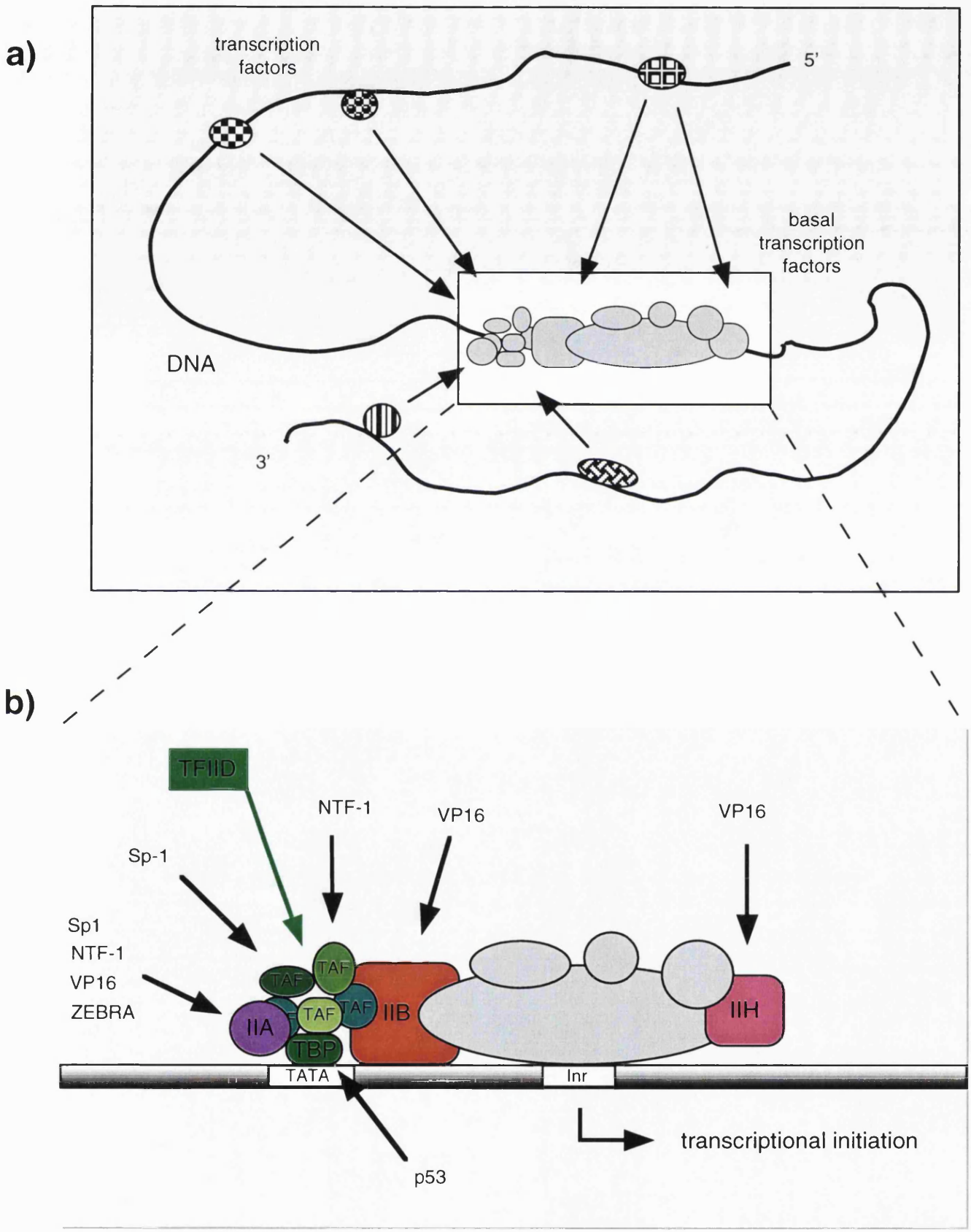
In accordance with this hypothesis a rising number of physical interactions between the TAF_{II}s and transcriptional activators are being reported (**figure 1.4**). Multiple contacts between activation domains and TAF_{II}s can strongly increase TFIID binding to the TATA element and synergistically activate transcription (Chen *et al.*, 1994a ; Jacq *et al.*, 1994 ; Sauer *et al.*, 1995). Cloning and reconstitution of the TFIID complex even reveals differential TAF requirements for distinct transcriptional activators. For example, the activator NTF-1 requires a complex containing TBP, $hTAF_{II}250$ and $hTAF_{II}150$, whereas the activator Sp1 additionally requires $hTAF_{II}110$ (Chen *et al.*, 1994a). A C-terminal activation domain of VP16 has been shown to interact with $TAF_{II}140$ and antibodies which disrupt this interaction impede transcriptional activation without affecting basal transcription (Goodrich *et al.*, 1993).

Figure 1.4

Interaction of transcriptional activators with the RNA polymerase II transcription initiation complex.

a. Depiction of the pre-initiation complex (highlighted in white square) in a DNA context showing postulated interactions with transcriptional regulators (represented by black/white patterned ovals) both up- (5') and down-stream (3') of the core promoter thus illustrating the concept of pre-initiation complex-regulator interactions across long stretches of DNA sequence.

b. The fully assembled minimal pre-initiation complex (see figure 1.1) highlighting the members believed, to date, to form transcription-promoting interactions with transcriptional activators bound to distant enhancer elements. TFIID is composed of multiple TAFs and TBP (green shaded ovals) and are with TFIIB (IIB - orange square) and TFIIH (IIH - pink square) bound to the TATA and Initiator (Inr) motifs on the DNA template (grey bar). TFIIA (IIA - purple circle) is a general transcription factor associated with TBP independently of the remaining pre-initiation complex and is probably only required for activated transcription. Postulated interactions of transcriptional activators with members of the pre-initiation complex are illustrated with black arrows.


a)
transcription factors
5'
basal transcription factors
DNA
3'
b)
TFIID
NTF-1
VP16
VP16
Sp-1
Sp1
NTF-1
VP16
ZEBRA
TAF
TAF
TAF
TAF
IIA
IIB
IIH
TBP
TATA
Inr
transcriptional initiation
p53

The glutamine-rich activation domain of Sp1 binds selectively to a glutamine-rich domain of hTAF$_{II}$110, and generally, mutations in the activation domains of the transcription factors, reveals a tight correlation between TAF binding and transcriptional activity (Hoey *et al.*, 1993 ; Gill *et al.*, 1994).

Positive Cofactors (PCs) are another group of factors believed to be involved in activation. They are not part of the TFIID complexes and they do not appear to stimulate basal transcription but rather enhance the response to transcriptional activators. Their mode of action is not clear but are found to interact with TFIIA (Maldonado & Reinberg, 1995).

TAF$_{II}$s and PCs are integral components of transcriptional activation, however, several laboratories have also demonstrated direct interactions between activators and non-TFIID basal transcription factors (**figure 1.4** ; Tjian & Maniatis, 1994). Mutations in TFIIB have been defined that disrupt interaction with VP16 and causes defects in activated but not basal transcription (Roberts *et al.*, 1993). TFIIH has also been shown to interact directly with VP16 as well as the tumour suppressor protein and transcription factor p53 (Xiao *et al.*, 1994). The precise role of TFIIA in transcription has been a source of much controversy but it is likely to be a stimulator of transcription possibly through TAFs. TFIIA interacts directly with *Drosophila* TAF$_{II}$110 and appears to contribute directly to the process of activation as it significantly enhances transcriptional activation by several activators including Sp1, VP16, NTF-1 and Zta (Maldonado & Reinberg, 1995). Additionally, the activator ZEBRA, a non-acidic activator from the Epstein-Barr virus (EBV), can stimulate the formation of a TFIID-TFIIA-TATA element complex (Chi *et al.*, 1995).

Mechanics of activation

Biophysical study of the dTAF$_{II}$42/dTAF$_{II}$62 complex suggests that TFIID contains a (dTAF$_{II}$42/dTAF$_{II}$62)$_2$ heterotetramer which could interact with H2B-like TAF$_{II}$s to form a histone-like octamer (Xie *et al.*, 1996 ; Hoffmann *et al.*, 1996 ; see above). It may well be possible that the interactions of regulative factors with the histone-like TAFs will serve to modulate this type of complex within TFIID. The TAF$_{II}$s which are believed to potentially adopt this histone-like structure have also been reported to interact with transcriptional activators. The isoleucine-rich activation domain of NTF-1 binds dTAF$_{II}$62 (Chen *et al.*, 1994a) while p53 and NF-κB/p65 activation domains bind dTAF$_{II}$42 (or its human homologue hTAF$_{II}$31) and dTAF$_{II}$62 (or its human homologue hTAF$_{II}$80 ; Lu & Levine, 1995). The VP16

activation domain interacts with dTAF$_{II}$42 (or its human homologue hTAF$_{II}$31 ; Goodrich *et al.*, 1993 ; Klemm *et al.*, 1995). It can be speculated whether interactions of these TAF$_{II}$s with the transcriptional activators will modify interaction of the histone structure with DNA.

Activator induced structural changes in TFIID have been demonstrated in the TFIID-promoter complex to be manifested by downstream extension of the TFIID footprint to well beyond the transcription start site and have been correlated with the increased recruitment of other general factors (Horikoshi *et al.*, 1988a ; Horikoshi *et al.*, 1988b). This suggests that binding of transcriptional activators can cause substantial rearrangements in the relative positions of TFIID subunits and DNA. It could be speculated that activator induced changes in TAF$_{II}$-DNA interactions allow the presumptive histone octamer-like substructure within TFIID to engage DNA and thus stabilise the pre-initiation complex.

It is as yet unresolved whether the interaction of transcriptional activators with TFIID is primarily, as believed by some, a recruiting function to bring TFIID to the promoter (Abmayr *et al.*, 1988 ; Workman *et al.*, 1988 ; Lieberman & Berk, 1994 ; Sauer *et al.*, 1995) or whether the interaction concerns already TATA-bound TFIID. The recent evidence concerning the possible histone-like structure of TFIID (see above) may suggest that TFIID can interact with the TATA element within chromatin on its own (Hoffmann *et al.*, 1996) while it may possibly be dependent upon activating factors to initialise the pre-initiation complex formation. The question is how TFIID can gain access, whether this is dependent upon activators or whether this is an inherent property of the complex itself. The histone-like structure found within TFIID may provide access by virtue of competition with chromatin structures as has been suggested by experiments reported above.

Recent studies performed on the yeast holoenzyme, which does not appear to contain TFIID, shows that this complex too may possess chromatin-disrupting activity. The yeast SWI/SNF complex has been found to be a stoichiometric and integral component of the yeast Pol II holoenzyme and endows the holoenzyme with the ability to disrupt nucleosomes (Wilson *et al.*, 1996). SWI/SNF is a highly conserved yeast complex that contains approximately ten proteins including many identified by mutations that affect transcription *in vivo* (Peterson & Tamkun, 1995). The complex is a DNA-stimulated ATPase and disrupts nucleosomal arrays in an ATP dependent manner *in vitro* (Peterson & Tamkun, 1995). This alteration in chromatin structure can facilitate binding of activator proteins or TBP to their target sites on nucleosomal templates *in vitro* (Côté *et al.*, 1994 ; Imbalzano *et al.*, 1994). Transcriptional defects caused by loss of SWI/SNF function can be alleviated by mutations in histones and other proteins that affect chromatin (Struhl, 1996).

So possibly despite TFIID having gained access to within the chromatin, the remaining holoenzyme may still need to perturb chromatin structures in order to join it. It is even possible that TFIID may be more or less permanently integrated in the chromatin (Hoffmann *et al.*, 1996) at active genes waiting for holoenzyme, or individual basal transcription factors, to access it. It seems from the above that the holoenzyme will be able to perturb chromatin structure and there is also evidence that activators *in vivo* can perturb chromatin structure in the absence of a functional TATA element and transcription (Struhl, 1995 ; Struhl, 1996). All in all, this suggests that the individual elements involved in transcriptional initiation could potentially gain access and stabilise each other during assembly within a chromatin structure, to form an active pre-initiation complex.

The cell cycle

DNA replication (S-phase) and cell division (M-phase) are the two most active and most easily observed "phases" of the replicative cell cycle. They are separated by two gap periods (G1 and G2) during which cells are believed to assess their capacity to progress onto the next phase. Non-replicating, or quiescent, cells are believed to enter a separate status, distinct from cell cycle-arrest, and referred to as G0. Distinct phases of differential gene expression accompany the progression of the cell cycle as their products are required for the sequential mechanistic tasks of cellular division (Kamb, 1995 ; see **figure 1.5** and accompanying legend).

The cyclin-dependent kinases

Cyclin-dependent kinases (CDKs) are a family of kinases which phosphorylate serine and threonine residues and are believed to mediate the downstream consequences of cell cycle progression (Nigg, 1995). The activity of the CDK family members, and its temporal regulation, is crucial to the progression of the cell cycle. In eukaryotes a variety of CDKs appear to be required to regulate different cell cycle stages. In mammals several CDKs have been described and are defined by number (CDK1, 2, 3 ...) while CDK1 is most frequently referred to as CDC2 for historical reasons. They are well conserved (40-70% identity) and also show extensive similarity with other serine/threonine protein kinases within their catalytic domains (Lees, 1995). The precise control of CDK activity is believed to be accomplished by a combination of modulatory effects including regulation via control of the availability of cyclin activating subunits, phosphorylation events on conserved sites in CDKs, and by exposure to a number of polypeptides that function to inhibit them (Lees, 1995 ; Morgan, 1995 ; Nigg, 1995 ; Pines, 1995).

Cyclin regulation

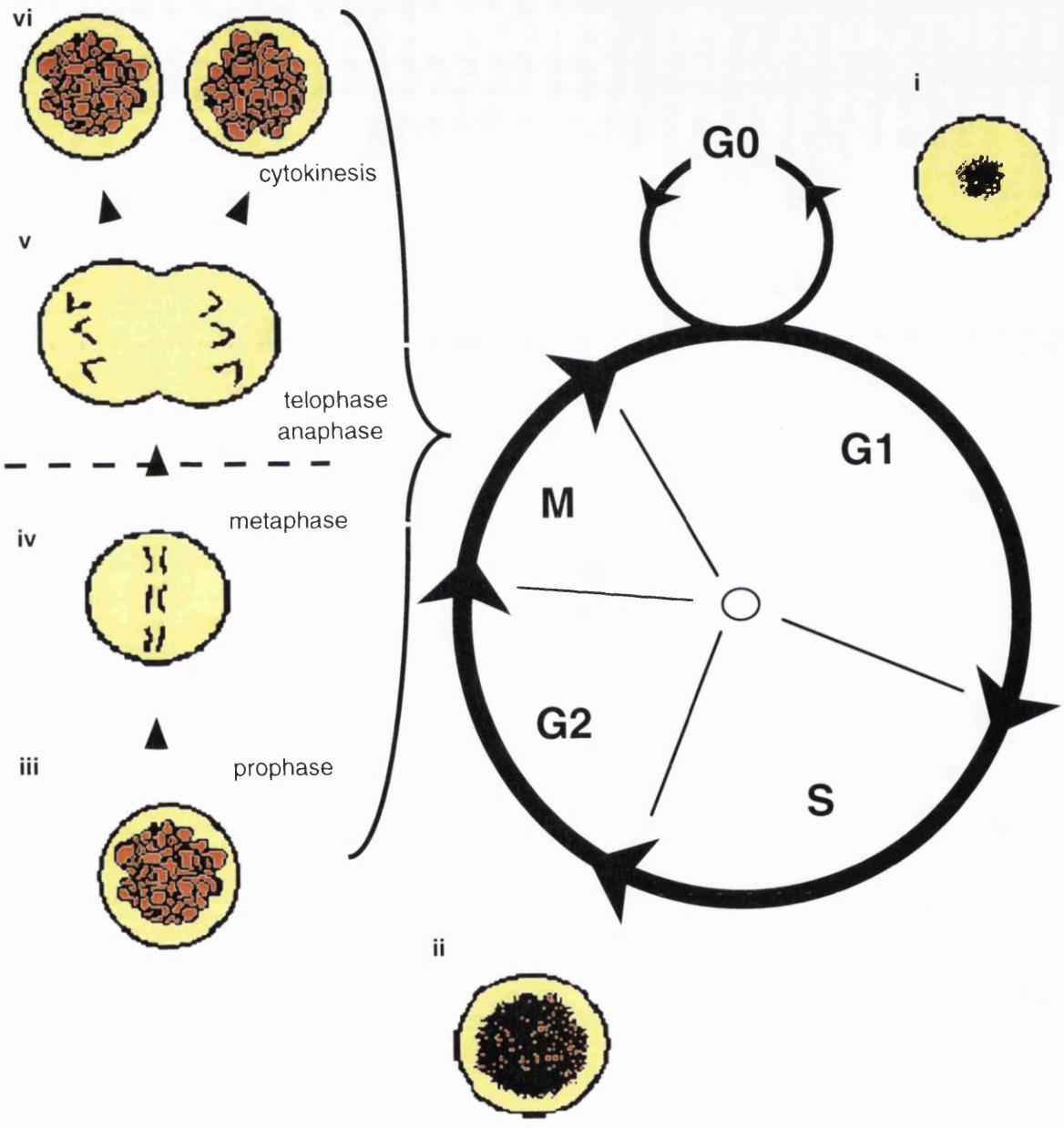
The cyclical activities of the members of the CDK family are differentially activated along the consecutive phases of the cell cycle (Nigg, 1995). Monomeric CDKs have almost no kinase activity and they require the binding of regulatory subunits known as cyclins for full activation (Lees, 1995). Formation of different cyclin/CDK complexes at appropriate stages during the cell cycle is crucial for the cells ability to control proliferation and is thus targeted by a number of regulatory

Figure 1.5
The cell cycle.

Cell division is not only required by a growing embryo but is also widespread in the adult mammalian body to counter lost cells by wear and tear or by programmed cell death. An adult human must manufacture millions of new cells each second for efficient maintenance. If all cellular division is halted, by for example ionising radiation, the body will die within a few days.

The complete cell cycle can take from eight minutes in a fly embryo cell to more than a year in mammalian liver cells. It is divided into the two most visibly distinct periods : Mitosis and the Interphase. During Interphase the cell assesses its capacity to divide and commits itself to do so by initiating the replication of its DNA and by doubling its mass. Mitosis is the more visibly active of the two periods and involves the separation of chromosomes to opposite ends of the cell followed by fission into two cell bodies.

Many cells in the mammalian body are in a state of non-commitment to growth and division called quiescence or G0 (Gap-0 phase). Commitment to a round of cellular replication occurs during G1 (Gap-1 phase) which is either entered from a previous round of replication or from G0. During G1 the cell monitors its environment and its own size and takes the decision whether or not to commit to DNA replication. At G1 a depiction of the nucleus (i) displays normal diploid DNA content which as the cell enters and passes through S (Synthesis phase) doubles (ii). During G2 (Gap-2 phase) the cell prepares for cellular division (Mitosis). G2 phase provides a safety gap allowing the cell to ensure that DNA replication is complete before entering Mitosis (M). The Gap phases separate M from S and during these the cell doubles its mass. Young embryos have no Gap-phases and so halve in size for each division. (iii) In the prophase of M, the nuclear envelope breaks down and the contents of the nucleus condenses to form visible chromosomes. The cell's microtubules reorganise to form the mitotic spindle which will eventually separate the chromosomes. (iv) In metaphase the chromosomes align themselves on the mitotic spindle, and there is a brief pause in visible activity. (v) In anaphase and telophase the chromosomes move to the poles of the spindle where they reform intact nuclei. (vi) Finally, by cytokinesis, or cellular fission, two daughter cells are created.


vi
cytokinesis
v
telophase
anaphase
iv
metaphase
iii
prophase
i
G0
G1
M
G2
S
ii

systems (Lees, 1995 ; Müller, 1995). Cyclin-CDK complexes have been implicated in the regulation of initiation and completion of DNA replication and cellular division (see below) and at a major control point called START, defined in yeast. At START the cell commits itself to a further round of DNA replication rather than the alternative fates of quiescence or, in yeast, mating (Pines, 1995). In mammalian cells the restriction point (R) can be thought of as a rough equivalent to START in yeast and has equally been implicated with specific cyclin-CDK regulation (Zetterberg *et al.*, 1995).

The CDKs, being stable proteins, are believed to be in excess of their cyclin partners and so cyclin abundance thus appears to be rate-limiting for progression through the different stages of the cell cycle (Pines, 1995). The cyclin family of protein factors is defined by homology in an approximately 100 residue region called the cyclin box which is the well conserved region that binds the CDKs. The best defined members are cyclins D, E, A and B, although other cyclins have been identified such as C, F, H and G whose roles are, as yet, relatively unclear (Pines, 1995).

Enzymatic measurements of the monomeric CDKs versus the cyclin-bound forms have indicated that cyclin binding leads to a 40,000-fold increase in CDK-kinase activity (Connel-Crowley *et al.*, 1993). Crystal structure analysis of the monomeric CDK2 (De Bondt *et al.*, 1993) compared to that of the structure of the cyclin A-CDK2 complex (Jeffrey *et al.*, 1995a) makes it evident that the binding of cyclin A causes dramatic structural changes within CDK2. This includes the rearrangement of the catalytic residues within the active site to a conformation which more closely resembles the E-K-D triad conformation (single letter amino acid code) found in other eukaryotic serine/threonine kinases that do not depend on a regulatory subunit for activation (Lees, 1995).

In vitro, cyclins are able to bind a large variety of CDKs and *vice versa* (Pines, 1995). Most of the cyclin-CDKs phosphorylate the same basic consensus sequence *in vitro* : (K/R)-S/T-P-X-(K/R) where the basic residues are preferred but not essential (Nigg, 1993 ; Pines, 1995). However the situation found *in vivo* contrasts to this apparent polygamy and only a handful of specific CDK-cyclin complexes have been described, and they sometimes appear to display differential substrate specificities (Nigg, 1993 ; Pines, 1995).

Since CDK activity is so dependent upon the presence of cyclin partners, cyclin turnover by the combination of transcription and protein degradation is a major target of CDK regulation. The transcriptional regulation of cyclin genes is poorly understood. Expression of the D-type cyclins is induced by cytokines and provides a possible mechanism whereby such growth factors can promote cell cycle progression and a good example of how proliferation-promoting extracellular factors could

influence the progression of the cell cycle (Matsushime *et al.*, 1991). There is some evidence that cyclin-CDK complexes promote the expression of later cyclins in mammalian cells and it is possible their cyclical expression patterns are due to interdependent cyclin regulatory cascades (Schulze *et al.*, 1995 ; see DRTF1/E2F section).

The better understood mechanism of cyclin protein level control is that of cyclin degradation. The cyclins can be roughly divided into the G1 and G2 groups, according roughly to the cell cycle phase in which they exert their function. The G1 cyclins, D and E, contain P-E-S-T sequences (single letter amino acid code) in a region C-terminal to the cyclin box which ensures their relatively short half-life (roughly 30 minutes) such that their level is determined by their rate of transcription (Pines, 1995). Cyclin Ds arise in mid-G1 and are required through to late G1 where they are rapidly turned over (Baldin *et al.*, 1993 ; Quelle *et al.*, 1993). Cyclin E accumulates at the G1/S-phase transition, and is degraded rapidly once the cells are in S-phase (Ohtsubo *et al.*, 1995).

The G2 cyclins, A and B, accumulate at the G1/S transition and the S-phase respectively (Girard *et al.*, 1991 ; Pines & Hunter, 1991), but both possess a region called the destruction box, loosely conserved between G2 cyclins, which targets them for destruction in an ubiquitin-dependent manner during mitosis (Glotzer *et al.*, 1991 ; Irniger *et al.*, 1995 ; King *et al.*, 1995 ; Tugendreich *et al.*, 1995 ; Murray, 1995).

CAK regulation

Post-translational modifications of CDKs add a further step to their regulation. Cyclin binding shifts an inhibitory "T loop" domain in CDKs to expose the T160 amino acid residue (single letter code) within the loop thus making it more accessible for phosphorylation by the CDK-Activating Kinase, CAK (Jeffrey *et al.*, 1995a). Phosphorylation of the conserved threonine residue is critical for the activity of CDK complexes (Fisher & Morgan, 1994). It has been proposed that T160 phosphorylation in CDK2 by CAK induces further conformational change to fully expose the catalytic cleft in order that substrates may bind (Jeffrey *et al.*, 1995a).

CAK is a multimeric enzyme complex composed of a distantly related cyclin-CDK pair, cyclin H-CDK7 (Fesquet *et al.*, 1993 ; Poon *et al.*, 1993 ; Solomon *et al.*, 1993 ; Fisher & Morgan, 1994 ; Makela *et al.*, 1994). CAK activity is constant throughout the cellular proliferation stages of the cell cycle and is therefore unlikely to be rate-limiting for cell cycle progression (Brown *et al.*, 1994 ; Tassan *et al.*, 1994). The presence of a T176 on CDK7 (equivalent to T160 in other CDKs) however, raises the possibility that CAK itself may be regulated by yet another

threonine kinase especially since mutation of this residue severely reduces CAK enzymatic activity (Fisher & Morgan, 1994 ; Labbe *et al.*, 1994). As a member of TFIIH, CAK may also have a direct role in the regulation of the basal transcription complex (see Transcription section).

A third component of CAK, the appropriately named MAT1 (Menage-A-Trois) has recently been isolated in starfish and *Xenopus* and is structurally related to the ring-finger family of proteins (Devault *et al.*, 1995). It has been implicated in the stabilisation of cyclin H-CDK7 complexes and may provide a mechanism for regulation of CAK heterotrimeric activity (Devault *et al.*, 1995).

Wee1/CDC25 regulation

Another mode of CDK regulation is through phosphorylation of a conserved tyrosine residue, tyrosine 15, in CDC2 of fission yeast. Tyrosine 15 is located within the ATP binding region of the enzyme (Atherton-Fessler *et al.*, 1993 ; Dunphy, 1994) and phosphorylation may sterically hinder substrate access to the catalytic site. Wee1 tyrosine kinase phosphorylates CDC2 on tyrosine 15 while its own activity is intricately regulated by an upstream network of kinases and phosphatases (Dunphy, 1994). Many of these processes have been genetically defined in fission yeast and, as yet, mammalian homologues of these genes have not been found. The kinase responsible for the phosphorylation of threonine 14 in CDC2 in higher eukaryotes has not yet been identified however its activity has been detected in both *Xenopus* extracts and in human cells (Atherton-Fessler *et al.*, 1994 ; Kornbluth *et al.*, 1994). In mammalian cells the cyclin B-CDC2 complex accumulates in an inactive form through S- and G2-phases, because the protein kinase activity of the CDC2 is inhibited by phosphorylation on threonine 14 and tyrosine 15 (Dunphy, 1994).

The CDC25 family of phosphatases, CDC25A, B and C, are tightly regulated enzymes and are responsible for dephosphorylation, and hence activation, of CDKs phosphorylated on both threonine 14 and tyrosine 15 (Dunphy, 1994 ; Hoffmann & Karsenti, 1994a). CDC25A is expressed in G1 and is essential for the G1 to S-phase transition (Hoffmann *et al.*, 1994b ; Jinno *et al.*, 1994).

The opposing activities of Wee1 kinase and CDC25C phosphatase govern the timing of mitosis (Pines, 1995). Tyrosine phosphorylation may allow the accumulation of inactive pools of cyclin-CDK complexes that can be rapidly activated by CDC25. CDC25 can be phosphorylated and activated itself by CDC2 and CDK2 to form a positive feedback loop to amplify CDK activity (Hoffmann *et al.*, 1993 ; Hoffmann *et al.*, 1994b ; Ogg *et al.*, 1994). In conjunction with the control that tyrosine phosphorylation provides for CDK regulation, this modification allows a mechanism whereby other signalling pathways can be integrated with cell cycle

control. For example signals from DNA damage appear to be mediated via CDC25 and prevent its phosphatase activity (Terada *et al.*, 1995) and a recent report linked the Raf1 kinase and the CDC25A providing a possible connection to receptor-mediated events and the cell cycle machinery (Galaktiokov *et al.*, 1995). Although this tyrosine residue is invariant in all CDKs, the importance of tyrosine phosphorylation as a regulator of CDK activity has only been well established for CDC2 and CDK2 kinases (Terada *et al.*, 1995). Recent work, however, has shown that CDK4 also contains phosphotyrosine suggesting that it too may be subject to regulation by Wee1 and CDC25 (Terada *et al.*, 1995).

The protein phosphatase PP2A is intimately concerned with preventing the premature activation of CDC25C, and perhaps the other types of CDC25 too. When PP2A is inhibited by adding okadaic acid to cells or frog egg extracts, CDC25C is activated and in turn causes the premature activation of cyclin B-CDC2, the mitotic kinase (Clarke *et al.*, 1993a).

Inhibitor regulation

A key mechanism in regulating the activity of the cyclin-CDK complexes involves a group of recently defined Cyclin-CDK Inhibitors (CDIs). To date two families of inhibitors, based on sequence similarity and mode of action, have been identified : p16 and p21 (Sherr & Roberts, 1995).

The p16 family of inhibitors include p16 (*Ink4a*) and three related polypeptides : p15 (*Ink4b*), p18 (*Ink4c*) and p19 (*Ink4d* ; Serrano *et al.*, 1993 ; Guan *et al.*, 1994 ; Hannon & Beach, 1994 ; Kamb *et al.*, 1994 ; Chan *et al.*, 1995 ; Hirai *et al.*, 1995) which are structurally related to each other. A hallmark for this family is their specificity for binding to, and inhibiting, CDK4 and CDK6. p16 will dissociate cyclin D-CDK4 complexes and p18 and p19 appear to have similar properties (Parry *et al.*, 1995). Recent findings suggest that because the p16 family of inhibitors can also bind preformed complexes of cyclin D and CDK4 and 6 *in vitro*, they may also inhibit interaction of the kinase complexes with their substrates (Hirai *et al.*, 1995).

The p21 (*Kip1/Waf1/Cip1* - hereon referred to as *Waf1*) family of inhibitors comprises three distinct gene products : p21, p27 and p57 which share an amino-terminal conserved region shown to be important for interaction with, and inhibition of cyclin-CDK complexes (Polyak *et al.*, 1994a ; Harper *et al.*, 1995 ; Lee *et al.*, 1995 ; Matsuoka *et al.*, 1995). p21 is able to inhibit all the G1 cyclin-CDK complexes and to a lesser extent cyclin B-CDC2 complexes (Harper *et al.*, 1995 ; Matsuoka *et al.*, 1995).

The p21 family of inhibitors can prevent the CDK activation by CAK but this may not be their primary mode of inhibition as they can also bind to CAK-phosphorylated cyclin-CDK complexes and inhibit their activity (Sherr & Roberts, 1995). In addition p21 also binds and inhibits the proliferating-cell nuclear antigen (PCNA), a regulatory unit of DNA polymerase δ, the principal replicative DNA polymerase (Waga *et al.*, 1994), and thus, a direct target of the DNA replication machinery.

The variety of different inhibitor molecules discovered suggests that each may have an independent specialised role to play in the cell cycle. The large number of inhibitors may reflect the requirement to respond to a variety of different signalling pathways. The importance of these CDIs in cell cycle control has been inferred from the observations that inhibitory proteins from both families (specifically : p15 and p21) have been implicated with TGFβ-induced cell cycle arrest (Hannon & Beach, 1994 ; Polyak *et al.*, 1994b) and that expression of p21 can be induced by DNA damage (El-Deiry *et al.*, 1993 and see below).

CDKs and the cell cycle

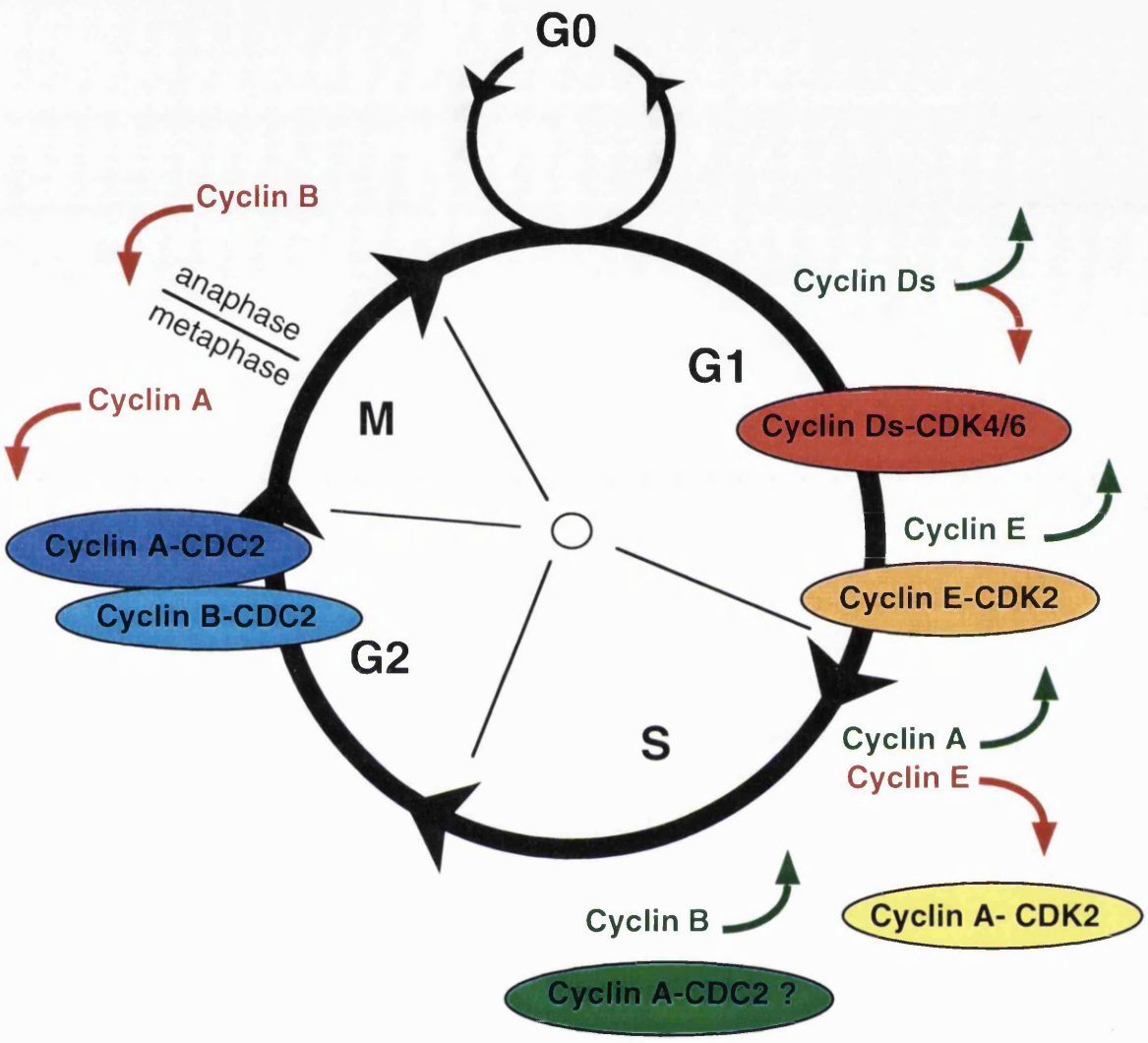
The orderly progression of the cell cycle depends on the timely activation of specific cyclin-CDK complex activities (Lees, 1995 ; Pines, 1995 and **figure 1.6**). The regulation of these proliferative processes will ultimately arise from extracellular cues. Particularly critical are the cues that regulate the passage of cells through the G1 phase of the cell cycle and into S-phase (Müller, 1995). Mammalian cells remain responsive to these cues for most of G1, until they pass through the restriction point after which they become committed to enter and complete S-phase (Pardee, 1989). It is clear that many of the known key regulatory proteins of the cell cycle are induced in or shortly before S-phase. This bias is probably a reflection of the fact that this is the period during which most cell-cycle checkpoints operate and that the induction of DNA synthesis is a major target of these checkpoints (Müller, 1995).

G1 to S

D-type cyclins have a very short half-life (approximately 30 min) and their synthesis is highly growth-factor dependent, when growth factors are withdrawn cyclin D synthesis ceases immediately suggesting that the D-type cyclins may act as growth factor sensors (Matsushime *et al.*, 1991). Cyclin D-dependent kinase activity arises in mid-G1 and is required through to late G1 for progression into S-phase (Baldin *et al.*, 1993 ; Quelle *et al.*, 1993). Overexpressing cyclin D genes, however,

Figure 1.6
Cyclin-CDK complexes during the progression of the cell cycle.

Depiction of the synthesis (green arrows) and destruction (red arrows) of cyclin family members during the progression of the cell cycle. Active cyclin-CDK complexes detected a various stages of the cell cycle are depicted in coloured ovals. Cyclin B is only degraded after the metaphase/anaphase transition.


G0
Cyclin B
anaphase
metaphase
Cyclin A
M
G1
Cyclin Ds
Cyclin Ds-CDK4/6
Cyclin E
Cyclin E-CDK2
Cyclin A-CDC2
Cyclin B-CDC2
G2
S
Cyclin A
Cyclin E
Cyclin B
Cyclin A- CDK2
Cyclin A-CDC2 ?

only moderately accelerates cells into S-phase (Quelle *et al.*, 1993) while induction of high levels of cyclin D1 at G1/S can functionally antagonise cellular proliferation by preventing S-phase entry (Quelle *et al.*, 1993 ; Baldin *et al.*, 1993 ; Atadja *et al.*, 1995) suggesting that the physiological effect of cyclin D1 may depend on the timing and the levels at which it is expressed. This has led to the hypothesis that nuclear clearance of cyclin D1 represents a prerequisite for S-phase entry (Baldin *et al.*, 1993). D-type cyclins are able to bind several different CDK partners : CDK2, 4, 5 and 6 (Xiong *et al.*, 1992 ; Bates *et al.*, 1994 ; Meyerson & Harlow, 1994). Of these, their main and consistent partner *in vivo* appears to be CDK4, and in many cell types CDK2, 5, and 6 are not associated with cyclin D (Matsushime *et al.*, 1992). CDK4 is unusual amongst the CDKs in that it associates with its partner cyclin for only a short period of the cell cycle, in late G1 and early S-phase (Matsushime *et al.*, 1992).

The cyclin E family are induced in late G1. Overexpressing E-type cyclin genes only moderately accelerates entry into S-phase (Ohtsubo & Roberts, 1993), but if both D- and E-type cyclin genes are overexpressed, there is a marked additive acceleration through G1 phase and into S-phase (Resnitzky *et al.*, 1994 ; Resnitzky & Reed, 1995a) suggesting that both cyclins control different rate-limiting events. *Drosophila melanogaster* mutated in *cyclin E*, arrest in development with their cells locked in late G1 phase (Knoblich *et al.*, 1994). Cyclin E forms an active protein kinase complex exclusively with CDK2 and the formation of this holoenzyme is seen as a crucial step in triggering progression from G1 to S-phase and the complex appears to be the target of TGFβ growth repression in mammalian cells (Koff *et al.*, 1993). TGFβ treatment activates the p27 CDI which is present in a latent form in untreated cells and is structurally related to p21 in the N-terminus (Polyak *et al.*, 1994a), the part of the protein which interacts with the cyclin-CDK complex. p27 binds and inhibits cyclin E-CDK2 and thus blocks the cell in late G1 phase (Polyak *et al.*, 1994b).

S-Phase

Genes encoding cyclin A and CDC2 are both activated around the G1/S transition and reach peak expression levels in late S-phase (Pines, 1995). Once the cells have entered S-phase the main cyclin-CDK complex present is cyclin A-CDK2 and there is some evidence that it is required for DNA replication (Girard *et al.*, 1991). For example, a good substrate for cyclin A-CDK2 *in vitro* is the replication factor RF-A (Dutta & Stillman, 1992). RPA helicase activity is enhanced after phosphorylation with cyclin A-CDK2 *in vitro*, and the site of phosphorylation is the same as that found on RF-A in S-phase cells (Dutta & Stillman, 1992). Inhibition of either cyclin A (Girard *et al.*, 1991 ; Pagano *et al.*, 1992) or CDK2 function (Pagano

et al., 1993 ; Tsai *et al.*, 1993) will prevent entry into S-phase and induction of cyclin A in fibroblast cells advances them prematurely into S-phase (Resnitzky *et al.*, 1995b) suggesting role in the G1/S transition. Low cyclin A-kinase activity has been correlated with the complexing of p27 and *vice versa* (Resnitzky *et al.*, 1995b) suggesting a regulatory role. Antisense *cyclin A* treatment or anti-cyclin A antibodies when microinjected into cells at G2 will prevent mammalian cells from entering mitosis (Pagano *et al.*, 1992).

G2 to M-Phase

Genes encoding cyclin B and CDC25C are both induced in S-phase and are expressed at maximum levels in G2 (Pines, 1995). The cyclin B-CDC2 complexes are the primary active protein kinases in mitosis, accumulating in the cytoplasm at interphase and then rapidly activated and translocated into the nucleus at the beginning of mitosis (Pines & Hunter, 1991). During metaphase cyclin B-CDC2 complexes are associated with the mitotic spindle (Pines & Hunter, 1991). Cyclin B is degraded at the metaphase-anaphase transition, thus inactivating CDC2. This event is necessary for exit from mitosis and mutant B-type cyclins that cannot be degraded stall cells at that point (Glotzer *et al.*, 1991 ; Murray *et al.*, 1989 ; Surana *et al.*, 1991). Rapid degradation of A-type cyclins is also essential for the exit of cells from mitosis (Irniger *et al.*, 1995 ; King *et al.*, 1995 ; Tugendreich *et al.*, 1995).

The cell cycle and cancer

Cancer is not a disease of the cell cycle, but rather a communications breakdown between up- and down- stream events of it. The cell cycle ensures the timely activation of a multitude of "engineering" genes to facilitate a perfect replication into two viable daughters. Due to the extreme complexity of this endeavour, inappropriate timing of cell cycle events is more likely than not to result in cell death. As such, a tumour cell will still retain a basically well functioning cell cycle machinery which responds to the mechanical demands of the proliferative process. Cancer cells still progress through the four broadly defined phases of the cell cycle, however, they tend to lack the ability to modulate these phases in response to external factors and to DNA damage, most obviously at the G1-S and G2-M checkpoints (Pines, 1995 ; Kamb, 1995).

Mutation of cell cycle components

Studies of naturally occurring tumours have found a relatively low abundance of mutations in the central components of the cell cycle. The cell cycle genes found to be deregulated in cancer thus far are only most of the ones coding for the p16 family of CDK inhibitors and two of the cyclin D family members (Pines, 1995 ; Kamb, 1995 ; see below). Interestingly, these are all components that have been associated with the G1 to S-phase transition (see cell cycle section), a reflection, possibly, of the important checkpoints in this period where the cell commits itself to a new round of replication (Müller, 1995).

The particular gene families involved are also interesting in that they have been associated with the integration of the cell cycle and extracellular cues, and are so reflecting such a loss often observed in neoplasms. p16 preferentially inhibits CDK4 and 6, CDK4 being the favoured partner of growth factor-inducible cyclin D (see cell cycle section), and p15 expression is strongly increased in response TGFβ-induced cell cycle arrest (Hannon & Beach, 1994). These proteins have thus been associated more with "communicative" roles, rather than with integral members of the cell cycle machinery (Pines, 1995 ; Lees, 1995). The levels of other cyclins are often raised in transformed cells and while there are some associations between non-D cyclins and the tumourigenic state, their raised levels are likely to be more of consequence of the greater proliferative rate than a cause of the neoplastic state itself (Pines, 1995)

p16-type CDI family

The gene for p16 (*Ink4a*) maps to human chromosome 9p12 close to a familial melanoma susceptibility locus (Kamb *et al.*, 1994) and is rearranged, deleted, or mutated in a majority of gliomas, leukaemias and melanomas (Kamb *et al.*, 1994 ; Nobori *et al.*, 1994). Indeed, *Ink4a* is a leading candidate for the melanoma susceptibility gene and now frequently referred to as a tumour suppressor. However, recently it has emerged, that *Ink4a* mutations appear to be more common in the establishment of cell lines, and less so than previously thought, in primary tumours (Pines, 1995). This issue is awaiting clarification. *Ink4b* (coding for p15) has also to some extent been associated with human tumours, while *Ink4c* (coding for p18) mutations appear to be rare (Zariwala *et al.*, 1996).

D-type cyclin family

There are three types of D cyclin : 1, 2 and 3 which are cell type specific (Sherr, 1993). Most cells express D3, and either D1 or D2, but not all three (Sherr, 1993). *Cyclin D1* has been identified as the *PRAD1* proto-oncogene (Motokura *et al.*, 1991) and as the most likely candidate for the *Bcl-1* proto-oncogene (Withers *et al.*, 1991). *Cyclin D2* has been shown to be the mouse *Vin-1* proto-oncogene (Hanna *et al.*, 1993) while human *cyclin D3* has not yet been oncogenically associated.

Deregulation of cyclin D1 expression occurs in several types of human cancer (Pines, 1995). Since it often results from a specific chromosomal abnormality, this over-expression is likely to be important in the development of the disease. Overexpressing D-type cyclin genes alone is not sufficient to transform a cell and only moderately accelerates cells into S-phase (Quelle *et al.*, 1993) which may be a general reflection of the requirement for many oncogenes to coorporate in tumourigenesis (Hunter, 1991). Cyclin D1 is also implicated in virally induced tumours in mice, and transgenic models based on the ectopic expression of cyclin D1 mirror features of naturally occurring tumours (Bates & Peters, 1995). By these criteria, *cyclin D1* has the hallmarks of a cellular proto-oncogene .

Other cyclins

There is some evidence that cyclin E differs in normal compared with transformed cells. Cyclin E levels are increased in a large fraction of tumours and some tumours have mutation in *cyclin E* (Keyomarsi *et al.*, 1994). However, there is no evidence that *cyclin E* itself is a proto-oncogene (Pines, 1995). Cyclin A has been implicated in oncogenesis. In one clonal hepatoma caused by a hepatitis B virus

(HBV), the site of integration was found to be the cyclin A gene (Wang *et al.*, 1990) causing the disruption of its N-terminus including the destruction box (Wang *et al.*, 1992) the resulting chimaera being unable to be broken down in the normal fashion during mitosis. The factors affected by such deregulation are not known and documented changes of *cyclin A* in cancer cells are still relatively rare (Pines, 1995).

The retinoblastoma gene

The fundamental importance of the periodically regulated CDK kinase activities for the cell cycle is very established (see cell cycle section). It seems clear from the above section, that there is a general lack of oncogenic involvement in the most basic cell cycle machinery in tumourigenesis such as cyclins and CDKs. Most of these components are mainly involved in the running of the carefully timed events that facilitate downstream cellular replication events. These factors and their downstream targets would be expected to remain relatively unaltered in tumour cells which still require this basic "service". The cell cycle factors which are modulated in tumourigenesis appear to be the ones involved in "talking" to the outside of the cycle, such as cyclin Ds. The downstream effectors of these cell cycle factors are therefore of particular interest. One of the favoured physiological targets of cyclin D-CDK complexes is the retinoblastoma susceptibility gene product (Kato *et al.*, 1993 ; Dowdy *et al.*, 1993).

Relatively few clear downstream target systems of CDK kinase activity have yet been identified to link the cell cycle machinery with the gene products that carry out the actual task of cell division. The retinoblastoma susceptibility gene product-associated pathways probably represents the best described system that demonstrates cell cycle regulation over a factor fundamentally interlinked with the control of the proliferative state.

The Retinoblastoma gene and cancer

The retinoblastoma susceptibility gene (*RB*) was first identified as the double-allele mutation in retinoblastoma, a rare childhood cancer, and the single allele mutation associated with susceptibility to the disease (Knudson Jr, 1971). This type of cancer occurs both as an inherited disease as well as sporadically - without inherited predisposition (Whyte, 1995). Inheritance of a defective allele of *RB* provides a strong predisposition for developing retinoblastoma. Loss of the remaining functional allele of *RB* appears to be a rate-limiting event for tumour initiation (Whyte, 1995).

Mutation of *RB* also occurs in many other types of human tumours, not via germline mutations but via alterations to both alleles of *RB* during tumourigenesis (Whyte, 1995). The tumours are of widely diverse origin including osteosarcomas, small cell lung carcinomas, breast carcinomas, prostate carcinomas and bladder carcinomas. Most striking are small cell lung carcinomas where loss of *RB* occurs in virtually 100% of tumours (Weinberg, 1991 ; Cowell & Hogg, 1992). It is not known why different tumours are differentially associated with *RB* mutation (Whyte, 1995). Due to *RB*'s link to the neoplasmic state, it has been termed a tumour suppressor gene, a label given to gene products that hold back a cell from its normal growth potential and which are essential to the control of the proliferative state. Microinjection of *RB* into tumour cell lines deficient for this gene results in an increase in the number of cells in G1 and a reversal of the tumourigenic state (Hinds *et al.*, 1992 ; Goodrich *et al.*, 1991) and attempts to co-express the *RB* gene product stably with a selectable marker, generally result in greatly decreased number of cellular colonies (Templeton *et al.*, 1991 ; Muncaster *et al.*, 1992 ; Qian *et al.*, 1992 ; Hinds *et al.*, 1992 ; Qin *et al.*, 1992 ; Hiebert, 1993 ; Fung *et al.*, 1993). These experiments tally with the perceived role of *RB* as a tumour suppressor gene.

By the technique of homologous recombination, *RB* knockout mice have been created (Clarke *et al.*, 1992 ; Jacks *et al.*, 1992 ; Lee *et al.*, 1992). Interestingly mice which are heterozygous for the *RB* mutation do not develop retinoblastomas but develop tumours of the brain and pituitary gland. This could either be an effect of laboratory knockouts or a reflection of subtly different roles of *RB* in mice and humans. Mice with germline mutations in both alleles of *RB* die *in utero* at approximately day 13.5 of development with abnormalities reported in blood, liver and brain (Clarke *et al.*, 1992 ; Jacks *et al.*, 1992 ; Lee *et al.*, 1992). This indicates that apart from a role in tumour suppression, *RB* also contributes to the process of cell differentiation, although not in all cell types. *RB* loss is thus tolerated by many cell types, as also demonstrated by the absence of *RB* in many cell lines.

The *RB* gene product (pRb) is a 105 KDal nuclear phosphoprotein (Kouzarides, 1995 and see below). A number of cellular proteins have been reported to associate with it including cyclins, kinases, and a substantial number of transcription factors. Binding of pRb to these factors can modulate their function (Kouzarides, 1995). Naturally occurring point mutations and deletions of pRb found in tumours all seem to abolish its function. pRb is the target of CDK-cell cycle dependent phosphorylative control likely to be mediated by cyclin Ds (Kouzarides, 1995 and see below).

Amongst the factors that interact with pRb, the transcription factor complex DRTF1/E2F is the best characterised, and arguably, the most important target identified to date (La Thangue, 1994). DRTF1/E2F induces transcription of a large

range of genes associated with the entry into S-phase and is inhibited by one phospho-form of pRb. Overactivated DRTF1/E2F will on its own force quiescent cells into S-phase, unaccompanied by the usually observed increase in CDK activity (Degregori *et al.*, 1995), thus apparently by-passing an entire section of cell cycle control mechanisms (see DRTF1/E2F section). This seems to highlight the centrality of this transcription factor as a target for regulation in the control of cell cycle progression.

The p53 gene

The only documented tumour suppressor gene more widely mutated in human tumours than *RB* is *p53* (Hollstein *et al.*, 1991). p53 is a repressor of a different nature than pRb as rather than being an active participant of the cell cycle, it appears only to be required when cells are distressed and in danger of acquiring mutation (see below). Strikingly, one of its modes of action involves G1 arrest and it seems to mediate this through the phosphorylation status of pRb, and thus indirectly targeting the activity of DRTF1/E2F (see below).

p53 and cancer

One can live without *p53*, but not for long. *p53*-deficient mice develop normally but are extremely susceptible to tumour formation and die early from cancer (Donehower *et al.*, 1992) with 70% developing tumours by six months of age (Jacks *et al.*, 1994 ; Williams *et al.*, 1994). The closest human equivalent is Li-Fraumeni syndrome in which one *p53* allele is mutated and predisposes victims to grossly abnormal cancer susceptibility (Srivastava *et al.*, 1990). No humans cases with double allele *p53* mutations have been reported, possibly due to consequential death *in utero.* Mutation of *p53* represents one of the most common known genetic events in human cancer accounting for around 60% of all tumours (Hollstein *et al.*, 1991 ; Hainaut, 1995). *p53* is subject to mutation in a multitude of cancer types, and in certain ones, mutational frequencies approach 90% (Harris, 1993). It thus seems that while p53 is dispensable for normal growth, its loss dramatically predisposes to body to tumourigenesis.

Cancer is a multistep process by which cells acquire increasingly abnormal proliferative and invasive behaviour (Nowell, 1976 ; Fearon & Vogelstein, 1990). Inactivation of p53 favours genetic instability that culminates in gene amplifications,

aneuploidy and other chromosomal aberrations. These abnormalities contribute to the clonal evolution of cancer cells and tumour progression (Carder *et al.*, 1993 ; Smith & Fornace Jr, 1995b). Many tumours are believed to embrace *p53* mutation as part of this "maturation" process (Graeber *et al.*, 1996).

The connection between DNA damage and p53 tumour suppression is indeed a close one. On induction of DNA damage, the half-life of the p53 gene product is increased, the protein accumulates in the nucleus (Maltzman & Czyzyk, 1984 ; Kastan *et al.*, 1991) and latent forms of p53 in the cell are activated (Hupp *et al.*, 1995 ; Macleod *et al.*, 1995). Many forms of genotoxic stress, including ultraviolet light, gamma radiation and genotoxic chemicals, induce p53 accumulation and are also associated with cell cycle G1 arrest (Tishler *et al.*, 1993 ; Zhan *et al.*, 1993). Although the biochemical signals involved are unclear (see below), the common denominator does seem to be actual DNA damage because p53 accumulation can be induced by intracellular DNA cleavage by microinjected bacterial restriction enzymes (Lu & Lane, 1993). When p53 is overexpressed artificially, it can block progression through the G1 phase of the cell cycle (Diller *et al.*, 1990 ; Martinez *et al.*, 1991 ; Mercer *et al.*, 1990) and it can also inhibit oncogene-mediated transformation (Finlay *et al.*, 1989 ; Eliyahu *et al.*, 1989 ; Baker *et al.*, 1990). There is thus a perceived connection between p53 and DNA-damage induced cell cycle arrest. Under certain conditions, some cells adopt a different p53-related strategy in response to UV radiation or other trauma (Lowe *et al.*, 1993a ; Lowe *et al.*, 1993b ; Clarke *et al.*, 1993b) : They commit suicide by initiating a set of steps culminating in cell death or apoptosis.

The tumour-suppressive activity of p53 is thus associated with two activities : The arrest of the cell cycle in G1, and apoptosis. It is suggested that p53 exerts its function in response to DNA damage or under conditions unfavourable for DNA synthesis. Its tumour suppressive powers are exerted by halting the cell cycle, to allow the cell to repair its DNA prior to S-phase entry, or to induce apoptosis, if progression of DNA replication is likely to entail mutation. The study of the molecular interactions of p53 has given us some clues as to how this might be achieved.

p53 and DNA damage

It seems clear that both p53 activity and its transcription is induced by DNA-damaging agents. Very little is known about the induction of the p53 gene but some evidence seems to indicate that activation of latent p53 in the cell is the rate-limiting contributor to the p53 response (Hupp *et al.*, 1995 ; Hupp & Lane, 1995).

p53 may directly or indirectly detect damaged DNA by binding with high affinity to single stranded DNA fragments (Brain & Jenkins, 1994). p53 could respond to changes in nucleotide metabolism as its induction is blocked by caffeine (Kastan *et al.*, 1991). DNA damage could be signalled by the double-stranded DNA dependent kinase which phosphorylates p53 and is activated by a broad range of DNA alterations. Mutation at one of these phosphorylation sites, serine 15, partially impairs p53 cell cycle arresting activity (Fiscella *et al.*, 1993).

p53 is sensitive to redox conditions and many genotoxins generate highly damaging reactive oxygen species (Hainaut & Milner, 1993), suggesting that the cells capacity to handle oxidative stress will affect p53 function. It has been suggested that hypoxia could induce p53 expression (Graeber *et al.*, 1994). Low levels of oxygen found in internal sections of tumours with low blood-supply result in p53-dependent programmed cell death (Graeber *et al.*, 1996). Mutants of *p53* or overexpression of the apoptosis-inhibitor protein Bcl-2, substantially reduce this cell death, giving these cells a survival advantage over cells with intact *p53* (Graeber *et al.*, 1996). Cells with *p53* mutation could then actually evolve in the surrounds of apoptotic cell death.

DNA binding of p53

Virtually all mutations of p53 abolish its ability to bind specific DNA sequences and activate the expression of adjacent genes (Pietenpol *et al.*, 1994 ; Hainaut, 1995). Most target genes have only recently been discovered and it seems that they are broadly concerned with cell birth (control of G1/S) and cell death (regulation of apoptosis), as elaborated upon below.

In the presence of a cognate recognition site, p53 behaves as a strong activator of transcription while it represses many genes without the p53 DNA binding element (Liu *et al.*, 1993 ; Ragimov *et al.*, 1993), possibly through an inhibitory interaction with the TATA-box binding protein, TBP (Prives & Manfredi, 1993). In *in vitro* re-constitution systems, p53 cooporates with TBP and TFIID in binding at *trans*-activating promoters containing both the p53 element and a TATA box. In the absence of a p53 element, p53 inhibits TBP-, but not TFIID-driven transcription (Chen *et al.*, 1993).

G1 arrest

The best characterised pathway of p53-induced G1 arrest is that of its induction of p21 (El-Deiry *et al.*, 1993). Its gene, *waf1*, maps to chromosome 6p21.2, and its promoter has a p53 response element which means that wild-type, but not most mutant p53, are able to enhance *waf1* transcription (El-Deiry *et al.*, 1993). p21 is

a potent inhibitor of G1 cyclin-CDK activity thought to phosphorylate, and inactivate, the tumour suppresser pRb, and thus promotes the repression of the S-phase stimulating transcription factor DRTF1/E2F (see below).

In transformed cells there is little or no p21 associated with any of the cyclin-CDK complexes (Xiong *et al.*, 1993), in part, probably due to mutation in *p53*. Searches have so far failed to find mutations of *waf1* in naturally occurring tumours (Shiohara *et al.*, 1994) and in accordance with this, *waf1* knockout mice undergo normal development, without the propensity for developing tumours such as seen with *p53* knockouts (see above). However, *waf1*-null mice are significantly deficient in their cells ability to arrest in G1 in response to DNA damage (Deng *et al.*, 1995). Apoptotic functions appear to be normal suggesting that p21 function is not required by p53 for this purpose (Deng *et al.*, 1995). This could suggest that p53 function in tumour suppression is orientated primarily toward apoptosis rather than to G1 arrest, indeed, several oncogenes, such as *Bcl2*, specifically inhibit apoptosis (Strasser *et al.*, 1993 ; elaborated upon in the Discussion chapter).

There has been a report that p53 is able to stimulate transcription from the *RB* promoter at low input doses of p53 expression plasmid in cotransfection assays. Transcription is repressed at high input doses and a p53 binding site is found in the *RB* promoter (Osifchin *et al.*, 1994). This work does not seem to have been followed up however, but the induction of pRb by p53 could represent a more direct method of generating pRb-mediated cell cycle arrest, one which could be maintained by the concomitant induction of *waf1* which would repress the inactivation of induced pRb by phosphorylation.

Other systems have also been found which potentially provides p53 with great authority over G1. *Gadd45* is a member of a set of genes induced at growth arrest and by DNA damage and is strictly p53-dependent (Zhan *et al.*, 1994a). It can suppress cell proliferation in association with other Gadd genes and may be effectors of G1 arrest (Zhan *et al.*, 1994b). p53 also binds RPA, a crucial replication factor and essential for replication entry. Binding prevents RPA associating with single stranded DNA and may affect the onset of S-phase (Dutta *et al.*, 1993). Cyclin D1 is induced in mid-G1 and is required for S-phase entry but its overexpression at the G1/S transition has been shown to inhibit S-phase entry (see Cell Cycle section). A recent report suggests that p53 induces the accumulation of cyclin D1 and that this is necessary for the induction of p53-mediated cell arrest (Del Sal *et al.*, 1996).

Apoptosis

Under certain conditions cells adopt a different strategy to cope with trauma, they commit suicide. We are now beginning to get an idea of how p53 manages to cause cell cycle arrest, but the mechanisms underlying the ability to cause apoptosis are as yet obscure. Deletion of *waf1* does not affect radiation-induced apoptosis in the mouse (Deng *et al.*, 1995) and it is even claimed by some that the transcriptional activation properties of p53 are dispensable for apoptosis (Caelles *et al.*, 1994 ; Sabbatini *et al.*, 1995 - elaborated upon in the Discussion chapter).

p53 up regulates the Bax gene but down regulates bcl-*2* (Miyashita *et al.*, 1994). The bcl-2 protein promotes cell survival and the Bax polypeptide promotes apoptosis by dominant-negative inhibition of bcl-2. It is likely that the intracellular ratio of bcl-2 to Bax controls the susceptibility to apoptotic stimuli, and that p53 induces cell death by tilting this equilibrium (Miyashita *et al.*, 1994).

MDM2

The MDM2 (murine double minute) gene is upregulated by p53 and the MDM2 gene product binds p53 in the N-terminus and blocks its *trans*-activation properties (Chen *et al.*, 1994b). This autoregulatory loop may overcome a p53-dependent G1 block to permit re-entry into the cell cycle after DNA repair.

The MDM2 oncogene was originally identified as a gene that is amplified and overexpressed in a tumourigenic derivative of mouse 3T3 cells (3T3DM cell line) with the amplified sequences located on extra chromosomal double minute particles (Cahilly-Snyder *et al.*, 1987). When overexpressed in immortalised rodent cells, the MDM2 gene is capable of transforming these cells (Farkharzadeh *et al.*, 1991). Overexpression of MDM2 can immortalise primary rat embryo fibroblasts and cooporate with an activated ras gene to transform these cells (Finlay, 1993). Amplification and overexpression of the MDM2 gene has been detected in a number of human sarcomas (Landanyi *et al.*, 1993 ; Oliner *et al.*, 1992) indicating that this oncogene plays a role in human carcinogenesis. MDM2 is able to overcome p53 suppression of transformed cell growth (Finlay, 1993) and MDM2 proteins can physically associate with p53 *in vivo* (Momand *et al.*, 1992 ; Olson *et al.*, 1993) and can inhibit p53-mediated transcriptional activation (Momand *et al.*, 1992 ; Oliner *et al.*, 1993). The MDM2 gene produces several splice-forms that differ in their ability to complex with and modulate the transcriptional activation of p53 (Haines *et al.*, 1994).

The complexity of p53 activity

Other p53 protein interactions, which are less well defined, illustrate the complexity of p53 activity. p53 function has been associated with DNA dynamics by the demonstration of binding to single stranded nucleic acids, DNA helicases, and exerting DNA strand re-association activity (Hainaut, 1995 ; Smith & Fornace Jr, 1995b). At least some of these properties are altered in p53 mutants, suggesting that the loss of p53 function associated with cancer is a complex, multifaceted phenotype.

In contrast with the wild-type p53, many mutant p53 proteins accumulate in cancer cells. This suggests that cancer cells select for *p53* mutations, and many mutants have gained an oncogenic phenotype so that some mutant p53 actually enhance the tumourigenicity of cells, as shown by experiments on p53 deficient cells (Dobashi *et al.*, 1993). Thus another facet of p53 activity is its activity as a putative oncogene.

The transcription factor DRTF1/E2F

The *RB* and *p53* tumour suppressor genes are the best defined genes of their class and their mutant forms are much more prevalent than any other gene mutations in human neoplasia (Whyte, 1995 ; Hainaut, 1995). A fascinating common feature between these two anti-oncogenes is their targeting to down-regulate the activity of the S-phase promoting transcription factor DRTF1/E2F : pRb directly and p53 via p21 and regulation of pRb. The importance of the regulative powers of p53 and pRb in the control of cellular proliferation is underlined by their selective inactivation by tumour promoting oncoviruses (see below).

E1a-like activity background

The E1a protein is an adenovirus oncoprotein which induces the expression of early viral genes, such as *E2a* (Berk *et al.*, 1979 ; Jones & Shenk, 1979) as well as some cellular genes (Nevins, 1982 ; Kao & Nevins, 1983 ; Stein & Ziff, 1984).

Some mammalian cells have an "E1a-like" activity of their own (Imperiale *et al.*, 1984). When a range of cell lines are infected by *E1a*-lacking and wild-type adenovirus, a correlation is observed between the requirement for E1a for E2a expression, and the degree of growth control of the cell line. That is, cell lines derived from malignant tumours (such as HeLa) have a lower dependency upon E1a and so are described as harbouring a low, but detectable, level of "E1a-like" activity. Cell lines displaying greater levels of growth control have no detectable "E1a-like" activity (Imperiale *et al.*, 1984). The activity is also detected in F9 embryonal carcinoma (F9EC) cells which exhibit many features of the transformed phenotype *in vitro* and are tumourigenic *in vivo* (Rudnicki & McBurney, 1987). These cells can be induced to differentiate to parietal endoderm cells (F9PE), a process which accompanies a reversion of the transformed phenotype (Rudnicki & McBurney, 1987). This differentiation process is found to be accompanied by a reduction of the E1a-like activity, to a level more associated with cell lines of non-malignant origin (Imperiale *et al.*, 1984). These observations prompted a search for the E1a-like activity, an activity deemed likely to be intimately involved with the status of cellular regulation of proliferation and state of differentiation.

The E1a-like activity in F9EC and F9PE cells correlated with the transcriptional activity from the *E2a* promoter in extracts from these cells (La Thangue & Rigby, 1987). This correlates again with the presence of factors binding to the *E2a* promoter in F9EC but not in F9PE extracts (La Thangue & Rigby, 1987). An

E1a-dependent *E2* promoter-binding Factor (E2F) was observed to be induced during the adenovirus lytic cycle in HeLa cells and bind co-operatively to two appropriately spaced GC-rich regions (TTTCGCGC) termed E2F-sites (Kovesdi *et al.*, 1986). This activity was present in uninfected cells but at greatly reduced levels. Characterisation of the binding activity on a single 5' E2F site from the *E2a*-promoter defined the Differentiation Regulated Transcription Factor (DRTF1) which resolves in gel retardation assays as three complexes : a, b and c (La Thangue *et al.*, 1990). This DNA binding activity is reduced dramatically as F9EC cells differentiate into F9PEs and so may, at least in part, account for the E1a-like activity (La Thangue *et al.*, 1990).

The DRTF1 and E2F DNA binding activities were originally thought to be distinct factors, but earlier detected differences can now to some extent be attributed to the larger section of the viral *E2a* promoter used to define E2F (Kovesdi *et al.*, 1986). Subsequent studies have found the dual GC-rich region to have an augmentative role in the infection process due to the influence of another viral gene product, orf 6/7 (open reading frames 6/7 ; see below). However E2F has become a general term for binding to the *E2a* promoter and is frequently referred to in the literature even where single E2F sites are used (for example : Reichel, 1992 ; Wu & Levine, 1994 ; Wu *et al.*, 1995), where DRTF1 is technically the more correct term. This is further confused by the naming of a family of polypeptide components from E2F as the E2F family (see below). In this report, therefore, for the sake of compromise and clarity, single site E2F DNA binding activity will be referred to as DRTF1/E2F and dual site binding will be referred to specifically as dual site E2F activity.

DRTF1/E2F

DRTF1/E2F DNA binding activity is regulated during cell cycle progression (Mudryj *et al.*, 1991 ; Shirodkar *et al.*, 1992 ; Schwarz *et al.*, 1993) where it peaks at the G1/S-phase transition. The pivotal role played by DRTF1/E2F in the G1/S transition is emphasised by the observation that cloned members from the complex, such as E2F-1 and E2F-5 (see below), can singularly promote S-phase entry of growing and quiescent cells, thus apparently by-passing any upstream cell cycle regulatory signals (Johnson *et al.*, 1993 ; Qin *et al.*, 1994 ; Beijersbergen *et al.*, 1994 ; Vairo *et al.*, 1995). This suggests that the G1 regulatory mechanisms of the cell cycle must rely heavily upon regulation of DRTF1/E2F activity.

DP and E2F families

Two cloned families of DNA binding proteins which are components of the DRTF1/E2F factor have been intimately linked with the control of the proliferative state. The DP family is exemplified by DP-1 (DRTF1 Polypeptide-1 ; Girling *et al.*, 1993a ; Girling *et al.*, 1993b), the other is the E2F family exemplified by E2F-1 (Helin *et al.*, 1992 ; Kaelin *et al.*, 1992 ; Shan *et al.*, 1992).

DP-1 and E2F-1 polypeptides were predicted (Girling *et al.*, 1993a), and demonstrated (Bandara *et al.*, 1993 ; Krek *et al.*, 1993 ; Helin *et al.*, 1993b), to synergistically form the DNA-binding activity of DRTF1/E2F by heterodimerisation, on the basis of a similarity in DP-1 to the second helix of the E2F-1 DNA binding domain. DP-1/E2F-1 heterodimers will bind DNA with much greater affinity than E2F-1 will bind on its own (possibly as a homodimer) while DP-1 alone has very little affinity for the E2F-site (Bandara *et al.*, 1993 ; Krek *et al.*, 1993 ; Helin *et al.*, 1993b).

Additional human cDNAs with significant homology to *E2F-1* have been identified : *E2F-2* and *E2F-3* (Lees *et al.*, 1993 ; Ivey-Hoyle *et al.*, 1993) as well as *E2F-4* and *E2F-5* (Ginsberg *et al.*, 1994 ; Sardet *et al.*, 1995 ; Itoh *et al.*, 1995) which represent a structurally and functionally distinct branch of the *E2F* family (see below). *E2F-1*, *-2* and *-3* are all proto-oncogenes (Xu *et al.*, 1995). *E2F* cDNAs have also been cloned from a variety of other species including mouse (*mE2F-1* : Li *et al.*, 1993a ; *mE2F-2* : Adams & Kaelin, 1995 ; *mE2F-5* : Buck *et al.*, 1995), Drosophila melanogaster (*dE2F* : Ohtani & Nevins, 1993) and chicken (*chE2F-1* : Pasteau *et al.*, 1995). Murine *E2F-1* is 86% identical to human *E2F-1* and both *chE2F-1* and *dE2F* show high levels of homology to the mammalian *E2F*s.

Recent isolations of *DP-2* (*Xenopus* : Girling *et al.*, 1994) and *DP-3* (mouse : Ormondroyd *et al.*, 1995, and human : Wu *et al.*, 1995 ; Zhang & Chellappan, 1995 : Mistakenly called *DP-2* in the literature) suggests that DP-1 is, like E2F-1, a member of a highly related family of transcription factors. Additional complexity is provided by *DP-3* which has multiple alternatively spliced transcripts α, β, δ, γ (Ormondroyd *et al.*, 1995 ; Wu *et al.*, 1995 ; Zhang & Chellappan, 1995).

E2F-containing promoters and trans-activation

Heterodimerisation of E2F and DP family members appears to be essential for efficient transcriptional activation (Bandara *et al.*, 1993 ; Krek *et al.*, 1993 ; Helin *et al.*, 1993b). Almost all E2F and DP family member heterodimer-combinations (some murine and some human) have been tested *in vitro*, and all of these can *trans*-activate

E2F-site dependent reporter plasmids (Ormondroyd *et al.*, 1995 ; Wu *et al.*, 1995). It still remains to be seen how many of the heterodimer combinations actually can be accounted for *in vivo*.

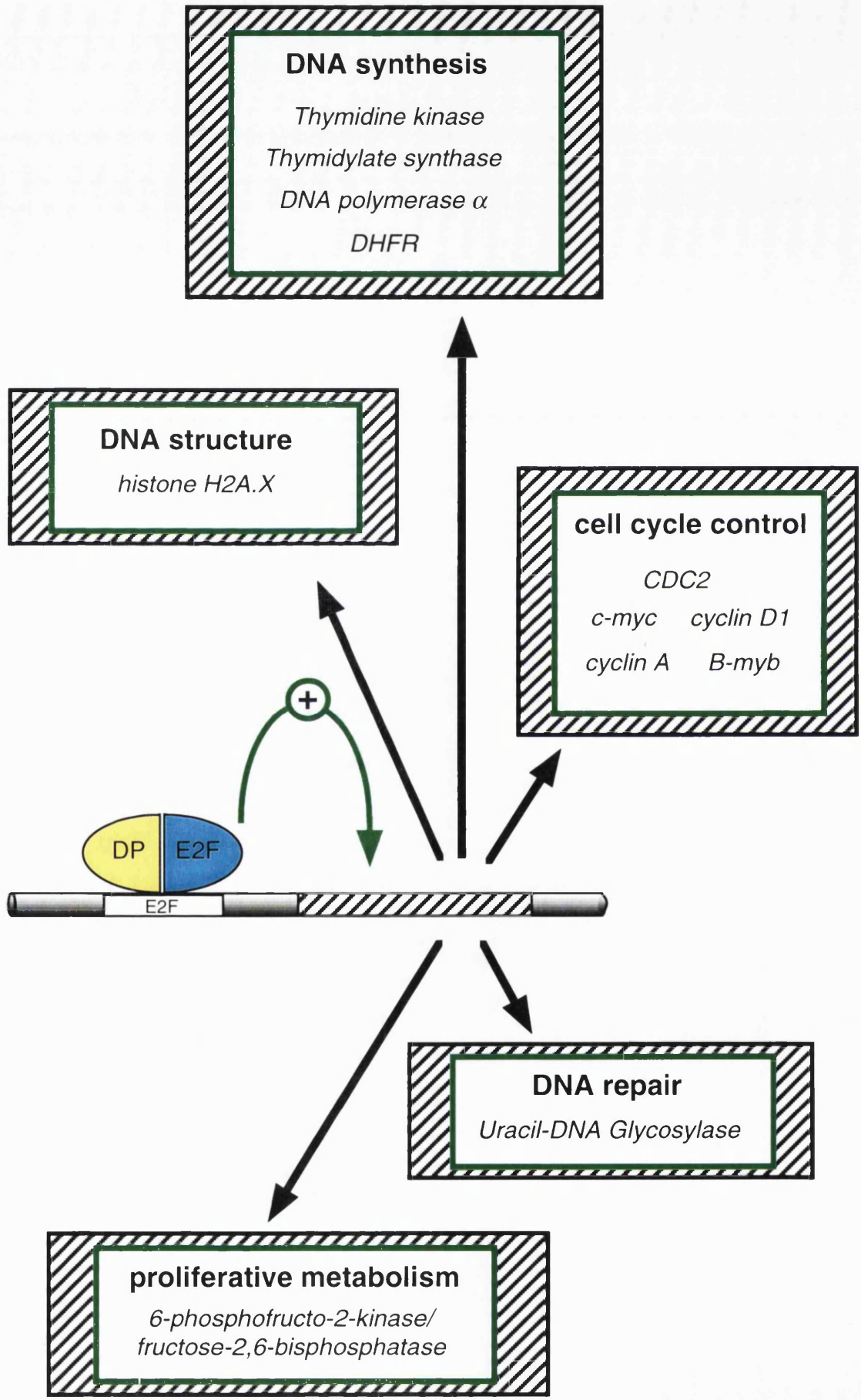
The phenomenon of S-phase induction by E2F-1 is linked to its ability to activate transcription since activation-deficient E2F-1 protein cannot display the same effect (Johnson *et al.*, 1993 ; Shan & Lee, 1994 ; Qin *et al.*, 1994). Apart from highlighting the importance of DRTF1/E2F in the control of G1/S phase transition, it certainly highlights the importance of the E2F binding site (consensus : 5' TTT(G/C ; G/C)CG(G/C) 3' ; Adams & Kaelin, 1995). This significance is reflected in the gradual revelation of E2F sites in the promoters of a plethora of cell cycle-associated genes. They include factors involved in cell cycle control and DNA synthesis, as well as those of the proliferative metabolism, DNA structure and DNA repair (below and **figure 1.7**).

Genes encoding enzymes required for DNA synthesis containing the E2F site include *dihydrofolate reductase* (*DHFR* ; Blake & Azizkhan, 1989 ; Slansky *et al.*, 1993), *thymidine kinase* (Ogris *et al.*, 1993 ; Dou *et al.*, 1994), *thymidylate synthase* (Joliff *et al.*, 1991) and *DNA Polymerase* α (Pearson *et al.*, 1991). Genes encoding cell cycle regulators are *CDC2* (Dalton, 1992 ; Yamamoto *et al.*, 1994), *c-myc* (Hiebert *et al.*, 1989 ; Hara *et al.*, 1993 ; Ishida *et al.*, 1995), *cyclin A* (Schulze *et al.*, 1995), *cyclin D1* (Herber *et al.*, 1994) and *B-myb* (Lam & Watson, 1993). The E2F sites within the *DHFR* promoter are necessary and sufficient to render *DHFR* expression cell-cycle and serum-dependent (Slansky *et al.*, 1993). Those in the *CDC2* and *B-myb* promoters are required for the transcriptional repression in G0 and early G1 suggesting a role for DRTF1/E2F in transcriptional repression (Dalton, 1992 ; Lam & Watson, 1993 ; Yamamoto *et al.*, 1994 ; see below). The mouse histone *H2A.X* gene is also stimulated trough an E2F site (Yagi *et al.*, 1995) as well as *6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase* which is found in proliferating but not quiescent cells (Darville *et al.*, 1995). Its gene product catalyses the generation and degradation of fructose-2,6-bisphosphate, a potent stimulator of glycolysis. The cyclin-like DNA repair enzyme, *Uracil-DNA Glycosylase*, is regulated by an E2F-site in a cell cycle dependent manner. Its gene product may have a negative effect on E2F-mediated transcriptional activity (Walsh *et al.*, 1995).

Recent work has raised the possibility of variant E2F sites which still involve DRTF1/E2F. In particular an overlapping inverted repeat sequence of the E2F site (5' TTTCGCGCCAAA 3') that is 100% conserved and found near the major transcription start sites in the promoters of three mammalian genes encoding *dihydrofolate reductase*. All forms of DRTF1/E2F heterodimer and homodimers tested bind and *trans*-activate more strongly to the inverted repeat (Wade *et al.*, 1995) but its physiological relevance still has to be discerned.

Figure 1.7
Gene targets of the E2F-site targeting transcription factor DRTF1/E2F.

Illustration of the genes, and the subclasses of their polypeptide products, which posses E2F sites within their promoters and are believed to be induced by DP/E2F family heterodimer transactivation, or repressed by heterodimer bound pocket proteins. The DP-E2F heterodimer is depicted as an oval (yellow half : DP, cyan half : E2F) bound to an E2F site on the DNA template (grey bar) upstream of an mRNA encoding, E2F-site responsive gene (striped box). Black arrows point to categories of such identified genes (striped boxes).


DNA synthesis
Thymidine kinase
Thymidylate synthase
DNA polymerase α
DHFR
DNA structure
histone H2A.X
cell cycle control
CDC2
c-myc
cyclin D1
cyclin A
B-myb
+
DP
E2F
E2F
DNA repair
Uracil-DNA Glycosylase
proliferative metabolism
6-phosphofructo-2-kinase/
fructose-2,6-bisphosphatase

DP / E2F heterodimers

The existence of multiple E2F and DP family members raises the possibility that different E2F/DP heterodimer combinations have differential roles such as binding to subtly different E2F promoters or possessing different temporal activities. E2F-1 mRNA abundance varies in a cell cycle dependent manner, being absent or low in G0, and maximal in S-phase (Kaelin *et al.*, 1992 ; Li *et al.*, 1993a). In T-cells, E2F-1 mRNA does not become detectable until the G1/S boundary, suggesting that E2F family members other than E2F-1 are responsible for contributing to the DRTF1/E2F activity observed prior to S-phase (Kaelin *et al.*, 1992). Temporal variations in the formation of these heterodimers, in conjunction with their promoter specificity, would be expected to give rise to different kinetics of activation of particular target genes. Compatible to this idea, E2F-1 *trans*-activates the *DHFR* and *DNA polymerase* α promoters, but not the *c-myc* (Li *et al.*, 1993a). Under normal circumstances, peak expression of the DHFR and DNA polymerase α occurs in late G1 and S-phase, whereas peak c-myc expression occurs at the G0/G1 boundary (Pearson *et al.*, 1991 ; Slansky *et al.*, 1993 ; Kaelin *et al.*, 1992 ; Li *et al.*, 1993a ; Kelly *et al.*, 1983). It is thus tempting to speculate that there are more than one DRTF1/E2F system of complexes, possibly with different temporal regulation patterns and specificity.

The human and murine *E2F-1* promoters have been cloned and confer serum stimulated activity on reporter plasmids that parallels the changes observed in endogenous *E2F-1* mRNA levels (Neuman *et al.*, 1994 ; Adams & Kaelin, 1995). E2F binding sites within these promoters are necessary for cell cycle dependent promoter activity and act as repressive elements in G0 and early G1 (Adams & Kaelin, 1995). E2Fs -1, -2 and -3, can all *trans*-activate the *E2F-1* promoter, at least when over-produced, suggesting that E2F-1 expression may be dependent upon the coordinated activity of other E2Fs and that E2F-1 may positively autoregulate its promoter (Adams & Kaelin, 1995). The newly cloned *E2F-4* and *E2F-5* show a unique pattern of expression in synchronised human keratinocytes : Their mRNAs are maximal in mid-G1 phase before E2F-1 expression is detected. This suggests that E2F-4 and -5 may contribute to the regulation of early (G1) events including the G0/G1 transition (Sardet *et al.*, 1995) and may possibly even induce the expression of E2F-1.

Transcriptional activation

E2F-1 carries the *trans*-activation domain of the heterodimer and in accordance with this it has been observed that this domain can interact with TBP *in vitro* (Hagemeier *et al.*, 1993b). A fusion of LexA-E2F-1 has also been shown to photo-cross-link with TBP when both factors are bound to a transcriptionally responsive RNA polymerase II promoter (Emili & Ingles, 1995). Mutations within the activation domain of LexA-E2F-1 that impairs its ability to *trans*-activate transcription *in vitro* also reduced the cross-linking to TBP. TFIIB can also be cross-linked but this is seen to be promoter independent. TFIIA, in contrast to TFIIB, strongly inhibits the promoter-dependent cross-linking of LexA-E2F-1 to TBP (Emili & Ingles, 1995). In general it can be said that the means employed by the DRTF1/E2F to modulate the activity of the basal transcription factors is still quite unclear.

The pocket proteins and DRTF1/E2F regulation

The pRb gene product is a member of the pocket family of proteins and interacts with and represses the stimulating activity of DRTF1/E2F at E2F-site containing promoters (**figure 1.8**). Inactivation of *RB* is associated with tumour formation (see above) and a strong correlation has been made between the tumourigenic condition and unregulated DRTF1/E2F activity (see below). The only other well-defined tumour suppressor, p53, does not have a direct role in the running of the cell cycle but will cause G1 arrest under circumstances of cellular stress. It seems symptomatic of the pivotal importance of pRb, as a regulator of DRTF1/E2F, that part of p53 action appears to be the preservation of the pRb active form through the stimulation of the p21 gene (see above).

pRb is not the only inhibitor of basal DRTF1/E2F transcription, it has two family members, p107 and p130, which are more related to each other than to pRb (Ewen *et al.*, 1991 ; Hu *et al.*, 1990 ; Huang *et al.*, 1990 ; Kaelin *et al.*, 1990 ; Zhu *et al.*, 1993 ; Hannon *et al.*, 1993 ; Li *et al.*, 1993b). They all share a structural and sequence relationship, particularly in the "pocket" domain which associates with DRTF1/E2F and which lends name to this family of transcription factor inhibitors.

As the cell cycle progresses, a myriad of changing DRTF1/E2F complexes is observed (**figure 1.9** ; Whyte, 1995). Extracts from proliferating cells contain several DRTF1/E2F complexes including "free" or "basic" DRTF1/E2F complex, believed just to be composed of the E2F/DP heterodimer and held to be the transcriptionally active form. Larger complexes are also observed. Some of these complexes include pRb, p107, p130, cyclin A/CDK2 or cyclin E/CDC2, as well as additional complexes that have yet to be fully characterised (Whyte, 1995).

Figure 1.8
Activation of the DP/E2F heterodimer by pocket protein inactivation.

(**i**) The DP-E2F heterodimer is depicted as an oval (yellow half : DP, cyan half : E2F) bound to an E2F site on the DNA template (grey bar). Bound to the heterodimer is a pocket protein (Orange hat) which masks the transactivation domain of the E2F-moity of the heterodimer thus preventing transcriptional activation. Three scenarios are depicted showing how the heterodimer-binding and repressive activity of the pocket protein can be disrupted : (**ii**) The pocket protein can be bound by viral oncoproteins (Magenta "roof") which mask the regions required for heterodimer binding. (**iii**) During normal cell cycle progression, pocket protein activity is regulated by phosphorylation (green circle - P) which prevents heterodimer binding. (**iv**) Naturally occurring tumour-promoting mutations of the pocket protein pRb (Khaki star) have lost their ability to complex the heterodimer. (**v**) Any of the above circumstances which prevent pocket protein binding to the heterodimer causes generation of "free" DP/E2F heterodimer on the E2F site which promotes E2F-directed transcription of target genes.

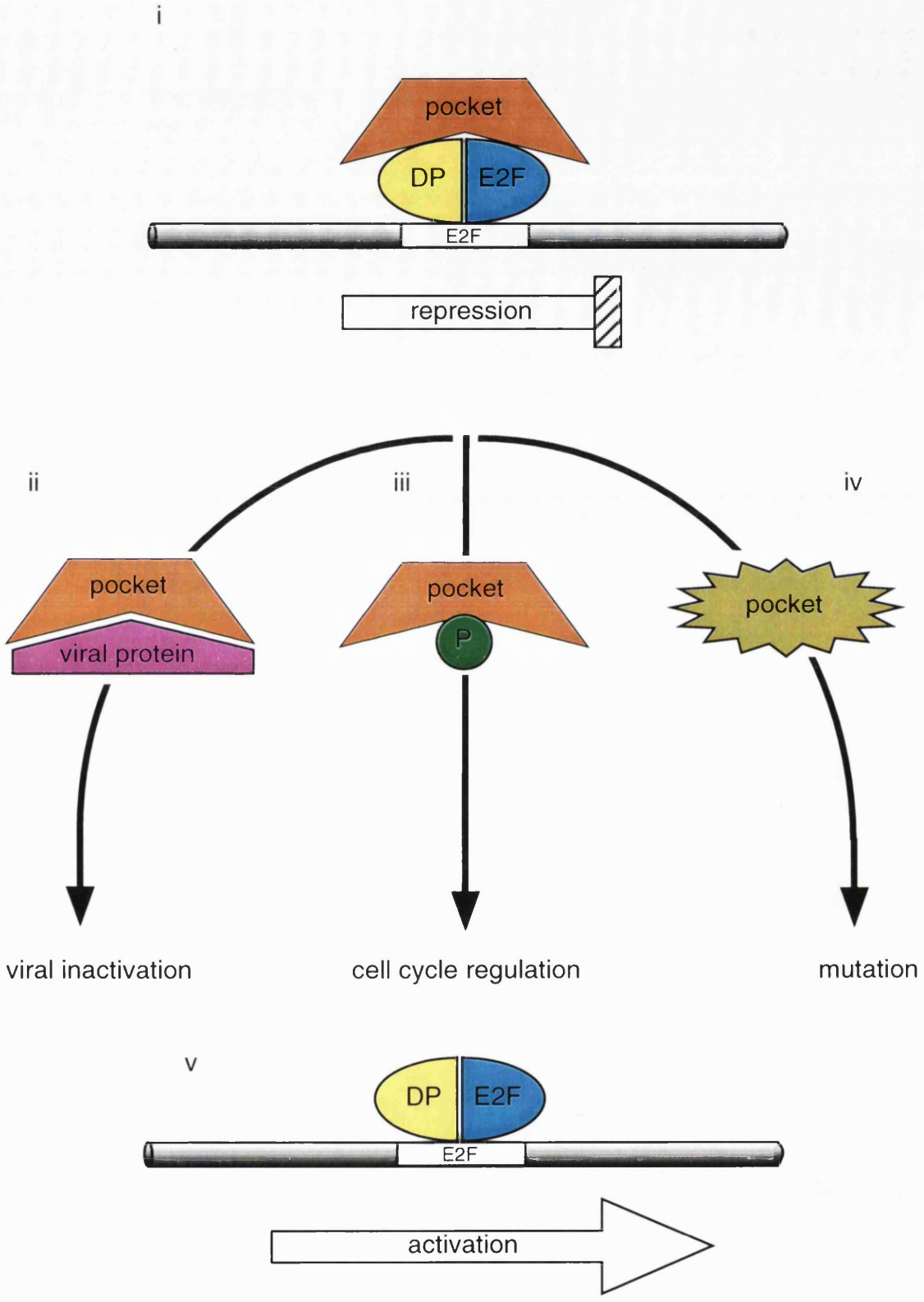
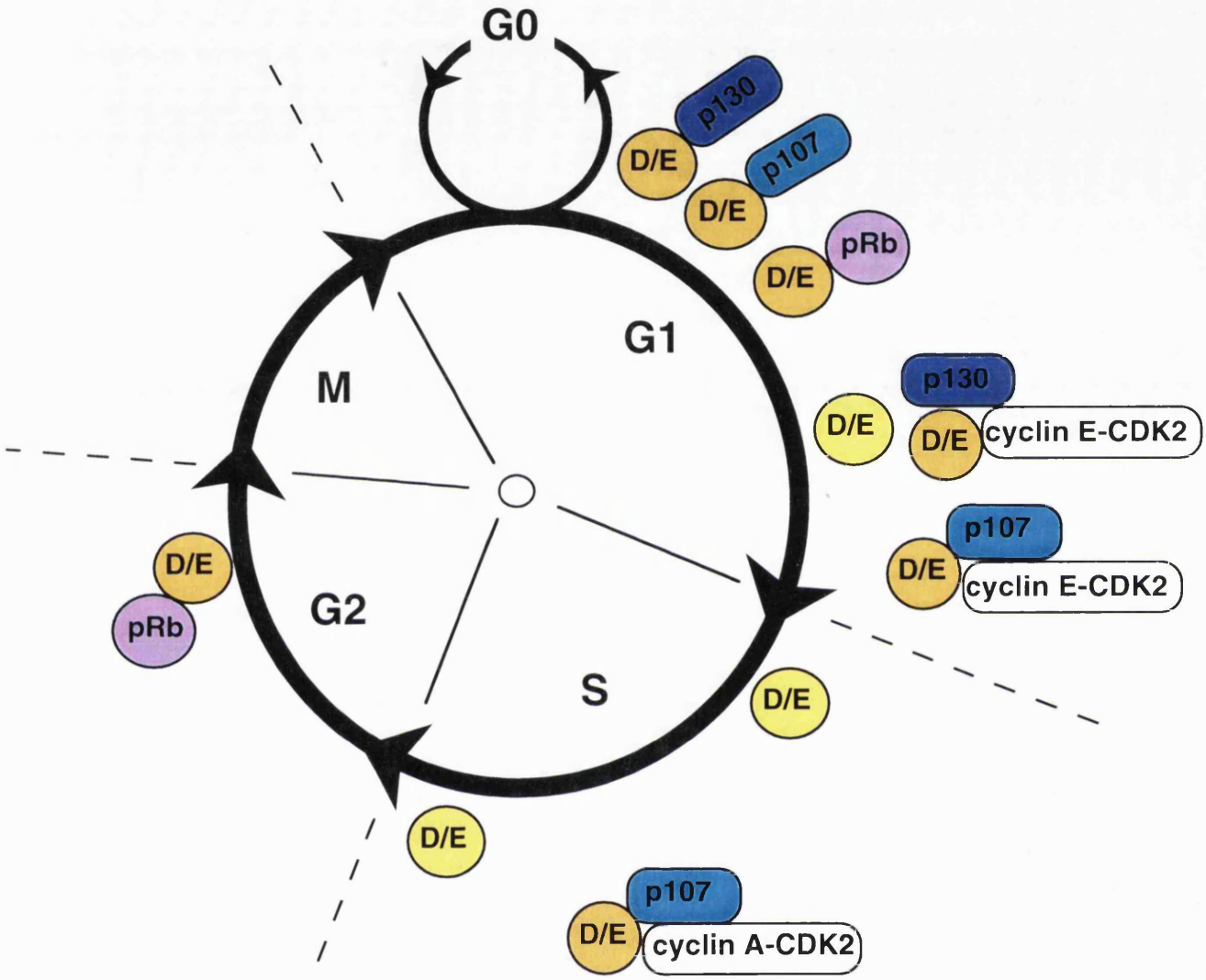

i
pocket
DP
E2F
E2F
repression
ii
iii
iv
pocket
viral protein
pocket
P
pocket
viral inactivation
cell cycle regulation
mutation
v
DP
E2F
E2F
activation

Figure 1.9
DRTF1/E2F complexes during the progression of the cell cycle.

Depiction of the exposure of the DP/E2F heterodimer (D/E : circles of light and dark shades of yellow) to regulatory pocket proteins (p130, pRb and p107) and with cyclin-CDK complexes during different stages of the cell cycle. From the middle of G1 to the end of S-phase free DP/E2F heterodimers are detected (light yellow circles) and coincide with enhanced expression from E2F-site regulated genes. Complexed/repressing DP/E2F heterodimers are depicted by dark yellow circles.


G0
G1
S
G2
M
p130
D/E
p107
D/E
pRb
D/E
p130
D/E
D/E
cyclin E-CDK2
p107
D/E
cyclin E-CDK2
D/E
D/E
pRb
D/E
p107
D/E
cyclin A-CDK2

p130 is found in the DRTF1/E2F complexes at G0 in growth arrested fibroblasts, and immediately following serum stimulation, until cells reach the G1/S boundary at which stage complexes that contain p107 are also observed (Adams & Kaelin, 1995 ; Whyte, 1995). In late G1, but not during G0, cyclins A and E and CDK2 become associated with p130/DRTF1/E2F. pRb is found in DRTF1/E2F complexes predominantly during G1, and a smaller amount is detected during S- and G2-phases (Adams & Kaelin, 1995 ; Whyte, 1995). The p107/DRTF1/E2F also contains cyclins A and E, and CDK2, and these complexes predominate through S-phase. The significance of the cyclins and CDK2 in the p130 and p107/DRTF1/E2F complexes is unknown but it is intriguing that p130 and p107 should bring cyclin/CDK complexes so proximal to the site of transcription (Adams & Kaelin, 1995 ; Whyte, 1995).

Retinoblastoma gene product inhibition of the DRTF1/E2F

Both DRTF1/E2F (Bandara *et al.*, 1991a ; Chellappan *et al.*, 1991) and the DP-1/E2F-1 heterodimer (Bandara *et al.*, 1993 ; Helin *et al.*, 1993a) have been shown to bind pRb, more specifically, via a C-terminal domain of E2F-1 aided by DP-1 (Bandara *et al.*, 1994). The E2F moiety appears to determine which pocket protein can bind to the heterodimer. E2F-1, -2 and -3 are associated *in vivo* only with pRb and not with p107 (Lees *et al.*, 1993) while neither E2F-4 or E2F-5 appear to interact with pRb at all (Beijersbergen *et al.*, 1994 ; Ginsberg *et al.*, 1994 ; Hijmans *et al.*, 1995). E2F family members cloned in *Drosophila melanogaster* (Ohtani & Nevins, 1993) and chicken (Pasteau *et al.*, 1995) have retained high homology in their pRb-family binding regions suggesting that such interactions are a fundamental aspect of DRTF1/E2F regulation.

Binding of pRb to DRTF1/E2F has been correlated with inactivation of E2F site dependent transcription, a repression not possible by *RB* mutations identified in naturally occurring tumour cells (Zamanian & La Thangue, 1992 ; Hiebert *et al.*, 1992). These mutations all affect the pocket domain of pRb and mutational analysis has indicated that both this domain, plus the carboxy-terminal sequences, are required for growth suppression (Qin *et al.*, 1992 ; Hiebert, 1993 ; Qian *et al.*, 1992 ; Goodrich *et al.*, 1991 ; Templeton *et al.*, 1991). pRb shows growth suppressive and tumour phenotype-reversing activity which can be reversed by the overexpression of E2F-1 (Qin *et al.*, 1995). An E2F-1 mutant that is unable to bind pRb, but which retains the ability to *trans*-activate, is likewise able to overcome a pRb-induced arrest suggesting

that E2F-1 does not merely displace other effectors from the pRb pocket in these assays (Qin *et al.*, 1995). Thus the tumour suppressive and G1 cell cycle arresting properties of pRb is intrinsically linked with its binding to and repression of DRTF1/E2F activity.

pRb and the transcriptional initiation complex

There are two sub-domains of the pRb pocket (A and B). Domain A is homologous to the C-terminus of TBP, and domain B is homologous to the C-terminus of a component of TFIIB (Hagemeier *et al.*, 1993a). This similarity seems to have a functional role since the pRb pocket and the C-terminus of TBP recognise identical domains within the same transcription factors. Both pRb and TBP recognise the activation domain of E2F-1 (Hagemeier *et al.*, 1993b ; see previous section), as well as c-myc (Rustgi *et al.*, 1991 ; Hateboer *et al.*, 1993) and PU.1 (Hagemeier *et al.*, 1993a). The homology between pRb and these two general transcription factors may explain how pRb can recognise and regulate the DP-1/E2F-1 heterodimer as well as so many other transcription factors. A proposed model suggests that by mimicking the structure of TBP and TFIIB, pRb has the potential to bind E2F-1 and interfere with its stimulatory interaction with the general transcription machinery (Kouzarides, 1995).

pRb and phosphorylation

The cyclical regulation of DRTF1/E2F *trans*-activation activity has been intimately associated with pRb. The retinoblastoma gene product migrates on SDS-polyacrylamide gels as a series of bands at approximately 105-115 KDal (Lee *et al.*, 1987) deemed to reflect differential states of phosphorylation and which fluctuate during the cell cycle (Buchkovich *et al.*, 1989 ; Chen *et al.*, 1989 ; Decaprio *et al.*, 1989). A hypo-phosphorylated form is both active and predominant in G0 (Ludlow *et al.*, 1989) becoming increasingly phosphorylated in late G1 with the state of hyper-phosphorylation being maintained until during or shortly after the following mitosis (Ludlow *et al.*, 1990).

In some cell lines where overexpression of pRb is unable to affect cell cycle progression, newly synthesised pRb is rapidly phosphorylated, suggesting an overactive inactivating kinase condition and co-expression of cyclin genes A, E, D1, D2 or D3 can rescue cells from the growth-suppressive effects of pRb correlating with an increase in pRb phosphorylation for all but cyclin D1 (Hinds *et al.*, 1992 ; Ewen *et al.*, 1993 ; Kato *et al.*, 1993 ; Dowdy *et al.*, 1993 : See Discussion). *E2F-1* overexpression can overcome G1 arrest caused by the inhibition of G1 CDK activity. This induction is not accompanied by a rise in either cyclin D-associated kinase

activity or CDK2 activity that is normally observed during the G1 phase of the cell cycle (Degregori *et al.*, 1995). These experiments suggest an involvement of cyclin-dependent kinase activity in the inactivating phosphorylation events on pRb. In accordance with these observations, cyclin D1/CDK4 , cyclin E/CDK2 and cyclin A/CDK2 have been shown to phosphorylate the DRTF1/E2F-pRb complex *in vitro* resulting in the dissociation of pRb (Hinds *et al.*, 1992 ; Dowdy *et al.*, 1993 ; Ewen *et al.*, 1993 ; Kato *et al.*, 1993 ; Dynlacht *et al.*, 1994 ; Suzukitakahashi *et al.*, 1995).

The D-type cyclins bind to pRb through an L-X-C-X-E motif in their N-termini (Dowdy *et al.*, 1993 ; Kato *et al.*, 1993). No L-X-C-X-E motif is present in cyclins A or E and neither cyclin A or E stably interact with pRb directly *in vivo* or *in vitro* (Hinds *et al.*, 1992). However, cyclins A and E are able to recruit CDK2 to the DRTF1/E2F DNA binding complex (Bandara *et al.*, 1991a ; Mudryj *et al.*, 1991 ; Bandara *et al.*, 1992 ; Lees *et al.*, 1992 ; Devoto *et al.*, 1992) and *in vitro* cyclin A-CDK2 or cyclin B-CDK2 complexes will phosphorylate and inhibit DRTF1/E2F-associated pRb (Dynlacht *et al.*, 1994). This suggests that these cyclins may exert an effect on pRb via the very transcription factor complex that it is targeting. It has been proposed that E2F-1 contains a cyclin A, but not cyclin E, binding domain at its N-terminus which may provide a platform for pRb phosphorylation by cyclin A (Krek *et al.*, 1994).

Cyclin D-CDK complexes have a very limited substrate specificity, and *in vitro* the best substrate found to date is pRb (Matsushime *et al.*, 1994). Cyclins D2 and D3 bind more stably to pRb *in vitro* but only cyclin D1 has been reported to co-immunoprecipitate with pRb from mammalian cell extracts (Kato *et al.*, 1993 ; Ewen *et al.*, 1993 ; Dowdy *et al.*, 1993). When CDK4, a kinase partner for D cyclins, is coexpressed with cyclins D1, D2 or D3, pRb has been reported to become phosphorylated and the interactions between pRb and the cyclins are destabilised. In these and other experiments examining the ability of cyclin D/CDK4 complexes to phosphorylate pRb, cyclin D1 was much less efficient than cyclins D2 and D3 (Kato *et al.*, 1993 ; Ewen *et al.*, 1993) but cyclin D1 is dispensable for G1 control in *RB*-deficient cells independently of CDK4 activity (Lukas *et al.*, 1995) suggesting that pRb and cyclin D1 do have some *in vivo* interaction.

The existence of two types of interactions of cyclin/CDK complexes with pRb may represent differences in initiation of pRb phosphorylation by the D cyclins and maintenance of pRb phosphorylation by the other cyclin/CDK combinations during cell cycle transition. Premature expression of cyclin D1 leads to immediate appearance of hyper-phosphorylated pRb, while premature expression of cyclin E does not (Resnitzky & Reed, 1995a). The D cyclins and the E1a/T/E7 viral transforming proteins require similar regions of pRb for their interactions and compete for binding to pRb (Ludlow *et al.*, 1990). Nonetheless, pRb is

phosphorylated in a cell cycle dependent manner in virally transformed cells (Ludlow *et al.*, 1990). One possible explanation is that cyclins such as A and E, co-mediate phosphorylation of pRb as the cell passes through the cell cycle. This would be in accordance with the observation that cyclin E or Ds alone are insufficient to accelerate the cell into S-phase, but that together there is a marked acceleration through G1 phase and into S-phase (Ohtsubo & Roberts, 1993 ; Quelle *et al.*, 1993 ; Resnitzky *et al.*, 1994).

p107 and p130 and DRTF1/E2F

p107 does not appear to interact with E2F-1, -2, or -3 under physiological conditions, although when its gene is overexpressed it can suppress E2F-1 dependent transcription (Lees *et al.*, 1993 ; Cress *et al.*, 1993 ; Dyson *et al.*, 1993). E2F-4 interacts with p107 and p130 but not with pRb (Ginsberg *et al.*, 1994 ; Vairo *et al.*, 1995 ; Beijersbergen *et al.*, 1994), whereas E2F-5 interacts preferentially with p130 (Hijmans *et al.*, 1995). The specificity of pocket protein binding appears to be predominantly mediated by the E2F-family subunit, but no genuine study of DP subunit contribution to pocket protein specificity has yet been reported.

DRTF1/E2F repression by p107 / p130

E2F-4 and -5 mRNAs are maximal in mid-G1 phase before E2F-1 expression is detected suggesting that their protein products, and p130/p107, may contribute to the regulation of early (G1) events including the G0/G1 transition (Sardet *et al.*, 1995). p107 binding can repress transcription from promoters containing E2F sites (Zamanian & La Thangue, 1993), and p107 overexpression results in growth arrest at G1 in some cell lines (Zhu *et al.*, 1993). Mutants of *p107* whose protein products are unable to bind DRTF1/E2F, also fail to repress E2F site-dependent transcription (Smith & Nevins, 1995a) but to date, there is no evidence for it to be a tumour suppressor gene and no mutations of it have been reported in tumour cells.

p130 can arrest cells in G1 (Vairo *et al.*, 1995) and DRTF1/E2F binding activity in quiescent cells has been shown to include p130 (Cobrinik *et al.*, 1993) or even be primarily composed of E2F-4 and p130 in association with a DP heterodimerisation partner (Vairo *et al.*, 1995). p130 effectively suppresses E2F-4 *trans*-activation, and co-expression of E2F-4 overcomes p130-mediated G1 arrest more efficiently than pRb-induced G1 blockade. Conversely, E2F-1 overrides a pRb-

block more efficiently than E2F-4 (Vairo *et al.*, 1995). Thus p130 and pRb appear to induce cell cycle arrest via biochemically distinct mechanisms that involve different E2F-family members at different temporal points in G0 and G1. *p130* has not been directly identified to be mutated in tumour cells, but it maps to a region of the genome that is frequently deleted in a variety of human tumours (Yeung *et al.*, 1993).

Regulation by phosphorylation

p107 phosphorylation levels are cell cycle regulated (Beijersbergen *et al.*, 1995). The first phosphorylation events coincide with increasing cyclin D1 protein levels and the phosphorylation pattern changes dramatically during G1 to S-phase of the cell cycle in fibroblasts (Beijersbergen *et al.*, 1995). It has been shown that cyclin D1/CDK4 complexes, but not cyclin E/CDK2 complexes, can phosphorylate p107 *in vivo* and that phosphorylation of p107 can be abolished by the overexpression of a dominant-negative form of CDK4. Phosphorylation of p107 results in the loss of ability to associate with E2F-4 and a p107-induced cell cycle block can be released by cyclin D1/CDK4 but not by cyclin E/CDK2 (Beijersbergen *et al.*, 1995). The cyclin A promoter contains an E2F site occupied in early G1 by a p107-containing DRTF1/E2F and ectopic expression of cyclin D1 triggers premature activation of the gene which again is blocked by p16 (Schulze *et al.*, 1995). It thus seems that p107 activity may be regulated by phosphorylation, possibly in a similar manner to pRb.

As opposed to pRb, p107 can make a direct interaction with cyclin A which seems to account for the observed DRTF1/E2F-p107-cyclin A/CDK2 complexes (Whyte, 1995). A recent report claims to disrupt p107/DRTF1/E2F complexes by phosphorylation on p107 by cyclin A/CDK2 (Suzukitakahashi *et al.*, 1995). The relative contributions of cyclin A and cyclin D to p107 regulation remain to be elucidated.

Relatively little is known about p130 phosphorylation, however the E2F-1 gene appears to be repressed by p130, and cyclin D-dependent kinase activity specifically activates the *E2F-1* promoter by relieving E2F site-mediated repression which again is inhibited by coexpression of p16 (Johnson, 1995). Phosphorylation seems to be cell cycle regulated with peak levels reached at S-phase (Baldi *et al.*, 1995 ; Mayol *et al.*, 1995). Cyclin E could have a role in the targeting of p107 or p130 by phosphorylation as it is found to be required for S-phase initiation in *RB*-deficient cells (Ohtsubo *et al.*, 1995).

Viral targeting of pocket proteins

Oncogenic DNA tumour viruses target mammalian cells to facilitate their own replication (Vousden, 1995). Most cells in adult mammals body are in a state of quiescence and therefore do not possess an activated replicative machinery for the virus to use. These viruses have thus evolved sophisticated invasive systems to induce the proliferative pathways of a quiescent cell by modulating the regulatory controls of its cell cycle. Historically these viruses have been invaluable in detecting the key regulatory mechanisms of the cell cycle (Vousden, 1995).

Three viral oncogenes, of independent evolutionary origin, target the transcriptionally inactive association of DRTF1/E2F with the tumour suppressor pRb and the inhibitors p107 and p130 in order to stimulate proliferation (**figure 1.8** and see below). The multi-viral targeting of DRTF1/E2F, with the effect of promoting its activity, serves as an evolutionary signature to the fundamentality of DRTF1/E2F in the control of the proliferative state and also underlines the central role occupied by pocket proteins for DRTF1/E2F regulation. DNA tumour viruses deregulate DRTF1/E2F activity as a contribution to the proliferative stimulation of quiescent cells, thereby providing an environment permissive for viral replication (Vousden, 1995).

The pRb-targeting tumour promoting viral oncoproteins include E1a from adenovirus (Whyte *et al.*, 1989), the E7 gene product from papilloma virus (Dyson *et al.*, 1989) and large T antigen from SV40 (Decaprio *et al.*, 1988 ; Dyson *et al.*, 1990), when pRb is in its hypo-phosphorylated form (Ludlow *et al.*, 1990). Any mutation that affects the ability of these oncoproteins to bind pRb, dramatically reduces their transformation potential (Decaprio *et al.*, 1988 ; Munger *et al.*, 1989 ; Whyte *et al.*, 1989) and the binding site of these oncoproteins maps to the region of *RB* that is most frequently mutated in tumours (Horowitz *et al.*, 1989 ; Hu *et al.*, 1990 ; Huang *et al.*, 1990 ; Kaelin *et al.*, 1990). In accordance with these observations, viral oncoproteins have been shown to target (Bandara & La Thangue, 1991b) and release transcriptionally active DRTF1/E2F from pRb (Zamanian & La Thangue, 1992 ; Hiebert *et al.*, 1992). This suggests that the small DNA tumour viruses may stimulate cellular proliferation by binding to and sequestering pRb in a manner that mimics the loss of pRb in naturally occurring tumours. These observations could appealingly explain how E1a-like activities were observed in cells associated with high proliferation and hence higher levels of non-pRb complexed DRTF1/E2F (see above).

The release of DRTF1/E2F transcriptional activity is believed to activate E2F-site containing promoters in the viral genome of adenovirus. The reason for targeting of pocket proteins by viral infection seems, at least in part, to be to release active DRTF1/E2F which has such a fundamental role in the stimulation of proliferation. This is supported by adenovirus which apart from targeting pocket proteins, also targets uncomplexed DRTF1/E2F (Vousden, 1995). The adenovirus polypeptide, orf 6/7, product of *E4*, changes DRTF1/E2F specificity from single cellular E2F sites to viral palindromic E2F sites as those found in the *E2a* promoter (Huang & Hearing, 1989 ; Marton *et al.*, 1990). This has been demonstrated for the DP-1/E2F-1 heterodimer (Bandara *et al.*, 1994 ; Helin & Harlow, 1994) and possibly occurs via an interaction with DP-1 (Cress & Nevins, 1994). This effect could explain the earlier discrepancies between the DRTF1 and E2F characterisation studies (see above). Unlike the adenovirus wherein the E2F site was originally defined, the SV40 and HPV genomes do not contain E2F sites suggesting that the release of active DRTF1/E2F may be the more important activity for cellular subjugation (Adams & Kaelin, 1995).

As well as for pRb, p107 and p130 bind to regions of E1a that are conserved among the various serotypes of adenovirus and are also present in the large T antigens of the polyoma family of viruses and the E7 proteins of papillomaviruses (reviewed in (Dyson & Harlow, 1992 ; Levine, 1993 ; Vousden, 1995).

Pocket proteins as trans-repressors at E2F sites

Recent studies on the pocket protein family have promoted an increasing perception of them as more than just inhibitors of the E2F site, but possibly as harbourers of more general *trans*-repressive regulative powers that affect other activating or general transcription factors (**figure 1.10**).

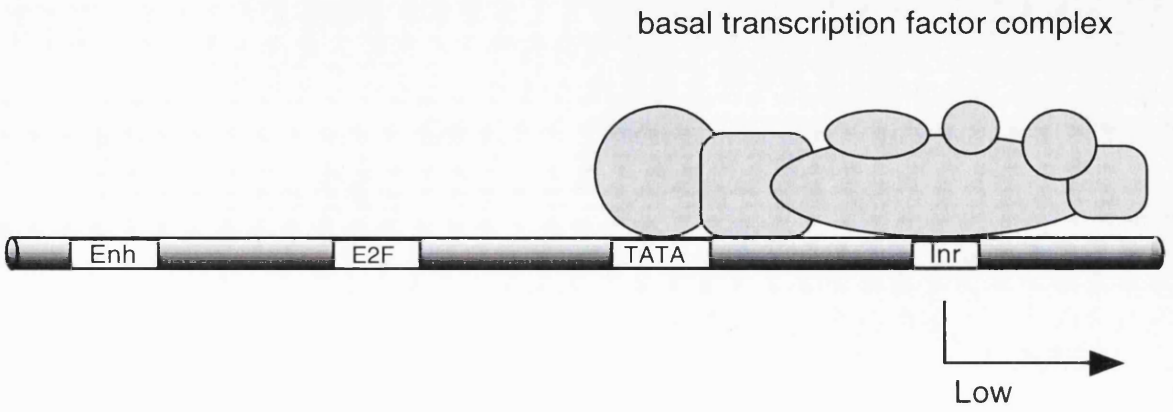
p107 and p130

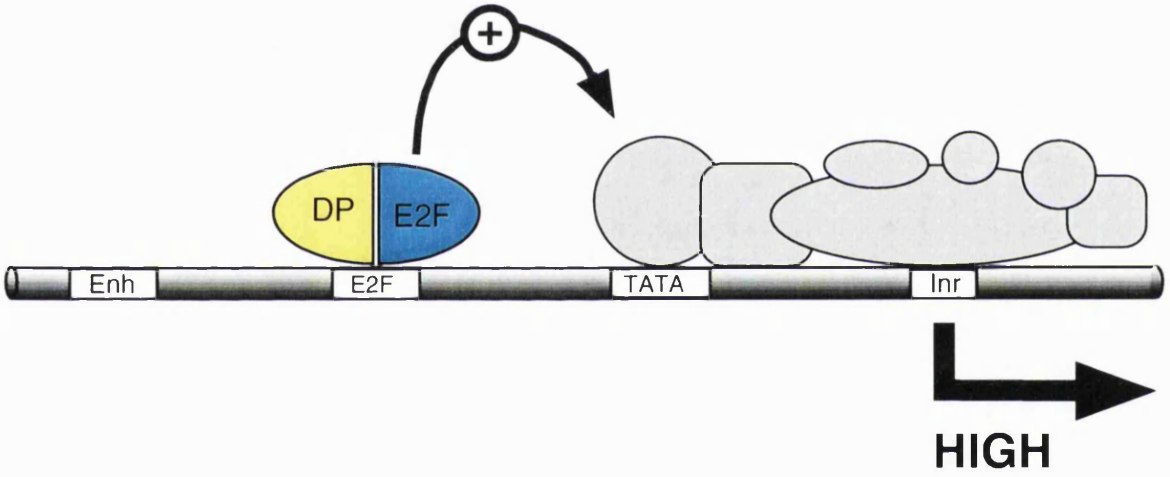
Studies involving p130 and p107 have provided good circumstantial evidence of E2F-site mediated *trans*-repression of promoters in G0 and G1 respectively. In several cases it seems that certain genes in question actually succumb to general transcriptional repression mediated by the E2F site occupied by pocket protein-bearing DRTF1/E2F. This is opposed to a pocket protein occupancy that merely inactivates the DRTF1/E2F contribution to the promoter activity irrespective of any other concomitant basal or activated transcription taking place due to other transcription factors on the same promoter.

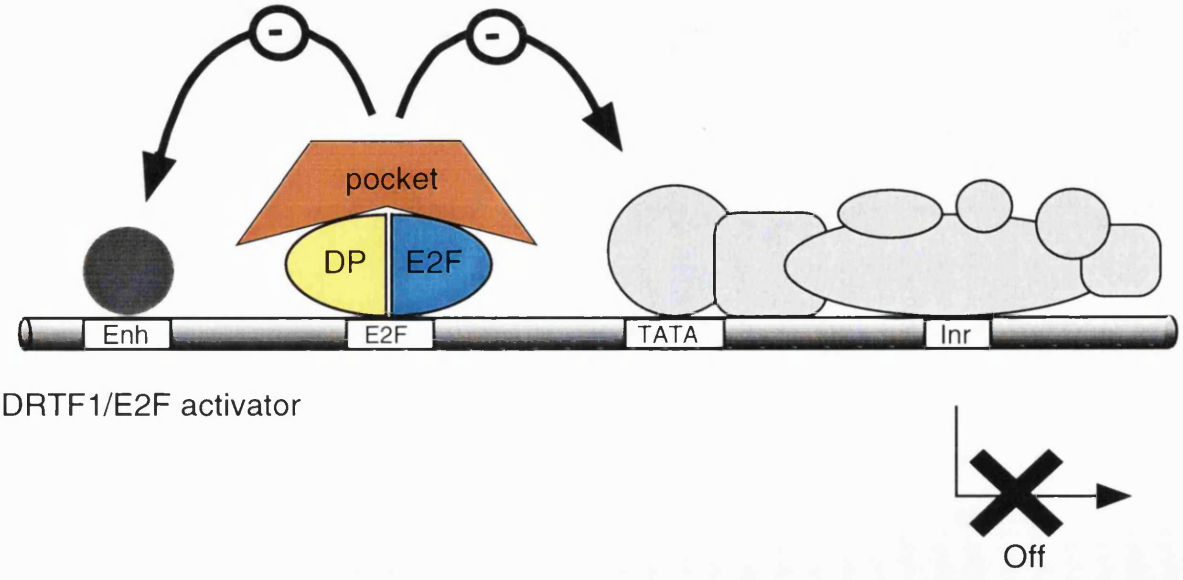
Figure 1.10

Transrepressional properties of pocket proteins bound to DP/E2F heterodimers.

(**i**) Depiction of a E2F site-containing promoter stretch of DNA (grey tube) possessing also an enhancer element (Enh) as well a TATA and Initiator (Inr) motif bearing the basal pre-initiation complex (grey body). In the absence of activation from DP/E2F heterodimer or other activators the basal transcription factor complex will display a minimal level of transcriptional activity. (**ii**) If the E2F site is occupied by the DP-E2F heterodimer (yellow and cyan oval) the transactivation domain in the E2F moiety will promote a high level of transcriptional activity by interaction with members of the basal transcription factor complex. (**iii**) If the DP/E2F heterodimer is bound by a pocket protein (Orange hat), not only is the transactivational activity of the E2F prevented, but the pocket protein will also actively repress the basal transcription factor complex so as to prevent basal levels of transcriptional activity. Furthermore the pocket protein may suppress the activities of certain enhancer elements containing non-DRTF1/E2F transcriptional activators.


basal transcription factor complex
Enh
E2F
TATA
Inr
Low


+
DP
E2F
Enh
E2F
TATA
Inr
HIGH


-
-
pocket
DP
E2F
Enh
E2F
TATA
Inr
non-DRTF1/E2F activator
Off

Cyclin A expression is usually repressed during G1 and induced at S-phase entry (Schulze *et al.*, 1995). This has been shown to be mediated by a DRTF1/E2F complex absent of pRb but including cyclin E and p107, which bind to a specific E2F-varient site in the cyclin A promoter. Mutation of this site releases repression during G1 and removes G1/S specific induction indicating that this site has a fundamental role in cyclin A regulation with possible trans-repressive activity mediated by p107 (Schulze *et al.*, 1995). In the *CDC2* promoter a one-base deviant E2F site appears to be occupied in a G0/G1-specific manner, disappearing at S-phase entry and coinciding with initial stimulation of CDC2 expression (Tommasi & Pfeifer, 1995). The site interacts with a subset of E2F-4/p130-bearing DRTF1/E2F present at G0 and mutational analysis indicates that the element is involved in suppressing *CDC2* activity in quiescent cells (Tommasi & Pfeifer, 1995). Overexpression of p130 inhibits *CDC2* promoter activity and the entry of quiescent L1 cells into S-phase (Wolf *et al.*, 1995). Similarly, *E2F-1* is transcriptionally repressed through E2F sites in G0 and early G1 correlating with the presence of p130 in DRTF1/E2F and p130 will inhibit transcription from the E2F-1 promoter (Johnson, 1995). The E2F site in the *B-myb* promoter has been shown to be occupied by DRTF1/E2F only in G0 and G1, and to be complexed to p107 (Zwicker *et al.*, 1996). All binding is removed as cells enter S-phase, and B-myb expression is induced precisely as the E2F site occupancy is lifted, indicating a generally repressive role only by DRTF1/E2F at this gene (Zwicker *et al.*, 1996).

These are examples of DRTF1/E2F complexes which appear to be solely involved in repression and raises the possibility of *trans*-repression on the whole promoter since suppression of expression is so complete. The role of the basal DP/E2F heterodimer in these complexes could thus be solely to provide a DNA binding platform for the pocket protein to access and generally repress a promoter. Such all-powerful *trans*-repression could be imagined to be essential in tightly regulated cell cycle genes to quench any basal transcription activity residual to the actual pocket protein repression of the E2F site alone.

pRb

Reintroduction of *RB* into Saos2 cells (which are normally deficient for pRb) causes a G1 arrest and characteristic cellular swelling. Co-expression of the cellular transcription factor E2F-1 can overcome these effects independently of the ability of E2F-1 to bind to pRb (Qin *et al.*, 1995). Mutational analysis shows that the ability of E2F-1 to bind to DNA is necessary and sufficient to block the formation of large cells by pRb whereas the ability to induce S-phase entry requires a functional *trans*-activation domain as well. The ability of the E2F-1 DNA-binding domain alone to

block one manifestation of pRb action is consistent with the notion that pRb-E2F complexes actively repress general transcription upon binding to certain E2F-responsive promoters (Qin *et al.*, 1995). These findings support the model in which DRTF1/E2F-pRb complexes generally repress the transcription of certain E2F containing promoters, rather than merely representing inactivated or sequestered DRTF1/E2F.

A chimaera made in which the E2F-1 *trans*-activation domain was replaced with the pRb pocket can, in a DNA binding and pocket-dependent manner, mimic the ability of pRb to repress transcription and induce cell cycle arrest (Sellers *et al.*, 1995). In contrast, a transdominant negative E2F-1 mutant that is capable of blocking DRTF1/E2F dependent *trans*-activation, does not (Sellers *et al.*, 1995). Fusion of pRb to a heterologous DNA binding domain unrelated to that of the E2F family, likewise generates a *trans*-repressor protein (Sellers *et al.*, 1995 ; Weintraub *et al.*, 1995).

These results suggest that growth suppression by pRb is due, at least in part, to *trans*-repression mediated by the pocket domain brought proximal to the core promoter by a carrier : DRTF1/E2F at the E2F site. Progression into S-phase thus seems to require both alleviation of transcriptional repression and transcriptional activation of certain DRTF1/E2F dependent genes. Possession of an E2F site could potentially cause the promoter of a gene to become a target of general transcriptional repression by pRb-DRTF1/E2F complexes that will not only prevent E2F-site stimulated transcription, but also inhibit any basal, or unrelated-transcription factor promoted transcription.

Mechanisms of trans-repression

The molecular basis of *trans*-repression by pRb has been probed by fusing a range of transcription factors, that do or do not interact with pRb, with a Gal4-DNA binding domain (Weintraub *et al.*, 1995). In *trans*-activation assays on Gal4 binding sites upstream of a reporter gene, pRb was found to be unable to inhibit any fusion proteins except the very strongest pRb-binders : E1A and E2F-1. pRb-inhibition of weak pRb-binding transcription factor fusion proteins (c-myc, Elf-1 and PU.1) was however achieved if pRb was targeted to the promoter either artificially (through a LexA binding site) or via E2F-1. Transcription factor fusion proteins that do not form pRb interactions (VP16, CTF or SP1) were still not affected (Weintraub *et al.*, 1995). These results suggested that pRb only gains access to the promoter via strong interacting transcription factors such as E2F-1, and once there will not inhibit the general transcriptional complex but rather target specific transcription factors. pRb can form a complex with the E2F-1/DP-1 heterodimer simultaneously with PU.1 and will prevent PU.1 from interacting with TFIID *in vitro* while not interacting with

TFIID itself (Weintraub *et al.*, 1995). The pRb pocket shows amino acid sequence similarity to TBP and TFIIB (see above) suggesting that it might mimic components of the basal transcription complex and sequester susceptible transcription factors at the promoter such that DRTF1/E2F-bound pRb will act as a *trans*-repressor not by targeting the basal transcriptional complex, but by compromising the activities of other adjacently bound activators.

It is also possible that trans-repressive activity of pRb is due to inhibition of the general transcription apparatus. If pRb harbours amino acid sequence similarity to TFIIB and TBP and can interact with polypeptides that bind them, then it could easily be imagined that if it was to be brought into proximity with the pre-initiation complex, interference with factors that normally interact with TFIIB or TBP would result. Evidence of direct pocket protein inhibition on the basal transcription apparatus has already been put forward for Pol I (Cavanaugh *et al.*, 1995) and Pol III (White *et al.*, 1996) which leaves the door open for similar investigations on Pol II. Enlightenment on this topic is eagerly awaited and likely soon to be forthcoming as it has the potential of dramatically transforming our interpretation of the roles of both the pocket proteins and DRTF1/E2F in the cell cycle.

DRTF1/E2F-independent cell cycle control by p107

Growth arrest mediated by pRb and p107 are not identical. There is some evidence that p107 is able to exert repressive effects independently of DRTF1/E2F. Two domains of p107 have been identified that independently are able to block cell cycle progression (Zhu *et al.*, 1995a). One domain corresponds with the sequences needed for interaction with DRTF1/E2F, and the other corresponds to the interaction domain for cyclin A or cyclin E complexes. In the cervical carcinoma cell line C33A, which has previously been shown to be sensitive to p107 but resistant to pRb growth suppression, only the cyclin binding domain is active as a growth suppressor (Zhu *et al.*, 1995a). Similarly, pRb inhibition of the *c-myc*, *fibronectin* and *thymidine kinase* promoters requires the presence of E2F sites while p107 inhibition does not (Dagnino *et al.*, 1995).

A possible explanation for these effects is provided by the observation that the cyclin interaction domain of p107 is structurally and functionally related to the p21 family cyclin/CDK interaction domain (Zhu *et al.*, 1995b). And, like the p21 family of CDK inhibitors, p107 can inhibit the phosphorylation of target substrates by cyclin

A/CDK2 and cyclin E/CDK2 complexes by binding to these complexes. Interactions between p107 or p21 with cyclin/CDK2 complexes are mutually exclusive (Zhu *et al.*, 1995b). In cells treated with DNA-damaging agents, elevated levels of p21 cause a dissociation of p107/cyclin/CDK2 complexes to yield p21/cyclin/CDK2 complexes (Zhu *et al.*, 1995b).

Objectives and achievements of this study

Interest in DRTF1/E2F rose immensely in the few years prior to the undertaking of this project. The then putative transcription factor complex had been demonstrated to be bound by the tumour suppressor pRb, as well as by cyclins and CDKs, and most of these interactions, in turn, shown to be inhibited by viral oncoproteins. Furthermore, a plethora of E2F DNA binding sites were being discovered in the promoters of genes which had already been associated with cell cycle progression. DRTF1/E2F thus had all the hallmarks of a transcription factor which potentially could bridge the fields of transcription and the cell cycle and thus illustrate how the cell cycle cascades exert their control over the specific transcriptional events that drive cellular division.

At the time that this study was initiated, *DP-1* had just been cloned (Girling *et al.*, 1993), and apart from confirming it as a participant in the DRTF1/E2F DNA binding activity, very little was known about its function. It was deemed important to determine DP-1s nature and contribution to DRTF1/E2F.

The results presented in this thesis propose the existence of two new levels of DRTF1/E2F regulation : Negative targeting by phosphorylation by the cell cycle pathways, and inhibition by the tumour suppressor p53. The proposed regulatory mechanisms are novel in several ways. Both target DP-1 as opposed to the E2F-moiety of the transcription factor complex, and they both affect the DNA binding activity of DRTF1/E2F, as opposed to its *trans*-activating activity. Both the cell cycle and p53 have been implicated before in the regulation of DRTF1/E2F activity, but only via pRb. The two new levels of regulation proposed in this thesis concern the direct targeting of DP-1, and hence DRTF1/E2F, and are independent of pRb.

The results highlight DP-1 as a target for the regulation of the DNA binding activity of DRTF1/E2F, suggesting that the regulation of the *trans*-activating activity of DRTF1/E2F is concerned predominantly with the E2F moiety of the complex. The results suggest that the cell cycle can both inhibit and stimulate the activity of DRTF1/E2F and that the two major tumour suppressors, pRb and p53, both target its activity directly and independently. Overall, these new possible pathways of regulation serve again to highlight the central importance of DRTF1/E2F in the regulative processes of cellular proliferation.

Chapter 2

Experimental protocols

All chemicals, unless otherwise indicated, were supplied by BDH/Merck Chemicals, UK. Radiochemicals were provided by Amersham International, UK. Final concentrations, where appropriate, are indicated in "curly cues" ({}).

Cell growth and extraction

Culture of F9EC and NIH-3T3 cells

All cells were grown as adherent monolayers in Dulbecco's modification of Eagles Medium supplemented with 10 % ($^{V}/_{V}$) foetal calf serum (FCS - Gibco BRL), 4mM L-glutamine (Gibco BRL) and antibiotics : 10mg/ml streptomycin and 100U/ml penicillin (Gibco BRL), at 37°C in a 5% CO_2 / H_2O-saturated atmosphere. F9-Embryonic Teratocarcinoma cells (F9EC cells : Bernstine *et al.*, 1973) and NIH 3T3 mouse fibroblast cells (Jainchill *et al.*, 1969) were both cultured in 100mm tissue culture dishes (NUNC) and were typically seeded at $1x10^6$ or $5x10^5$ for 48 or 72 hours of growth respectively. Cells were passaged by washing with sterile phosphate buffered saline (PBS) and treated with trypsin (Gibco BRL - dissolved in FCS-free DMEM, 0.05%$^{W}/_{V}$) at room temperature to displace the cells from the polystyrene. Trypsinisation was arrested by the addition of complete media and the resulting cellular suspension was gently pelletted by centrifugation (in a Sorval RT6000D at 4K), resuspended into fresh media, counted with a haemocytometer and re-plated.

F9EC cell differentiation

F9EC cells were induced to differentiate to parietal endoderm-like cells (F9PE) by additionally supplementing the media with 0.05μM retinoic acid, 1mM dibutyryl-adenosine 3'5'-monophosphate and 0.1mM isobutyl methylxanthine according to Strickland & Mahdavi (1978). Cells were harvested by microextraction (see below) at 3, 5 and 7 days after introducing the differentiating agents. F9EC cells for differentiation were seeded at the lower densities 2-3 x 10^5, per 100mm dish, into differentiating medium because total cellular arrest, associated with the new cellular

phenotype, does not occur for all cells in the first round of replication, but rather only by about day five. Seeding at extra low densities ensured that the cells did not over-grow in this period. During the seven-day period the cells were regularly re-fed as directed by the colour indication of the medium.

Serum starvation of NIH-3T3 cells

To arrest the growth of NIH 3T3 cells they were allowed to reach confluence in normal media and then serum starved by growing them in the same medium, but containing only 0.1% foetal calf serum, for 72 hours. The cells were then re-exposed to full media and harvested at different time points thereafter.

Cryo-storage of cells

Live cells were subjected to long-term storage by resuspending pre-confluent cells in media containing 10% DMSO. This suspension was then aliquoted out into cryotubes (NUNC) with each tube typically containing $2x10^6$ cells. The tubes were then placed at -80°C in a polystyrene box insulated with cotton wool to ensure gentle freezing over-night. The following day the tubes were transferred to liquid nitrogen.

Cell thawing was performed by transferring the cryotubes from liquid nitrogen to dry ice and then snap thawing in a 37°C water bath with "flicking". The cellular suspension was then placed in a 50ml falcon centrifuge tube and its volume doubled with warm (37°C) complete media. The suspension was gently swirled for a few minutes and the volume was then doubled again, and again, after further mixing. This precaution was taken to ensure that all cells were completely thawed prior to centrifugation. The cells were then pelletted by gentle centrifugation at room temperature to remove the DMSO and usually all ($2x10^6$) were plated out onto one 100mm dish. Cell survival after thawing was normally very good (close to 100%). Typically, the cells required one extra day of growth for full recovery and would not be used for any experimentation unless passaged at least once.

Phosphate Buffered Saline (PBS)

136mM NaCl	pH adjusted to 7.2 at 25°C
2.7mM KCl,	Stored at 4°C or room temperature
4mM Na_2HPO_4	
1.8mM KH_2PO_4	

PBS was prepared by services at the National Institute for Medical Research. In later experiments complete PBS tablets (Sigma), made to similar specifications, were used.

Metabolic labelling

^{32}P

Radio-phosphate labelling of F9EC cells was initiated at around 40 hours post-passage of $1x10^6$ cells. At this stage the monolayers were virtually confluent at about $5x10^6$ cells per 100mm tissue culture dish. The medium was replaced with phosphate-free DMEM (built up from Gibco BRL basal DMEM and all supplements except sodium phosphate) for one hour under normal growth conditions and then this media was replaced with 3ml of phosphate free DMEM containing 3mCi of ^{32}P-orthophosphate (*in vivo* cell labelling grade 10mCi/ml). The cells were incubated under normal conditions for a further three hours and then harvested in LSL buffer (see below).

^{35}S

Methionine radio-labelling was initiated on F9EC cells at around 32 hours post-passage of $1x10^6$ cells per 100mm dish. These cells were not pre-incubated in methionine-free media but were just washed once with methionine-free DMEM (built up from Gibco BRL basal DMEM and all supplements except L-methionine) and provided with 10ml methionine-DMEM containing 500 μCi L-^{35}S-Methionine (*in vivo* cell labelling grade 10mCi/ml) and incubated under normal growth conditions for a further 16 hours.

Mock-labelling

Isotopic cell labelling was accompanied by mock-labelling procedures, lacking isotope but with otherwise identical treatments, to control for any effects due to the labelling procedure.

Microextracts

Cultured cells

Asynchronous confluent cells (approximately $1x10^7$ per 100mm dish for F9EC cells and $5x10^6$ for NIH-3T3 cells), approximately 48 hours post-passage, were washed with around 5ml room temperature PBS and scraped off into another 1ml of PBS. The cellular suspensions were placed in eppendorf tubes and cooled down on

wet ice for about five minutes and then microcentrifuged for 2 minutes, 4°C at 13K (15g). The supernatant was discarded and the cellular pellet was snap frozen on dry ice and either stored indefinitely at -80°C or immediately micro-extracted.

Animal tissue

BALB-C mice (Harlan, UK) were sacrificed by standard procedures. Different tissue types were immediately isolated and frozen in pre-cooled eppendorf tubes on dry ice. Separate tissues were then placed in a mortar containing liquid nitrogen and ground to a fine powder using a pestle. Particular tissues types proved to be especially hard nuts to crack, but special care was always taken to ensure that the fragments and powder were always kept "moist" with liquid nitrogen. Tissue powders were stored at -80°C. Microextracts were made from the powder as described below using visually estimated equivalent quantities to tissue culture cell pellets.

Extraction

Whole cell microextracts were prepared as described by Schöler *et al.* (1989) except that sonication was replaced by freeze-thawing three times. Cell pellets were gently resuspended in 100-200µl microextraction buffer (MEB : Containing protease inhibitors) at 4°C depending on the size of the pellet. A typically large pellet of approximately 20µl, would be resuspended in 200µl microextraction buffer. 200µl was the maximum volume still deemed to ensure the rapid freeze/thawing events central to the extraction procedure. Suspensions were transferred to dry ice until the solution was frozen, then thawed by 30°C incubation and flicking followed again by snap-freezing. This was done three times in total and samples were then centrifuged for 10 min at 13K (15,000g) in a microfuge at 4°C.

The resulting supernatants were pooled, mixed and aliquotted into 100-200µl samples, assayed for protein concentration (see below) and stored at -80°C. The extracts were thawed a maximum of five times before being discarded and only "virgin" extracts were used for gel retardation or immunoprecipitation assays.

1xMicroextraction buffer (MEB)

20mM Hepes pH 7.8 Stored at 4°C
450mM NaCl
0.2mM EDTA
25% Glycerol ($^{V}/_{V}$)

A $^{1}/_{100}$ dilution of 100x PIC and PMSF to 0.5mM, from a stock of 0.1M stored in isopropanol (propan-2-ol) at -20°C, were added just prior to use.

1x Protease Inhibitor Cocktail (PIC)

0.5µg/ml Leupeptin (Sigma) Stored at -20°C as a 100x stock
0.5µg/ml Protease inhibitor (Sigma)
1µg/ml Chymotrypsin/Trypsin inhibitor (Sigma)
40µg/ml Bestatin (Sigma)
0.5µg/ml Aprotinin (Sigma)
0.5mM DTT

Low salt lysis (LSL) extractions

Asynchronous confluent cells (radio-labelled or "cold") were washed in 5mls PBS at room temperature. 500µl of 4°C LSL Buffer was added and the plate was incubated at 4°C or on wet ice for 30 minutes with regular swirling. The resulting cellular debris in suspension were pipetted off (no scraping), placed in a pre-cooled eppendorf and microcentrifuged at 4°C for 10 minutes at 13K (15g). Supernatants were treated and stored as the microextracts (see above) albeit usually aliquotted into 300µl portions. Radiolabelled extracts for immunoprecipitations were always used fresh without freezing.

Low Salt Lysis Buffer (LSL Buffer)

50mM Tris-HCl pH 8.0 Stored at 4°C
150mM NaCl
0.1% NP40 (Sigma) or IGEPAL (Sigma ; $^{V}/_{V}$)

A $^{1}/_{100}$ dilution of 100x PIC and PMSF to 0.5mM, from a stock of 0.1M stored in isopropanol (propan-2-ol) at -20°C, were added just prior to use.

Protein concentration estimation

Protein concentrations were determined by the method of Bradford (1976), using the Bio-Rad protein concentration assay reagent. 1-10µl of protein solution was mixed with 1ml Bradford reagent (diluted 1:5 in dH_20) and incubated at room temperature for 10-15 minutes. The optical density was then 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 through a manually plotted standard curve. New protein standard measurements in the range 1, 3, 6, 9, 15, 22 and 30µg BSA was performed for every protein estimation. All measurement reactions were performed in triplicate and the mean value used while single deviant measurements were ignored. For the most accurate measurements, sample concentrations were aimed at a near-linear 5-15µg/reaction mixture-range.

The concentrations of precious purified fusion protein were often too low to employ the Bradford assay and so visual estimations were made through the degree of Coomassie staining after SDS-PAGE (see below) relative to my own standards (GST protein) or commercial markers (Sigma).

Antibodies

Immunisations

Antibodies were raised to peptide epitopes by immunising mice or rabbits with peptide coupled to BSA using the agent M-makimidobenzoyl N-hydroxy succinimide (MBS). Care was taken during the preparations of the samples to keep them as clean and pure as possible.

1ml of 25mg/ml MBS in DMF (dimethyl formamide) solution was added slowly, 50µl at a time, to 10mls of a 10mg/ml solution of high grade BSA (Sigma) in PBS. The addition was performed in a 37°C room with constant stirring and several-minute gaps between additions, avoiding high local concentrations. After addition, the solution was transferred to room temperature and stirred for a further 30 minutes. This procedure activates the BSA so that it will make covalent interactions with added peptide. However MBS is toxic and so had to be removed from the BSA. This was achieved by running the mixture through a G25 Sephadex (Pharmacia) column (2 by 10 cm) and collecting 1ml fractions. BSA runs through this column faster than MBS and so the aim was to use early fractions of activated BSA eluted containing a minimal of MBS contamination. The eluted fractions were assayed for protein content using the Bradford reaction (see above) and the direct optical densities of the fractions were also read at 280nm which indicates both protein and MBS concentration. From these readings fractions with BSA and minimal MBS contamination were identified and pooled. Typically around 74mg low-MBS BSA was recovered in a volume of about 7.5ml.

Peptides were synthesised by services at the National Institute for Medical Research and supplied in a lyophilised form. Peptide solutions prepared to 2nmol/µl in 0.1M Tris-HCl pH 7.6 were added in equal volume to the activated BSA. This mixture was then diluted with PBS to make up to a volume that allowed for 3.5ml per rabbit to be immunised. This consisted of five 700µl aliquots (500µl injection, 200µl needle space) per animal. Typically the mixture was diluted to allow for six rabbits to be injected (two per peptide type) providing around 1.6mg BSA-peptide per 500µl immunisation. Finally 35µl of 1M DTT was added per ml to de-toxify any remaining MBS and the total solution was mixed overnight at room temperature to allow the BSA to couple fully to the peptide. The samples were then aliquoted into 1.4ml and frozen at -20°C until immunisation.

Half Sandy Lop rabbits were immunised by the Biological Services at the National Institute for Medical Research. The antigen sample was mixed in equal portions with Non-Ulcerative Freunds Adjuvant (NUFA - 500µl for Rabbits and 50µl

for mice) and a typical immunisation procedure was a course of three injections over a period of ten weeks. Test bleeds were assayed by immunoblotting and gel retardation and, if positive, the animals would receive a final boost followed two days later by their terminal bleed-out by cardiac puncture under general anaesthesia.

Blood samples (test bleeds typically 2mls, terminal bleeds typically 70-90mls) were incubated at 37°C for one hour to initiate coagulation, and then at 4°C for 12-16 hours. They were then centrifuged in a microfuge or benchtop centrifuge at full power until all coagulate had been pelletted. Serum was stored at -20°C or at 4°C supplemented with NaN_3 (0.05% $^{w}/_{v}$).

Monoclonal antibody generation

As described in Chapter 4.

Epitope affinity purification of polyclonal antibody reagents

4 g of activated Sepharose (CNBr- Cyanogen bromide activated Sepharose-4B, Pharmacia) was resuspended in 20mls 1mM HCl to remove preservatives and initiate activation. The suspension was shaken vigorously until no lumps remained. The Sepharose was washed with 400mls 1mM HCl in a Sinta funnel followed by 400mls 10mM sodium phosphate buffer pH 7.5. Drained Sepharose was then scraped into a 50ml Falcon tube and mixed with 2mg peptide dissolved in 10ml 10mM sodium phosphate buffer (pH 7.5). The tube was then rotated on a "wheel of death" (cell mixer) for 2 hours at room temperature, or overnight at 4°C, so that the Sepharose could covalently couple with the peptide. The suspension was then poured back through the funnel and rinsed with a little 0.1 M Tris-HCl pH 8.0. The Sepharose was placed back into a Falcon tube and mixed with 20ml of the same buffer on the "wheel of death" for two hours at room temperature or overnight at 4°C. The Sepharose was washed again with around 100mls 0.1M Tris-HCl pH 8.0 and 100mls of PBS.

Rabbit polyclonal antibodies were raised to DP-1 synthetic peptides as described above. A peptide-Sepharose bead volume of around 7ml was placed in a sealed column unit (Pharmacia C-16/20) with a chamber diameter of 16mm. Eventual elution of the specifically bound antibody was carried out at low pH and so prior to antibody binding the column was washed with that same buffer, 0.1 M Glycine pH

2.5. The pH was then brought back to neutral by washing with sodium phosphate buffer and then PBS, as monitored using pH indicator strips. The pH of the antisera was checked to be close to neutral and hence optimal for antigen-binding. Any variance was adjusted with 1M Tris-HCl buffer pH8.0.

Purification was carried out by passing 1ml antisera over the column at room temperature and low flow-rate (approximately 0.25ml/min) followed, at the same rate, by PBS until all sera was clearly within the bed of beads. The apparatus was then turned up-side-down and the sera eluted at high flow rate (approximately 6.0ml/min) using PBS. A sample of the flow-through was kept as a control. After all remaining visible sera had left the column, it was washed with a further 30ml of PBS. The bound antibodies were then eluted with 0.1M glycine pH2.5 at medium flow-rate (approximately 2.0ml/min) into $^1/_4$ volume preservation/neutralisation buffer (1M Tris-HCl pH 8.0 / 0.2% ($^W/_V$) NaN_3). A series of 1.0-1.5 fractions were collected which were all assayed by gel retardation and immunoblotting assays. The column was washed with sodium phosphate buffer and then with PBS/0.05% NaN_3. It was then sealed in that buffer and stored at 4°C.

Enzyme-linked immuno-sorbent assays

"Immunosorp" microtitre plates from NUNC were used to covalently adhere peptide antigen to microtitre wells. Peptide antigen was diluted to 1µg/100µl with sensitising buffer and 50µl was placed in each microtitre well (hence 500ng peptide per well) and incubated for one hour at 37°C or overnight at 4°C. The wells were then washed three times in PBST (PBS plus 0.05% ($^V/_V$) Tween-20 Sigma), usually by sequential "dunking" in three $2dm^3$ beakers. The activated polystyrene was then blocked by the addition of 100µl per well of PBST containing 1% BSA (PBST/BSA). This was incubated for a minimum of one hour at room temperature and often stored thereafter for up to a month at 4°C in a humidity chamber. The plates were washed in PBST again. The primary antibody was diluted in PBST/BSA, often ten-fold for polyclonal antisera, and 50µl loaded which was sequentially diluted by 50% "down" the microtitre plate wells, eight fold. Hybridoma supernatants were added neat. The primary antibody was allowed to bind for one hour at room temperature after which the plates were washed in PBST. For the secondary, anti-mouse or anti-rabbit alkaline phosphatase conjugated antibody (Promega), typically a $^1/_{10,000}$ dilution was made in PBST/BSA. 50µl was aliquated per well and incubated at room temperature for one hour. The plates were then washed again with an additional two washes in PBS only.

Fresh substrate was made up (p-nitrophenyl phosphate tablets in PBS - Sigma) and 100µl was dispensed per well. The plate was incubated at 37°C until colour developed (15-60 minutes) and the optical densities of the reaction products were measured at 400nm on a Titertek Multiscan.

Sensitising buffer

18mM Sodium carbonate (Na_2CO_3) Stored at room temperature
32mM Sodium bicarbonate ($NaHCO_3$)

Peptide dephosphorylation

Phosphorylated peptide D was dephosphorylated for ELISA analysis of monoclonal 32.3 affinity by the following reaction :

10µl	Peptide (300ng/µl)
2µl	Calf Intestinal Phosphatase (CIP 20U/µl - Boehringer Mannheim) or dH_2O as a control.
2µl	CIP Buffer (Boehringer Mannheim)
6µl	dH_2O

The reaction was allowed to run at 37°C for 12-16 hours. 10µl of the reaction mixture, and a further control : 1:2 dH_2O diluted peptide, were diluted by $^1/_{2500}$ with sensitising buffer and 50µl of this mixture was loaded into each microtitre well (30ng per well). Low peptide levels were chosen for this experiment to highlight any phosphorylative changes.

Immunoprecipitation and re-immunoprecipitation

Immunoprecipitations from cellular lysates mostly employed Low Salt Lysis (LSL) cell extracts since it was deemed likely that in these low-stringency samples, even weak protein-protein interactions would remain undisrupted.

The LSL lysate (aliquot of 300µl, see above) was rapidly thawed at 30°C and microcentrifuged at 13K (15g) for two minutes at 4°C to remove cellular precipitates which tend to form as a result of the freezing. 250µl of supernatant was removed and added to 20µl unpurified polyclonal antisera, 100µl affinity purified polyclonal antisera (DRTF1/E2F gel retardation positive fractions), 100µl monoclonal hybridoma supernatant or 10-50µl of purified monoclonal antibody (0.7mg/ml) :

Depending on the strength of the signal sought. Typically each assay was performed in duplicate when a specific peptide was available. The samples would then be mixed with antibody and an equal volume of peptide (2nmol/µl), one specific (the peptide to which the antibody was raised) and one non-specific (an equivalently sized un-related peptide) and the total mixtures incubated on wet ice for one hour.

150µl of 10% ($^{v}/_{v}$) protein A-Sepharose conjugate (Boehringer Mannheim, in PBS/0.05% NaN_3 - stored at 4°C) was then added directly to the antibody-cell extract mixture, or if a particularly weak signal was expected, the protein A was first pelletted by centrifugation (20 seconds in microfuge at 13K) and the cell extract was added to the 15µl moist pellet. The former was then turned on the "wheel of death" for one hour at 4°C while the latter, due to its smaller volume, was incubated in wet ice with regular agitation by hand .

For non-radioactive immunoprecipitations the beads were then washed three times in the same tube (900µl each) with LSL buffer containing protease inhibitors while radioactive samples were washed five times with one or two tube changes. Washing was carried out by 20 second 13K 4°C microfuge centrifugations, supernatant removal, and LSL buffer resuspension. For western blotting the beads were sucked dry with a syringe and 1xSDS loading buffer was added (see below). For weak signals two immunoprecipitations were pooled for one loading.

When the immunoprecipitate was required for gel retardation assays, the beads were sucked almost-dry and exposed to 12µl gel retardation eluting buffer containing salmon sperm DNA and mutant E2F site (see gel retardation section below) but also the peptide that the antibody of the primary immunoprecipitation was raised to at 3.3nmol/µl. This suspension was incorporated directly into the protocol of the gel retardation assay with 10 minutes incubation at 30°C followed by addition of radioactive probe and further incubation (see below).

For re-immunoprecipitations, most of the supernatant from the last wash was removed and 40µl LSL buffer containing specific peptide (4nmol/µl) was added to elute the antigen off the bead. The mixture was incubated for 10 minutes at 30°C and then centrifuged to recover the supernatant containing the eluted protein complexes. To the beads was then added another 160µl LSL buffer containing 2nmol/µl specific peptide which was incubated again for 10 minutes at 30°C, and the new supernatant recovered. The two elutants were pooled, the secondary antibody added and the immunoprecipitation procedure repeated.

Gel retardation elution buffer

50mM Tris-HCl pH 7.9 Stored at -20°C
6mM $MgCl_2$
0.2mM EDTA
1mM DTT
15% Glycerol ($^{V}/_{V}$)
166 ng/µl Salmon sperm DNA
25ng/µl Mutant E2F site DNA oligonucleotide (60/62)
3.3nmol/µl Peptide

Immuno-depletion and de-phosphorylation

Immunodepletion was carried out by four consecutive immunoprecipitations (see above) by purified monoclonal antibody 32.3 (0.7mg/ml ; 50µl per round) on 300µl LSL extract. Immunoprecipitates and extract supernatants were sampled by immunoblotting at each stage.

The de-phosphorylation treatment of immuno-depleted extract (stage four) was set up as follows :

20µl	Stage four immuno-depleted F9EC LSL extract
4µl	CIP (Boehringer Mannheim : 18U/µl)
4µl	CIP buffer (Boehringer Mannheim)
12µl	dH_2O

The total reaction mixture was added to 40µl SDS-loading buffer or allowed to incubate at 37°C for 60 minutes longer before addition to the loading buffer. The effects of the reaction was assayed by immunoblotting.

Peptide epitopes

The following peptide epitopes were used in this study :

Peptide	Origin	Sequence	Rabbit
A	DP-1 3-15	(C)KDASLIEANGELK	004
17	DP-1 206-220	(C)EVERQRRLERIKQKQ	153
24 ‡	DP-1 323-337	(C)RSLVPKALEPYVTEM	090
26 ‡	DP-1 352-336	(C)STSNGTRLSASDLSN	086
D	DP-1 385-400	(C)RVETPVSYVGEDDDDD	099 †
C	Control	(C)DYSTRRPLSPSNQLQEKHV	-
V1 ‡	E2F-5 106-123	(C)VGAGCNTKEVIDRLRC	053
V2 ‡	E2F-5 192-203	(C)IPEMGQNGQKKY	056
VC ‡	E2F-5 320-335	(C)NEGVCDLFDVQILNY	2197

Peptide A is also called "Angel". Peptide C was the initially predicted to be the C-terminus of DP-1 but later found to be the code from the wrong frame. "V", stands for "5" (E2F-5) and "Vickey" (Buck *et al.*, 1995). Cysteine residues were added to each N-terminus for the eventuality of having to perform a gluteraldehyde-based coupling on them. A phosphorylated version of peptide D (D_P) was also employed and is described in Chapter 6, figure 6.3. † The monoclonal antibody producing hybridoma cell line 32.3 was also raised to this peptide. ‡ These peptides were designed, and antisera against them raised, by myself.

Electrophoretic analysis

SDS PAGE

Proteins from MEB or LSL cell extracts (described above) were analysed by SDS-PAGE (Laemmli, 1970) using the Bio-Rad Mini Protean II system and 10% denaturing acrylamide gels with a low-acrylamide/low-pH stacking gel. The samples were prepared in SDS-PAGE sample buffer and heated at 100°C for 5 minutes prior to loading. Gel were electrophorised at 80V until the dye-front was "stacked" and then at 150V until the dye-front ran off the gel or longer. High molecular weight markers (Sigma) were usually included in one of the lanes and for radioactive samples ^{14}C-methylated "Rainbow-markers" were used. Gels containing radioactive samples were vaccum dried immediately after running while gels containing large amounts of specifically purified protein were stained for one hour at room temperature on a rotating platform with Coomassie stain buffer and then de-stained for a further hour with de-stain buffer.

5xSDS PAGE gel running buffer

250mM Tris-HCl (not pH'ed) — Stored at room temperature
2M Glycine
0.5% SDS ($^{W}/_{V}$)

2x SDS PAGE loading buffer base

250mM Tris-HCl (pH6.8) — Stored at room temperature
20% ($^{V}/_{V}$) Glycerol
4% SDS ($^{W}/_{V}$)
aprx. 0.01% ($^{W}/_{V}$) Bromophenol Blue

β-Mercaptoethanol was added to 5% prior to use and the total mixture kept at 4°C for maximum one month.

Stacking gel

850µl 30% Acrylamide / 0.8% Bis-acrylamide {5.1% / 0.186%} ($^{W}/_{V}$)
1ml 0.5M Tris-HCl pH6.8 {0.1M}
50µl 10% SDS {0.1%} ($^{W}/_{V}$)
3.05ml dH_20
~
20µl 25% APS ($^{W}/_{V}$)
10µl TEMED (Sigma)

Resolving gel

3.3ml 30% Acrylamide / 0.8% Bis-acrylamide {10% / 0.27%} ($^{W}/_{V}$)
5.0ml 1.5 M Tris-HCl pH8.8 {0.75M}
100µl 10% SDS {0.1%} ($^{W}/_{V}$)
1.5ml dH_20
~
40µl 25% APS ($^{W}/_{V}$)
30µl TEMED (Sigma)

Tris-HCl, Acrylamide, APS and TEMED were stored at 4°C. APS solution was stored at most for one month. Total gel mixtures were made up fresh each time while APS and TEMED were added last and in quick succession.

Coomassie stain

0.1% ($^{W}/_{V}$) Brilliant Blue	Stored at 4°C and re-used
25% ($^{V}/_{V}$) Methanol	
5% ($^{V}/_{V}$) Acetic acid	

De-stain

30% ($^{V}/_{V}$) Methanol	Stored at room temperature.
10% ($^{V}/_{V}$) Acetic acid	

Immunoblotting

Western immunoblotting was carried out based on the methods described by Towbin *et al.* (1979) and Burnette (1981). Protein samples were run alongside protein molecular weight markers (Sigma) on a 10% SDS-PAGE mini-gel (Bio-Rad Mini Protean II System) prepared according to the manufactures instructions as above. Each lane was typically loaded with 40-80µg of cell MEB- or LSL-extract.

Polypeptides were transferred onto 0.45μm pore nitrocellulose membrane (Bio-Rad), at 400mA for one hour, using a Bio-Rad mini protein Trans-Blotter cooled by dry ice and under constant stirring with a magnetic bar. Blotting efficiency was estimated by the visualisation of transferred proteins by staining the membrane with a 5% Ponceau-S solution (Sigma), washing with dH_2O and destaining with PBS. The nitrocellulose membrane was blocked in PBS with 10% Milk protein (Tesco or Safeway) for one hour at room temperature on a moving platform, washed with PBS and incubated with primary antibody diluted in BSA buffer overnight at 4°C. Superior results were generally found with a long 4°C incubation of the primary antibody, especially if this was a polyclonal, followed by a shorter room temperature incubation of the secondary antibody. Ascites fluid was typically diluted $^1/_{500}$, unpurified antisera $^1/_{200}$, affinity purified antibodies $^1/_{100}$, and 32.3 concentrate (0.7mg/ml) $^1/_{20}$, in BSA buffer, while hybridoma supernatants were employed neat. For those primary antibodies raised to synthetic peptide epitopes, an equal volume of 2nmol/μl specific or non-specific peptide was added in parallel treatments as a control for antibody activity. After incubation, blots were washed three times for ten minutes with PBS/0.1% NP-40 (Sigma) or Igepal (Sigma) and incubated with secondary antibody, either anti-rabbit alkaline phosphatase conjugated antibody (Promega) diluted $^1/_{7500}$ in BSA buffer or anti-mouse alkaline phosphatase conjugated diluted $^1/_{1000}$ (Dakopatts) or $^1/_{7500}$ (Promega). The secondary antibody was incubated for 3-6 hours at room temperature after which the blots were washed three times ten minutes with PBS 0.1% NP-40/Igepal as above, washed with PBS alone, and then incubated with substrate reaction buffer : NBT/BCIP in alkaline phosphatase buffer. Reactions were terminated by washing with 1% acetic acid. The colour reactions were typically allowed to run for 20-60 minutes at room temperature.

10x Blotting buffer

250mM Tris base (not pH'ed)	Stored at room temperature
2.0M Glycine	

BSA buffer

PBS	Stored at 4°C
5% BSA ($^W/_V$)	
0.05% NaN_3 ($^W/_V$)	

1xAlkaline phosphatase buffer

100mM Tris-HCl (pH9.0-9.5)	Stored at room temperature
50mM $MgCl_2$	
100mM NaCl	

The colour reagents were added separately to the following concentrations just prior to use :

330µg/ml NBT	:	Each stored as 50mg/ml stocks at -20°C in 70%
330µg/ml BCIP		DMF and neat DMF respectively.

In later experiments "Sigma Fast" BCIP/NBT tablets were used and, against manufacturers instructions, dissolved in alkaline phosphatase buffer as opposed to the recommended dH_20 as the reaction would then proceed faster.

Gel retardation assays

Gel shifts were performed essentially as described by La Thangue *et al.* (1990). Each gel retardation reaction was made up to a final volume of 20µl and reaction mixtures generally contained reaction buffer and an E2F site DNA binding component, whether it be cellular extract, immunoprecipitates from cell extracts or *in vitro* translated DP and E2F family members. Typically 8µg protein from MEB or LSL cell extracts were used per reaction and a total volume of reticulolysate (for *in vitro* translations) of less than 8µl, above which inherent E2F site DNA binding activity appears. Additionally 2µg of sonicated salmon sperm DNA (Sigma) was included to remove non-specific DNA binding activities, as well as 300ng of mutant E2F site oligonucleotide (60/62 - see below) to compete out a non-DRTF1/E2F binding activity on the E2F probe (71/50 - see below and La Thangue *et al.*, 1990).

Antibodies were included at various concentrations depending on activity (see legend specifications). For those antibodies that were raised against peptide epitopes, the antibody activity could be controlled for by the inclusion in the reaction mixture of the same volume (as the antibody) of either specific (the peptide the antibody was raised to) or non-specific peptide (an unrelated similar sized peptide) at 2nmol/µl in corresponding reactions. Other components were also added to the reaction mixtures, such as purified proteins, to assay their effect on DRTF1/E2F DNA binding. All mixing and addition procedures, as well as periods between incubations, were performed on wet ice.

The reaction mixtures were incubated at 30°C for 10 minutes prior to addition of labelled probe ("71/50" or "P" - see below) unless otherwise indicated. Approximately 6ng of ^{32}P-α-GTP-labelled oligonucleotide probe (see below) was

added and the reaction was incubated at 30°C for a further 10 minutes. Reaction mixtures were loaded on to a non-denaturing 4% polyacrylamide Tris-acetate EDTA (TAE) gel run at 4°C with buffer (1xTAE) recirculation. Electrophoresis was performed at 500 volts for 2 min and then 150V for 100 minutes. The gel retardation gels were pre-run at 150V for at least 2-4 hours so that the current would drop and settle to below 30mA. This indicates that initial resistance, and hence heat-generation, in the gel has been overcome. The gels were subsequently vaccum-dried and exposed to X-ray film (Fuji or Kodak) at -80°C for around 12 hours, or to phospho-imager cassettes for 1-2 hours at room temperature.

50 x TAE Stock

2M Tris base — Stored at room temperature
1M Glacial acetic acid (5.71% $^{V}/_{V}$)
50mM EDTA

10x concentration working dilution : pH'ed to 8.3 at 4°C with acetic acid and also stored at room temperature.

TAE non-denaturing gel

6.6ml 30% Acrylamide/1.5% Bis-acrylamide {4.0% / 0.2%} ($^{W}/_{V}$)
5.0ml 10xTAE (pH8.3/4°C)
38.4ml dH_20
~
125µl 25% APS ($^{W}/_{V}$)
60µl TEMED

Tris-HCl, Acrylamide, APS and TEMED were stored at 4°C. APS was stored at most for one month. Total gel mixtures were made up fresh each time while APS and TEMED were added last and in quick succession.

4xBandshift reaction buffer

200mM Tris-HCl pH 7.9 — Stored at -20°C
24mM $MgCl_2$
0.8mM EDTA
4mM DTT
60% Glycerol ($^{V}/_{V}$)

Oligonucleotides for gel retardation

Synthetic oligonucleotides were prepared at the National Institute for Medical Research using an Applied Biosystems automatic synthesiser and supplied heat deprotected in ammonia. The oligonuleotides used in this study were :

71/50 : The wild type binding sequence for DRTF1/E2F derived from the adenovirus (Ad5) E2a promoter from -71 to -50 (also called 19/20 ; La Thangue *et al.*, 1990).

5'-GATCTAGTTTTCGCGCTTAAATTTGA-3'
3'-ATCAAAAGCGCGAATTTAAACTCTAG-5'

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

5'-GATCTAGTTTTCGATATTAAATTTGA-3'
3'-ATCAAAAGCTATAATTTAAACTCTAG-5'

P : The ATF binding site oligonucleotide was derived from sequences -58 to -39 of the adenovirus (Ad5) E4 promoter (Gillinari *et al.*, 1989).

5'-GATCTAACCGTTACGTCATTTTTT-3'
3'-ATTGGCAATGCAGTAAAAAACTAG-5'

Preparation of labelled oligonucleotides

Complementary strand oligonucleotides were ethanol precipitated, dissolved in dH_2O and the concentrations estimated using the formulae presented below. Equal moles of each oligonucleotide were then mixed and placed in a 100°C heating block for five minutes, after which the block was turned off and allowed to cool gently at room temperature to below 30°C.

300ng of double stranded oligonucleotide with a 5' cytosine overhang were incubated with 30µCi of (α-^{32}P)-GTP and 2U of labelling grade Klenow enzyme (Boehringer Mannheim) in labelling buffer (Boehringer Mannheim - restriction enzyme buffer H) in a 20µl reaction. The reaction was allowed to proceed for 30 to 60 minutes at room temperature after which 120µl dH_20 was added and the oligonucleotides were then purified by standard phenol / phenol-chloroform and

chloroform extraction using 100µl of each. 30µl sodium acetate (pH5.2 / 3M) was added with 300µl absolute ethanol at -20°C to make a precipitating mixture of approximately 0.2M sodium acetate in 66% ethanol. The mixture was incubated either for 30 minutes on dry ice or overnight at -20°C. The precipitated oligonucleotide was pelletted by microcentrifugation at 10,000g (13K) for 30 minutes, washed in 1ml of 70% ethanol (-20°C) respun, allowed to dry on the bench and resuspended in 50µl of H_2O. 1µl, corresponding roughly to 6ng of labelled oligonucleotide, was used per gel retardation reaction. Typically 1µl of this final labelling solution would have a cpm in excess of 200.

Molecular biological techniques

Concentration estimations

The concentration of nucleic acid solutions was determined spectrophotometrically using quartz cuvettes employing the formulae below (Sambrook *et al.*, 1989).

1 A260nm=50µg/ml double-stranded DNA
1 A260nm=40µg/ml single-stranded DNA
1 A260nm=20µg/ml double-stranded oligonucleotides

Bacteria

The *E. coli* strain DH5α was used for plasmid DNA preparation and fusion protein production. Bacterial colonies were maintained on LB plates stored at 4°C or in LB media containing 15% ($^{v}/_{v}$) glycerol and stored at -80°C.

Large scale growth of bacteria for DNA plasmid or fusion protein preparations were initiated from single colonies on agar plates.

1xLB-Broth media

1% Bacto-tryptone ($^{w}/_{v}$)	Stored at 4°C, and at room
0.5% Bacto-yeast extract ($^{w}/_{v}$)	temperature after opening of a new
1% NaCl ($^{w}/_{v}$)	bottle.

Premixed LB (Sigma) powder was used in later experiments. Ampicillin was added at the time of use to 50-100µg/ml.

Agar plates

1.5% Agar in LB broth. ($^{w}/_{v}$)	pH adjusted to 7.5 with NaOH followed by autoclaving.

The set agar was melted in a microwave oven and when cooled enough to be comfortably held by the gloved hand (approximately below 55°C), ampicillin was added to 50-100μg/ml. The plates were poured into 100 mm dishes and flamed with a Bunsen burner to remove bubbles. The ampicillin plates were stored at 4°C for a maximum of two months. Prior to use the plates were dried at 37°C for two hours with the lids removed.

Transformation of bacteria

Competent DH5α *E.coli* bacteria were obtained either in super-competent format commercially (Promega or Gibco) or courtesy of Chandi Patel, National Institute for Medical Research.

The competent cells were stored at -80°C and a desired aliquot was allowed to thaw on wet ice. The transformation of plasmids into DH5α was accomplished using 50ng of plasmid DNA mixed gently with 200μl of competent cells (1ng with 20μl cells for super-competent cells) and incubated on wet ice for 30 min. The cells were heat shocked by transferring reactions to 42°C for 30 seconds or 37°C for two minutes. The mixture was allowed to cool down again on wet ice and 800-1000 μl of warm (37°C) LB-broth was added (no ampicillin) and incubated for a further 60 minutes at 37°C with shaking. 200μl broth was subsequently spread onto agar plates (with ampicillin) and incubated overnight at 37°C, lid facing down.

Large scale plasmid preparation (Maxiprep)

The large scale preparation of plasmid DNA was either kindly carried out by Chandi Patel at the National Institute for Medical Research using standard $CsCl_2$ methology (Birnboim, 1983 ; Sambrook *et al.*, 1989) or by myself using the kit by Quiagen (QIA filter plasmid maxi kit 25), according to manufacturers instructions.

In vitro transcription-translation

Carried out using Promega TNT T3/T7/SP6 coupled reticulocyte lysate system according to manufacturers instructions. 1μg vector DNA per 50μl reaction mixture incubated for 90 minutes. Radioactivity labelled protein was made by the inclusion of 40μCi of L-^{35}S-Methionine (*in vivo* labelling grade - 10μCi/μl) and absence of "cold" methionine in the amino acid mixture. Reticulolysate controls were subjected to the same reagents and incubations as the actual samples bar the presence of plasmid DNA. All samples were stored at -20°C.

Bacterially expressed fusion protein purification

Transformed bacteria (*E.coli* DH5α) from glycerol stocks or colonies were innoculated into 5mls of LB-Broth with ampicillin (100μg/ml) and incubated, while shaking at 37°C, until reaching stationary phase. Cultures were subsequently diluted 1/10 with 50mls of fresh LB-broth/ampicillin and grown to stationary phase. Pre-warmed 500mls of LB broth/ampicillin was then innoculated with the 50ml culture and grown for one hour shaking at 37°C after which isopropyl-β-D-thiogalactopyranoside (IPTG, Sigma) was added to a final concentration of 0.1mM and grown for three to six hours at 37°C or overnight at 30°C or room temperature. Induction conditions depended on the fusion protein to be induced. After the induction period the cultures were cooled on wet ice and then pelletted by centrifugation for 10 minutes, 5K, 4°C in Sorval RC5C or Du Pont SLA3000. Pellets were either used for extraction immediately or stored at -80°C.

Glutathione S-transferase recombinant protein

Fusion proteins were purified essentially as described by Smith & Johnson (1988) but with some modifications. Bacterial pellets were resuspended in 10mls PBS containing lysozyme (Sigma - 10mg/ml) while kept cool on wet ice and then sonicated briefly (single 5 second burst) on ice. Bacterial debris were pelletted by centrifugation and re-centrifugation at 10K 4°C for 5 minutes each in a Du Pont SS34 rotor and syringe-mesh (Qiagen) filtered as required. 300μl of glutathione beads (33% $^{v}/_{v}$) in suspension (PBS/0.05%NaN_3) were added to the supernatant and mixed at 4°C on the "wheel of death" for 30-60 minutes. The suspension was placed in a 50ml Falcon tube and washed once with 50mls PBS containing 0.5% NP-40 (or Igepal) and twice with PBS only, by spinning in a bench-top centrifuge (RT6000B Sorval) at 4°C 6K for 5minutes each time. The washed beads were placed in an eppendorf tube at 4°C.

At this stage either the beads were retained for pull-down studies (stored at 4°C in PBS/0.05% NaN_3) or the GST-fusion proteins were released with glutathione. This was done by pelletting the beads by a 20 second spin in a microfuge at 13K,

removing the supernatant and adding instead 150µl of freshly made up 5mM reduced glutathione in 50mM Tris-HCl pH8.0. This suspension was allowed to rest on wet ice for 1-2 minutes before spinning down and recovering the supernatant containing the eluted fusion protein. This was repeated up to eight times and a 20µl sample from each elution tested by 10% SDS-PAGE. Fusion proteins were stored at -20°C or -80°C.

Histidine tagged protein

Using the histidine-Nickel chelation purification system (Qiagen) it was possible to extract large amounts of bacterially expressed protein to a high degree of purity due to the 6M salt conditions of the extraction procedure. The denaturing conditions of the extraction had to be followed by gentle and gradual reduction of salt concentration in order for the protein to regain its tertiary structure.

The bacterial pellet was resuspended in 10ml denaturing buffer and gently stirred for two hours at room temperature. $MgCl_2$ was added to a final concentration of 5mM and cellular debris were cleared by repeated centrifugation at 10K for 5 minutes at 4°C (SS-34 Du Pont). 800µl (50% $^{v}/_{v}$) of nickel chelate affinity resin (Qiagen) was added to the supernatant and rotated on a wheel of death for one hour at room temperature. The resin was then pelletted and washed stepwise (Sorval RT6000B at 4°C, 5 minutes, 6K) in 50ml Falcon tubes with two 50ml volumes each of denaturing buffer pH 8.0, denaturing buffer pH 6.4 and renaturing buffer containing 1M, 0.1M, and 0M guanidine hydrochloride respectively. The resin was then placed in an eppendorf tube and either stored in PBS/0.05% NaN_3 or sequentially eluted with 100-200µl Imidazole buffer. Eluted his-tagged fusion protein was assayed and stored as for GST-fusions.

Denaturing buffer

100mM Sodium phosphate pH8.0 and pH6.3 Stored at 4°C
10mM Tris base
6.0M Guanidine hydrochloride
30mM imidazole

Renaturing buffer

25mM Sodium phosphate pH 7.0 Stored at 4°C
300mM NaCl
10mM β-mercaptoethanol
1M, 0.1M, or 0M Guanidine hydrochloride respectively

Imidazole buffer

150mM Imidazole — Stored at 4°C
50mM Tris-HCl pH 7.9
150mM NaCl

Polypeptide size estimations from amino acid length

Average amino acid molecular weight taken as 136Dal, water lost by condensation as 18Dal. Estimated Dal size of a polypeptide of αα residues in length :

$$= \quad (\alpha\alpha \times 136) - [(\alpha\alpha - 1) \times 18]$$

The additional size of chimaeric GST fusion proteins was taken as 26 KDal (Smith & Johnson, 1988).

Fusion protein pull-down assays

300µl LSL F9EC extract was micro-centrifuged at 13K (15g) 4°C for two minutes and 250µl of supernatant was added to the pellet of a 150µl 10% ($^{V}/_{V}$) suspension of fusion protein-bearing Sepharose beads. This mixture was rotated on a wheel of death for two hours at 4°C after which the Sepharose was washed three times by centrifugation in a microfuge (4°C, 13K, 20 seconds) using RIPA buffer containing protease inhibitors. The final Sepharose pellet was resuspended in SDS-loading buffer and analysed by SDS-PAGE.

RIPA buffer

150mM NaCl — Stored at 4°C
1.0% NP-40/Igepal ($^{V}/_{V}$)
0.5% DOC ($^{W}/_{V}$)
0.1% SDS ($^{W}/_{V}$)
50mM Tris-HCL (pH8.0)

A $^{1}/_{100}$ dilution of 100x PIC and PMSF to 0.5mM, from a stock of 0.1M stored in isopropanol (propan-2-ol) at -20°C, were added just prior to use.

Reagent gifts

Plasmids

GST plasmid construct
pGEX-2T from Promega.

pRb plasmid construct
The GST-Rb contains cDNA sequence from amino acid residue 379 to 928 of pRb in pGEX-2T (Kaelin *et al.*, 1991).

E2F-1 plasmid constructs
The human E2F-1 cDNA contains the full length sequence from amino acid residues 1-427 in Sp72 (Kaelin *et al.*, 1992) and is *in vitro* translatable. E2F-1 amino acid residues 89-437 in pGEX-2T made up the GST-E2F construct (Helin *et al.*, 1992).

DP-1 plasmid constructs
The *in vitro* translatable DP-1 plasmid and the his-tagged mouse DP-1 fusion plasmid both contained the full length cDNA sequence encoding amino acid residues 1-410. GST-DP-1(B) (Girling *et al.*, 1993a) contains mouse sequence from 84 to 410. All were kindly provided by Rowena Girling.

DP-2 plasmid construct
In vitro translatable full length *Xenopus laevis* DP-2 (Girling *et al.*, 1994) a kind gift from Rowena Girling.

DP-3 plasmid constructs
The *in vitro* translatable mouse DP-3 α, β, γ and δ were kindly provided by Liz Ormondroyd and Susana De La Luna (Ormondroyd *et al.*, 1995).

p53 plasmid construct
Histag-p53 full length mouse vector (pH6-mmp53wt - unpublished) generously donated by Gunnar Weidt and Wolfgang Deppert (Universität Hamburg).

ATF construct
In vitro translatable full length ATF construct made by Panayotis Tassios (Unpublished).

Antibodies

Anti-mdm2

Monoclonal ascites fluid SMP14 against mouse MDM2 was a kind gift from Stephen Pixley and David Lane (Department of Biochemistry, University of Dundee).

Anti-p53

Monoclonal hybridoma supernatant 421 and 248 against human/mouse p53 was a kind gift from Julian Gannon (I.C.R.F., Clare Hall).

Anti-DP-1

Antisera to peptide A (rabbit 4), peptide 17 (rabbit 153) and peptide D (rabbit 098 and 099) were designed, prepared for immunisations and generously provided by Rowena Girling.

Chapter 3

Immunochemical characterisation of DP-1

The project was initiated by the screening of a large range of polyclonal antisera raised to different regions of the predicted DP-1 amino acid sequence. This was done by employing immunoblotting and gel retardation techniques in an attempt to characterise the nature and role of the DP-1 polypeptide product in cellular extracts.

Preparation of immunochemicals

Upon commencing studies, DP-1 had recently been cloned from E2F-site affinity purified DRTF1/E2F, and different antisera had been raised to small peptides (15-20 residues - see Experimental Protocols) representing different regions of the cDNA-predicted DP-1 protein sequence (Girling *et al.*, 1993a). A major advantage of raising antiserum to peptides is that any activity of it can easily be controlled for by the inclusion of the peptide in the binding reaction. If the antiserum activity is abolished upon addition of the peptide, the effect must be specifically dependent on epitopes shared between the peptide and the antigen under study. Such effects were referred to as "specific". Antibody effects that were not abolished by the peptide against which they were raised were referred to as "non-specific". A further control, widely employed in the studies presented in this thesis, is the inclusion in a parallel experiment of a peptide of similar size, containing unrelated sequence, which controls for the eventuality that the competition by the specific peptide is a general consequence of changing the chemical balance of the reaction mixture and not due to epitope competition.

Several of the antisera raised to peptides derived from DP-1 identified a large range of polypeptides in immunoblots on F9EC cell (from which DP-1 was cloned ; Girling *et al.*, 1993a) microextracts. Many of these polypeptides were in the size range of around 46KDal, and their reactivity could be specifically competed out by inclusion of the specific but not a control peptide. DP-1 was cloned from microsequence data from an E2F DNA-binding site purified 46KDal polypeptide (Girling *et al.*, 1993a), and so particular attention was paid to polypeptides

specifically identified in this size region. Noteworthy examples were the antisera raised against three peptides : A, 17 and D, which represent N-terminal, central, and C-terminal domains of murine DP-1 respectively (**figure 3.1a** and Experimental Protocols).

Immunoblotting with antiserum raised against peptide A on microextract prepared from F9EC cells revealed a range of polypeptides (**figure 3.1b** lane 2). Inclusion of the specific peptide "A" in the primary antiserum incubation mixture abolished the recognition of some of these (compare to lane 1) suggesting that these polypeptides contain epitopes similar to ones found in the peptide A region of DP-1.

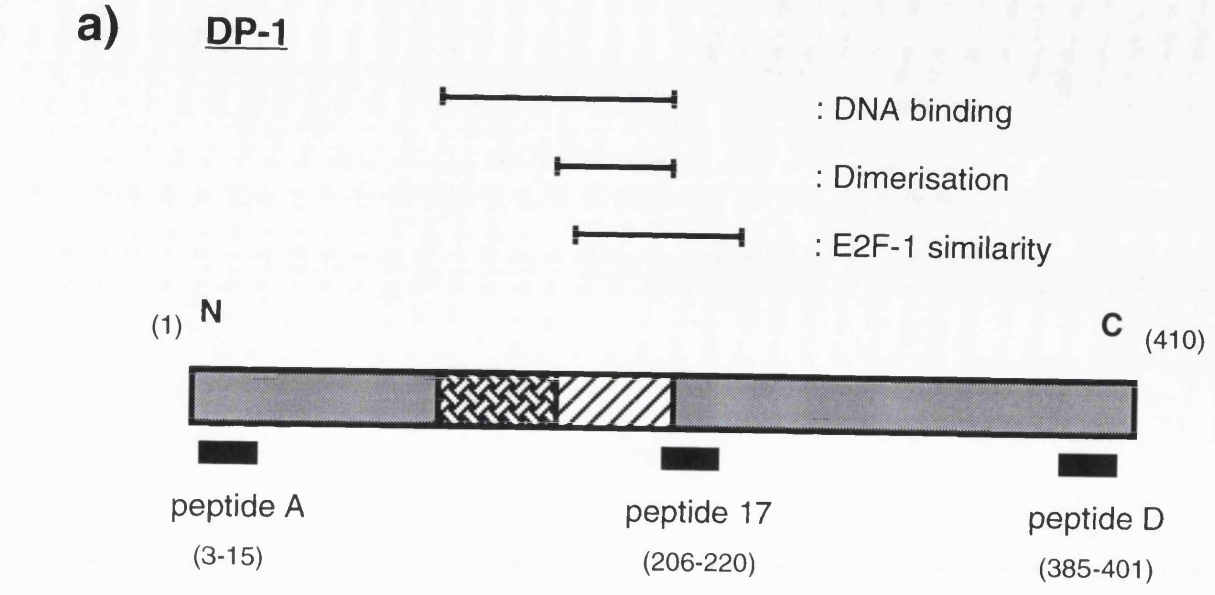
In order to facilitate the identification of DP-1 polypeptides in cellular extracts I decided to epitope affinity-purify promising antisera such as those against peptide A (αA), peptide 17 (α17) and peptide D (αD). Epitope affinity-purification isolates the antibodies in a polyclonal antisera that bind to an immobilised peptide, typically the peptide the antisera was raised against. This has three advantages : Firstly, like a monoclonal, any effects of the resulting purified antibody fractions can be assigned to factors carrying very specific epitopes. Secondly, in the case of immunoblots, epitope-purified and monoclonal antibodies provide much "cleaner" and simpler polypeptide recognition patterns than an unpurified polyclonal antibody. Thirdly, removal of the antibodies that do not bind to the peptide of interest can reveal, in the case of an immunoblot, specifically identified polypeptides, in for example cell extracts, which would otherwise be obscured by non-specific ones. All three polyclonal reagents (αA, α17 and αD) displayed a great degree of non-specific polypeptide recognition in immunoblots as seen when recognition patterns of F9EC cell microextract were tested in the presence of specific peptide compared to the presence of a control peptide (αA: **figure 3.1b**, lanes 1 and 2 ; α17: **figure 3.2a**, lanes 1 and 2 ; αD: **figure 3.2b**, lanes 3 and 4).

For the purification of a given antisera, the peptide used for the original immunisation was covalently cross-linked to Sepharose beads and placed in a column. The serum was then passed, at neutral pH, over the peptide-beads which were then washed, followed by elution at low pH of the epitope-bound peptide-specific antibodies into a preservation buffer (see Experimental Protocols, Chapter 2). For the affinity purification of anti-peptide A serum (αA) the specific polypeptides identified by competition in F9EC cell extract by anti-peptide A serum (lanes 1 and 2, **figure 3.1b**), were absent when the same cell extract was tested with the same serum that had been passed over a peptide A Sepharose column (lanes 3 and 4). The activity that had been retained by the column re-appeared in the first low-pH elution fractions (lane 5/6, fraction number 3 and 4) and was fully eluted off the column by around fraction 11 (lane 13).

Figure 3.1
Epitope-affinity purification of antisera to DP-1 peptides.

a. *Analytical peptides representing distinct regions of DP-1.*
Representation of the DP-1 polypeptide indicating the N- and C-termini and the locations of its DNA binding domain, E2F-family dimerisation region and the area of similarity to E2F-1. Also shown (in black boxes) are the locations of three peptides (A, 17 and D) used to raise antibodies to DP-1 and widely employed in this study. Amino acid residue numbers are indicated in brackets.

b. *Epitope affinity purification of antisera to peptide A assayed by immunoblotting.*
Immunoblot (IB) of seven loadings of F9EC microextract (lanes 1/2, 3/4, 5/6 *etceteras* : 40µg total protein each). Each gel-lane was cut in half, to make 14 lanes, and probed with different antibody preparations. Lanes 1 and 2 were probed with rabbit polyclonal antisera to peptide A (load), in the presence of peptide A (A : lane 1), which eliminated the recognition of a subset of cellular polypeptides, or in the presence of a control peptide (C : lane 2). Lanes 3 and 4 were probed with the same anti-sera after it had been passed over a peptide A-Sepharose column (flow-through) again in the presence of peptide A (lane 3) and peptide C (lane 4). Lanes 5 to 14 were probed with successive glycine buffer pH 2.5-eluted fractions 3 to 12 (f3 to f12) to assay the antibodies that had been retained on the peptide A Sepharose. These fractions were assayed at $^1/_{50}$ dilution. The epitope affinity purified antibodies identify three polypeptides in the extract : A singlet p65 and a doublet p55. A non-specifically recognised polypeptide is indicated by *.


a)
DP-1
: DNA binding
: Dimerisation
: E2F-1 similarity
(1) N
C (410)
peptide A
(3-15)
peptide 17
(206-220)
peptide D
(385-401)

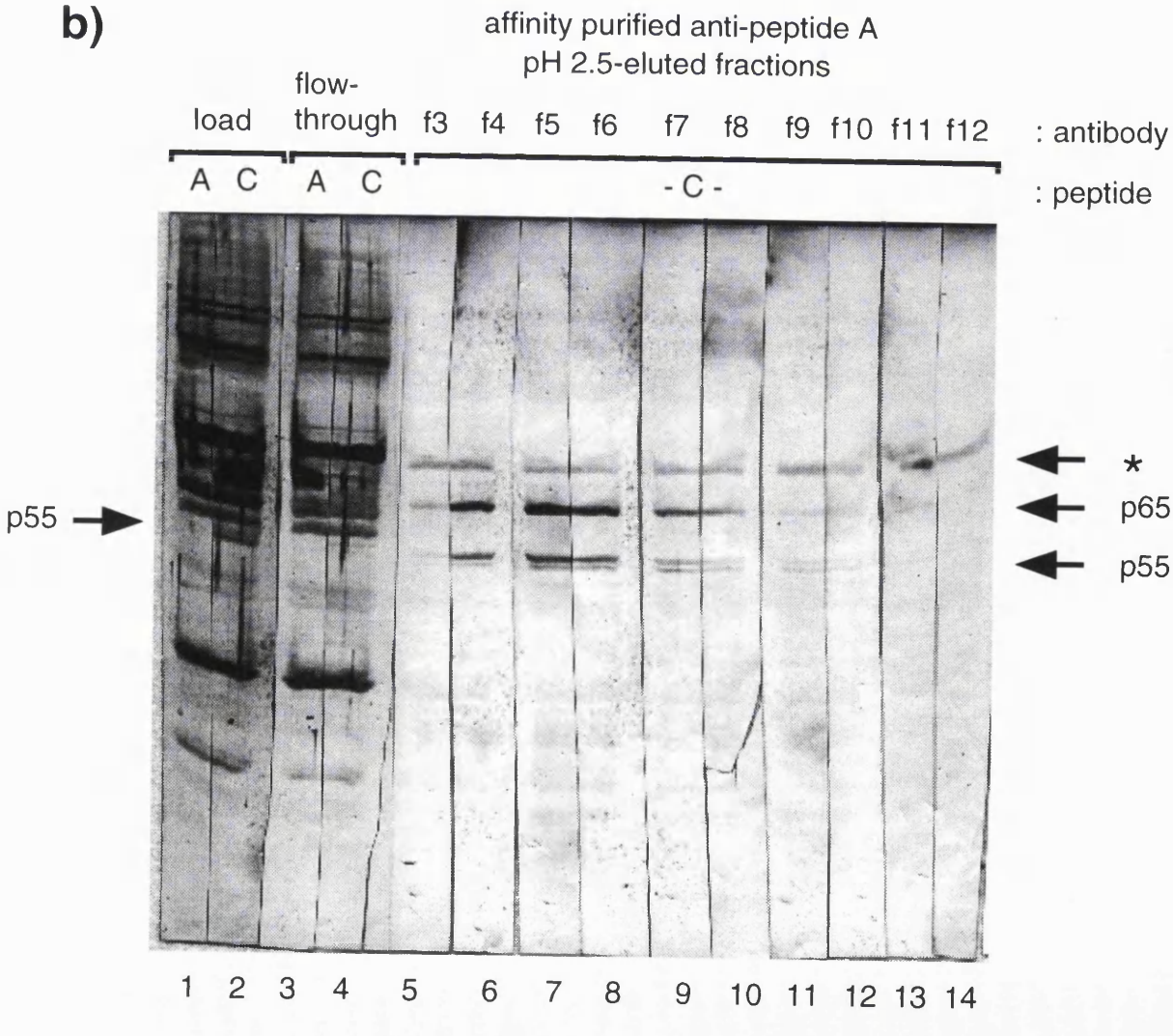

b)
affinity purified anti-peptide A
pH 2.5-eluted fractions
load
flow-through
f3 f4 f5 f6 f7 f8 f9 f10 f11 f12
: antibody
A C A C
- C -
: peptide
p55
*
p65
p55
1 2 3 4 5 6 7 8 9 10 11 12 13 14

Figure 3.2

Polyclonal antisera raised to different peptide locations in DP-1 identify similarly-migrating polypeptides.

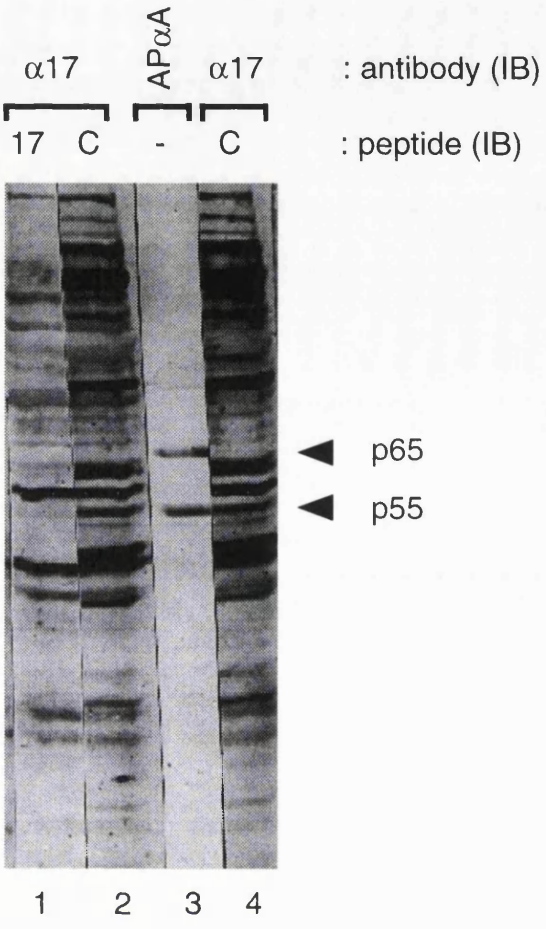
a. *Polypeptides specifically identified by antisera against DP-1 peptide 17 in an immunoblot migrate similarly to peptides identified by epitope affinity purified antisera to DP-1 peptide A.*

Each gel-lane (lanes 1/2 and 3/4) was loaded with 40μg of total F9EC cell microextract protein. After immunoblotting (IB) each gel-lane was cut in half and lanes 1, 2 and 4 probed with polyclonal antiserum to peptide 17 (α17). Lanes 2 and 4 were probed in the presence of a control peptide (C) while lane 1 was probed in the presence of peptide 17 (17). Lane 3 was probed with epitope affinity purified anti-peptide A antiserum (APaA). The 65KDal singlet, and the 55KDal doublet polypeptides identified by APαA are indicated as p65 and p55.

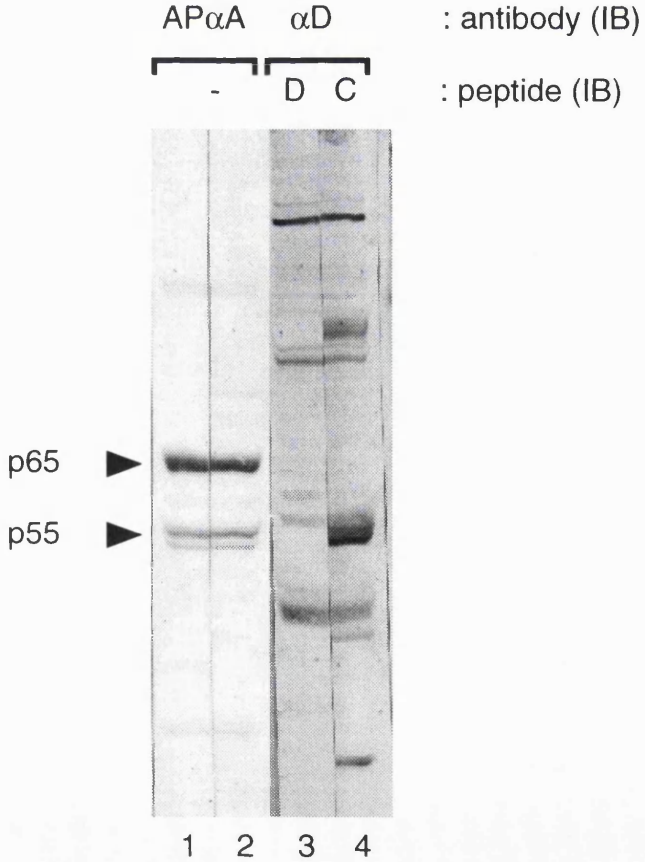
b. *Polypeptides specifically identified by antisera against DP-1 peptide D in an immunoblot migrate similarly to peptides identified by epitope affinity purified antisera to DP-1 peptide A.*

Each gel-lane (lanes 1/2 and 3/4) was loaded with 40μg of total F9EC cell microextract protein. After immunoblotting (IB) each gel-lane was cut in half and lanes 3 and 4 probed with polyclonal antiserum to peptide D (αD). Lane 4 was probed in the presence of a control peptide (C) while lane 3 was probed in the presence of peptide 17 (D). Lanes 1 and 2 were probed with epitope affinity purified anti-peptide A antiserum (APαA). The 65KDal singlet, and the 55KDal doublet polypeptides identified by APαA are indicated as p65 and p55.

a)


α17
APαA
α17
: antibody (IB)
17 C
-
C
: peptide (IB)
p65
p55
1 2 3 4

b)


APαA
αD
: antibody (IB)
-
D C
: peptide (IB)
p65
p55
1 2 3 4

Peptide affinity-purified antibody preparations such as this, provided an invaluable tool in the characterisation of DP-1 by providing clear and specific immunoblots and immuno-precipitations.

Two cellular polypeptides recognised by antibodies raised to different regions of DP-1

The peptide-affinity purification of antisera raised to peptide A, as assayed by immuno-blotting (**figure 3.1b**), demonstrated three potential identities for the DP-1 polypeptide in its cellular format : A doublet at 55KDal (p55 ; lanes 6-12) and a singlet at 65KDal (p65 ; lanes 6-12). A slower migrating polypeptide detected on the immuno-blots (indicated by *) is unlikely to have been recognised by an antibody that is specific to a peptide A-epitope as it was recognised to an equal intensity by all fractions including fraction 3 (lane 5) which was partially diluted by the pre-elution wash buffer. It is thus likely to be a cross-reaction of the secondary anti-rabbit-Ig antibody and so irrelevant to this study.

The recognition of a polypeptide band in an immunoblot of a cell extract by a single antibody reagent is however not enough evidence to ascribe to it an identity of a particular polypeptide. Thus the p55-doublet and p65 polypeptides, although carrying a DP-1 epitope as demonstrated by affinity-purified anti-peptide A antibodies (APαA), could not be certified as *DP-1* products at this stage. Comparison of the recognition pattern in immunoblots of F9EC cell extract using APαA with those of the specific polypeptide band sizes recognised by antisera such as anti-peptide 17 (**figure 3.2a**, lanes 3 and 4) and anti-peptide D (**figure 3.2b**, lanes 2 and 4), suggested that common peptides might be recognised by the three antibody reagents. For this reason, more epitope affinity purifications were carried from these promising antisera, anti-peptide D and anti-peptide 17 (αD and α17), to make the reagents APαD and APα17 respectively.

As the first step in the characterisation of these new antibody reagents, a histidine-tagged full-length murine DP-1 encoding gene was expressed in bacteria and the induced protein purified by its affinity to nickel-ion chelated Sepharose under denaturing conditions (see Experimental Protocols). This type of purification procedure yields exceptionally pure recombinant protein (**figure 3.3a** ; lane 2) and provided a means for testing the ability of the antibody reagents to specifically recognise DP-1 protein in an immunoblot assay. The three epitope affinity-purified antisera, APαA, APα17 and APαD, representing N-terminal, central and C-terminal regions of murine DP-1 respectively (**figure 3.1a**, and Experimental Protocols), were all found to specifically recognise histidine-tagged full length murine DP-1 in an

Figure 3.3

Epitope-affinity purified antibodies to DP-1 peptides specifically recognise recombinant DP-1 protein under the conditions of an immunoblot.

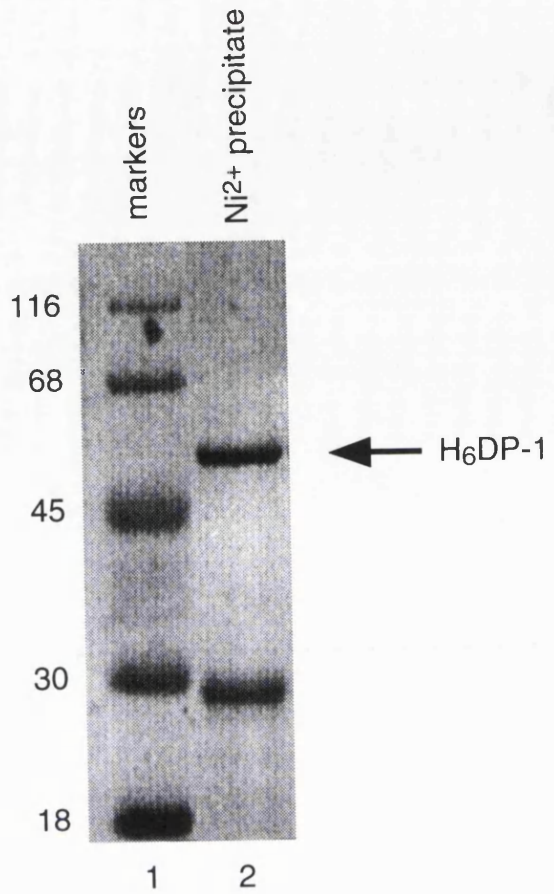
a. *Coomassie-stained SDS-PAGE of purified bacterially expressed histidine-tagged DP-1 protein.*

Each Nickel bead elution step would employ 100µl of imidazole buffer (see Experimental Protocols) and 20µl of a third elution step is presented here in lane 2. Lane 1 displays protein molecular weight markers indicated by number representing KDal.

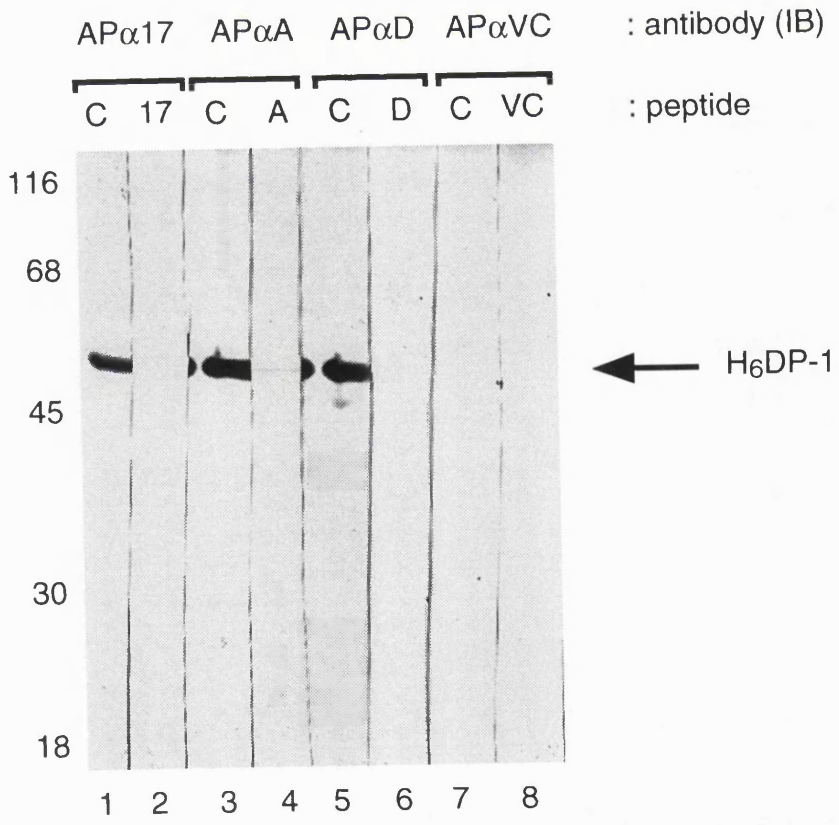
b. *Immunoblot to assay the specificity of epitope affinity-purified antibodies, raised to DP-1 peptides, against bacterially purified DP-1.*

Approximately 100ng of histidine-tagged, bacterially expressed and purified full length DP-1 (H_6DP-1) was loaded per gel lane (lanes 1/2, 3/4, 5/6 and 7/8). After immunoblotting (IB), each gel lane was cut into two and each pair probed with a different epitope affinity purified antibody (APαA, APα17, APαD and APαVC respectively). For each pair, one primary antibody incubation mixture included the original peptide that the antisera was raised to (even lanes : e.g. peptide 17 [17] for APα17 in lane 2) or a control peptide C (odd lanes : e.g. peptide C [C] - in lane 1). The migration of molecular weight markers in the same experiment are indicated by number indicating KDal.

a)


markers
Ni2+ precipitate
116
68
45
30
18
H6DP-1
1
2

b)


APα17
APαA
APαD
APαVC
: antibody (IB)
C 17 C A C D C VC
: peptide
116
68
45
30
18
H6DP-1
1 2 3 4 5 6 7 8

immunoblot (**figure 3.3b** ; lanes 1, 3, and 5). This recognition was in each case abolished by the inclusion of the specific peptide (the peptide to which the original antisera was raised and with which the reagent was purified) with the primary antibody (lanes 2, 4 an 6), indicating that the recognition of the fusion protein was directly due to the primary antibody. The non-competed primary antibody incubation mixtures contained equivalent concentrations of a control unrelated peptide (peptide C ; lanes 1, 3 and 5) to ensure that competition by the other peptides was truly specific. Recognition of DP-1 was not accomplished by an affinity purified antibody raised to a C-terminal peptide (VC) region from the E2F-5 transcription factor (APαVC ; lanes 7 and 8) assayed in the same experiment. This indicated that the recognition of the fusion protein by the other reagents was dependent on the ability of the primary antibody to recognise a murine DP-1 epitope and not due to non-specific protein-protein interactions that can occur if too much protein is immunoblotted. Thus the three anti-DP-1 reagents were found to be potentially good reagents with which to identify cellular DP-1 protein under the conditions of an immunoblot.

The three antibody reagents were assayed on F9EC cell extracts by immunoblotting adjoining gel lanes to determine if any identified polypeptide would be recognised by more than one antibody (**figure 3.4a**). The epitopes contained within a peptide against which antisera is raised, are not necessarily unique to the polypeptide that that peptide originates from. Therefore to ascertain the identity of a polypeptide in a cellular extract recognised by an antibody reagent, it should ideally be identified by at least two antisera specific to different regions within that polypeptide. The APαA reagent was compared to APα17 (lanes 2 versus 1 respectively). It can be seen that the affinity purification of anti-peptide 17 antisera provided a much less suitable reagent than APαA. The APα17 reagent recognised a wealth of polypeptides, possibly because there are many polypeptides in F9EC cells which carry similar epitopes to ones found in the peptide 17-region of DP-1 (lane 1). Despite the wealth of polypeptides recognised by APα17, two were found to almost perfectly mimic the recognition of the 55KDal migrating doublet detected by the APαA reagent (**figure 3.4a**, lane 2). The 65KDal polypeptide (p65) recognised by APαA was not recognised by APα17 indicating that the p65 does not contain both 17 and A epitopes, as the p55 doublet appears to do.

The APαD reagent recognised the p55 doublet in F9EC extracts while not identifying any of the non-p55 polypeptides recognised by either APαA or APα17 (**figure 3.4a** ; lane 3). The recognition pattern of the p55 doublet by APαD was slightly different from those of APα17 and APαA which more efficiently recognised the slower-migrating member, p55U (Upper), than the faster migrating form, p55L (Lower). APαD preferentially recognised p55L, but still, albeit weakly, identified p55U.

Figure 3.4

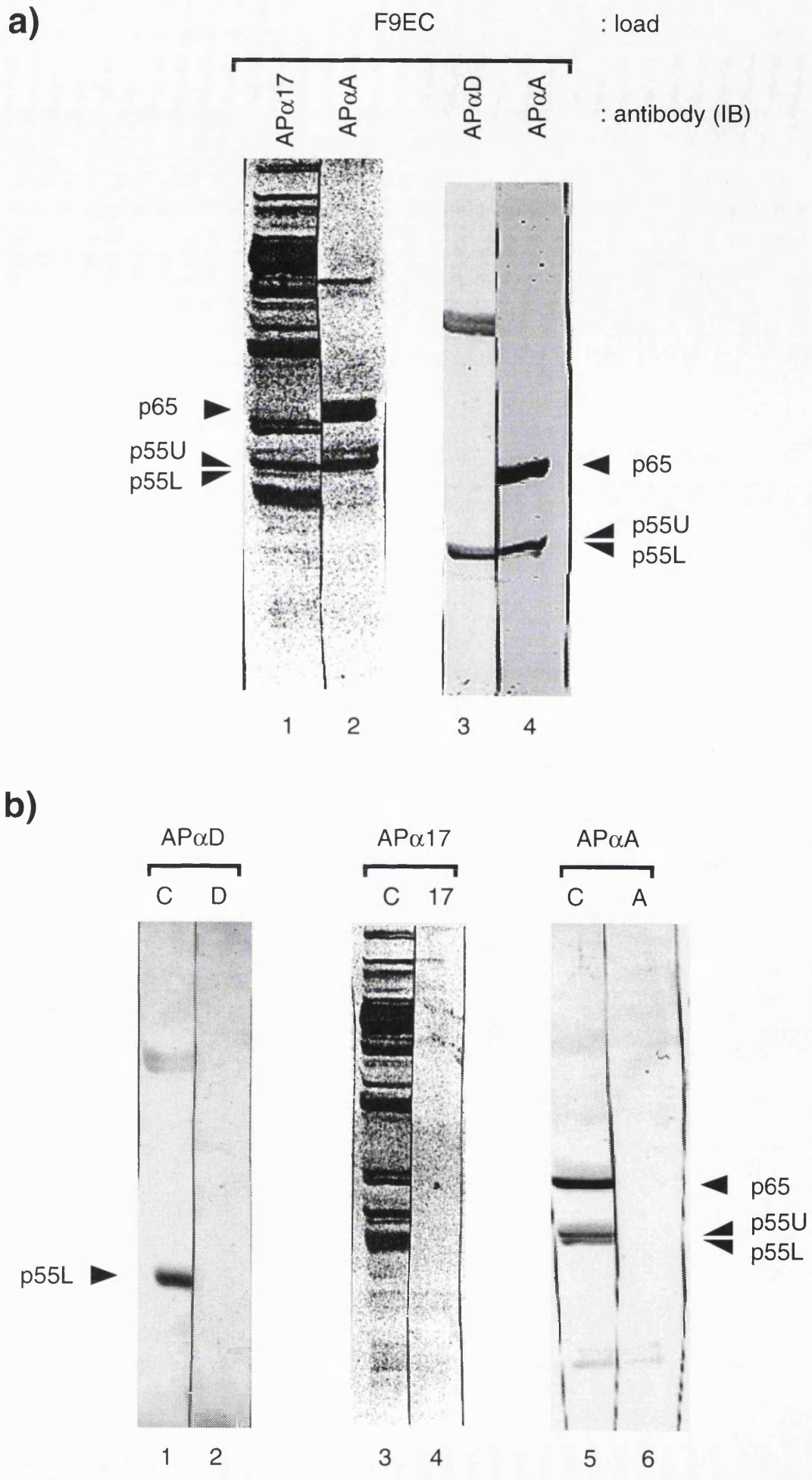
Two polypeptides are specifically identified by several purified antisera that are specific to different amino acid locations in DP-1.

a. *Immunoblot on cellular F9EC extract to compare the recognition patterns of different epitope affinity purified antibodies raised to DP-1.*

For each gel-lane (lanes 1/2 and 3/4) 40μg of total F9EC microextract protein was loaded, immunoblotted (IB), and each then cut in half and probed with different antibodies. All primary antibody incubations were performed in the presence of peptide C. The 65KDal singlet, and the 55KDal doublet polypeptides identified by APαA are indicated as p65 and p55. Lanes 1/2 and 3/4 were not from the same experiment and were electrophoresed for differing lengths of time.

b. *Immunoblot on cellular F9EC extract to control for the specificity of recognition of polypeptide patterns by different epitope affinity purified antibodies to DP-1.*

For each gel-lane (lanes 1/2, 3/4 and 5/6) 40μg of total F9EC microextract protein was loaded, immunoblotted (IB), and each then cut in half and probed with different antibodies. Each gel-lane was probed with one antibody reagent, one half in the presence of a control peptide (lanes 1, 3 and 5) the other in the presence of the peptide that the antibody reagent was originally raised to (lanes 2, 4 and 6). The 65KDal singlet, and the 55KDal doublet polypeptides identified by APαA are indicated as p65 and p55. The different gel-lanes were not from the same experiments and were electrophoresed for differing lengths of time.


a)
F9EC
: load
APα17
APαA
APαD
APαA
: antibody (IB)
p65
p55U
p55L
p65
p55U
p55L
1
2
3
4
b)
APαD
APα17
APαA
C
D
C
17
C
A
p55L
p65
p55U
p55L
1
2
3
4
5
6

The binding to all polypeptides by these three reagents in F9EC cell extracts, including the p55 doublets, was absent in the presence of their respective specific peptides, as opposed to control peptides, in the primary antibody incubation mixtures of the immunoblot (**figure 3.4b**; odd lanes, control peptides ; even lanes, specific peptides). This was to be expected given that, by definition, the only antibodies present in the peptide affinity purified preparations are the ones that will bind to their respective specific peptides. Thus each antibody reagent (APαA, APα17 and APαD) identified the same polypeptide pattern in F9EC cell extracts, in the presence of control peptide C, as in **figure 3.4a** (**figure 3.4b**, lanes 1, 3 and 5). But inclusion of the respective specific peptide in the primary antibody incubation mixtures completely abolished any recognition (lanes 2, 4 and 6).

The study indicates that all the polypeptides recognised by APαA, APα17 and APαD bear peptide A-, 17- or D-containing epitopes respectively while only two appear to carry all three epitopes : p55U and p55L, as these polypeptides are identified by all three antibody reagents. Non-p55 polypeptides recognised by any one of the three reagents are unlikely to represent DP-1 as they do not share more than one epitope with this protein. They are likely to be cross-reactions caused by the presence of similar epitopes in other related, or unrelated, polypeptides. Since DP-1, by definition, carries all three epitopes, it is very likely that the p55 doublet represents the protein products of the DP-1 gene. Thus only the p55 doublet in a cell extract (**figure 3.4a**) and bacterially expressed full-length DP-1 (**figure 3.3b**) are recognised by all three DP-1 antibody reagents.

It cannot be completely ruled out, however unlikely, that non-DP-1 polypeptides exist that contain all three DP-1-epitopes. The most likely candidates for such polypeptides would be other DP-family members. The cloning, subsequent to these early studies, of DP-family members DP-2 and DP-3α, β, γ and δ (see Introduction) allowed a comparative analysis of the equivalent peptide A-, 17- and D-regions of these proteins (**figure 3.5**). It is clear from this that both peptide A and peptide D are quite unique in their sequence as far as the sequences of other known DP-family members are concerned. Indeed one member, DP-2, does not even possess the N-terminus that carries the peptide A region in DP-1. Of slightly more concern is peptide 17 which shares a great deal of homology with similar regions in DP-2 and -3. If this region really is so homologous in DP-family polypeptides then that might explain why APα17 specifically recognises so many polypeptides in a cell extract (**figure 3.4b**). Some of the polypeptides bound by APα17 may be, as yet, uncloned DP-family members. The identity of the p55 doublet as being that of DP-1 is however

Figure 3.5

The DP-1 peptides are distinct from equivalent regions in other DP-family members.

Comparisons of the amino acid sequences of DP-1 peptides with equivalent sequences in other DP-proteins.

For each DP-1 peptide (amino acid residue representative letters displayed in blue) a line-up with equivalent regions in other DP-family members is presented. The amino acid residues of these sequences are displayed in blue if they are identical to the DP-1 sequence, and in red, if different.

DP-1 Peptide A (amino acids 3-15)

DP-1	K	D	A	S	L	I	E	A	N	G	E	L	K
DP-2	-	-	-	-	-	-	-	-	-	-	-	-	-
DP-3	K	N	V	G	L	P	S	T	N	A	E	L	R

DP-1 Peptide 17 (amino acids 206-220)

DP-1	E	V	E	R	Q	R	R	L	E	R	I	K	Q	K	Q
DP-2	E	M	E	K	Q	R	R	I	E	R	I	K	Q	K	S
DP-3	E	I	E	K	Q	R	R	I	E	R	I	K	Q	K	R

DP-1 Peptide D (amino acids 385-400)

DP-1	R	V	E	T	P	V	S	Y	V	G	E	D	D	D	D	D	D
DP-2	R	G	E	T	P	C	W	F	-	-	D	D	D	E	D	D	E
DP-3	R	G	E	T	P	C	S	F	N	D	E	D	E	E	D	E	E

very strengthened by its recognition by the two other antibodies, specific to different regions on DP-1. But ultimately, it still cannot be excluded that as yet uncloned DP-family members are cross reacting with all three antibody reagents. This possibility was thus borne in mind whenever interpreting data.

DP-1 was originally cloned from a purified 46KDal polypeptide (Girling *et al.*, 1993a). This does not necessarily contradict the 55KDal size defined by these immuno-studies as the inherent prolonged handling of cell extracts and fractions during a purification procedure cannot exclude some protein degradation. The recognition of both forms of the p55 doublet by all three antibodies, including two recognising the terminal ends of DP-1, does however suggests that one form of p55 is not a degraded version of the other. If this was the case, one would expect recognition of one of the terminal regions by APαA or APαD to be abolished in the lower form of p55.

The DP-1 doublet is associated with DRTF1/E2F

DP-1 is a common DNA-binding member of the transcription factor complex DRTF1/E2F (see Introduction), an idea enhanced by the capacity of several unpurified antisera raised to different locations of DP-1 to affect DRTF1/E2F activity in gel retardation assays of the E2F binding site with F9EC extract (**figure 3.6**). αA (**figure 3.8**) and αD (**figure 3.6a**) both shifted the E2F-site complex very efficiently in the presence of a control peptide (lane 2 in both) but not the peptide the sera was raised to (lane 1 in both). Similarly, antisera raised by myself against two other DP-1 peptide locations, peptide 24 (amino acids 323-337 ; **figure 3.6b**) and peptide 26 (amino acids 352-366 ; **figure 3.6c**) both specifically affected DRTF1/E2F, α24 by supershifting the complex, like αA and αD, and α26 by apparently disrupting its DNA binding activity. Both effects were again sensitive to their respective specific peptides (lanes 1) but not to an unrelated control peptide (lanes 2). αA, αD, α24 and α26 all appeared to affect all three DRTF1/E2F complexes : *a*, *b* and *c*. The *b* and *c* complexes are believed to be composed of uncomplexed, and thus transcriptionally active, heterodimer, while the *a* complex is composed of heterodimers bound by other factors such as pocket proteins and cyclins. The antisera against peptide 17 (α17) did not affect the mobility nor the DNA binding activity of DRTF1/E2F (**figure 3.6d**). This could either be due to the 17-epitopes being obscured within the complex or because the specific antibodies in the serum only recognise the epitopes under denaturing conditions and not when DP-1 is in a non-denatured tertiary conformation.

Figure 3.6

Differential effects of antisera raised to different peptide regions in DP-1 on the DRTF1/E2F complex E2F-site DNA-binding activity in gel retardation assays.

a. *Antisera to peptide D specifically shifts the DRTF1/E2F complex in gel retardation assays.*

Phospholabelled E2F-site DNA probe was mixed with 4μg of total protein from F9EC cell microextract, exposed to 6μl of antisera raised against peptide D (αD) and loaded into each of lanes 1 and 2. Included in the reaction mixture of lane 1 was peptide D, and in that of lane 2, a control peptide C at approximately 0.2nmol/μl. Gel-retarded sub-complexes of DRTF1/E2F (a and b/c) are indicated.

b. *Antisera to peptide 24 specifically shifts the DRTF1/E2F complex in gel retardation assays.*

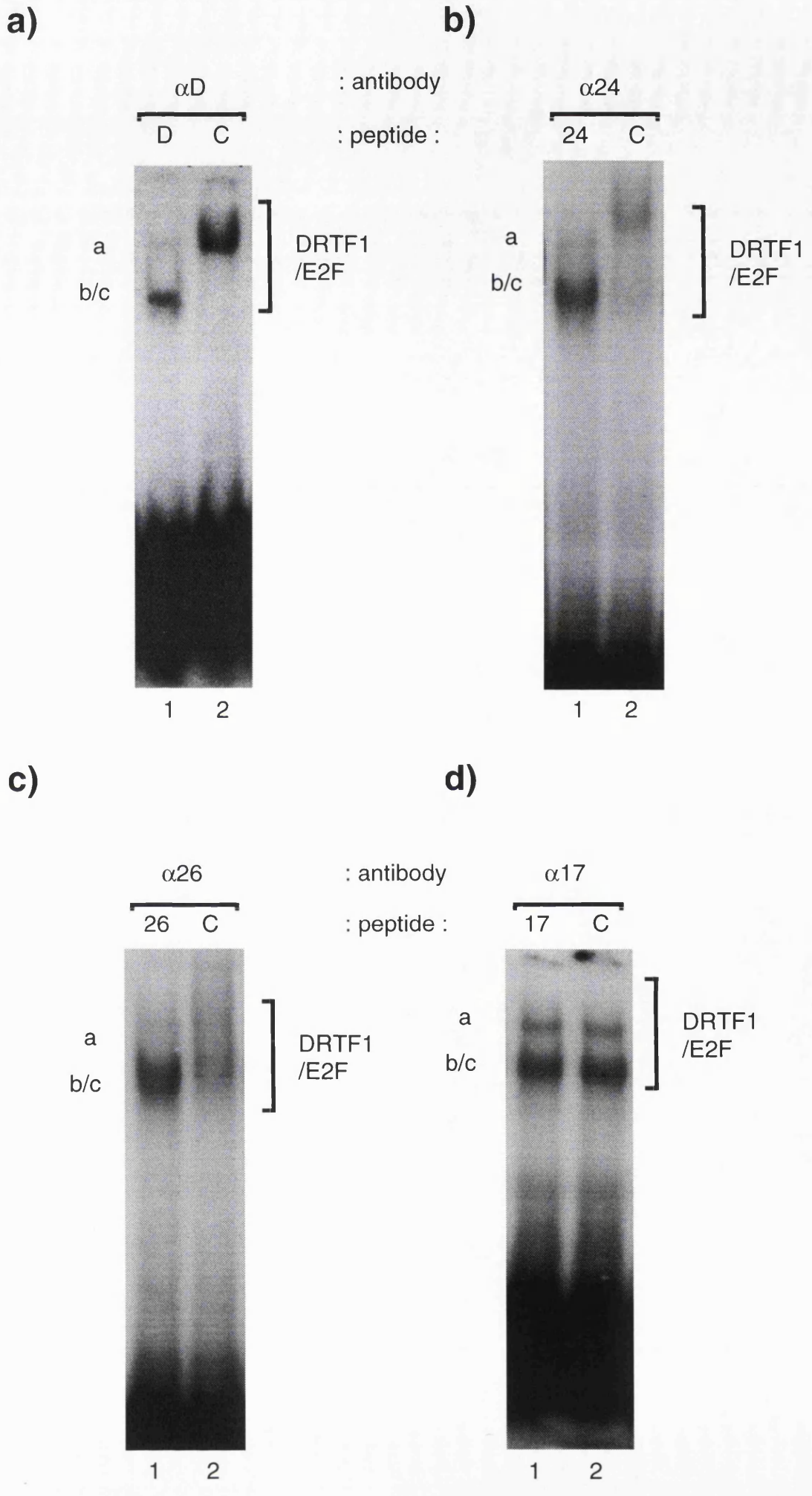
Phospholabelled E2F-site DNA probe was mixed with 4μg of total protein from F9EC cell microextract, exposed to 6μl of antisera raised against peptide 24 (α24) and loaded into each of lanes 1 and 2. Included in the reaction mixture of lane 1 was peptide 24, and in that of lane 2, a control peptide C at approximately 0.2nmol/μl. Gel-retarded sub-complexes of DRTF1/E2F (a and b/c) are indicated.

c. *Antisera to peptide 26 specifically disrupts the DRTF1/E2F complex in gel retardation assays.*

Phospholabelled E2F-site DNA probe was mixed with 4μg of total protein from F9EC cell microextract, exposed to 6μl of antisera raised against peptide 26 (α26) and loaded into each of lanes 1 and 2. Included in the reaction mixture of lane 1 was peptide 26, and in that of lane 2, a control peptide C at approximately 0.2nmol/μl. Gel-retarded sub-complexes of DRTF1/E2F (a and b/c) are indicated.

d. *Antisera to peptide 17 does not affect the DRTF1/E2F complex in gel retardation assays.*

Phospholabelled E2F-site DNA probe was mixed with 4μg of total protein from F9EC cell microextract, exposed to 6μl of antisera raised against peptide 17 (α17) and loaded into each of lanes 1 and 2. Included in the reaction mixture of lane 1 was peptide 17, and in that of lane 2, a control peptide C at approximately 0.2nmol/μl. Gel-retarded sub-complexes of DRTF1/E2F (a and b/c) are indicated.


a)
b)
αD
: antibody
α24
D C
: peptide :
24 C
a
b/c
DRTF1
/E2F
1 2
c)
d)
α26
α17
26 C
17 C

If the purified antibody reagents, prepared in this study from some of the above antisera, recognise DP-1, they may likewise be expected to affect the migration of DRTF1/E2F in a gel retardation assay. It was important to ascertain that the purified antibody preparations would still recognise DP-1-containing DRTF1/E2F such that a closer association be made between the p55 observed in the immunoblots and DRTF1/E2F.

This association, between the p55 doublet as identified by purified anti-sera, and DRTF1/E2F, was made by similar, though weaker, supershifts in gel retardation assays with the same purified antisera (**figure 3.7a**). APαA (lanes 1 and 2) and APαD (lanes 3 and 4) both created a more slowly migrating form of DRTF1/E2F in gel retardation assays (indicated by * in lanes 2 and 4 - the slower migrating form in lane 4 is clear on the original autoradiograph) the formation of which was prevented by the inclusion in the reaction mixtures of the specific peptides that the antibodies were raised to (lanes 1 and 3), as opposed to control peptides. The third reagent shown to recognise the p55 doublet in immunoblotting assays, APα17, was unable to affect the gel retardation complex. This was not surprising as the unpurified antisera from which it was made did not have this activity either (**figure 3.6d**).

The super-shifting activity of the purified antisera was relatively weak and not all fractions possessed even any (see below). To clarify the observation that the epitope purified antibodies do recognise DRTF1/E2F, the APαA reagent was used to immunoprecipitate E2F DNA binding activity (**figure 3.7b**). A fraction of APαA, that possessed gel retardation activity, was mixed with F9EC LSL-extract and the antibodies within precipitated with protein A-Sepharose after an incubation period. The antibody-precipitate was eluted with peptide A and assayed by gel retardation (see Experimental Protocols). This immunoprecipitation would isolate both the *a* and the *b/c* forms of DRTF1/E2F in the presence of a control peptide (lane 2) but not in the presence of peptide A (lane 1). APαD was able to specifically re-immunoprecipitate E2F binding activity from this APαA-precipitate (Chapter 6 and **figure 6.1b**), suggesting not only that this reagent can also recognise DP-1 in a native DNA binding complex, but also that both APαA and APαD can recognise the same DP-1-bearing complex (DRTF1/E2F).

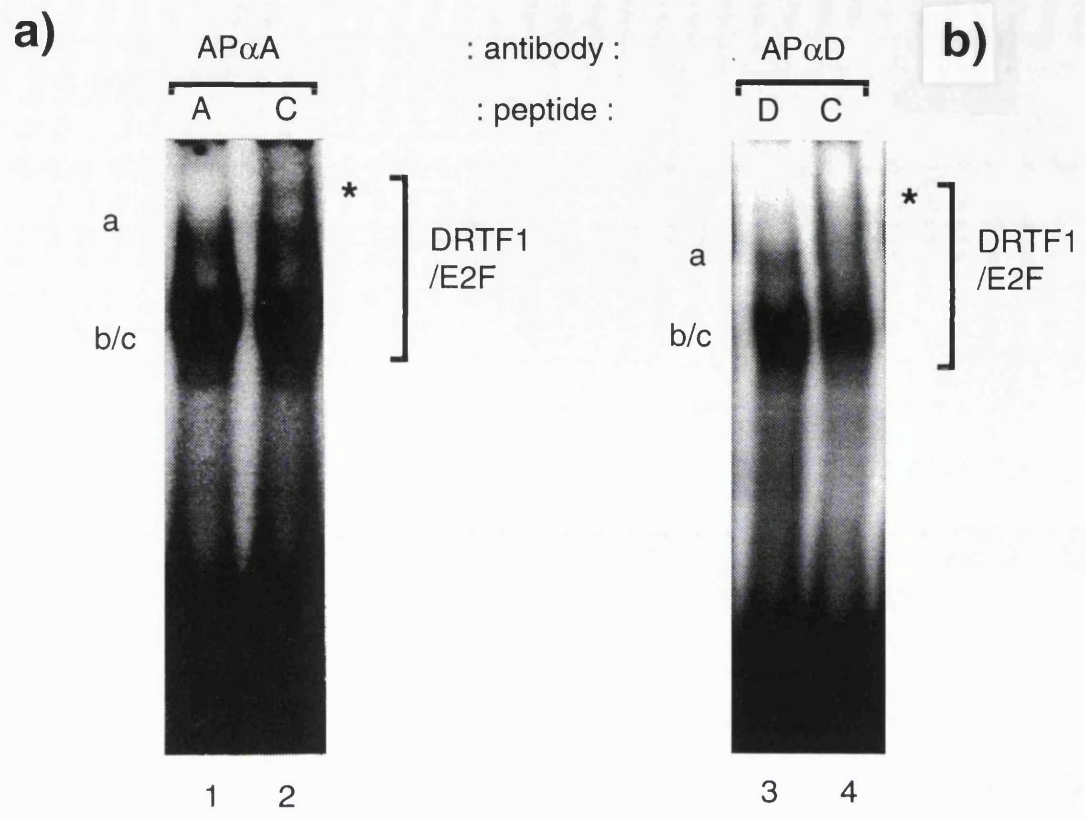
Only a relatively few fractions of epitope affinity-purified polyclonal antisera against DP-1 actually retained the ability to affect DRTF1/E2F mobility in gel retardation assays while many more would be able to identify the p55 doublet in an immunoblot. This indicated that the polyclonal sera contained different populations of antibodies with different affinities to the peptide epitope, and that only some had the ability to recognise DP-1 in its native DRTF1/E2F-participating form, as opposed to the de-natured version in an immunoblot. The gel supershifts of the few positive fractions were also relatively weak compared to the original anti-sera which would

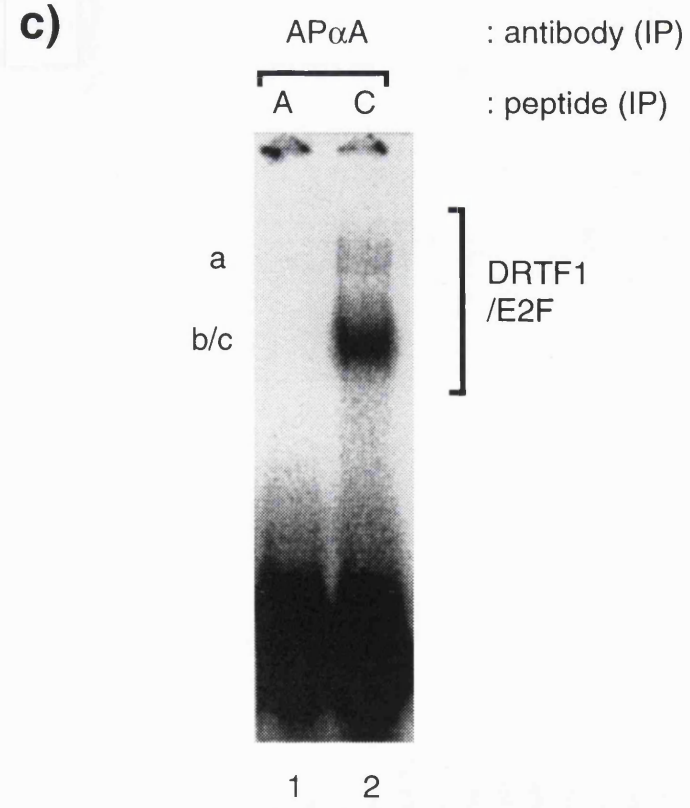
Figure 3.7
The p55 DP-1 doublet is immunogenically linked to DRTF1/E2F.

a. *Affinity purified anti-peptide A specifically binds to DRTF1/E2F in a gel retardation assay.*
A gel retardation assay in which 4µg protein of F9EC microextract was loaded with labelled E2F site DNA probe in each lane. Included in both reaction mixtures was 12µl epitope affinity purified antisera to peptide A (APαA) as well as peptide A (A) in lane 1 and peptide C (C) in lane 2 at approximately 0.2nmol/µl. Gel-retarded complexes of DRTF1/E2F are indicated (a and b/c) and a supershifted complex by *.

b. *Affinity purified anti-peptide D specifically binds to DRTF1/E2F in a gel retardation assay.*
A gel retardation assay in which 4µg protein of F9EC microextract was loaded with labelled E2F site DNA probe in each lane. Included in both reaction mixtures was 12µl epitope affinity purified antisera to peptide D (APαD) with peptide D (D) in lane 1 and peptide C (C) in lane 2 at approximately 0.2nmol/µl. Gel-retarded sub-complexes of DRTF1/E2F are indicated (a and b/c) and a supershifted complex by * (this latter complex is clear in the original autoradiograph).

c. *Affinity purified anti-peptide A will specifically immunoprecipitate E2F-site DNA binding activity detectable by gel retardation.*
Gel retardation positive fractions of APαA were used to immunoprecipitate (IP) from F9EC LSL extract in the presence of peptide A (A) or peptide C (C). Precipitated complexes were released with peptide A and assayed by gel retardation for binding to labelled E2F site DNA. Lane 1 contains the precipitation performed in the presence of peptide A and lane 2 in the presence of peptide C.


a)
APαA
: antibody :
: peptide :
A C
a
b/c
*
DRTF1
/E2F
1 2
b)
APαD
D C
a
b/c
*
DRTF1
/E2F
3 4


c)
APαA
: antibody (IP)
: peptide (IP)
A C
a
b/c
DRTF1
/E2F
1 2

often shift almost all DNA binding activity, suggesting that a lot of this activity was lost during the purification procedure (**figure 3.8**). Original antisera (αA) clearly affected the mobility of DRTF1/E2F in a manner sensitive to the presence of peptide A (compare lanes 1 and 2). The flow-through antisera (FT), that had passed through the peptide A-column was completely devoid of any such activity suggesting that that had been retained on the column (lanes 3-6). However, the fractions eluted at low pH, despite containing activity that recognised the p55 doublet in an immunoblot (see **figure 3.1b**), contained either no or very weak (e.g. **figure 3.8**, lane 9, indicated with *) gel retardation activity (antibody-fraction loadings were three times higher to take dilution effects into account). Several attempts, using high salt and detergent buffers, were made to elute any activity which might have been retained on the column after the low pH elution, but none were successful. The peptide A-Sepharose column was used extensively for the purification of APαA but never showed any obvious signs of loosing binding activity. This could suggest that lost gel retardation activity in the eluted fractions was not due to it being retained irreversibly on the column as this might be expected to lower the resin binding efficiency over time. The most likely explanation is that the low pH elution procedure caused loss of gel retardation activity, despite elution being made straight into a neutralising buffer (see Experimental Protocols).

These problems, however, should not detract from the fact that the purified antibodies can interact with DRTF1/E2F thus making correlation between the reagents that almost exclusively identify cellular DP-1, p55, in immunoblots, and an ability to bind to the transcription factor complex that DP-1 is a part of. The results thus suggest that the form of DP-1 characterised in immunoblots is also one that may participate in DRTF1/E2F.

The p55 doublet parallels DRTF1/E2F behaviour during cellular differentiation

Further evidence that the p55s identified by the purified polyclonal antibodies are involved in DRTF1/E2F comes from differentiation studies using F9EC cells. The DNA binding activity of DRTF1/E2F is down regulated as F9EC cells differentiate (**figure 3.9a**) as previously reported (see Introduction and La Thangue & Rigby, 1987 ; La Thangue *et al.*, 1990). Since DP-1 is part of DRFT1/E2F DNA binding activity, the abundance of the p55 doublet in these extracts, as identified by the purified antibodies, was investigated.

Figure 3.8

Gel-retardation super-shifting activity is lost during the affinity purification procedure of peptide antisera.

Gel-retardation profile of the epitope-affinity purification procedure of antisera to the DP-1 peptide A.

Every reaction mixture loaded in this gel-retardation assay contained 4μg of total F9EC cell extract protein exposed to phospholabelled E2F DNA sites. Lanes 1 and 2 additionally contained 4μl antisera raised to peptide A (αA) in the presence of peptide A (A, lane 1) or peptide C (C, lane 2) at approximately 0.2nmol/μl. The αA antisera was passed over a peptide A column (see main text and Experimental Protocols) and flow-through (FT) fractions collected. The same volume (4μl) was added to the reaction mixtures loaded in lanes 3 to 6 from flow-through fractions 2 to 5 respectively (FT2-5). Specific peptide A-recognising antibodies retained on the peptide A column were eluted at low pH and fractions 3 to 10 (f3 to f10) were added to reaction mixtures in lanes 7 to 14 respectively. Three times the volume, 12μl, of eluted fractions were added to account for dilution effects estimated from the total volume of immunoblot-positive fractions (**figure 3.1b**) versus the volume of antisera they had been made from. Gel-retarded complexes of DRTF1/E2F are indicated (a and b/c) and a supershifted complex by *.

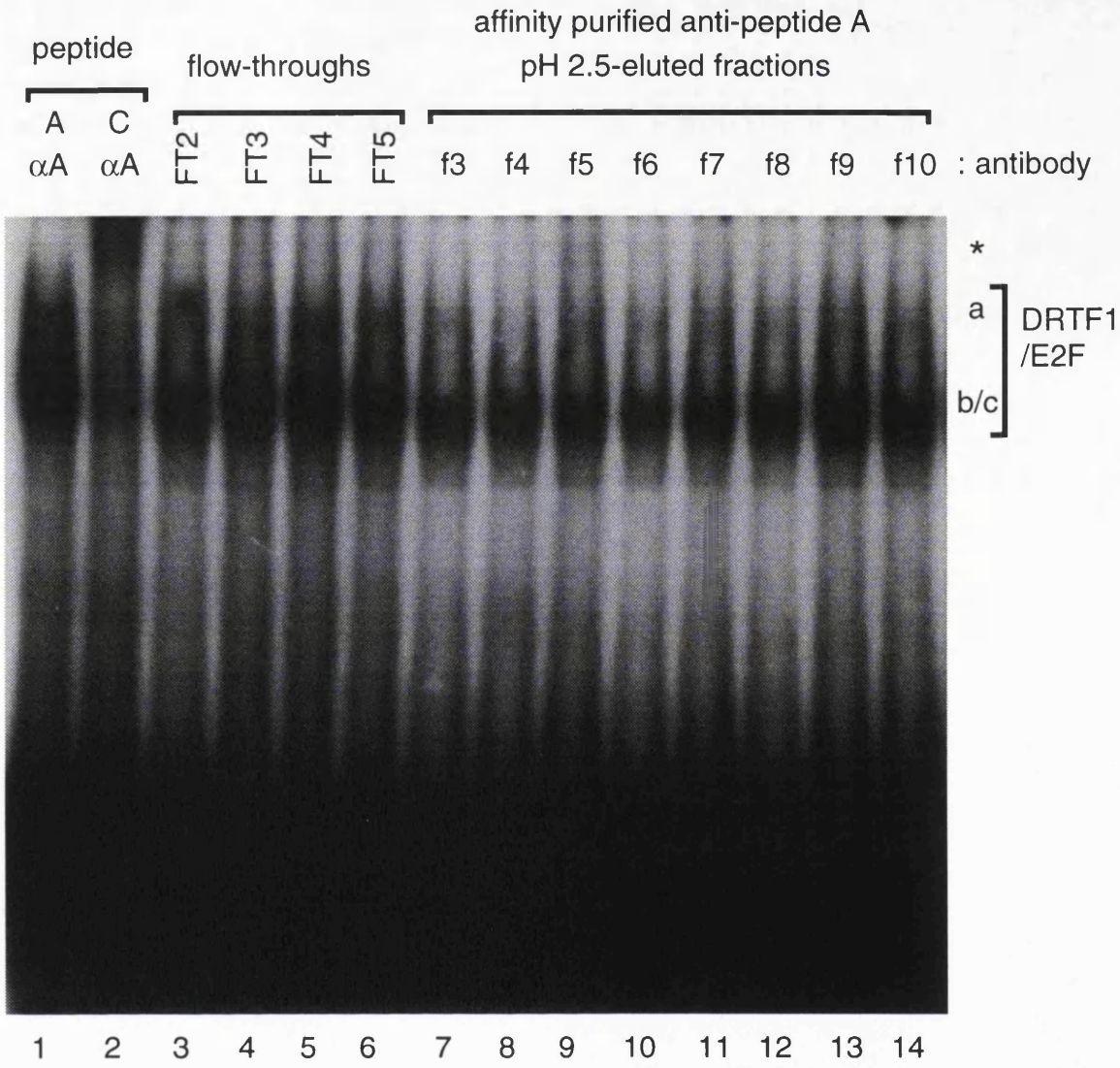
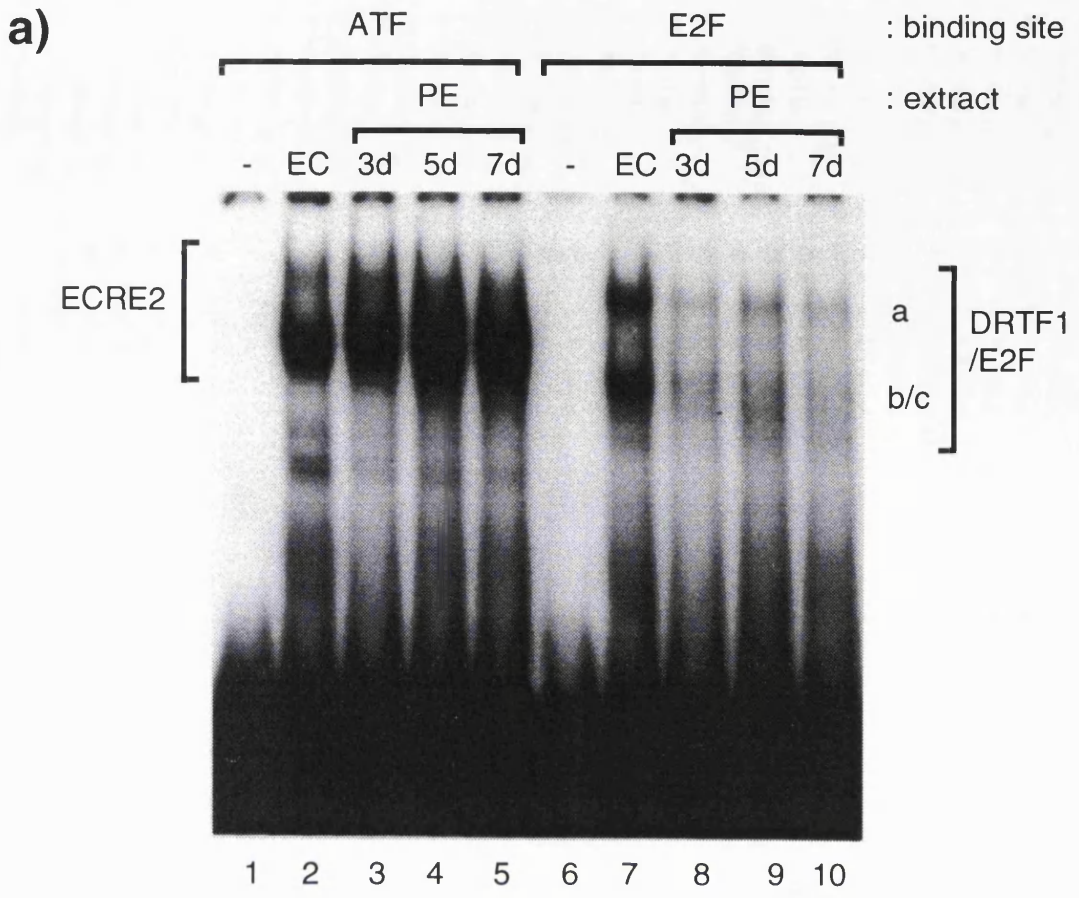

peptide
flow-throughs
affinity purified anti-peptide A
pH 2.5-eluted fractions
A
C
αA
αA
FT2
FT3
FT4
FT5
f3
f4
f5
f6
f7
f8
f9
f10
: antibody
*
a
b/c
DRTF1
/E2F
1
2
3
4
5
6
7
8
9
10
11
12
13
14

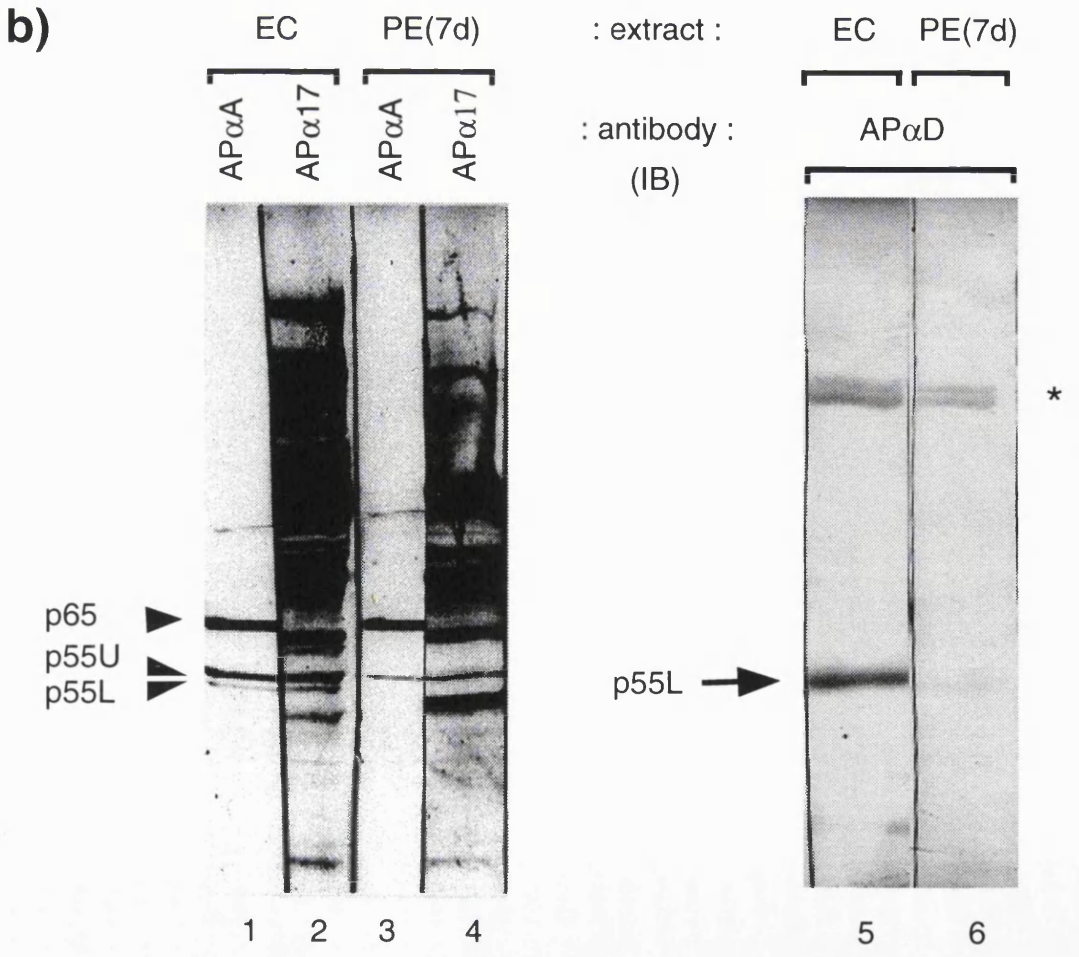
Figure 3.9

The regulation of DRTF1/E2F DNA binding activity during cellular differentiation is mirrored by DP-1 p55-doublet abundance.

a. *Gel retardation assay of undifferentiated and differentiated F9EC cell extracts for E2F and ATF DNA site binding activity.*
Gel retardation assay of DNA binding activity to labelled E2F (lanes 6-10) and ATF (lanes 1-5) sites in F9EC cell (EC ; lanes 2 and 7) microextracts and F9PE cell (PE ; λανεσ 2, 4, 5 ανδ 8, 9, 10) microextracts, three, five and seven days post-differentiation reagent exposure (3d, 5d and 7d). Lanes 1 and 6 contained probe only (-). Gel-retarded sub-complexes of DRTF1/E2F are indicated (a and b/c).

b. *Immunoblot of extracts of undifferentiated and differentiated F9EC cells probed with affinity purified antibodies raised to different regions of DP-1.*
Equal protein amounts of F9EC (EC, lanes 1/2 and 5) and seven-day differentiated F9PE (PE(7d), lanes 3/4 and 6) cell microextracts were loaded in separate gel wells and the blotted lanes cut in half and probed with different affinity purified antibody reagents (APαA, lanes 1 and 3 ; APαD, lanes 5 and 6 ; APα17, lanes 2 and 4). * indicates a non-DP-1 antibody cross-reaction. (IB =Immunoblot).


a)
ATF
E2F
: binding site
PE
PE
: extract
- EC 3d 5d 7d - EC 3d 5d 7d
ECRE2
a
b/c
DRTF1
/E2F
1 2 3 4 5 6 7 8 9 10


b)
EC
PE(7d)
: extract :
EC
PE(7d)
APαA
APα17
APαA
APα17
: antibody :
(IB)
APαD
p65
p55U
p55L
p55L
*
1 2 3 4
5 6

F9EC cells were allowed to differentiate into parietal endoderm cells (F9PE) by retinoic acid-induced differentiation over a seven-day period (see Experimental Protocols). Microextracts were made at 3-, 5- and 7-days post-exposure and equal cell extract protein loadings assayed for E2F site binding activity by gel retardation (**figure 3.9a**, lanes 6 to 10). Extract integrity was controlled for by the binding activity of an unrelated DNA binding protein factor, ECRE2 (lanes 1 to 5), whose affinity to an ATF DNA site is known not to be affected by the differentiation process (Tassios & La Thangue, 1990). The experiment clearly showed that E2F site DNA binding activity was rapidly reduced as the cells embarked upon the differentiation process (lanes 7-10), while binding activity to the ATF site was not down-regulated (lanes 2-5). Immunoblots with the same extracts, probed with the purified antibody reagents, revealed that the abundance of the p55 doublet correlated with the down-regulation of DRTF1/E2F (**figure 3.9b**). Equal amounts of total cell-extract protein was loaded in each lane yet p55U was reduced while p55L disappeared completely from differentiating cells (lanes 3, 4 and 6). APαA and APα17 recognise both p55 forms in F9EC cell extract (lanes 1 and 2) and demonstrated a clear reduction of the p55 doublet in F9PE extracts (lanes 3 and 4). APαD, which preferentially recognises the p55L form, failed to identify DP-1 at all in F9PE extracts (lane 5 compared to lane 6) confirming the observation using the other two purified antibodies. A slower-migrating non-DP-1 polypeptide recognised by APαD (indicated by * : lane 6) was unaffected by the differentiation process and thus served as a useful internal control for loading-levels and extract integrity. This was similarly supported by the unchanging recognition pattern of the unspecific polypeptides such as p65 identified by APαA (lanes 1 versus 3), and the wealth of unspecific polypeptides bound by APα17 (lane 2 versus 4).

The data presented in this chapter thus suggests that the p55 polypeptide doublet represents the physiological DP-1 gene product and proposes that it may be intimately linked with the regulation of DNA-binding activity of DRTF1/E2F in F9EC cells. The elucidation of the DP-1 protein as a doublet by denaturing polyacrylamide gel electrophoresis assay is a novel observation and laid the path for further biochemical analysis.

Chapter 4

32.3: Monoclonal antibody to DP-1

Immunisations and screening

A monoclonal antibody was raised against the C-terminal DP-1 peptide D (the same peptide used for raising the polyclonal antisera, αD, used for making the APαD reagent). The C-terminus of DP-1 had provided a good response in rabbit polyclonal antisera and so it was hoped that a similar response could be elicited in mice from which to make a monoclonal reagent. Monoclonal antibodies will often display very specific activities and it was hoped that such a reagent might provide additional information about DP-1.

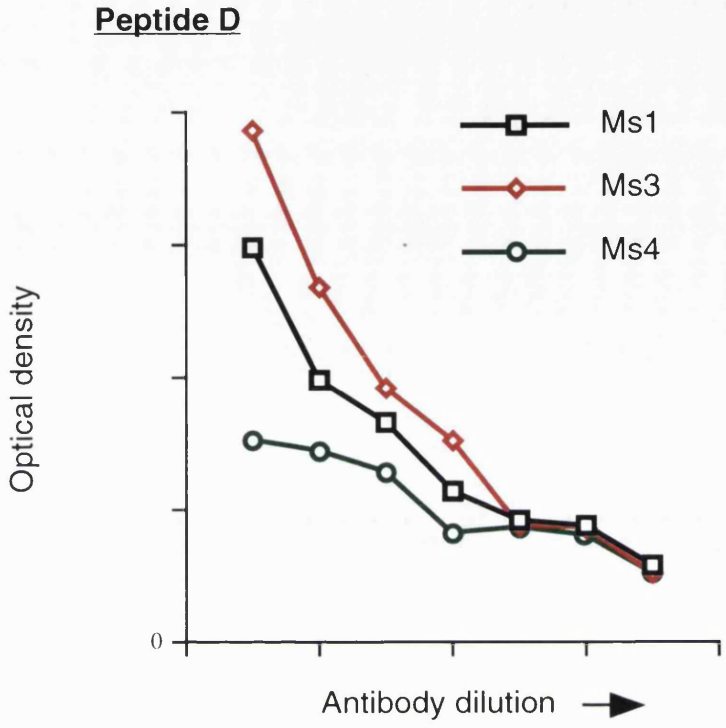
Four mice were immunised with BSA-coupled peptide D (see Experimental Protocols) and anti-sera test bleeds were assayed by ELISA, immunoblotting and gel retardation. One mouse died during the immunisation program. ELISA assays were employed to quantitatively assess the immune responses to peptide D (**figure 4.1**). Immunosorbent microtitre wells were coated with equal amounts of peptide D or C and the binding activity of progressive dilutions of antisera assessed. The binding activity of the sera from the remaining three mice to peptide D (**figure 4.1a**), and control peptide C (**figure 4.1b**) indicated that the greatest response was from mouse number three (Ms3). The antisera from Ms3 was assayed in a gel retardation assay (**figure 4.2a**) and found to shift the DRTF1/E2F complex from F9EC extract very efficiently when not exposed to peptide D (lane 2). Exposure to peptide D (lane 1) completely abolished this activity suggesting specificity to DP-1 and DRTF1/E2F. However, when the sera was assayed in an immunoblot on the same extract, it did not seem to specifically identify a p55 polypeptide, or for that matter any polypeptide (**figure 4.2b**). A gel-lane (lanes 1 and 2) of F9EC microextract was blotted and probed with Ms3 sera in the presence of peptide D (lane 1) or control peptide C (lane 2). In a neighbouring lane (3), F9EC extract was probed with APαA, but the p55 that it identified did not seem to have a counterpart in lane 2 which was competed out in lane 1. This could simply have been because that non-specific bands were recognised by the Ms3 antisera which obscured a specific identification of the p55. Alternatively

Figure 4.1
ELISA screening of murine antisera for response to immunisation with DP-1 peptide D.

a. *ELISA screen of mouse anti-peptide D serum affinity for peptide D.*
Three antisera, from mice immunised with peptide D, were applied to immobilised peptide D at sequential 50% dilutions. The resulting antibody-antigen complexes were then exposed to equal concentrations of secondary alkaline phosphatase-conjugated antibody, substrate for a colour reaction added and reaction product formation measured by optical densitronomy.

b. *ELISA screen of mouse anti-peptide D serum affinity for peptide C.*
As for **a**. but using the unrelated, though similar sized, peptide C.

a)


Peptide D
Ms1
Ms3
Ms4
Optical density
0
Antibody dilution

b)

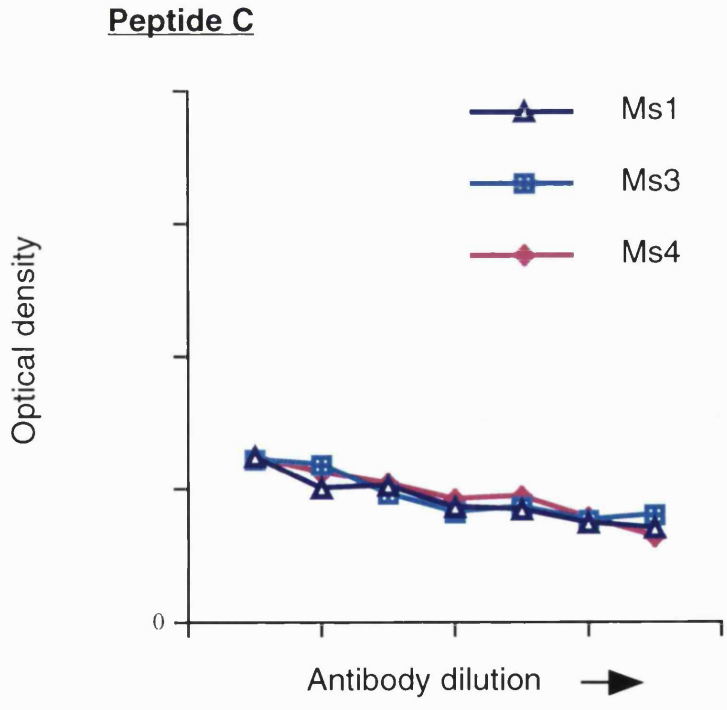

Peptide C
Ms1
Ms3
Ms4
Optical density
0
Antibody dilution

Figure 4.2

The Ms3 antisera shifts DRTF1/E2F in a gel retardation assay of F9EC cell extract yet fails to identify p55 in an immunoblot of the same extract.

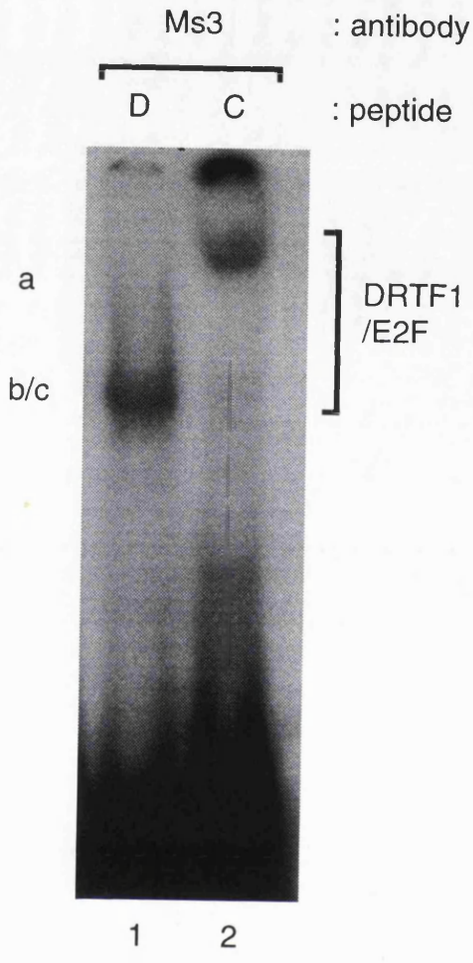
a. *Gel retardation assay of Ms3 antisera on F9EC DRTF1/E2F DNA binding activity.*

Both gel retardation assay reaction mixtures contained 4μg of total protein from an F9EC cell extract, labelled E2F site DNA probe, 8μl of Ms3 antisera and either peptide D (lane 1) or peptide C (lane 2) at approximately 0.2nmol/μl. Gel-retarded sub-complexes of DRTF1/E2F are indicated (a and b/c).

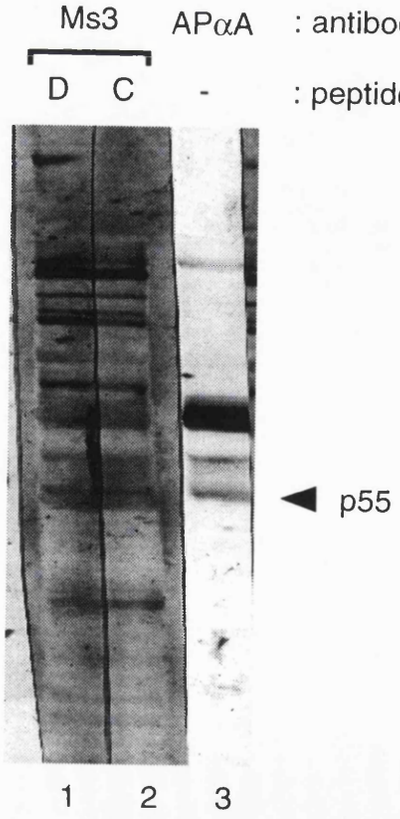
b. *Immunoblot of Ms3 antisera on F9EC cell extract.*

Two gel-lanes were each loaded with 40μg of total protein from an F9EC cell extract, blotted, and cut in half. Lanes 1 and 2 were probed with Ms3 antisera in the presence of peptide D (lane 1) or a control peptide (lane 2). Lane 3 was exposed to epitope affinity purified antiserum to peptide A (APαA). The DP-1 polypeptide doublet (unresolved in this particular immunoblot), p55, is indicated.

a)


Ms3
: antibody
D
C
: peptide
a
b/c
DRTF1
/E2F
1
2

b)


Ms3
APαA
: antibody
D
C
-
: peptide
p55
1
2
3

it is possible that the antisera would not recognise the peptide D epitopes in DP-1 under denaturing conditions. The gel retardation result was however so convincing and strong that on this justification alone an attempt to make a monoclonal cell line from Ms3 was made.

Hybridoma generation and maintenance was carried out in collaboration with Julian Gannon (ICRF Clare Hall) according to standard procedures (Harlow & Lane, 1988) while all hybridoma supernatant screening was performed by myself. Initial screening of polyclonal hybridoma supernatants was performed by ELISA against the peptide D that the mice had been immunised with. Early rounds of screening showed up potential positives which were subcultured into proto-monoclonal cultures which were further sub-cloned into monoclonal cultures on the basis of a similar selection. Thirty five monoclonal cultures were selected from the first monoclonal screen of approximately 2000 cultures and were grown up in 3ml wells and assayed again by ELISA, this time against a control peptide C as well as D. The absorbance of peptide D binding above the peptide C background is displayed in **figure 4.3**. During the expansion of the selected cultures in the 3ml wells, some cultures appeared to gain signal strength, while others waned or bound the control peptide as strongly as the specific peptide. The highest scorers (**figure 4.3**, white stars) were selected for screening by immuno-blotting against an N-terminally truncated bacterially expressed DP-1 GST-fusion protein. The GST-DP-1 fusion proteins were expressed in *E.coli* and purified from bacterial lysates using glutathione-coupled Sepharose, eluted with reduced glutathione buffer and assayed by SDS-PAGE and coomassie staining (**figure 4.4a** and see Experimental Protocols). The particular N-terminally truncated GST-DP-1 fusion was chosen because its expression was better than the full-length DP-1-GST while still containing the C-terminus in which the peptide D sequence is located. The size of the purified protein (**figure 4.4a**) corresponded with the predicted GST-truncated-DP-1 size of around 64KDal (see Experimental Protocols, Chapter 2, for the prediction formulae employed). The top ELISA-responding hybridoma supernatants (indicated by white stars, **figure 4.3**, bar two clones which died, number 3 and 6 indicated by †) and three low scorers (numbers 4, 15 and 22 - included as negative controls, indicated by black stars) were assayed in an immunoblot against GST-DP-1 (**figure 4.4b**). A very clear result emerged indicating that only clone, number 32, recognised the GST-DP-1 fusion protein under the denaturing conditions of an immunoblot (lane 6). Oddly enough, this was not the highest scorer in the ELISA assay. The result was internally and negatively controlled for by the lack of response from the other selected supernatants, and positively controlled by a sample of the original antisera from the sacrificed mouse (lane 10). The hybridoma-32 was itself subcultured further to ensure complete clonal purity, and from this the subclone 32.3, and finally 32.3.2, was selected and a cell line established.

Figure 4.3

ELISA screening of hybridoma supernatants identifies clones which produce antibody that recognise peptide D but not peptide C.

ELISA scores of hybridoma supernatants against peptide D above peptide C. Peptide D and peptide C coated microtitre well plates were assayed for reactivity to candidate hybridoma supernatants. The values obtained for peptide C were subtracted from those of peptide D. The highest scoring clones (white stars and black crosses) were selected for further analysis and some of the lowest (black stars) selected as negative controls. Black crosses indicates two cultures that were selected for further analysis but which died prior to further testing.

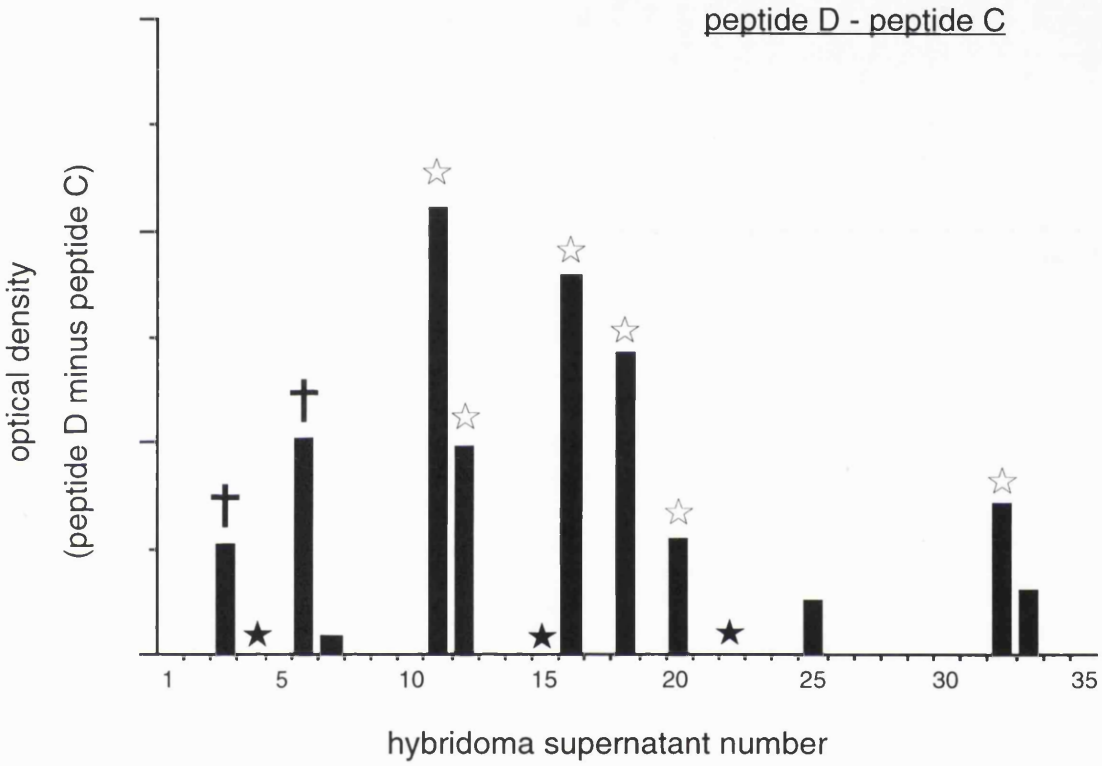

peptide D - peptide C
optical density
(peptide D minus peptide C)
1
5
10
15
20
25
30
35
hybridoma supernatant number

Figure 4.4

One hybridoma clone supernatant positively identifies bacterially expressed recombinant DP-1 in an immunoblot.

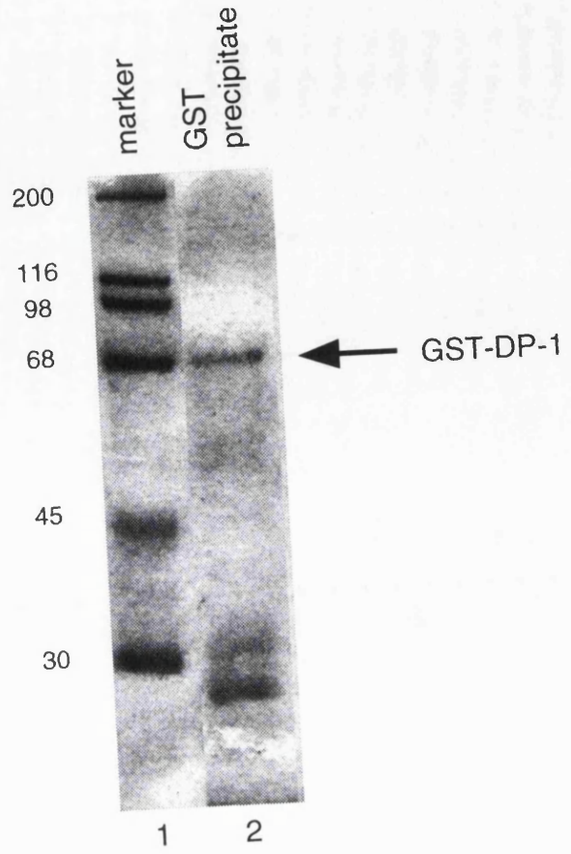
a. *SDS PAGE and coomassie stained purified N-terminally truncated DP-1 fused to glutathione S-transferase.*

SDS PAGE coomassie stain of purified bacterially expressed N-terminally truncated DP-1-GST fusion (GST-DP-1, lane 2) and standard molecular weight protein markers (lane 1). Protein marker sizes are indicated in KDal.

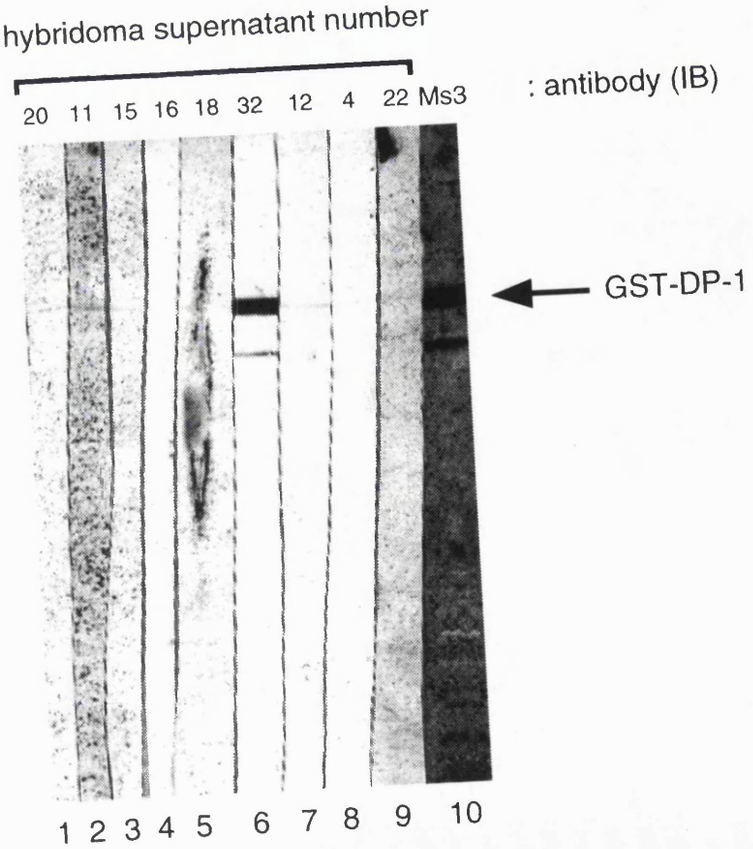
b. *Immunoblotting of bacterially produced and purified GST-DP-1 fusion protein probed with candidate hybridoma supernatants.*

Immunoblot (IB) of hybridoma supernatants against a bacterially expressed and purified GST-DP-1 N-terminally truncated fusion protein (approximately 25ng per lane). Ms3 indicates the serum positive control, serum from the mouse from which the hybridoma were made.

a)


marker
GST precipitate
200
116
98
68
45
30
GST-DP-1
1
2

b)


hybridoma supernatant number
20 11 15 16 18 32 12 4 22 Ms3
: antibody (IB)
GST-DP-1
1 2 3 4 5 6 7 8 9 10

Immunochemical properties of the monoclonal antibody 32.3

The potential benefits of the 32.3 hybridoma supernatant were assessed by immunoblotting F9EC extract with it and comparing its recognition pattern to APα17 (**figure 4.5a**). This experiment demonstrated that 32.3, as well as recognising bacterially purified DP-1, also recognised a single polypeptide in an F9EC cell microextract (lane 1) which was abolished by inclusion of peptide D in the primary incubation mixture (lane 2). Depending on blot conditions, 32.3 occasionally and weakly would recognise a slightly slower migrating polypeptide as well (lane 1 and 4). The major recognised polypeptide co-migrates with the cellular form of DP-1, as defined by APα17 (lane 3 compared to 4), and indeed only one of the two DP-1 forms : p55L. The faint slower migrating polypeptide did not co-migrate with p55U and so is unlikely to be DP-1. The fact that the Ms3 antisera could recognise GST-DP-1 in an immunoblot (**figure 4.4b**), and that a monoclonal derived from it recognised p55, suggests that Ms3 could not identify p55 in a cell extract due to an obscuring non-specific activity in cellular extracts (**figure 4.2b**).

In order to establish the identity of the polypeptide identified by 32.3 as that of p55L, a similar series of experiments were conducted upon 32.3 as those used to investigate the nature of the polyclonal epitope-affinity purified antibodies in Chapter 3. The identity of the 32.3 polypeptide as being truly that of p55L was strongly suggested by its disappearance in differentiated F9EC cells (**figure 4.5b**, compare lanes 1 and 2) as was also demonstrated for p55L by polyclonal purified antibodies (**figure 3.9b**). In accordance with recognition of DP-1 by 32.3, the antibody also supershifted the DRFT1/E2F complex in F9EC cell extracts (**figure 4.5c**). This effect was prevented by the inclusion in the reaction mixture of peptide D (lane 1) as opposed to a control peptide (lane 2) suggesting specificity. In agreement with this ability to recognise DP-1 in the non-denaturing conditions of a gel retardation assay, 32.3 would also immunoprecipitate E2F binding activity from the same cells (**figure 4.5d**) in the presence of a control peptide (lane 2) but not in the presence of peptide D (lane 1).

Other high ELISA scoring monoclonal hybridoma supernatants were tested in gel retardation assays because although they were unable to recognise the denatured form of the C-terminus of DP-1, it was possible that they would identify the native conformation found under the conditions of the gel retardation assay. However these assays were negative indicating that some of the ELISA positives were only able to recognise epitopes of peptide D in the context of that assay, and so were of limited benefit to the study of cellular DP-1 protein.

Figure 4.5

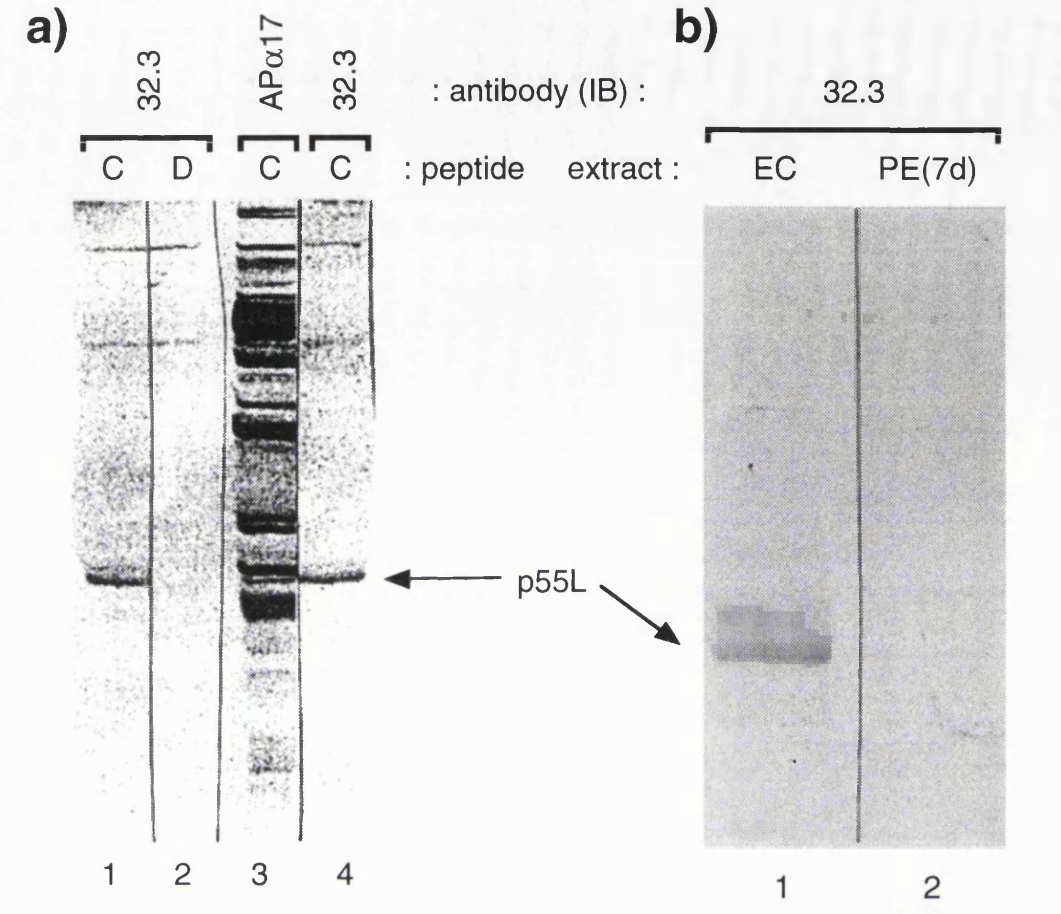
The first monoclonal against DP-1, 32.3, specifically recognises DRTF1/E2F in cell extracts but only identifies the p55L form in immunoblots of the same extracts.

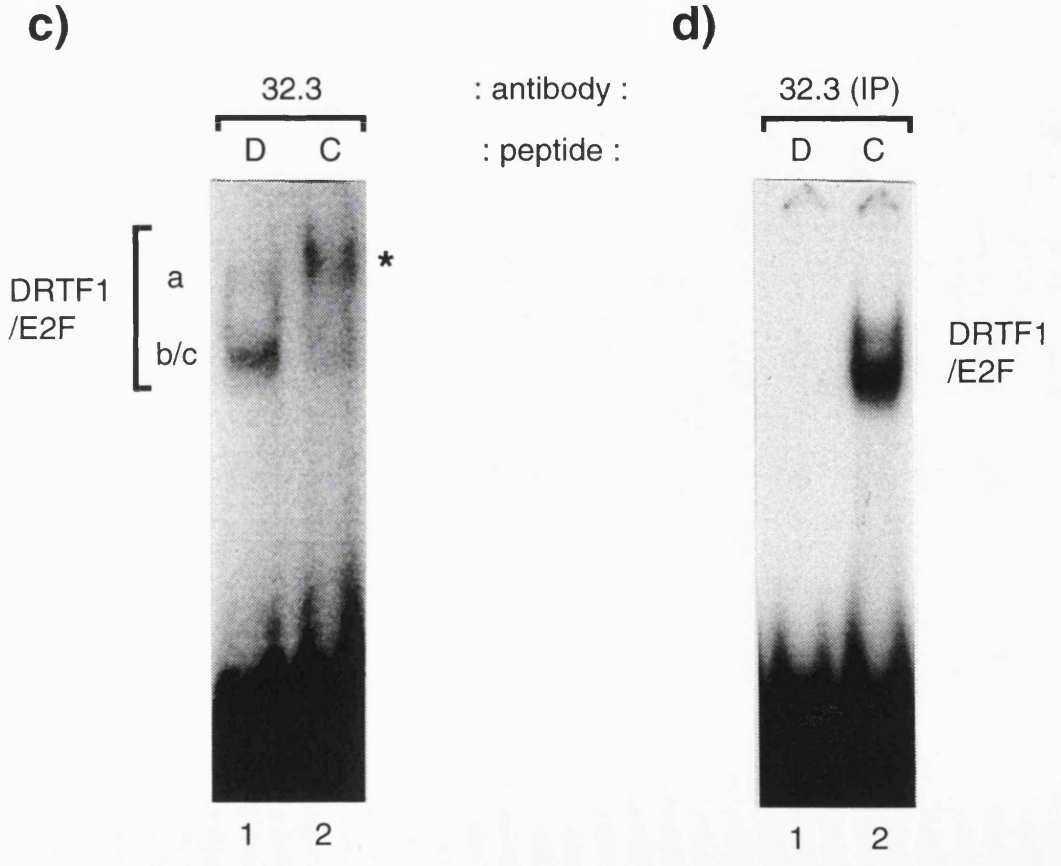
a. *Immunoblot with the monoclonal hybridoma supernatant 32.3 on F9EC extract.* Two gel-lanes were each loaded with 40µg total protein of F9EC microextract, blotted, cut in half, and each half subjected to different antibody treatments. Lane 3 was exposed to epitope affinity purified antiserum to DP-1 peptide 17 (APα17) while lanes 1, 2 and 4 were treated with the monoclonal hybridoma supernatant 32.3. Antibody exposure was performed in the presence of either a control peptide (C, lanes 1, 3 and 4) or the peptide D (D, lane 2) that the monoclonal 32.3 was raised to. (IB = immunoblot).

b. *Immunoblot with the monoclonal hybridoma supernatant 32.3 on F9EC and F9PE extract.*
Two gel-lanes were each loaded with 40µg total protein of F9EC (EC, lane 1) or F9PE (PE(7d), lane 2) microextract, blotted and each exposed to the 32.3 hybridoma supernatant. (IB = immunoblot).

c. *Gel retardation to assay the effect of the hybridoma supernatant 32.3 on E2F-site bound DRTF1/E2F from F9EC extract.*
F9EC microextract (4µg of total protein) was exposed in each reaction to labelled E2F DNA site and maximal 32.3 hybridoma supernatant volume (13µl out of a 20µl reaction mixture). Also included in the mixtures were either peptide D (D : lane 1) or peptide C (C : lane 2) at approximately 0.2nmol/µl. Gel-retarded sub-complexes of DRTF1/E2F are indicated (a and b/c) and * indicates supershifted complex.

d. *Gel retardation assay of monoclonal hybridoma supernatant 32.3-immuno-precipitate from F9EC cell extract.*
Hybridoma supernatant (100µl) was mixed with 300µl of LSL F9EC extract (approximately 3µg/µl) and immunoprecipitated (IP) by standard procedures in the presence of peptide D (D : lane 1) or peptide C (C : lane 2). The precipitates were eluted from the antibody-Sepharose complex by exposure to peptide D and the elutants assayed by gel retardation using labelled E2F DNA binding probe.


a)
32.3
APα17
32.3
: antibody (IB) :
C D C C : peptide
p55L
1 2 3 4
b)
32.3
extract : EC PE(7d)
1 2


c)
32.3
D C
DRTF1
/E2F
a
b/c
*
1 2
: antibody :
: peptide :
d)
32.3 (IP)
D C
DRTF1
/E2F
1 2

A monoclonal antibody producing cell line (32.3.2) was thus successfully made. 32.3 is a monoclonal antibody that will specifically identify the p55L form of DP-1 in an immunoblot on cellular extracts and also specifically binds to DP-1 in the context of DRTF1/E2F. Its properties indicated that it would be a powerful tool in the study of DP-1 as it could provide a means for distinguishing between the two immunochemically distinct forms of DP-1 : p55U and p55L. Indeed it proved to be an invaluable reagent in elucidating some of their respective properties.

Prior to making the monoclonal antibody against peptide D, two unsuccessful, and time-consuming, attempts were made to make monoclonals against two other DP-1 peptide locations.

Chapter 5

Characterisation of p55U and p55L

The availability of the polyclonal epitope affinity purified antibodies and the monoclonal 32.3 provided the means to differentiate between the two forms of the DP-1 protein and to ascertain their relative contributions to the DRTF1/E2F.

DP-1 DNA binding activity

The major function that we know to be associated with DP-1 is its contribution to the DRTF1/E2F DNA binding activity (see Introduction - Chapter 1 and Chapter 3). Thus an immediate question to ask, concerning the properties of p55U and p55L, was in relation to their respective DNA binding properties.

As part of the purification and cloning procedure of DP-1, F9EC crude cellular extracts were fractionated over heparin-Sepharose columns in order to isolate DNA binding activity. Fractions were assayed for E2F site binding activity using gel retardation assays, and positive fractions were further purified over E2F-site coupled Sepharose columns (Girling *et al.*, 1993a). Two representative heparin-Sepharose column fractions assayed for their E2F-DNA binding activity are displayed in **figure 5.1a**. Equal volumes of the two selected fractions were assayed by gel retardation in the presence of competing E2F DNA binding site (71/50) or an equivalent mutant site (60/62-see Experimental Protocols). Fraction number 19 (lanes 1 and 2) displayed no E2F-site DNA binding activity either in the presence of non-^{32}P-labelled wild-type E2F site (lane 1) or non-labelled mutant E2F site (lane 2). However fraction 39 (lanes 3 and 4) displayed a clear E2F-site DNA binding activity in the presence of un-labelled mutant E2F site (lane 4) which was abolished by the inclusion of wild-type E2F-site (lane 3) thus demonstrating the activity to be specific to the E2F site. This clearly demonstrates that fraction 19 has no detectable E2F-DNA binding activity while an equal volume loading of fraction 39 has.

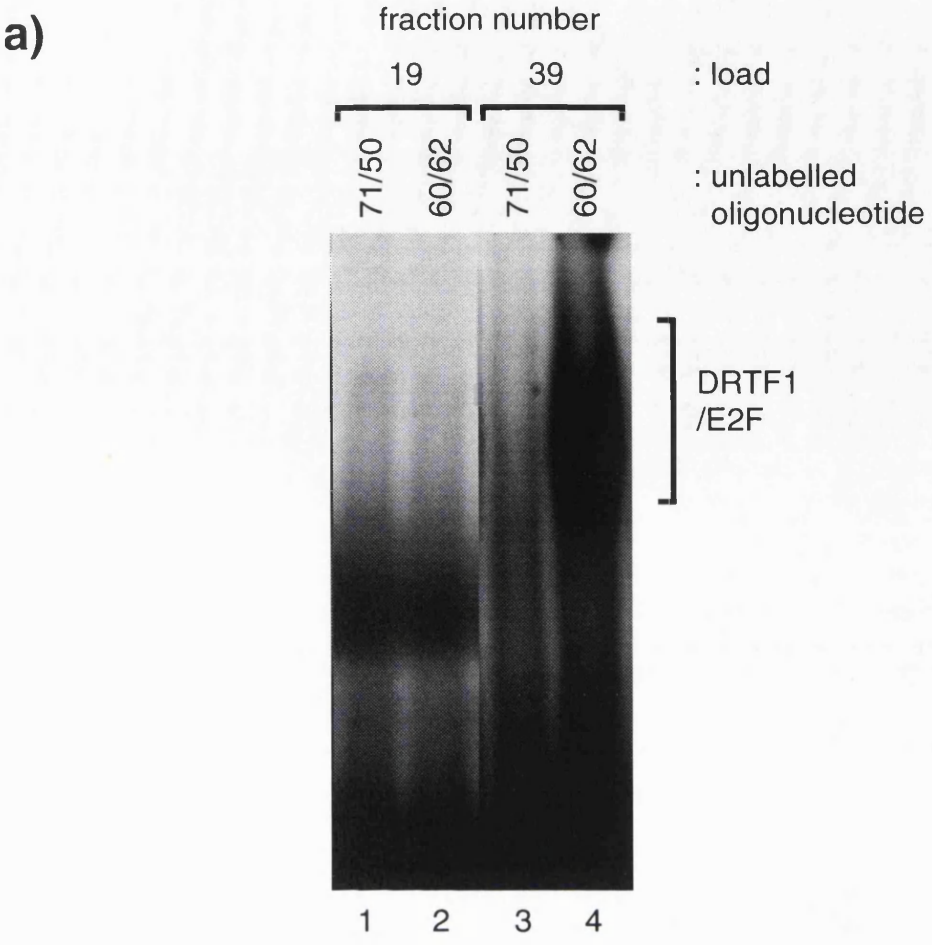
The p55 doublet as defined by immunoblotting (see Chapter 3) was found to be differentially distributed in the heparin-Sepharose fractionations of F9EC extract as exemplified by the two representative fractions in **figure 5.1b**. Equal volume loadings of the same fractions were assayed by immunoblotting with APαA and 32.3. APαA

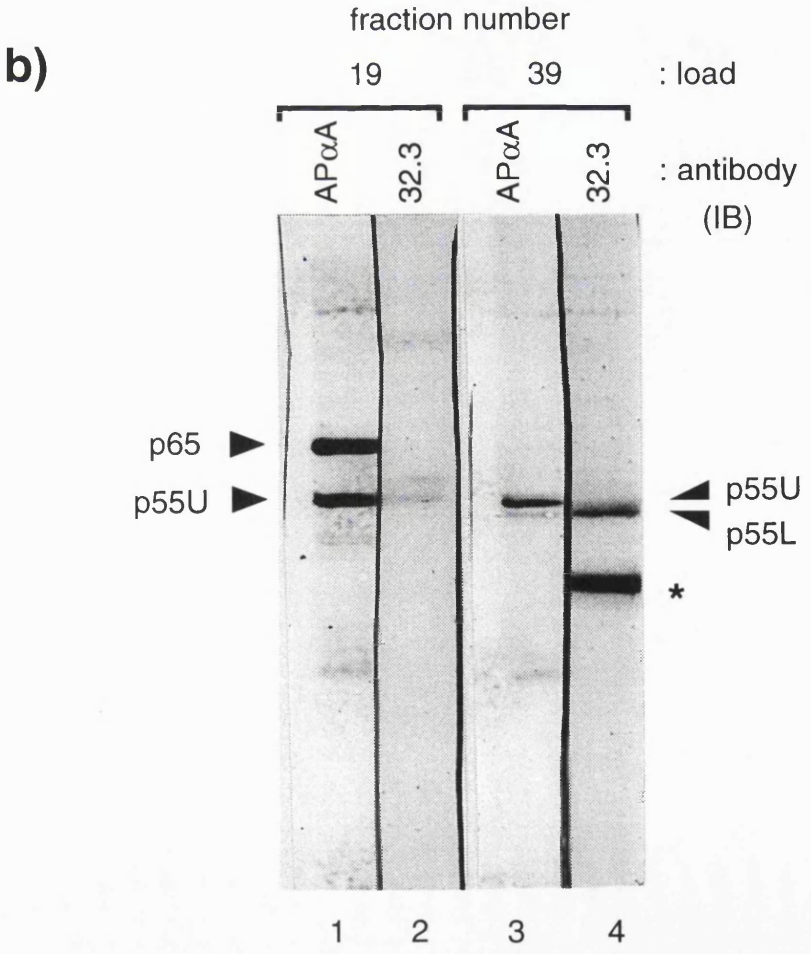
Figure 5.1

The DP-1 isoform p55L is associated with E2F-site DNA binding-positive cellular fractionates.

a. *Gel retardation assay of heparin Sepharose fractions of F9EC cell extract.*
This result exemplifies two fractions from a heparin Sepharose fractionation procedure, one containing E2F site DNA binding activity (fraction 39, lanes 3 and 4) the other not (fraction 19, lanes 1 and 2). Equal volumes (10µl) of each fraction were mixed with labelled E2F DNA binding site, as well as with unlabelled binding site (oligonucleotide : 71/50, lanes 1 and 3) or unlabelled mutant binding site (oligonucleotide : 60/62, lanes 2 and 4).

b. *Immunoblotting of heparin Sepharose fractions of F9EC cells positive and negative for E2F DNA binding activity.*
Equal volume loadings (approximately 40µl each) of heparin Sepharose fractions, positive (fracton 39 : lanes 3 and 4) and negative (fraction 19 : lanes 1 and 2) for E2F DNA binding activity, were immunoblotted (IB). The gel-lanes were cut in half and probed with different antibodies to DP-1. Lanes 1 and 3 with epitope affinity purified antisera to peptide A and lanes 2 and 4 with the monoclonal antibody 32.3. * indicates a possible degradation product.


a)
fraction number
19
39
: load
71/50
60/62
71/50
60/62
: unlabelled oligonucleotide
DRTF1 /E2F
1
2
3
4


b)
fraction number
19
39
: load
APαA
32.3
APαA
32.3
: antibody (IB)
p65
p55U
p55U
p55L
*
1
2
3
4

recognises both p55 forms of DP-1 (see Chapter 3) in whole cell microextracts, albeit p55U more strongly than p55L. 32.3 exclusively recognises p55L (**figure 4.5a**). In E2F binding positive heparin-Sepharose fractions, both p55U and p55L could be detected by APαA (lane 3), and accordingly the monoclonal antibody recognises the p55L form as well (lane 4). Curiously, 32.3 also recognised a faster migrating polypeptide (indicated by * : lane 4) which was never detected in non-fractionated extracts and thus likely to be a C-terminal-containing degradation product of DP-1 generated as a result of prolonged extract handling. Analysis of non-DNA binding fractions using the same antibody probes showed a complete absence of p55L as recognised by either APαA or 32.3 (lanes 1 and 2), but a clear presence of p55U and the non-DP-1 65KDal protein, p65 (lane 1 ; see Chapter 3). p65 was absent in the E2F-binding fraction (lane 3). APαA recognises p55L more weakly than p55U, however this difference could not explain the lack of identification of p55L in non-DNA binding fractions as 32.3 recognises p55L very strongly and detected nothing (lane 2). The experiment illustrated in **figure 5.1b** is merely a representative example of a general and very clear distinction between E2F-, and non-E2F-binding heparin-Sepharose fractions as regarding the presence of p55L or not. No non-DNA binding fractions contained p55L while DNA binding fractions would commonly contain both p55U and p55L (these data were elaborated upon in Sørensen *et al.*, 1996). Thus these experiments suggest that p55U can exist in a non-DNA binding state whilst p55L appears to be associated with DNA binding activity.

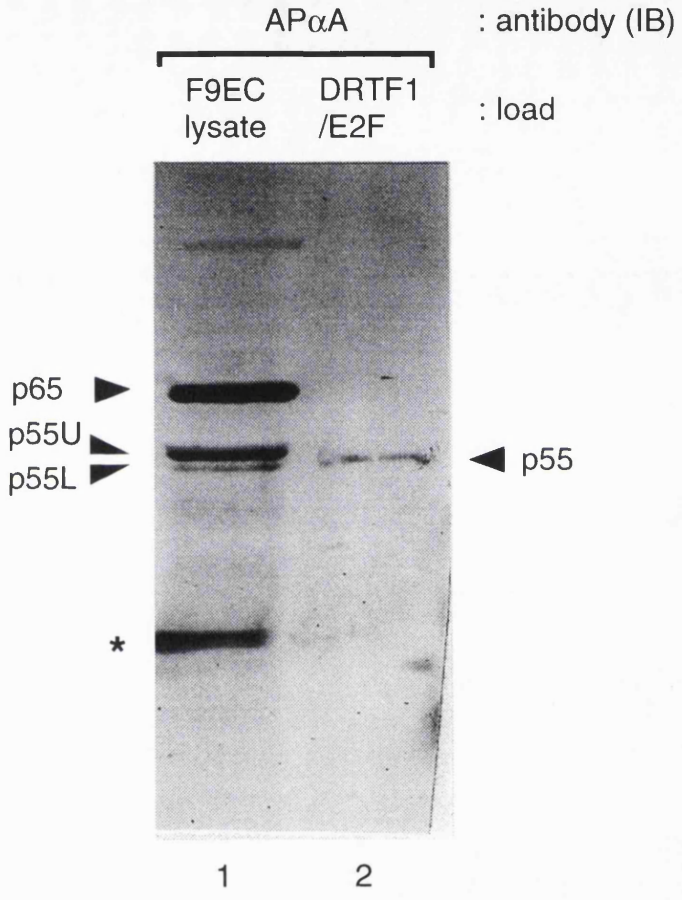
Various attempts were made to generate E2F-DNA binding activity from the non-DNA binding heparin-Sepharose fractions. Fractions were subjected to phosphatase treatment, heat treatment and detergent addition but with no effect. This was done in the eventuality that the p55U was inactive due to some enzymatic activity such as phosphorylation or due to interaction with some inhibitor. To test the possibility that the p55U form could not participate in DNA binding due to loss of some co-activator, non-DNA binding fractions were mixed with DNA binding ones, but again with no more generated E2F-site binding activity. An obvious "co-activator" candidate is the E2F-1 polypeptide which was also added to non-DNA binding fractions with no effect. This indicates that the species of DP-1 present in these fractions was inherently unable to participate in DNA binding activity.

To understand the role of the p55 doublet in DRTF1/E2F, samples of protein purified by E2F-site-bound Sepharose columns were immunoblotted with APαA and compared to the original F9EC extract from which they came (**figure 5.2**). The characteristic APαA recognition pattern is displayed in lane 1, thus the unspecific 65KDal polypeptide and the p55 DP-1 doublet (a known contaminant from a neighbouring lane is marked with *). Purified DRTF1/E2F probed with the same antibody (lane 2) had predictably lost the unspecific 65KDal band, confirming its

Figure 5.2

Just one form of DP-1 is involved in the DRTF1/E2F DNA binding complex.

Immunoblot assay of E2F-site purified DRTF1/E2F probed with APαA.
Gel lane 1 was loaded with 40μg F9EC lysate protein and lane 2 with 40μl E2F-site affinity purified, KCl-eluted DRTF1/E2F. The F9EC lysate was a sample of the original extract used for the DRTF1/E2F purification. Blotting was followed by probing with the antibody APαA. p55 indicates a single polypeptide which migrated with the p55U/L doublet but which could not with confidence be assigned to either. * indicates a known contaminant. (Immunoblot = IB).


APαA
: antibody (IB)
F9EC lysate
DRTF1 /E2F
: load
p65
p55U
p55L
p55
*
1
2

non-association with DRTF1/E2F or DP-1, but this highly purified fraction also contained only a single p55 polypeptide. The migration of the band could not be assigned with confidence to either p55U or p55L, probably due to the high KCl concentration of the sample eluting buffer, but it did appear to migrate more closely with p55L. The result, however, indicates that only one p55 polypeptide is associated with E2F-site DNA binding activity. Since the heparin-Sepharose fractions would only contain p55L in the DNA-binding-positive fractions, and not in non-DNA binding fractions (**figure 5.1b**), the possibility is thus raised that the single polypeptide observed in the purified DRTF1/E2F is in fact p55L.

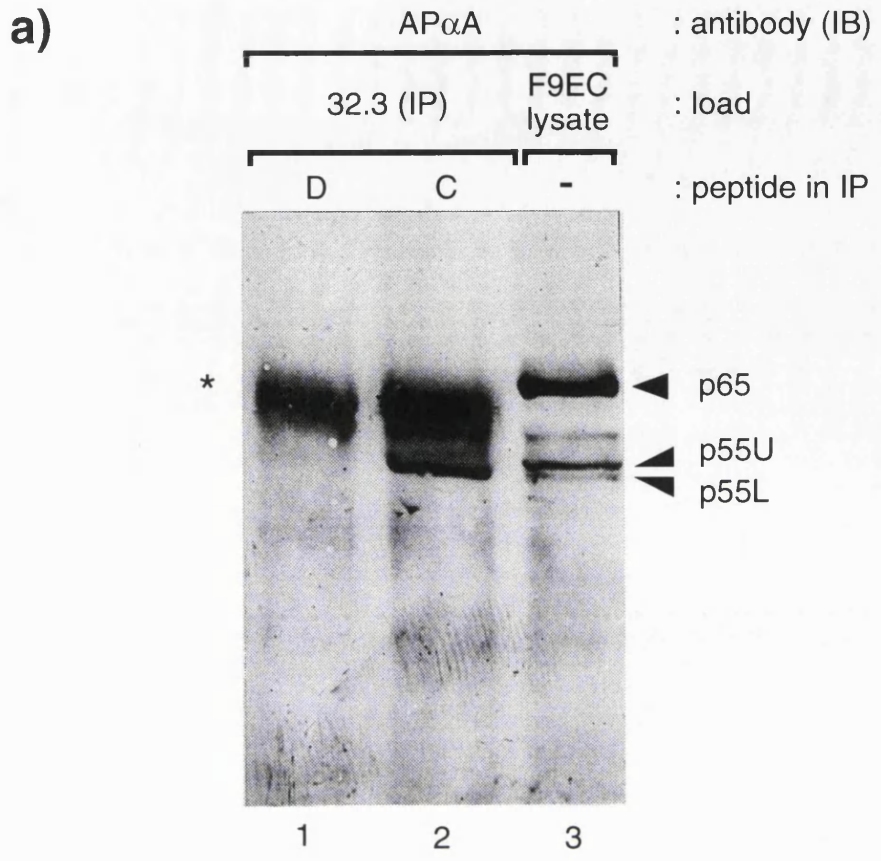
Further evidence suggesting p55L involvement in DRTF1/E2F in F9EC cells was provided by the 32.3 monoclonal antibody by virtue of its sole recognition of the p55L form. The previous chapter demonstrated by gel retardation (**figure 4.5c**) and immuno-precipitation (**figure 4.5d**) that 32.3 will bind to the DNA binding DRTF1/E2F transcription factor. 32.3 also recognises just the p55L form in immunoblots (**figure 4.5a**), but to confirm that this exclusive recognition was also the case under non-denaturing conditions, 32.3 was used to immunoprecipitate from F9EC extract and the immunoprecipitate immunoblotted with APαA which recognises both p55 forms (**figure 5.3a** and **5.3b**). The immunoprecipitation was performed in the presence of the specific peptide (peptide D - **figure 5.3a**, lane 1 ; **figure 5.3b**, lanes 1 and 2) or an unrelated peptide of similar size (**5.3a**, lane 2 ; **5.3b** lanes 3 and 4). Both immunoprecipitates and the F9EC extracts that they were made from (**5.3a**, lane 3 ; **5.3b**, lane 5) were immunoblotted with APαA which recognises both p55-forms (see Chapter 3). The large "shadows" (**5.3a** lanes 1 and 2 ; **5.3b** lanes 1-4 : labelled with *) were non-specific secondary antibody interactions with the 32.3-immunoglobulin used for the immunoprecipitation. The extract the immunoprecipitation was made from (**5.3a**, lane 3 ; **5.3b**, lane 5) clearly shows that both DP-1 forms were originally present. Immunoprecipitates made in the absence of competing peptide (**5.3a**, lane 2 ; **5.3b**, lane 3 and 4) contained p55L, as identified by APαA, and were not identified if the primary antibody incubation mixture contained specific peptide A (**figure 5.3b**, lane 3). Since 32.3 will bind to DNA binding DRTF1/E2F this results strongly suggests that p55L is a contributor to this complex.

When 32.3 hybridoma supernatants were purified (via protein A binding and HPLC) and concentrated to 0.7mg/ml preparations, it became clear that *all* detectable DRTF1/E2F activity in F9EC cells could be super-shifted by 32.3 in gel retardation assays (**figure 5.4a**). When the DNA-binding complex was exposed to 32.3 hybridoma supernatant, in (lane 2) and without (lane 1) the presence of specific peptide D, a partial super-shift was observed in the absence of competition (**figure**

Figure 5.3
p55L-DP-1 preferentially associates with the E2F DNA binding site.

a. *Immunoblot of monoclonal 32.3-immunoprecipitates from F9EC cell extract probed with APαA.*
Immunoprecipitation (IP) from F9EC LSL extract was performed using 32.3 in the presence of peptide D (D : lane 1) or peptide C (C : lane 2). The precipitates were immunoblotted (IB) next to the extract they were precipitated from (40μg. lane 3) and probed with APαA.

b. *Immunoblot of monoclonal 32.3-immunoprecipitates from F9EC cell extract probed with APαA with and without peptide competition.*
The same experiment as in **a.** except the gel lanes carrying the immunoprecipitates were cut in half after immunoblotting. They were still probed with APαA but one half (1 and 3) in the presence of peptide A and the other (lanes 2 and 4) in the presence of control peptide C.


a)
APαA
: antibody (IB)
32.3 (IP)
F9EC lysate
: load
D
C
-
: peptide in IP
*
p65
p55U
p55L
1
2
3

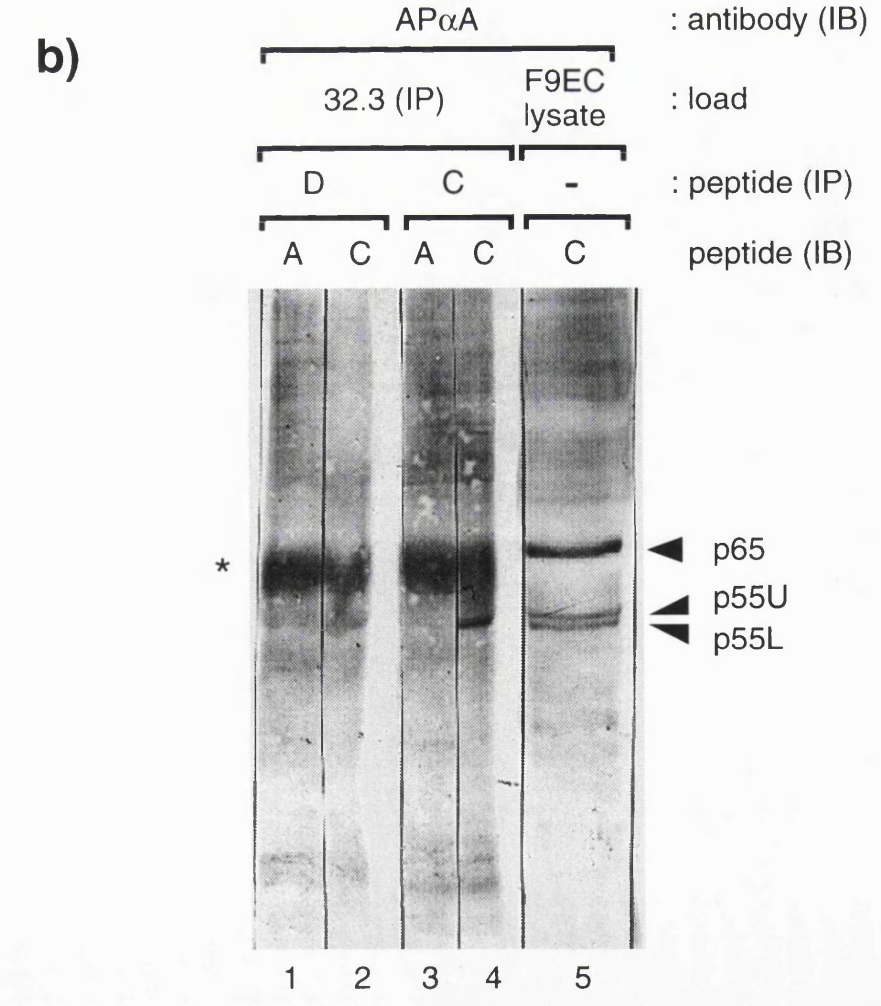

b)
APαA
: antibody (IB)
32.3 (IP)
F9EC lysate
: load
D
C
-
: peptide (IP)
A
C
A
C
C
peptide (IB)
*
p65
p55U
p55L
1
2
3
4
5

Figure 5.4

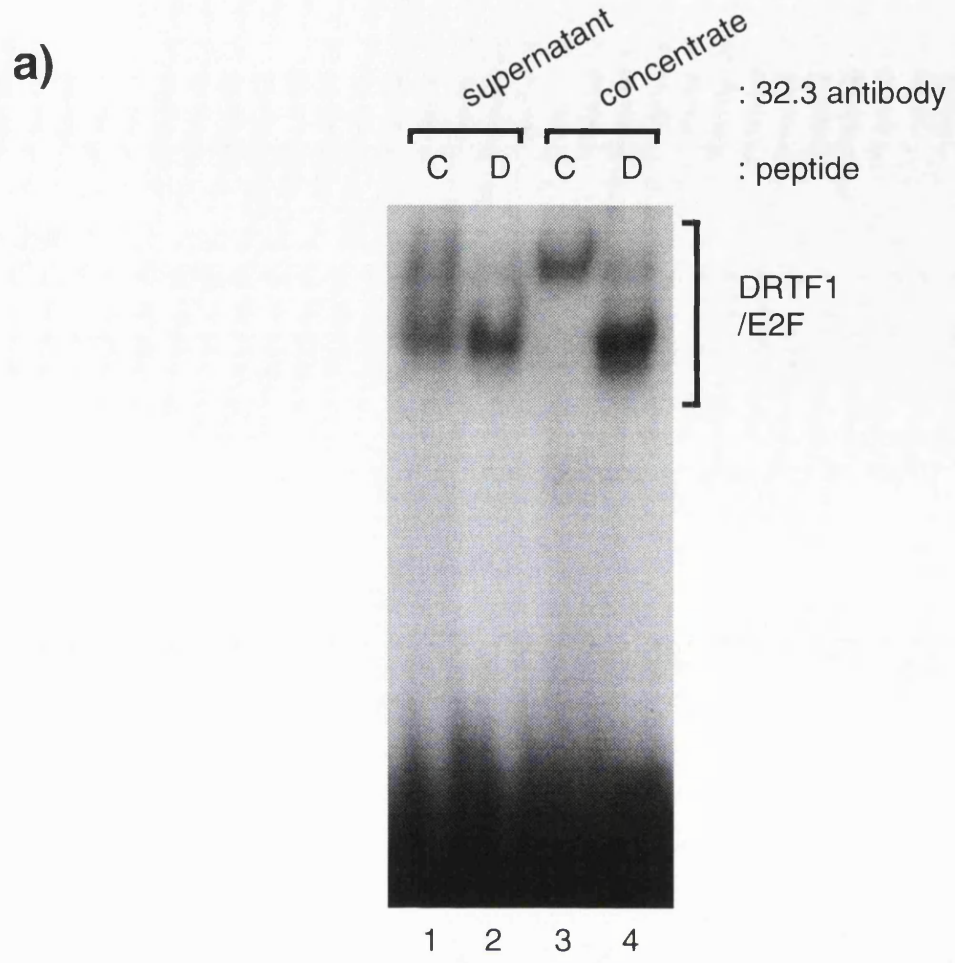
p55L is associated with all detectable DRTF1/E2F DNA binding activity in F9EC cells.

a. *Gel retardation assay of DRTF1/E2F in F9EC extract probed with different preparations of the 32.3 monoclonal antibody to DP-1.*

Four gel retardation reaction mixtures all contained 4μg of total F9EC microextract protein, labelled E2F site DNA probe, as well as maximal volume (13μl out of a 20μl reaction mixture) of 32.3 hybridoma supernatant (lanes 1 and 2) or concentrated 32.3 monoclonal antibody (0.7mg/ml - lanes 3 and 4). In each pair, the reaction mixture additionally contained peptide D (lanes 2 and 4) or control peptide C (lanes 1 and 3) at 0.2nmol/μl.

b. *Gel retardation assay of F9EC cell extracts sequentially depleted with the monoclonal 32.3.*

F9EC LSL extract was subjected to 32.3 monoclonal antibody sequential immuno-depletion (lane 2), and mock-depletion (lane 1) using PBS (as described in Experimental Protocols). Equal volumes (4μl) of the two were assayed by gel retardation using labelled E2F DNA binding site.


a)
supernatant
concentrate
: 32.3 antibody
C D C D
: peptide
DRTF1
/E2F
1 2 3 4
b)
mock-depleted
32.3-depleted
: F9EC extract
DRTF1
/E2F
1 2

5.4a). However the same treatment (lanes 3 and 4) using concentrated 32.3, appears to super-shift *all* detectable DRTF1/E2F activity (lane 3) when not exposed to competing peptide D (lane 4).

To substantiate this idea, F9EC extracts were subjected to immuno-depletion using the 32.3 monoclonal antibody (**figure 5.4b**). The extract was subjected to four consecutive immunoprecipitations using concentrated 32.3 (see Experimental Protocols, Chapter 2) and a "mock" depletion was carried out in parallel using PBS instead of antibody to control for dilution and handling effects. All detectable DRTF1/E2F activity was removed by the monoclonal (lane 2) while the control depletion procedure failed to affect DNA binding activity greatly (lane 1).

These experiments indicated that all detectable DRTF1/E2F activity in F9EC cells contains DP-1, and more specifically, the isoform p55L. It thus seems that the DNA binding activity of DP-1 results from the p55L isoform only. This is supported by the detection of just one DP-1 polypeptide in the E2F site purified fractions (**figure 5.2**). p55U thus appears to be an abundant form of DP-1 which however lacks the ability to participate in DNA binding complexes. The observations on non-fractionated cell extracts using 32.3 are important because the fractionation results could have been interpreted to suggest that p55U had lost some essential co-factor in non-DNA binding fractions. The results obtained with 32.3 however, suggest that p55U, in whatever complex status it may be in, does probably not participate in E2F site DNA binding.

Early suspicions that the two forms of DP-1, defined here, might be cell cycle regulated led to studies using serum starved NIH-3T3 cells (Bandara *et al.*, 1994). Cell-extracts were taken at different times after re-stimulation and assayed by gel retardation for E2F site DNA binding activity. As demonstrated by many other groups (see Introduction), the DRTF1/E2F activity is low at G0 and rises progressively to a peak at the G1/S boundary. DP-1 was demonstrated by super-shifting to be present at all stages of the cell cycle in the DRTF1/E2F complexes. When the same extracts were assayed by immunoblotting using the APαA reagent (the same as described in chapter 3), p55U was found to be present throughout the cell cycle while p55L only became detectable concomitantly with the induction of DRTF1/E2F activity at G1/S (Bandara *et al.*, 1994). This result beautifully supports both the perception of p55L as the DNA-binding form of DP-1, and also implicates the p55L, and hence the state of DP-1, with the cell cycle regulation of the DRTF1/E2F.

To ensure that the p55L, as defined by APαA, behaved in a similar manner when recognised by 32.3 in an immunoblot, a simple replica of the above experiment was performed. NIH-3T3 cells were allowed to grow to confluence and then deprived of full foetal calf serum concentration at 10% ($^{v}/_{v}$) by reduction to 0.1% ($^{v}/_{v}$) for 72 hours. The cells were restimulated and grown for a further 24 hours with duplicate

treatments harvested at four-hour intervals during this period. Such treatment arrests cells in G0 and re-stimulation pushes them into one complete round of replication in approximately 24 hours back to G1 (Bandara *et al.*, 1994). Three representative NIH-3T3 microextracts, zero, 16 and 24 hours post stimulation, were immunoblotted with the APαA and 32.3 antibody reagents (**figure 5.5a**). Lanes 1 and 2 display the respective recognition patterns of APαA and 32.3, identifying both p55 forms and the p55L form only, respectively, in asynchronous NIH 3T3 cells. The recognition pattern of p55 displayed by APαA was very similar to that reported by Bandara *et al.* (1994) with p55U present in all samples (lanes 3, 5 and 7), but with p55L absent at 0 hours (G0 ; lane 3), present at 16 hours (G1/S ; lane 5) and reduced again at 24 hours (lane 7). This recognition pattern was mirrored by 32.3 (lanes 4, 6 and 8) thus making a connection between the p55L identified by these two immunochemical reagents in this assay. The p55L as defined by 32.3 was not completely absent at zero hours which is likely simply to reflect higher affinity of 32.3 for this polypeptide, detecting levels of p55L so low that they were not picked up by the APαA reagent. It is difficult to completely arrest all cells by serum deprivation and this could explain why some p55L was detectable at zero hours by the more sensitive 32.3. Alternatively the more sensitive detection by 32.3 could have revealed that not all p55L is removed at G0. This is an area which is in need of further investigation.

As a control, the E2F DNA binding activity was assayed for each extract (**figure 5.5b**) and confirms low levels at zero hours (lane 2, relative to an asynchronous extract lane 1), high at 16 (lane 3) and reduced again at 24 (lane 4), again correlating with p55L abundance (**figure 5.5a**). Each of these samples was assayed in the presence of 32.3 and peptide D, which cancels out the antibody activity. When the peptide D was replaced by an unrelated control peptide (lanes 5-8) it was seen that all detectable activities were supershifted. This could suggest that DRTF1/E2F complexes at different stages of the cell cycle of NIH-3T3 cells all involve DP-1/p55L.

p55 association with E2F-1

The DRTF1/E2F DNA binding activity depends upon the heterodimerisation of an E2F family member and a DP family partner (see Introduction). Investigations were therefore undertaken to determine the nature of the DP-1/p55 isoform association with E2F family members.

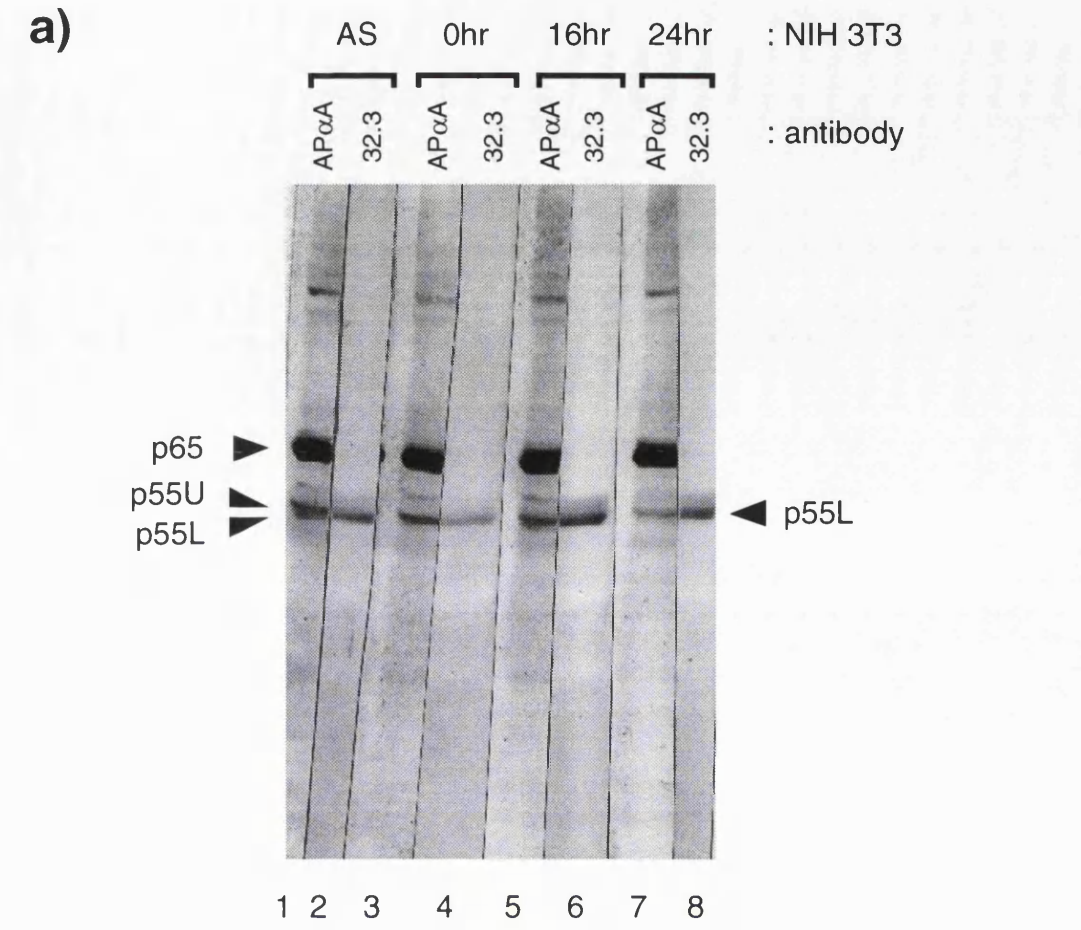
If DP-1/p55L is involved in DRTF1/E2F DNA binding, then it would be expected that this form of DP-1 would be able to form an association with one of the E2F family partners, such as E2F-1. An experiment was conducted to test this by assaying the ability of 32.3-bound DP-1 to associate with E2F-1. E2F-1 was *in vitro*

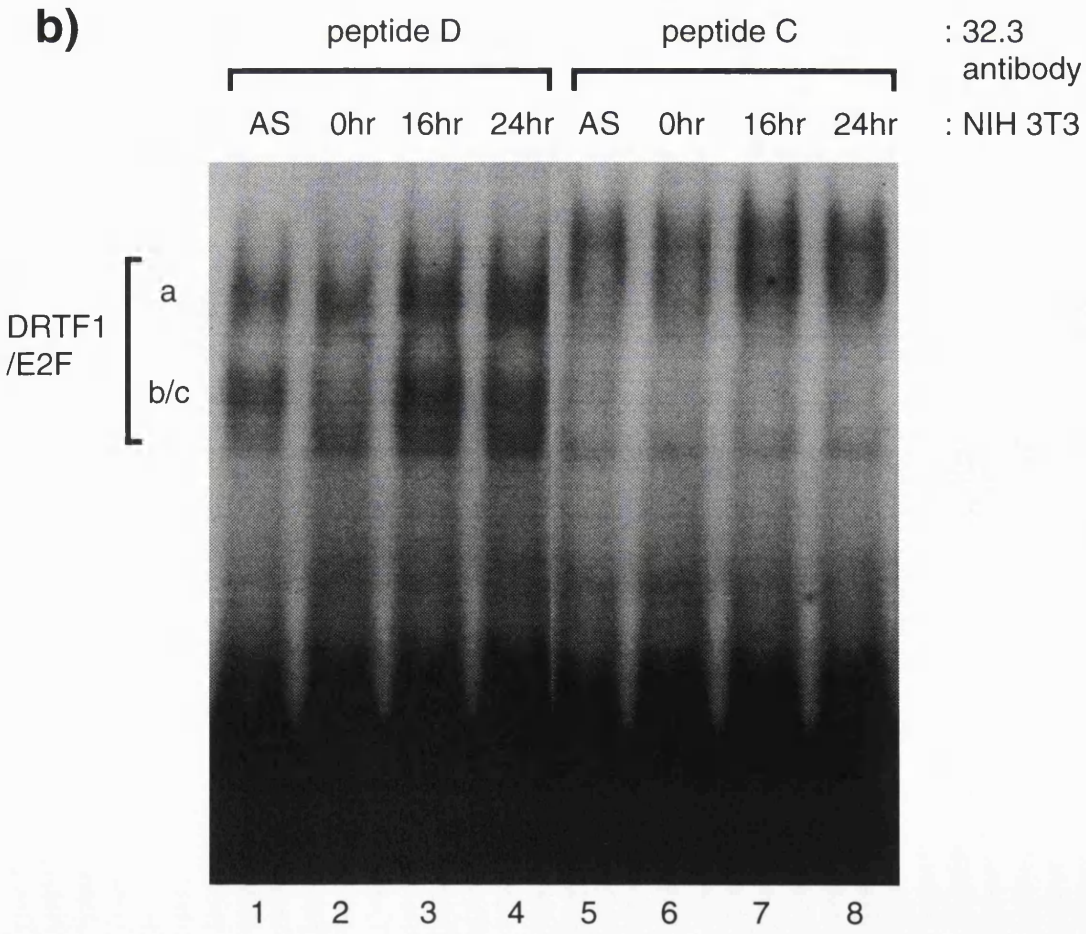
Figure 5.5

The abundance of p55L is regulated during the progression of the cell cycle and correlates with DRTF1/E2F DNA binding activity in NIH 3T3 cells.

a. *Immunoblot of NIH 3T3 cell microextracts from different stages post-serum depletion/stimulation.*
Microextracts (40μg of total extract protein) of asynchronous (AS) and zero (0hr), 16 (16hr) and 24 (24hr) hours post-serum deprivation/stimulation NIH 3T3 cells were immunoblotted. Each gel-lane was cut in half and treated with APαA (odd lane numbers) or 32.3 (even lane numbers).

b. *Gel retardation assay of NIH 3T3 cell microextracts from different stages post-serum depletion/stimulation probed with the monoclonal antibody 32.3.*
Total protein of 4μg from microextracts of asynchronous (AS) and zero (0hr), 16 (16hr) and 24 (24hr) hours post-serum deprivation/stimulation NIH 3T3 cells were assayed by gel retardation using labelled E2F site DNA probe. All reaction mixtures contained 5μl of concentrated 32.3 monoclonal (0.7μg/μl) in the presence of either peptide D (lanes 1 to 4) or peptide C (lanes 5 to 8) at 0.2nmol/μl. Gel-retarded sub-complexes of DRTF1/E2F are indicated (a and b/c) and * indicates supershifted complex.


a)
AS
0hr
16hr
24hr
: NIH 3T3
APαA
32.3
: antibody
p65
p55U
p55L
p55L
1 2 3 4 5 6 7 8


b)
peptide D
peptide C
: 32.3 antibody
AS 0hr 16hr 24hr AS 0hr 16hr 24hr
: NIH 3T3
DRTF1 /E2F
a
b/c
1 2 3 4 5 6 7 8

translated in the presence of ^{35}S-labelled methionine using a commercial reticulolysate (see Experimental Protocols). This was mixed with *in vitro* translated, non-labelled, DP-1 reticulolysate, or non-programmed reticulolysate reagent only. The mixtures were then subjected to immunoprecipitation using 32.3 and the precipitates analysed by SDS PAGE (**figure 5.6**). 32.3 immunoprecipitated E2F-1 in the presence of DP-1 (lane 1) but not in its absence (lane 2). The E2F-1 gene was slightly C-terminally truncated (see Experimental Protocols) and its protein product migration corresponded well to the predicted size of around 50KDal (**figure 5.6**, lane 1). The experiment indicated that the monoclonal 32.3 binds to a form of DP-1 which is able to engage in a heterodimeric interaction with E2F-1 *in vitro*. This suggests that p55L, being the only form that is bound by 32.3, will also heterodimerise with E2F-1.

To investigate this further, E2F-1-GST fusion protein, and GST protein alone, were expressed in and purified from *E.coli*, but allowed to remain attached to Sepharose-glutathione-beads (see Experimental Protocols). The beads were analysed by SDS-PAGE and Coomassie stained (**figure 5.7a** and **b**) to ensure that the attached polypeptide fusions were pure and of the predicted sizes. The N-terminally truncated E2F-1 fused to GST was predicted to be around 67KDal which corresponded well with the purified polypeptide indicated in **figure 5.7a**, lane 2. The purified GST protein alone migrated, as expected (Smith & Johnson, 1988), at around 26KDal (**figure 5.7b**). The fusion-protein bearing beads were then incubated with F9EC extract, washed, and immunoblotted with APαD (**figure 5.8**, see Experimental Protocols). Approximately a third total protein of GST alone was used relative to GST-E2F-1 to approximate equimolar GST domain exposure to the extracts. The F9EC lysate contained the lower form of p55, preferentially recognised by APαD (indicated : lane 1). Incubation of the extract with GST-E2F-1 (lanes 2 and 3) precipitated a polypeptide which was identified by APαD in the presence of a control peptide (lane 2) but not in the presence of peptide D (lane 3), and which co-migrated with p55L. No such polypeptide was observed in the same experiment when the precipitation was carried out with GST-protein alone (lanes 4 and 5). This indicates that p55L can interact with E2F-1.

This experiment did however not address the role of p55U in heterodimerisation and so similar experiments were carried out in which both APαD and APαA were employed for immunoblotting. APαA can identify both forms of p55 (see Chapter 3) and would potentially distinguish any differential affinity between the two for E2F-1. Unfortunately, despite many attempts, it never proved possible to detect either p55 form in a GST-E2F-1-precipitate using APαA. APαA generally detected p55U more strongly than p55L in an immunoblot (for example **figure 3.9b**, lane 1 and **figure 5.2**, lane 1). APαD detected p55L much more efficiently than

Figure 5.6

The monoclonal antibody 32.3 identifies a form of DP-1 that can interact with E2F-1 thus associating the DP-1 p55L form with one that can bind E2F-1.

SDS PAGE and autoradiographically analysed immunoprecipitates of in vitro translated protein.

50µl ^{35}S-methionine labelled reticulolysate *in vitro* translated E2F-1 was mixed with an equal volume of unlabelled DP-1 reticulolysate (lane 1) or plain untranslated reticulolysate (lane 2). The mixtures were incubated on wet ice for one hour and then subjected to immunoprecipitation (IP) by the monoclonal antibody 32.3. The immunoprecipitates were analysed by SDS-PAGE and autoradiography. The migration of standard molecular weight protein markers is indicated in KDal.

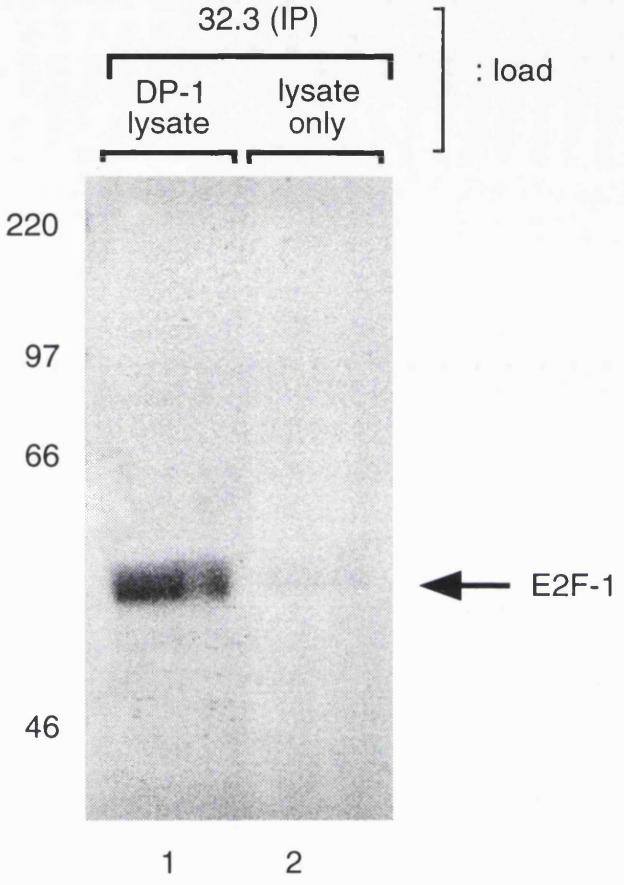

32.3 (IP)
: load
DP-1 lysate
lysate only
220
97
66
46
E2F-1
1
2

Figure 5.7

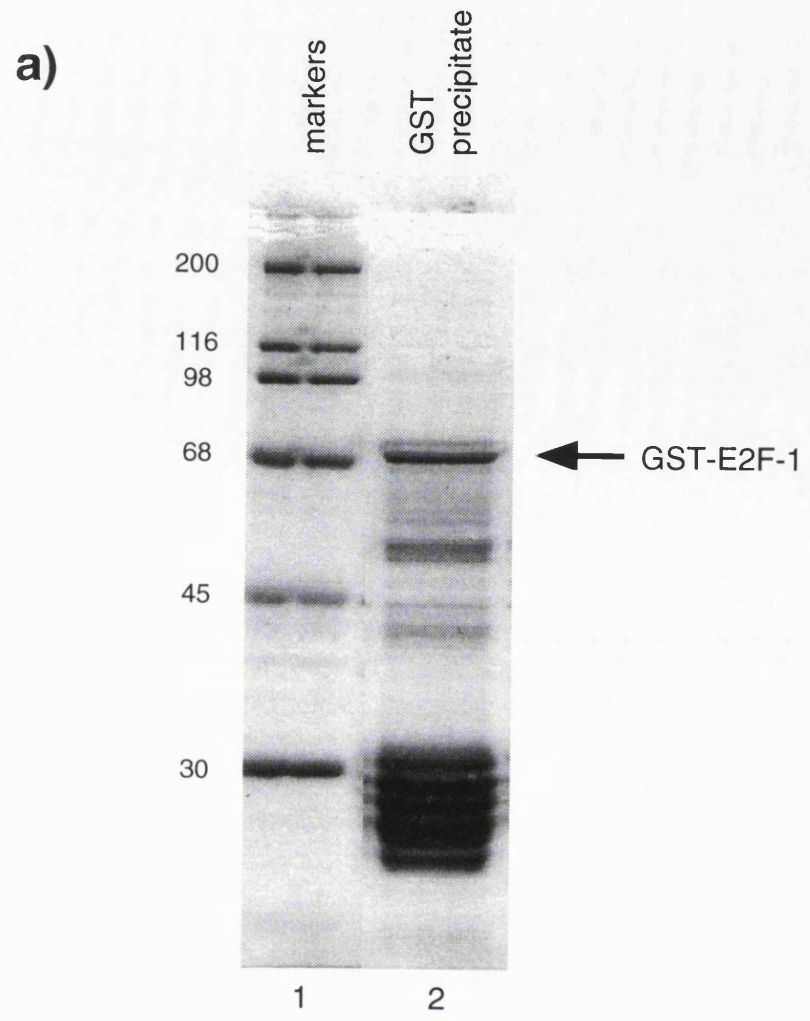
SDS PAGE analysis of bacterially expressed glutathione S-transferase fused recombinant E2F-1 protein-associated with Sepharose beads.

a. *SDS PAGE analysis of bacterially expressed glutathione S-transferase fused recombinant E2F-1 protein-beads.*

A 15µl sample (solid) of GST-E2F-1 recombinant fusion protein still retained on the glutathione beads with which it was purified (lane 2) was analysed by SDS PAGE and coomassie staining next to standard molecular weight protein markers labelled with their sizes in KDal (lane 1). The estimated full-length fusion protein is indicated (GST-E2F-1, see main text)

b. *SDS PAGE analysis of bacterially expressed glutathione S-transferase protein-beads.*

GST protein-only beads (15µl sample, solid, lane 2), made as a control for the GST-E2F-1 beads, analysed by SDS PAGE and coomassie staining next to standard molecular weight protein markers (lane 1) labelled with their sizes in KDal.


a)
markers
GST precipitate
200
116
98
68
45
30
GST-E2F-1
1
2

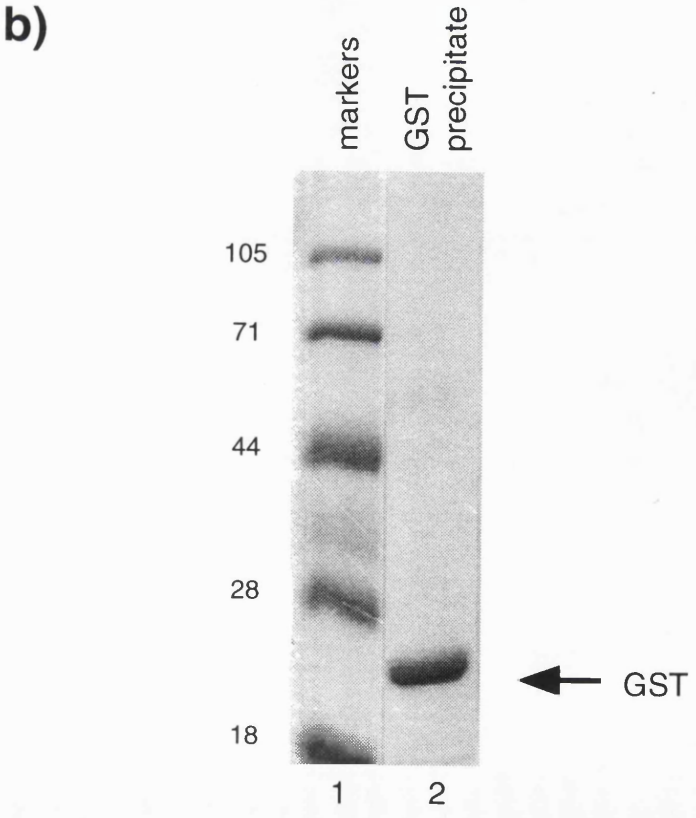
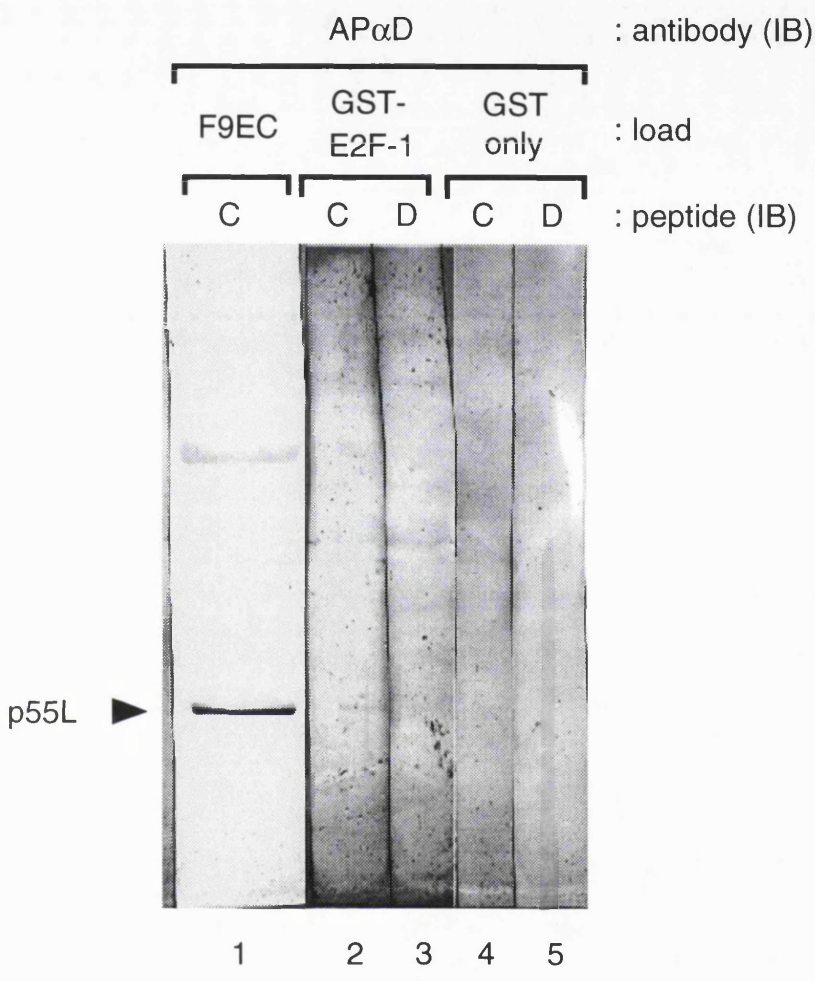

b)
markers
GST precipitate
105
71
44
28
18
GST
1
2

Figure 5.8

Bacterially expressed GST-E2F-1 fusion protein will complex DP-1 p55L.

Immunoblot of GST-protein bead precipitates probed with an antibody to DP-1.

Approximately 250ng of GST-E2F-1 bound to beads was mixed with LSL F9EC extract sample and then precipitated. Precipitates were analysed by immunoblotting (IB) with APαD. Gel-lanes were cut in half and probed in the presence of peptide C (lane 2) or peptide D (lane 3). As a control, around 80 ng GST protein only on beads was put through the same procedure (lanes 4 and 5). Also loaded was a 40μg sample of the extract the precipitations were made from (F9EC : lane 1). The DP-1 form, p55L, is indicated.


APαD
: antibody (IB)
F9EC
GST-
E2F-1
GST
only
: load
C
C
D
C
D
: peptide (IB)
p55L
1
2
3
4
5

APαA (**figure 3.4a**, lane 3 and 4) yet, at best, only barely detected this polypeptide in a GST-E2F-1 precipitate (**figure 5.8**, lane 2). Thus precipitated levels of p55L were probably too low to be detected by APαA, but p55U was never detected despite being strongly recognised by the same antibody. This could suggest that p55U is not complexed by E2F-1 under the conditions of this experiment.

Attempts to clarify the idea that p55U does not interact with E2F-1 was pursued with immunoprecipitation studies. Immunoprecipitation of the DP-1 form(s) with antibodies to E2F-1 was however unsuccessful, possibly a reflection of the low amount of E2F-1 in F9EC cells as these antibodies detected only a weak signal in F9EC extracts. Immunoprecipitations using HeLa cell extracts, the cell line from where E2F-1 was cloned (Helin *et al.*, 1992), also failed. A drawback of the GST-E2F-1 studies is that they did not involve native E2F family members due to the problems of E2F-1 detection *in vivo*. Another recently cloned E2F family member, E2F-5, was cloned from F9EC cells and is present in greater abundance in several cell lines, and so provided an opportunity to study the p55 association with other E2F partners *in vivo*.

p55 association with E2F-5

E2F-5 was cloned using DP-1 as a bait in a yeast two-hybrid detection screening of an F9EC cDNA library (Buck *et al.*, 1995). The predicted amino acid sequence was studied and three sites were selected on the basis of their high proportions of polar amino acid residues and their being different from equivalent regions in other known E2F-family members. Three peptides were thus synthesised representing these regions in E2F-5 : Peptide V1, peptide V2 and the C-terminal peptide, VC (see Experimental Protocols and **figure 5.9**). It can be seen that all three peptides bear high, but not complete homology to equivalent regions in E2F-4, but are all quite different from these regions in E2F-1, 2 and 3 (**figure 5.9**). The peptides were coupled to BSA and used for the immunisation of rabbits (see Experimental Protocols).

Antisera to peptide V1, V2 and VC (αV1, αV2 and αVC respectively) will all specifically disrupt the DRTF1/E2F complex from F9EC cells in a gel retardation assay indicating that antibodies within them interact with a component of the complex (**figure 5.10**). Each anti-sera did not affect DRTF1/E2F in the presence of their

Figure 5.9

The E2F-5 peptides are distinct from equivalent regions in other E2F-family members.

Comparisons of the amino acid sequences of E2F-5 peptides with equivalent sequences in other E2F-proteins.

For each E2F-5 peptide (amino acid residue representative letters displayed in blue) a line-up with equivalent regions in other E2F-family members is presented. The amino acid residues of these sequences are displayed in blue if they are identical to the E2F-5 sequence, and in red, if different.

E2F-5 Peptide V1 (amino acids 106-123)

E2F-5	V	G	A	G	C	N	T	K	E	V	I	D	R	L	R	C
E2F-4	V	G	P	G	C	N	T	R	E	I	A	D	K	L	I	E
E2F-3	C	S	L	-	S	E	D	G	G	N	A	G	Q	C	Q	G
E2F-2	R	G	M	-	F	E	D	P	T	R	P	G	K	Q	Q	Q
E2F-1	S	-	-	-	H	T	T	V	G	V	G	G	R	L	E	G

E2F-5 Peptide V2 (amino acids 192-203)

E2F-5	I	P	E	M	G	Q	N	G	Q	K	K	Y
E2F-4	I	P	E	-	G	L	N	G	Q	K	K	K
E2F-3	-	-	-	-	-	D	S	I	E	-	S	L
E2F-2	-	-	-	-	-	D	R	T	E	D	N	L
E2F-1	-	-	-	-	-	D	S	S	E	-	N	F

E2F-5 Peptide VC (amino acids 320-335)

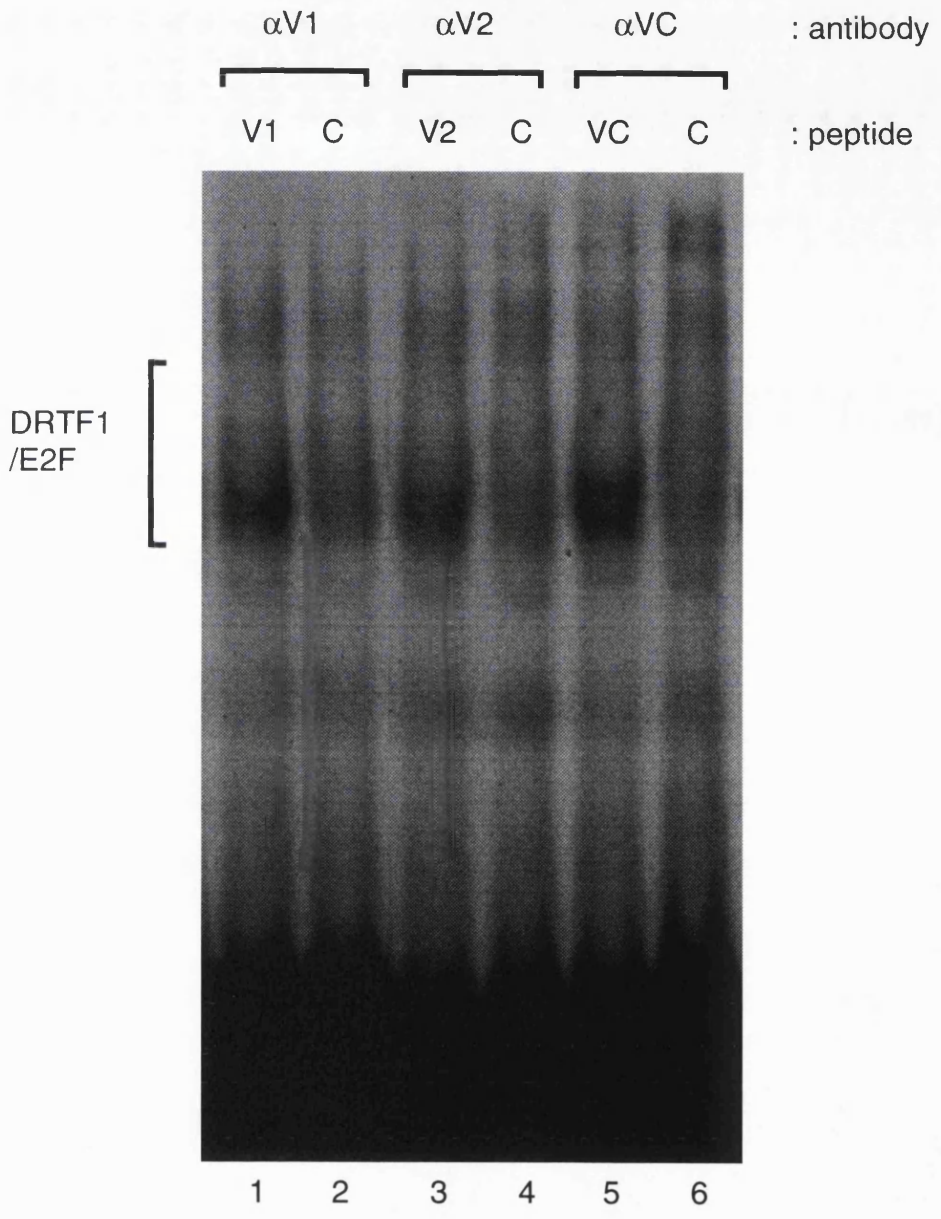
E2F-5	N	E	G	V	C	D	L	F	D	V	Q	I	L	N	Y
E2F-4	S	E	G	V	C	D	L	F	D	V	P	V	L	N	L
E2F-3	E	E	G	I	S	D	L	F	D	A	Y	-	-	D	L
E2F-2	G	E	G	I	S	D	L	F	D	S	Y	-	-	D	L
E2F-1	G	E	G	I	R	D	L	F	D	C	D	F	G	D	L

Figure 5.10

Antibodies to E2F-5 peptides disrupt the DRTF1/E2F complex in F9EC cells in gel retardation assays.

Gel retardation assay of F9EC extract probed with the E2F DNA binding site and antibodies to E2F-5.

F9EC microextract (4μg total extract protein each) was assayed in six reactions by gel retardation using the E2F site probe. Included in the reactions was the anti-peptide V1 antibody (αV1 : lanes 1 and 2 ; including either peptide V1, lane 1 : V1, or the control peptide C, lane 2 : C), the anti-peptide V2 antibody (αV2 : lanes 3 and 4, including either peptide V2, lane 3 : V2, or the control peptide C, lane 4 : C) and the anti-peptide VC antibody (αVC : lane 5 and 6, including either peptide VC, lane 5 : VC, or the control peptide C, lane 6 : C).


αV1
αV2
αVC
: antibody
V1
C
V2
C
VC
C
: peptide
DRTF1
/E2F
1
2
3
4
5
6

respective specific peptides that they were raised to (lanes 1, 3 and 5) but reduced the E2F-site binding activity of the same extract in the presence of a control peptide (lanes 2, 4 and 6). This indicates that in F9EC cells, just as with DP-1/p55L, E2F-5 appears to be a significant contributor to DRTF1/E2F activity, raising the possibility that the two factors interact in this cell line.

The same antisera were used to immunoblot F9EC extract in order to identify the physiological E2F-5 polypeptide. As with the analysis of DP-1 (Chapter 3) it was hoped that more than one of these antisera, raised to different regions of E2F-5, would identify an identically migrating specific polypeptide, thus suggesting E2F-5 identity as opposed to a specific cross-reaction. Unfortunately none of the antisera to peptide V1, V2 or VC identified even similarly-migrating polypeptides (**figure 5.11a** and **5.11b**). When αV1 antisera was used to probe an immunoblot of F9EC extract, several polypeptides would be identified in the presence of control peptide (**figure 5.11a**, lane 2) but not in the presence of the specific peptide V1 (lane 1). From the presumed length of E2F-5 (335 amino acid residues, Buck *et al.*, 1995) its physiological form was predicted to be an approximate size of 40KDal. Only one polypeptide specifically identified by αV1 was in this range (lane 2, indicated by *) and thus potentially E2F-5. Antisera to peptide V2 failed to identify any polypeptides in the presence of control peptide (**figure 5.11a**, lane 4) that were competed out by specific peptide V2 (lane 3). This was despite the property of αV2 to specifically recognise DRTF1/E2F in the same extract under the non-denaturing conditions of a gel retardation assay (**figure 5.10**). Antisera to peptide VC specifically identified a polypeptide of approximately 47KDal (see below and **figure 5.11b**, lane 5 and 6) which thus was different to the polypeptide identified by αV1 and hence, like it, therefore only a potential candidate for E2F-5.

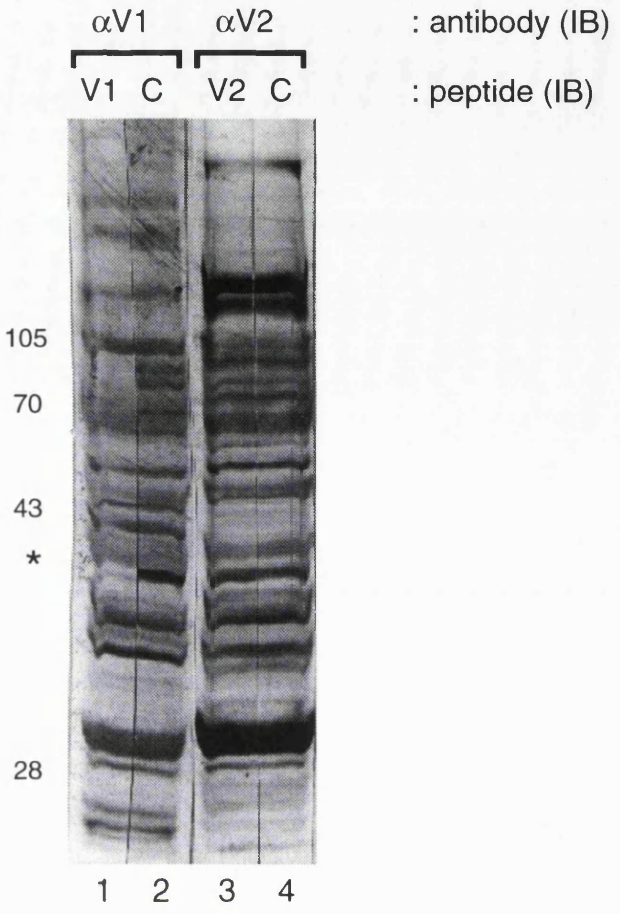
As an alternative strategy to identify the E2F-5 polypeptide, the different antisera raised against E2F-5 peptides were used to screen immunoprecipitates of the monoclonal antibody 32.3. Since E2F-5 was cloned by virtue of its interaction with DP-1 in F9EC cells, then a DP-1-interacting polypeptide that is recognised specifically by one of the antisera to E2F-5 in extracts from these cells would be very likely to be E2F-5. 32.3 was used in immunoprecipitation experiments using F9EC LSL cell extracts (**figure 5.11b**, see Experimental Protocols), with (lanes 1 and 2) and without (lanes 3 and 4) peptide D competition. Each precipitate was probed in an immunoblot by the polyclonal anti-E2F-5, αVC, with (lanes 1 and 3) and without (lanes 2 and 4) competition from the peptide VC. Only immunoprecipitates made in the presence of control peptide appeared to precipitate a polypeptide which again was solely recognised by αVC in the absence of competing peptide VC (lane 4). This polypeptide is thus likely to be E2F-5 by virtue of its mobility (around 47KDal) and by its co-migration with a polypeptide identified (in the absence of peptide VC) by

Figure 5.11
E2F-5 associates with DP-1 p55L.

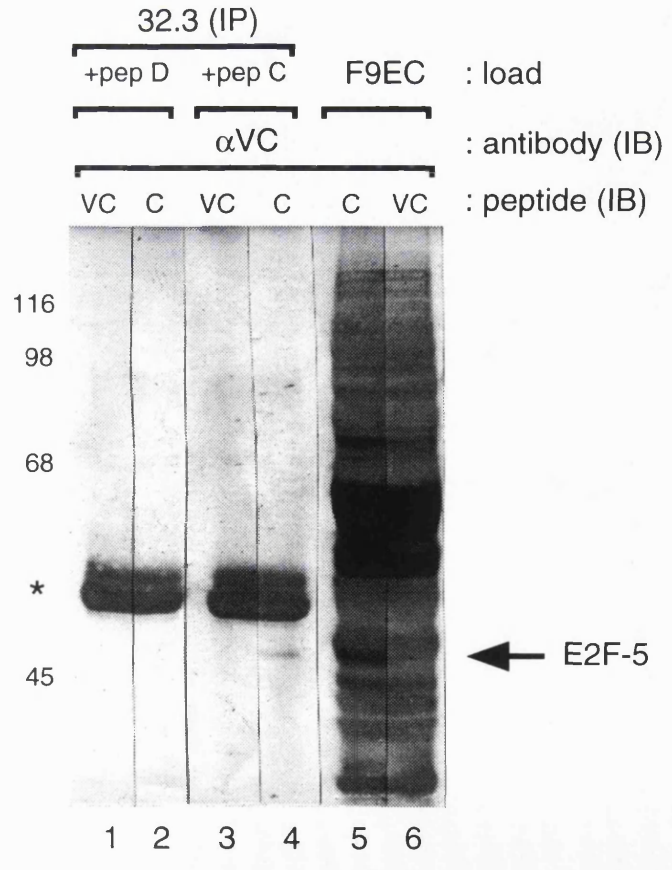
a. *Immunoblotting F9EC extract with antisera to E2F-5 peptides.*
F9EC microextract was loaded into two gel-lanes (40μg each) and immunoblotted (IB). Lanes 1 and 2 were probed with αV1 in the presence of peptide V1 (lane 1) or peptide C (lane 2). Lanes 3 and 4 were probed with αV2 in the presence of peptide V2 (lane 3) or peptide C (lane 4). Standard molecular weight protein markers are indicated in KDal. * indicates a specifically identified polypeptide in lane 2.

b. *The monoclonal antibody 32.3 will specifically immunoprecipitate E2F-5.*
Immunoprecipitations (IP) from F9EC LSL extract was carried out by 32.3 in the presence of either peptide D (pep D : lanes 1 and 2) or the control peptide C (pep C : lanes 3 and 4) and assayed by immunoblotting (IB) next to the F9EC extract (lanes 5 and 6) the immunoprecipitates were made from. The gel-lanes were then cut into halves and probed with E2F-5 anti-peptide VC anti-sera (αVC). Incubation mixtures included peptide VC (VC - lanes 1, 3 and 6) or peptide C (C - Lanes 2, 4 and 5). * indicates non-specific antibody cross-reaction with the large amounts of monoclonal antibody used in the immunoprecipitations. Standard molecular weight protein markers are indicated in KDal.

a)


αV1
αV2
: antibody (IB)
V1 C
V2 C
: peptide (IB)
105
70
43
*
28
1 2 3 4

b)


32.3 (IP)
+pep D
+pep C
F9EC
: load
αVC
: antibody (IB)
VC C VC C C VC
: peptide (IB)
116
98
68
*
45
E2F-5
1 2 3 4 5 6

the same antisera (αVC) in the F9EC cell extract that the immunoprecipitation was performed from (lane 5). The recognition of this polypeptide was specifically competed out by the presence of peptide VC (lane 6). Neither αV1 nor αV2 specifically identified 32.3 immunoprecipitated polypeptides. αVC antisera also specifically recognised GST-E2F-5 in an immunoblot (Elizabeth Allen - personal communication) adding to the certainty that the 47KDal polypeptide is E2F-5. It is not necessarily of concern that the polypeptide identified by αV1 had a migration closer to the predicted one for E2F-5 (40KDal) since physiological protein products are often post-translationally modified which adds to their molecular mass. The predicted size of DP-1, for example, is 48KDal (from the sequence of Girling *et al.*, 1993), while the actual cellular protein turned out to be 55KDal (see Chapter 3). The peptide VC displays a very high homology to the equivalent region in E2F-4 (see **figure 5.9**) which raises concerns about possible cross-reactivity to this closely-related family member. However physiological E2F-4 migrates as a heterogeneous set of polypeptides of the range 57 to 64KDal in an immunoblot (Ginsberg *et al.*, 1994) and so unlikely to be the 47KDal polypeptide identified here.

As well as identifying the physiological polypeptide form of E2F-5, this experiment also serves as *in vivo* confirmation that the p55L form of DP-1 can interact with at least one E2F-family member.

The interaction of DP-1 with E2F-5 in F9EC cell extracts was very clearly demonstrated by immunoprecipitation with 32.3. An investigation was undertaken to determine how prevalent this interaction is in naturally occurring animal tissues. Since both the antibodies to DP-1 and to E2F-5 were against murine forms, the animal of choice for this study was the mouse. Microextracts were made from nine different mouse tissues and subjected to immunoblotting with APαA (**figure 5.12a**). p55 could be detected in all tissues, bar striated muscle and liver (lanes 6 and 7), but due to the poor clarity of these blots, could not easily be assigned to either p55U or p55L. Very high total protein concentrations of these extracts had to be employed in order to detect polypeptides of interest, this however often had the effect of distorting the polyacrylamide gel somewhat. The same extracts, and F9EC cell culture microextract as a control, were assayed with αVC in an immunoblot (**figure 5.12b**). Each extract was probed in the presence of specific peptide VC (odd lane numbers) or in the presence of a control peptide (even lane numbers). In general the presence of E2F-5 correlated very well with that of p55 which supported the indication from the studies on F9EC cells that the two polypeptides may be partners of each other in a physiological context. The only notable exception to this was adopose tissue (from the "fat sack") and heart muscle which clearly contained p55 (**figure 5.12a**, lanes 1 and 4 respectively) but showed no evidence of containing E2F-5 (**figure 5.12b**, lanes

Figure 5.12
DP-1/E2F-5 heterodimers can be detected in multiple murine tissues.

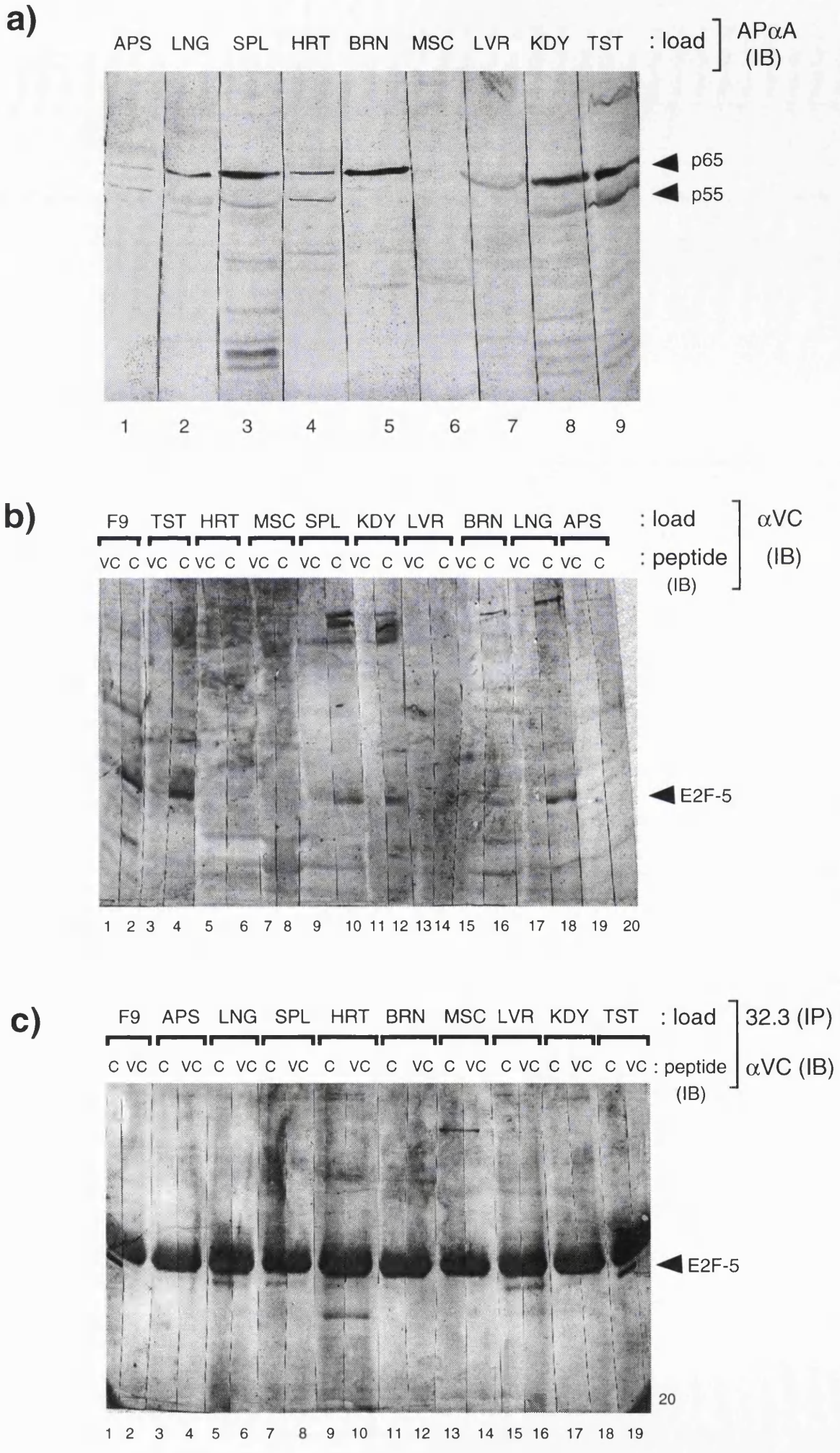
a. *Immunoblot of murine tissues probed with APαA.*
It is not possible to make accurate cell-protein concentration measurements from tissue extracts due to the great variation in blood levels. Cell protein concentrations were thus estimated from ponceau protein stains of immunoblots and loadings varied accordingly in subsequent blots to have an as equal as possible cell-protein loading in each lane. Different tissue extracts (see below for codes) were immunoblotted with APαA.

b. *Immunoblot of murine tissues probed with αVC.*
The cell protein loadings of different tissue extracts (see below for codes) were approximated as in **a.** and immunoblotted and probed with αVC. Even lanes were probed with competition from peptide VC, while odd lanes were probed in the presence of peptide C.

c. *Immunoblot of 32.3-immunoprecipitates from murine tissues with αVC.*
Each tissue extract (see below for codes) was subjected to immunoprecipitation with the monoclonal antibody 32.3. Since the extracts were made as microextracts and thus of high salt concentration (450mM NaCl), they were first diluted to 150mM salt and incubated for 30 minutes on wet ice. Immunoprecipitates were immunoblotted and probed with αVC. Even lanes were probed in the presence of peptide VC while odd lanes were probed with competition from peptide C.

Tissue abbreviations :

F9 : F9EC cell line
LNG : Lung
SPL : Spleen
APS : Adipose tissue
HRT : Heart
BRN : Brain
MSC : Striated muscle
LVR : Liver
KDY : Kidney
TST : Testes


a)
APS LNG SPL HRT BRN MSC LVR KDY TST : load
APαA (IB)
p65
p55
1 2 3 4 5 6 7 8 9
b)
F9 TST HRT MSC SPL KDY LVR BRN LNG APS : load
VC C VC C VC C VC C VC C VC C VC C VC C VC C : peptide (IB)
αVC (IB)
E2F-5
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
c)
F9 APS LNG SPL HRT BRN MSC LVR KDY TST : load
C VC C VC C VC C VC C VC C VC C VC C VC C VC : peptide (IB)
32.3 (IP)
αVC (IB)
E2F-5
20
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

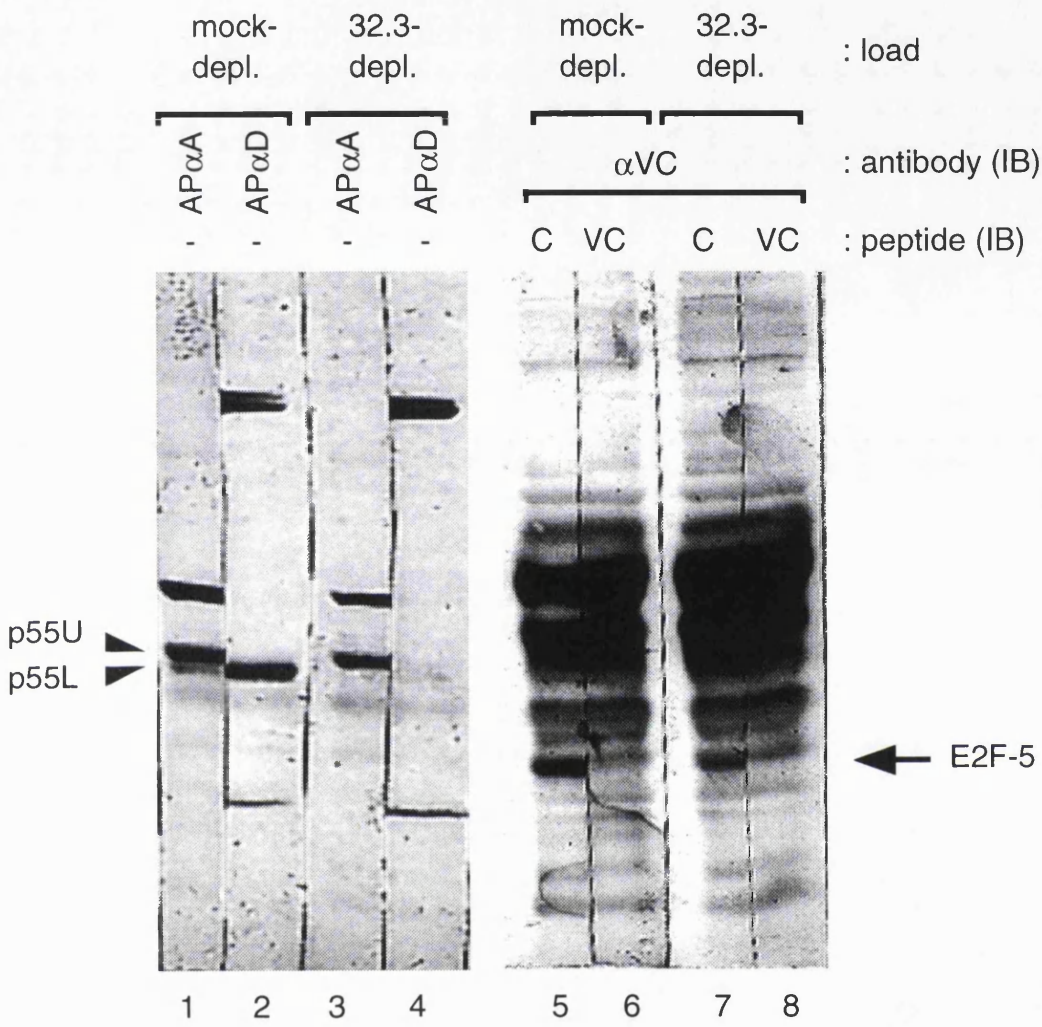
19/20 and 5/6 respectively). To determine if there was any interaction between p55L and E2F-5 in these tissues, each extract was subjected to immunoprecipitation by 32.3 and precipitates immunoblotted and probed with antisera to peptide VC (**figure 5.12c**). The αVC antisera was employed in the presence of a control peptide (odd lanes) or the specific peptide VC (even lanes) and demonstrated that the presence of p55 and E2F-5 in the same tissue extract correlates with their interaction as demonstrated in lung (lane 5/6), spleen (lane 7/8), brain (lane 11/12), kidney (lane 17/18) and testes (lane 19/20) tissue. A weak p55-E2F-5 interaction was detected in heart tissue (lanes 9/10) despite E2F-5 not being detected in that extract (**figure 5.12b**, lanes 5/6) suggesting a degree of enrichment as part of the immunoprecipitation procedure. The p55-E2F-5 interaction was never detected in adipose tissue (**figure 5.12c**, lanes 3/4), striated muscle (lanes 13/14) and liver (lanes 15/16). For striated muscle and liver this was most likely due to an absence of p55 in these extracts (**figure 5.12a**, lanes 6 and 7). It can however not be excluded that degradation processes in these extracts have destroyed p55 which had previously been present. Further experiments are needed to control for the intactness of other factors in these extracts. But to some extent this is provided by the non-DP-1 65KDal polypeptide detected in liver extracts (**figure 5.12a**, lane 7). It is possible that in these tissues there is a prevalence of non-DP-1-E2F-5 contributions to DRTF1/E2F. Adipose tissue clearly contains p55 (lane 1) yet no p55-E2F-5 interaction can be detected in it (**figure 5.12c**, lanes 3/4). This could suggest that in this particular tissue DP-1 interacts with a non-E2F-5 partner. This type of study is of course quite crude since that within a given organ there will be many cell types. It nevertheless serves to illustrate differential DP-/E2F- family contributions to different cell types in the broader sense of such a definition.

The fact that 32.3 will immunoprecipitate E2F-5 shows that p55L will make an *in vivo* interaction with an E2F family partner. This however does not address the question of whether p55U can complex with an E2F family member *in vivo* or not. Unfortunately it was not possible to probe this question by immunoprecipitation via αVC since the detecting antibody would have to be able to identify both forms of DP-1 and the only ones available that did so were rabbit antisera, the same species as αVC. This would result in an obscurring interaction between the secondary anti-rabbit antibody and αVC in the immunoprecipitate. The only non-rabbit primary antibody to DP-1 in the possession of the laboratory is 32.3 which of course only recognises the p55L and so would be unsuitable to test if p55U was to be precipitated. Several attempts were made to precipitate DP-1 forms from cell extracts using bacterially produced E2F-5 as was done for E2F-1, but these were all unsuccessful, probably due to the poor expression of E2F-5 as a GST fusion in bacteria.

In an attempt to determine if p55L is the only form of DP-1 that is associated with E2F-family members in F9EC cells, the p55L was depleted using the monoclonal 32.3 with resultant loss of all DNA binding activity (see **figure 5.4b**). The depleted and mock depleted extracts were assayed by immunoblotting to determine if E2F-5 had been removed concurrently with p55L depletion, thus excluding a p55U/E2F-5 association (**figure 5.13**). The depleted extract was clearly absent of detectable p55L (lanes 3 and 4) compared to the mock depleted extract (lanes 1 and 2) as detected by both APαA and APαD reagents. The same extracts however showed that not all detectable E2F-5 had been removed from the extract (lane 7 and 8). This indicates that not all E2F-5 in the cell is complexed to p55L, or for that matter involved in DNA binding, meaning that while the p55L-E2F-5 interaction can be clearly demonstrated, an interaction of p55U with E2F-5 cannot be excluded. However, if such an interaction takes place, it is likely to be non-DNA binding.

Figure 5.13
Not all E2F-5 in F9EC cell extracts is associated with DP-1 p55L.

Immunoblot of monoclonal antibody 32.3-depleted F9EC extracts probed with antibodies to DP-1 and E2F-5.
Monoclonal antibody 32.3-depleted (lanes 3/4 and 7/8) and mock-depleted (lanes 1/2 and 5/6) F9EC extracts were assayed by immunoblotting (IB - approximately 80μg protein each). Each gel-lane was cut into half and probed with affinity purified antibodies to DP-1 (lanes 1-4, odd : APαA ; even : APαD) or the E2F-5 anti-peptide VC antibody (αVC) in the presence of peptide VC (VC - lane 6 and 8) or peptide C (C - lane 5 and 7). DP-1 p55-isoforms, and E2F-5 polypeptides, are indicated.


mock-
depl.
32.3-
depl.
mock-
depl.
32.3-
depl.
: load
APαA
APαD
APαA
APαD
αVC
: antibody (IB)
- - - -
C VC C VC
: peptide (IB)
p55U
p55L
E2F-5
1 2 3 4 5 6 7 8

Chapter 6

Phosphorylation of DP-1

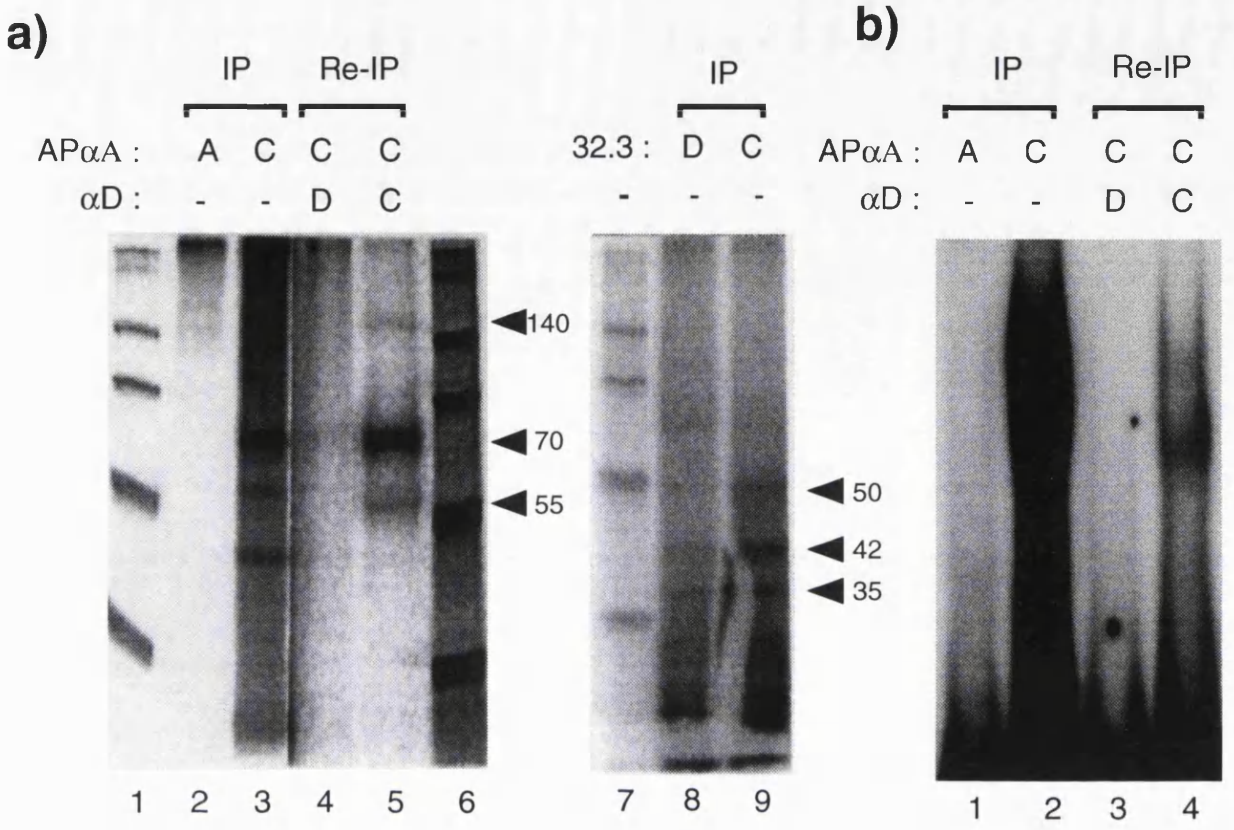
The difference between p55U and p55L is unlikely to be due to partial degradation of p55U since both isoforms are recognised by N-terminal, central and C-terminally targeted antibodies, suggesting that the polypeptide lengths and sequences are the same (see Chapter 3). For the same reasons the possibility that the two p55s are differential splice-forms of DP-1 is slight, though still finite. However, a more likely explanation for the physical and functional difference between the two forms of DP-1 is that of post-translational modulation, such as phosphorylation, since this type of modification is frequently observed in transcription factors (see Introduction).

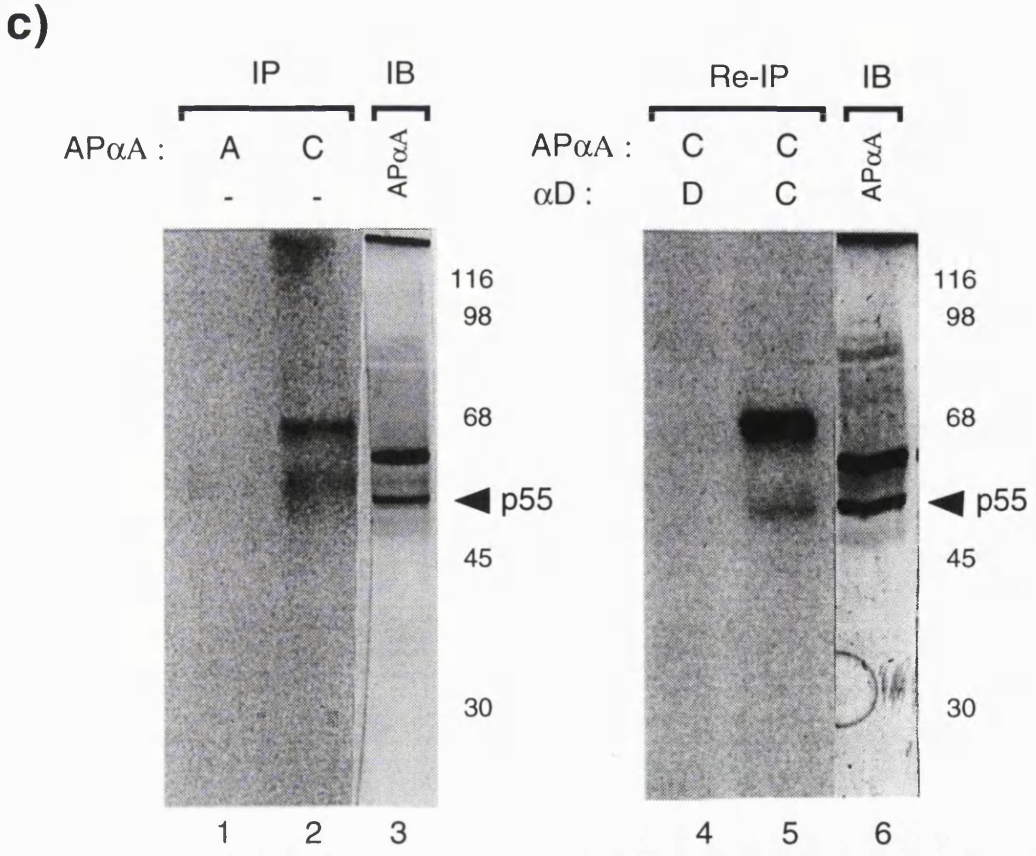
Differential phosphorylation of p55 isoforms

To investigate the possibility of differential phosphorylation between p55U and p55L, F9EC cells were grown in ^{32}P-ortho-phosphate containing media and extracts from these subjected to immunoprecipitation studies using antibodies to DP-1 (**figure 6.1a**). Two antibodies raised to opposite termini of DP-1 were employed to immunoprecipitate, and re-immunoprecipitate DP-1 from cell extracts to isolate ideally only DP-1 and any DP-1-associated factors. The extracts of mock-^{32}P-phosphate-labelled cells displayed no change in DRTF1/E2F DNA binding activity and so it was assumed that any observations made from extracts of these cells would be relevant to other studies on non-labelled cells. Fractions of APαA that harbour gel retardation activity immunoprecipitated a series of phospho-peptides (lane 3) in the presence of a control peptide, which were not precipitated in the presence of the peptide A to which the original antibody was raised (lane 1). These precipitates were released from the antibody-bound beads by incubation with peptide A, and the eluate subjected to re-immunoprecipitation with polyclonal αD sera (see Experimental Protocols). In the presence of peptide D (lane 4), αD re-precipitated no phosphorylated polypeptides, but an unrelated peptide instead (lane 5) allowed the re-precipitation of some of the phosphorylated polypeptides detected in the first precipitation step (lanes 4 to 6 are from a different gel and have run longer than lanes 1 to 3). Both APαA and αD (APαD) recognise both forms of DP-1 in immunoblots (see Chapter 3) and were assumed also to recognise both forms under non-denaturing

Figure 6.1
p55L and p55U are differentially phosphorylated.

a. *Immunoprecipitation analysis from ^{32}P-labelled F9EC cells using different antibody reagents to DP-1 assayed by SDS PAGE and autoradiography.*
LSL extract from ^{32}P-phosphate-labelled F9EC cells was subjected to immunoprecipitations which were assayed by SDS-PAGE and autoradiography. Gel retardation positive fractions of APαA were employed for the primary immunoprecipitation (IP) in the presence of peptide A (A : lane 2) or peptide C (C : lane 3). Non-competed immunoprecipitate (lane 3) was eluted with peptide A and the elutant subjected to re-immunoprecipitation (Re-IP) by polyclonal antipeptide D sera (αD) in the presence of peptide D (D : lane 4) or peptide C (C : lane 5). A single immunoprecipitation was also carried out using the monoclonal antibody 32.3 in the presence of peptide D (D : lane 8) or peptide C (C : lane 9). Lanes 1 to 3 were exposed for a shorter period of time than lanes 4 to 6. Standard molecular weight protein markers are loaded in lanes 1, 6 and 7 for reference between the gels.
b. *Gel retardation analysis of immunoprecipitates of DRTF1/E2F DNA binding activity using different antibody reagents.*
LSL F9EC extracts were subjected to immunoprecipitations which were assayed by gel retardation using labelled E2F DNA binding site. Gel retardation positive fractions of APαA were employed for the primary immunoprecipitation (IP) in the presence of peptide A (A : lane 1) or peptide C (C : lane 2). Precipitates were eluted using peptide A, and assayed for E2F site DNA binding activity. Non-competed eluted immunoprecipitates (lane 2) were subjected to re-immunoprecipitation (Re-IP) by polyclonal antipeptide D sera (αD) in the presence of peptide D (D : lane 3) or peptide C (C : lane 5), and eluted with peptide D.
c. *SDS PAGE/autoradiographically analysed immunoprecipitates from phospho-labelled F9EC cells compared to p55 as identified by immunoblottting of cell extract.*
Immunoprecipitations from labelled cells were assayed by SDS-PAGE and autoradiography exactly as described in legend **a.** with lanes 1, 2, 4 and 5 in **c.** corresponding to lanes 2, 3, 4 and 5 in **a.** Immunoprecipitates were assayed next to unlabelled F9EC LSL extract on the same denaturing gel (lanes 3 and 6) which was immunoblotted (IB) with APαA to compare immunoprecipitated polypeptides identified by autoradiography with those identified by immunoblotting in the extract.


a)
IP
Re-IP
APαA :
A C C C
αD :
- - D C
32.3 :
D C
- - -
140
70
55
50
42
35
1 2 3 4 5 6 7 8 9
b)
IP
Re-IP
APαA :
A C C C
αD :
- - D C
1 2 3 4


c)
IP
IB
APαA :
A C
- -
APαA
116
98
68
p55
45
30
1 2 3
Re-IP
IB
APαA :
C C
αD :
D C
APαA
116
98
68
p55
45
30
4 5 6

conditions, although this could not be proven conclusively. Certainly p55L must be brought down by both forms as DRTF1/E2F was specifically immunoprecipitated in a parallel control experiment where precipitates instead were assayed for gel retardation of the E2F site (**figure 6.1b** - the overexposed lane 2 is presented as such to illustrate the relative yields of the two precipitation steps).

Three phosphoproteins were detected in the re-immunoprecipitate (**figure 6.1a**, lane 5) of the approximate sizes of 55, 70 and 140 KDal (a weaker 40KDal band did not reproduce well on the scan). They were very likely to be either DP-1 or DP-1-Associated Factors (DAFs, see Chapter 7) since they had been specifically isolated individually by two antibodies with affinities to opposite ends of the DP-1 polypeptide, hence minimising the possibility of cross-reactions to irrelevant protein bodies. Thus the 55KDal phosphoprotein is very likely to be DP-1 by virtue of its size. Migration of this particular polypeptide was compared to DP-1 in F9EC extract by running immunoprecipitates and re-immunoprecipitates next to extract in the same gel and immunoblotting the extract with APαA (**figure 6.1c**). It seems clear from this that a phosphoprotein slightly larger than p55 obscures the 55KDal protein in the first precipitate (lane 2 compared to lane 3). This larger contaminant is removed by the re-precipitation making it clear that the 55KDal phosphoprotein co-migrates exactly with p55 as defined by APαA (lane 5 compared to lane 6). Due to the rather broad bands obtained by autoradiography, it is impossible to determine the identity of the p55 phosphoprotein as either that of p55U or p55L. All that could be concluded from this experiment was that DP-1 is likely to be a phosphoprotein.

In order to discern between the p55L and p55U forms in the phospho-immunoprecipitates, the p55L-specific monoclonal 32.3 was employed to immunoprecipitate p55L from phospholabelled cell extracts. 32.3 had been shown to be highly efficient at immunoprecipitating DP-1-p55L so that this can be visualised easily by immunoblotting (see **figure 5.3**). However the ^{32}P-phospho-labelled immunoprecipitate showed no evidence of a 55KDal sized protein (**figure 6.1a** ; lane 9). Other phosphorylated polypeptides were immunoprecipitated only in the absence of peptide D (compare lanes 8 and 9) indicating that the assay itself was successful. This suggested that p55L is relatively hypo-phosphorylated, and argues that the 55KDal band in the polyclonal re-immunoprecipitates is the p55U, which must then be hyper-phosphorylated relative to p55L.

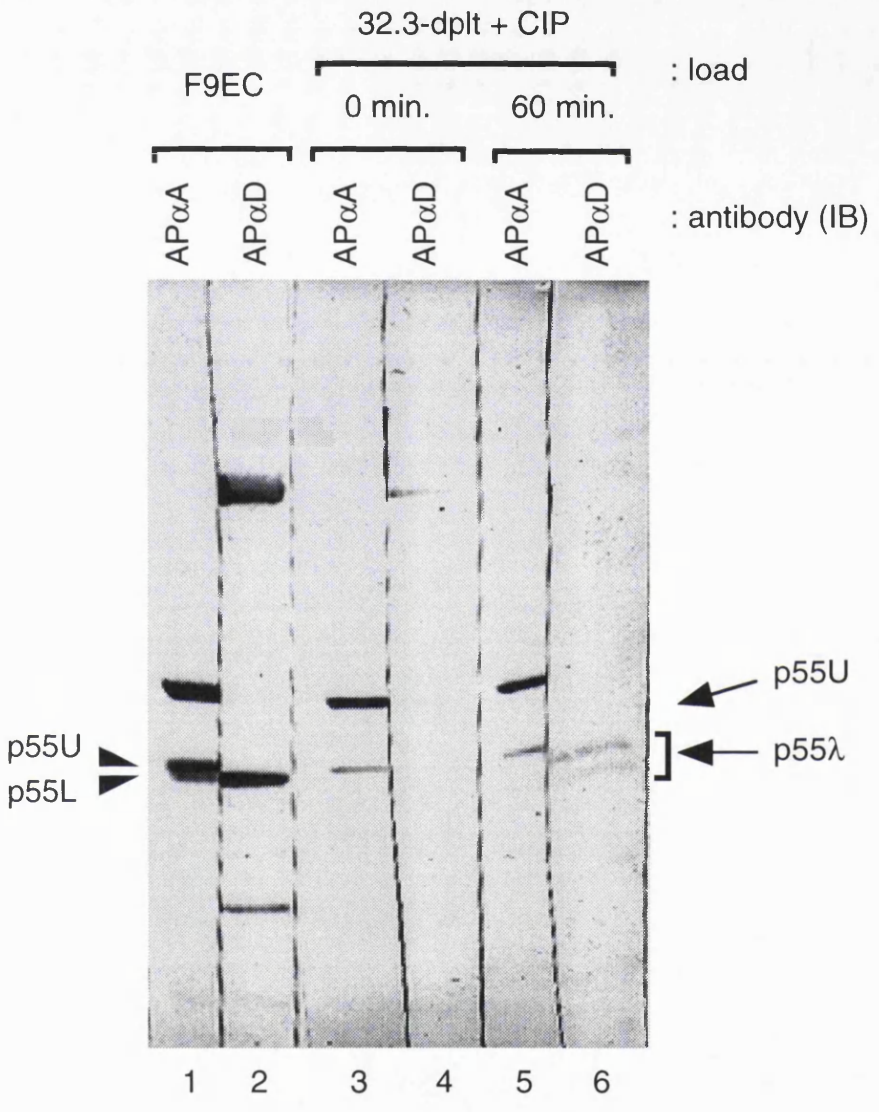
To investigate the notion that p55U is hyper-phosphorylated relative to p55L, phosphatase treatments were attempted on F9EC extracts which were then immunoblotted to detect any change in the ratio of p55U to p55L. If p55U is a more phosphorylated version of p55L, then it would be predicted that de-phosphorylation of cell extracts containing both would lead to an accumulation of p55L at the cost of p55U. Several different phosphatases were tried with a range of different buffers and

incubation procedures but it eventually became clear that any effects of phosphatase treatment on whole cell extracts were very subtle indeed, possibly due to poor access of phosphorylation sites on DP-1 for the particular phosphatases screened. To clarify any small accumulation of p55L, F9EC extract was sequentially immunodepleted with 32.3 so that only p55U could be detected by immunoblotting (same extract as presented in **figure 5.13**), since a marginal accumulation of p55L as a consequence of phosphatase treatment should be more easily detectable under these circumstances (**figure 6.2**). The 32.3-depleted F9EC extract was included in a de-phosphorylation reaction mixture (see Experimental Protocols) and either mixed with SDS PAGE loading buffer (lanes 3 and 4) or allowed to incubate for a further 60 minutes at 37°C before addition to the loading buffer (lanes 5 and 6). Both treatments, which only differed in length of incubation, were immunoblotted with APαA (lanes 3 and 5) and APαD (lanes 4 and 6). At 0 minutes incubation only p55U could be detected in the depleted extract, while at 60 minutes two new polypeptides were detected by APαD (lane 6). The new polypeptides, cautiously referred to as p55λ rather than p55L, were only recognised by APαD in accordance with its preference for p55L recognition and the preference of APαA for p55U. The more slowly migrating form of p55λ appeared to co-migrate with p55L (compare the relative migration of p55U and p55L in lane 1 and 2, with p55U and the upper p55λ in lane 5 and 6) suggesting that a de-phosphorylation event may have been responsible for the conversion of p55U to a form of p55 with a phosphorylation status similar to that of p55L. Although it is tempting to refer to the more slowly migrating form of p55λ as p55L, there is no guarantee that the two forms are identical as the former was created "artificially". The fastest migrating form of p55λ may be a hyper-de-phosphorylated version of p55 that never occurs in detectable amounts under normal cellular conditions. It is important to note that the detection of p55 by these two antibody reagents are only qualitative with respect to each other, not quantitative. Thus the apparent lack of reduction of p55U in lane 5 due to de-phosphorylation is not necessarily a concern. APαD is manifold more sensitive to p55L than APαA (see lane 1 versus lane 2) and so even a minor conversion of p55U to p55L will be detected by APαD but not necessarily by APαA. The results of this experiment support the hypothesis that the p55U is a hyper-phosphorylated version of p55L. It is not unusual to be able to detect different phospho-forms of a polypeptide by SDS PAGE and immunoblotting. This was for example how different phosphorylation states of pRb were initially observed (Buchkovich *et al.*, 1989 ; Chen *et al.*, 1989 ; Decaprio *et al.*, 1989) and the migration of these could also be modulated by phosphatase treatment (Buchkovich *et al.*, 1989).

Figure 6.2

p55L in cellular extracts can be immunodepleted and a p55L-like polypeptide re-generated by phosphatase treatment.

Immunoblot of immunodepleted and phosphatase treated F9EC extract.
F9EC extracts immunodepleted by the monoclonal 32.3 (dplt) were subjected to phosphatase treatment by exposure to Calf Intestinal Phosphatase (CIP) for 0 (lane 3/4) or 60 (lane 5/6) minutes (see Experimental Protocols). The mixtures were then analysed by immunoblotting (IB) next to a non-depleted, untreated, F9EC LSL extract (lane 1/2). The blotted gel-lanes were cut in half and each was either probed with APαA (lanes 1, 3 and 5) or APαD (lanes 2, 4 and 6). p55 isoforms of DP-1 are indicated (p55U, p55L) as well as a p55-like polypeptide (p55λ).


32.3-dplt + CIP
F9EC
0 min.
60 min.
: load
APαA
APαD
APαA
APαD
APαA
APαD
: antibody (IB)
p55U
p55L
p55U
p55λ
1
2
3
4
5
6

Some attempts were made to detect kinase activity in the DRTF1/E2F complex which might be responsible for phosphorylative differences between the two forms of DP-1. APαA/αD cold re-immunoprecipitates were washed into a kinase buffer containing peptide D (to elute the complex) and incubated with ^{32}P-labelled ATP. This approach however failed to attain any conclusive results.

Analysis of the role of the C-terminus of DP-1 in differential phosphorylation

The above evidence raises the possibility that the physical and functional differences between the DP-1 forms p55U and p55L, may be due to a greater degree of phosphorylation on p55U. This could suggest that the monoclonal antibody 32.3 will only recognise p55L because its epitope is altered in p55U by phosphorylation. The epitope recognised by 32.3 is contained within peptide D which represents a C-terminal section of DP-1. Analysis of the amino acid sequence of peptide D shows that it contains a threonine residue (T388) which forms part of a consensus cyclin-dependent kinase (CDK) site, as well as a protein kinase C (PKC) site (**figure 6.3a**). Both sites are conserved in *Xenopus laevis* DP-1 and DP-2, as well as in the murine DP-3 splice-forms (Girling *et al.*, 1994 ; Ormondroyd *et al.*, 1995). No other CDK sites in DP-1 are conserved in all these family members.

To investigate whether phosphorylation of peptide D will compromise recognition by 32.3, a phosphorylated version of peptide D, called Dp (**figure 6.3b**), was synthesised and antibody binding to it assessed by ELISA. Peptide Dp was synthesised at N.I.M.R. and mass spectrophotometrically analysed. Non-phosphorylated peptide could not be detected in the Dp preparation but a small contamination of singly phosphorylated peptide was observed. The 32.3 monoclonal was assayed at increasing dilution against microtitre plates coated with either peptide D or Dp (**figure 6.4a**). It was apparent from this result that the phosphorylation of peptide D reduced the recognition by 32.3 to a level comparable with the binding of an unrelated control antibody to the same peptides. The weak association observed between the control antibody and both peptides D and Dp was very similar suggesting that the levels of peptide coating the wells were equivalent. The APαD reagent recognises the p55U form of DP-1 weakly (**figure 3.4a**) and so the serum it was purified from, αD, was also tested in this assay (**figure 6.4b**) and was found to bind to peptide D strongly but also displayed some affinity to peptide Dp, albeit more weakly.

Figure 6.3

The C-terminus of DP-1 contains a conserved consensus CDK phosphorylation site.

a. *Peptide D contains conserved CDK and PKC consensus sites.*
The peptide D sequence from murine DP-1 (amino acid residues 385 to 400) is presented with bold and underlined residues being the ones conserved in Xenepus laevis DP-1 and DP-2, and in murine DP-3s. Consensus cyclin-dependent kinase (CDK) and protein kinase C (PKC) sites are highlighted.

b. *Peptide D_P is phosphorylated at threonine 388 and serine 391.*
The peptide D_P has the same sequence as peptide D, but is phosphorylated at its threonine (T) and serine (S) residues (green circles).

a) Peptide D

385 400

DP-1 : **R** V **E T P** V S Y V G E **D** D D **D** D

	T P	CDK consensus
R	T	PKC consensus
	X	Conserved in *Xenepus laevis* DP-1 and 2, and in mouse DP-3

b) Peptide D_P

Figure 6.4

Antibody binding to the C-terminal peptide of DP-1 is compromised by phosphorylation at its conserved potential CDK site.

a. *The monoclonal antibody 32.3 recognition of peptide D, detected by ELISA, is abolished by phosphorylation.*

ELISA assay on peptide D and peptide D_P with the monoclonal antibody 32.3 and a control antibody (polyclonal anti-peptide A). The assay of affinity of each antibody for a peptide was carried out by measuring the binding of sequential 50% dilutions of antibody relative to a constant concentration of peptide on the microtitre plates. Binding of 32.3 to peptide D (black line) is compared to binding to peptide D_P (red line). An unrelated antibody (K^-) is assayed against peptide D (green line) and peptide D_P (blue line).

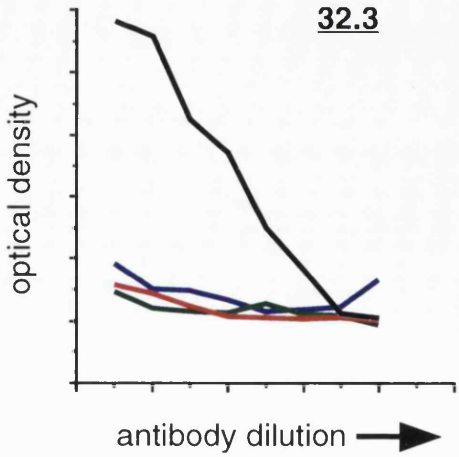
b. *The polyclonal antibody αD recognition of peptide D, detected using ELISA, is only partially abolished by phosphorylation.*

ELISA on peptide D and peptide D_P with the polyclonal antibody αD and a control antibody (polyclonal anti-peptide A). The assay of affinity of each antibody for a peptide was carried out by measuring the binding of sequential 50% dilutions of antibody relative to a constant concentration of peptide on the microtitre plates. Binding of αD to peptide D (black line) is compared to binding to peptide D_P (red line). An unrelated antibody (K^-) is assayed against peptide D (green line) and peptide Dp (blue line).

c. *ELISA analysis of the affinity of 32.3 to peptide Dp and the effect of dephosphorylating the peptide.*

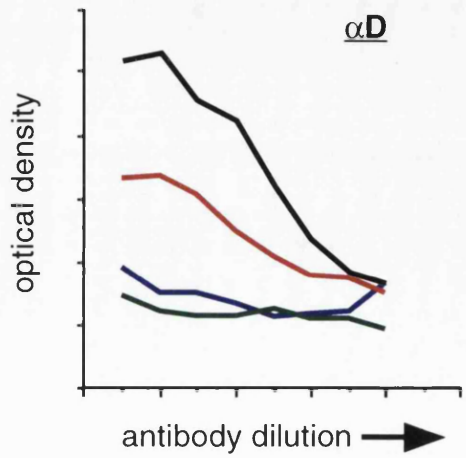
Peptides D, A and D_P were assayed for binding to the monoclonal antibody 32.3 (columns 1, 2 and 3 respectively : Averages of sequential antibody dilutions). In column 4, 5 and 6 the same peptides were subjected to incubation with Calf Intestinal Phosphatase (CIP) and in columns 7, 8 and 9, the same treatment, bar the phosphatase.

a)



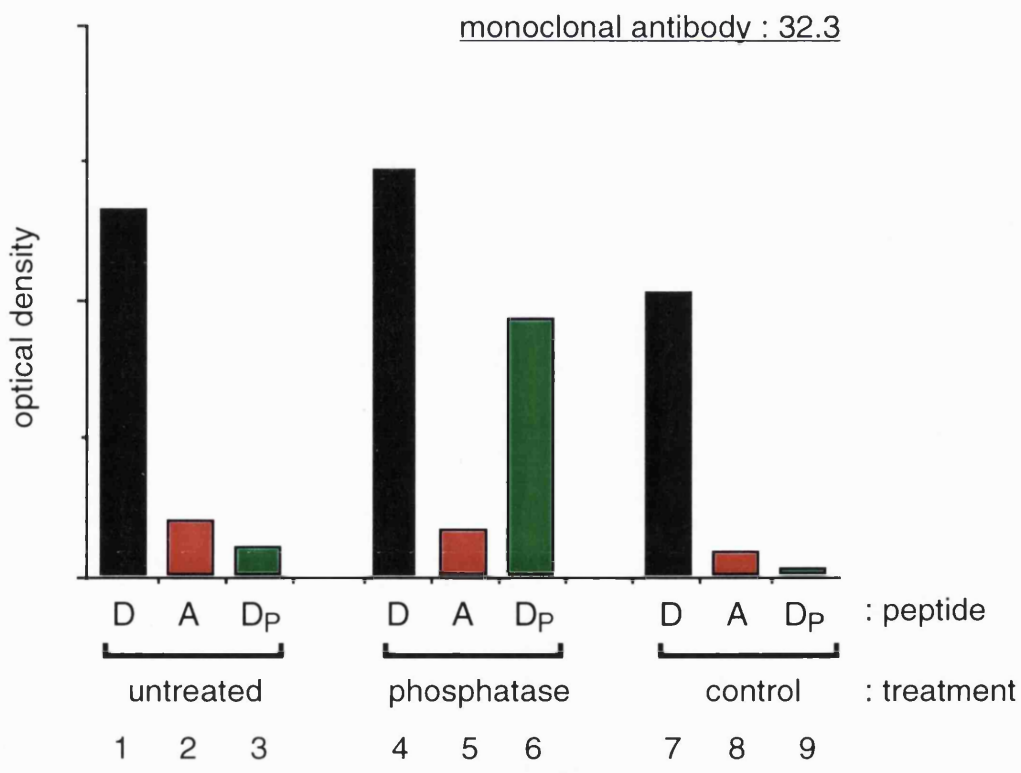
peptide	antibody
D	32.3
D_P	32.3
D	K-
D_P	K-

b)



peptide	antibody
D	αD
D_P	αD
D	K-
D_P	K-

c)



These results raise the possibility that the difference between the p55U and p55L may, in part, be due to the phosphorylation status of the C-terminus of DP-1. The migration of p55U could be modified by phosphatase treatment (**figure 6.2**) and so the binding of 32.3 to Dp was tested with and without treatment of the peptide by the same phosphatase (**figure 6.4c**). Binding of 32.3 to peptide Dp (column 3) was considerably lower than the binding to peptide D (column 1) and indeed comparable to its affinity to the completely unrelated peptide A (column 2). The three test peptides were all subjected to incubation with (columns 4, 5 and 6) and without (columns 7, 8 and 9) CIP phosphatase. The treatment had little effect on peptide D (column 4 versus 7) nor on the control peptide A (columns 5 versus 8) but greatly increased 32.3 binding to peptide Dp in the presence of phosphatase (column 6) but not without (column 9).

These results are in line with the observations on the recognition pattern of APαD and 32.3 on the p55 DP-1 doublet (see **figures 3.4a** and **4.5a**). 32.3 will exclusively recognise p55L, the supposed hypo-phosphorylated form of DP-1, and its recognition of peptide D is completely abolished upon phosphorylation. APαD has some affinity for p55U, though preferring p55L, and indeed, αD recognition of peptide D is only reduced, and not abolished, upon phosphorylation. This suggests that the polyclonal APαD may recognise more than one epitope within peptide D, some of which are not affected by phosphorylation. De-phosphorylation of the peptide Dp regenerates its affinity to 32.3, the antibody that preferentially recognises p55L, just as the de-phosphorylation of cellular extracts using the same phosphatase, generates a p55L-like migrating polypeptide.

These data imply that the absence of recognition of p55U by 32.3 is due to phosphorylation at the C-terminus of DP-1, and that this phosphorylation, at least in part, contributes to the migratory differences between p55U and p55L, and indeed, may even contribute to their functional differences. The limitations of parts of this study are clearly the observations made *in vitro* whose extrapolation into physiological relevance can only be an act of faith.

Chapter 7

DP-1 associated factors

From the studies into the phospho-forms of DP-1, it became clear that this protein is associated with at least three other phosphoproteins (**figure 6.1a**). This prompted an investigation into the identities of these DP-1 Associated Factors (DAFs). A re-immunoprecipitation experiment, mimicking the one performed on phospho-labelled cells and the one on un-labelled cells for gel retardation studies (**figure 6.1b**), was carried out with ^{35}S-methionine labelling and revealed at least six DAFs in a size range of 20 to 140KDal (**figure 7.1**). The extracts of mock-labelled cells displayed only a subtle reduction in total DRTF1/E2F DNA binding activity which suggested that the status of this complex was not greatly affected by the labelling procedures. This was reassuring to know if data from labelled-cell experiments was to be correlated with those from experiments involving non-labelled cells. The experiment was conducted exactly as described in Chapter 6, **figure 6.1**. The initial APαA immunoprecipitate showed that a few polypeptides had been bound in the presence of unspecific peptide (lane 3) which were not precipitated in the presence of specific peptide A (lane 2) and hence brought down as a direct result of APαA interaction. However, even though a polypeptide can interact with APαA, it may still not be DP-1 or a DAF, but just carry a peptide A epitope in a similar fashion to the 65KDal polypeptide detected in immunoblots with APαA (e.g. **figure 3.1b**). A 65KDal polypeptide may indeed have been immunoprecipitated by APαA (indicated by **+ figure 7.1**) in the absence of specific peptide competition (lane 3) but not in the presence (lane 2). Therefore, the re-immunoprecipitation of the APαA-precipitate with APαD, in the presence (lane 4) and absence (lane 5) of peptide D, ensures that the polypeptides exclusive to the re-precipitation (lane 5) are very likely to be either DP-1 or DAFs. When the re-immunoprecipitate for the ^{35}S-methionine experiment (lane 5) is compared to that of the ^{32}P-phosphate (lane 6), it is seen that some of the polypeptides display different levels of phosphorylation.

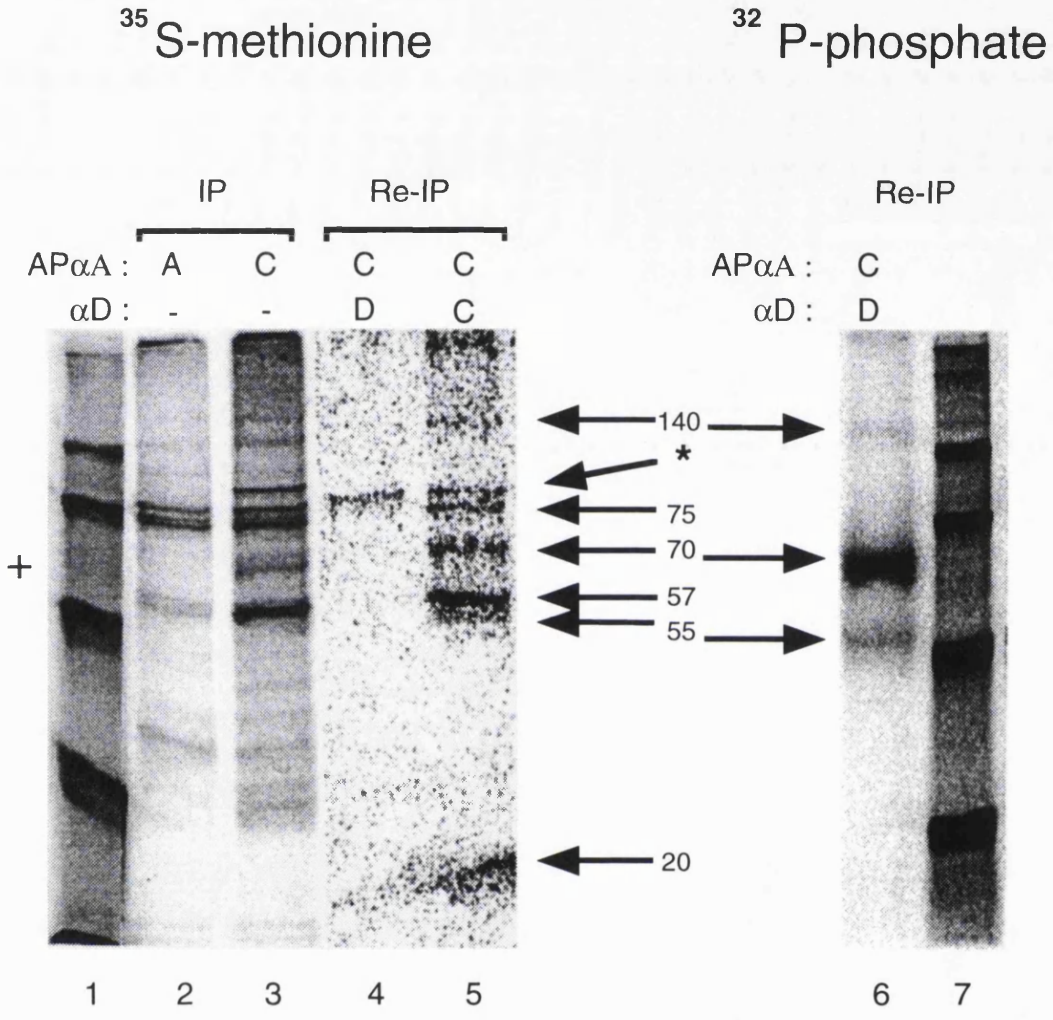
Some of the DAF polypeptide sizes observed can potentially be correlated to factors already associated with DRTF1/E2F (**figure 7.1**) in accordance with the immunoprecipitation of this DNA-binding complex using the exact same procedures (**figure 6.1b**). The 55KDal polypeptide is very likely to be DP-1 (see Chapter 3). The sizes of its potential E2F-family partners range from 47KDal for E2F-5 (Chapter 5) to 57 to 65KDal for E2F-1, 2, 3 and 4 (Kaelin *et al.*, 1992 ; Ivey-Hoyle *et al.*, 1993 ; Lees *et al.*, 1993 ; Ginsberg *et al.*, 1994). The 57KDal protein could thus be an E2F-

Figure 7.1

DP-1 is associated with a range of unidentified polypeptides in F9EC cells.

Immunoprecipitation analysis from ^{35}S-methionine labelled F9EC cells using different antibody reagents raised to DP-1.

^{35}S-methionine-labelled F9EC LSL extract was subjected to immunoprecipitations which were assayed by SDS-PAGE and autoradiography. Gel retardation positive fractions of APαA were employed for the primary immunoprecipitation (IP) in the presence of peptide A (A : lane 2) or peptide C (C : lane 3). Non-competed immunoprecipitate (lane 3) was eluted with peptide A and the elutant subjected to re-immunoprecipitation (Re-IP) by polyclonal antipeptide D sera (αD) in the presence of peptide D (D : lane 4) or peptide C (C : lane 5). The re-immunoprecipitation is compared to that of ^{32}P-phosphate labelled cells (from **figure 6.1a**) in lanes 6 and 7. Estimated polypeptide sizes are given in KDal and * indicates a non-specific protein as it is also re-immunoprecipitated in the presence of peptide D. + indicates a 65KDal sized polypeptide only immunoprecipitated in the first round.


35 S-methionine
32 P-phosphate
IP
Re-IP
Re-IP
APαA : A C C C
αD : - - D C
APαA : C
αD : D
+
140
*
75
70
57
55
20
1 2 3 4 5 6 7

family partner for DP-1. Alternatively both cyclin A and cyclin B migrate around 60KDal (Lees, 1995) and so could also be contenders. This leaves the 20, 70, 75 and 140KDal polypeptides without obvious identities. Although it may be that some associated polypeptides are degraded versions of larger factors, the possibility was raised that some of these proteins were as yet unidentified DP-1 binding factors. Since the antibody reagents involved in both immunoprecipitation steps both potentially would be precipitating p55U as well as p55L, it is possible that some of the DP-1 associated factors may be involved in non-DNA binding, non-DRTF1/E2F complexes.

p53 and DP-1

The tumour suppressor gene product, p53, has the capacity to arrest the cell cycle at the G1 phase (see Introduction, Chapter 1). Since the transcription factor DRTF1/E2F has been so intimately connected with the promotion of the cell cycle at G1 (see Introduction), the question was asked whether p53 might directly interact with this complex. To this end, a possible interaction between DP-1 and p53 was investigated since DP-1 appears to be a prominent member of DRTF1/E2F in some cell types.

A monoclonal antibody to p53 was used to immunoprecipitate from F9EC cell extracts and the precipitate probed in an immunoblot with APαA (**figure 7.2a**). The immuno-precipitate contained p55U as recognised by APαA by immunoblotting in the presence of control peptide (lane 3) but not in the presence of peptide A (lane 4). The p55U co-migrates precisely with p55U from the F9EC extract that the immunoprecipitation was made from (lane 2).

Gel retardation-positive APαA factions specifically immunoprecipitated the p53-binding protein, MDM2 (**figure 7.2b**) from NIH-3T3 cellular extracts. Immunoprecipitation using the anti-sera in the presence of peptide A (lane 1) did not precipitate the polypeptide identified by immunoblotting with a monoclonal to MDM2 in immunoprecipitations performed in the presence of a control peptide (lane 2). The fact that the immunoprecipitation by the antisera is inhibited, by the inclusion of the N-terminal DP-1 peptide A that the antisera was raised to, indicates that the polypeptide is likely to have been precipitated by virtue of at least a DP-1-containing complex and possibly a direct DP-1 interaction. The immunoprecipitated polypeptide co-migrated with a protein recognised by the same monoclonal to MDM2 in the NIH-3T3 cell extract that the immunoprecipitation was made from (lane 3), suggesting that the precipitated polypeptide is MDM2.

Figure 7.2

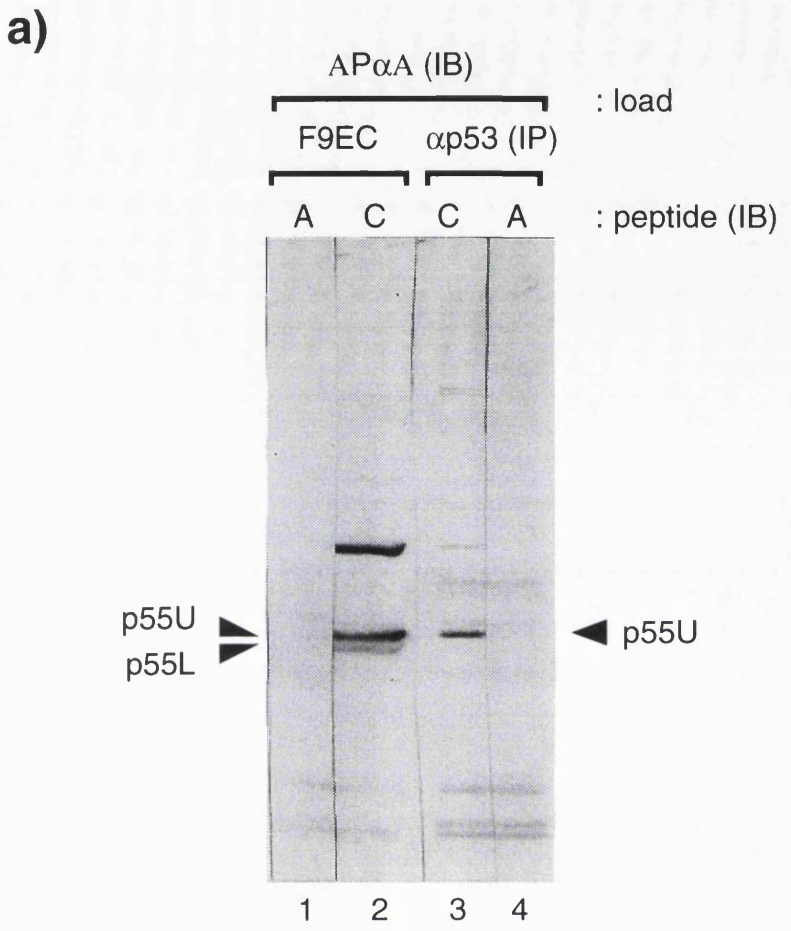
DP-1 forms an *in vivo* association with the tumour suppresser p53 and the p53-associated oncoprotein MDM2.

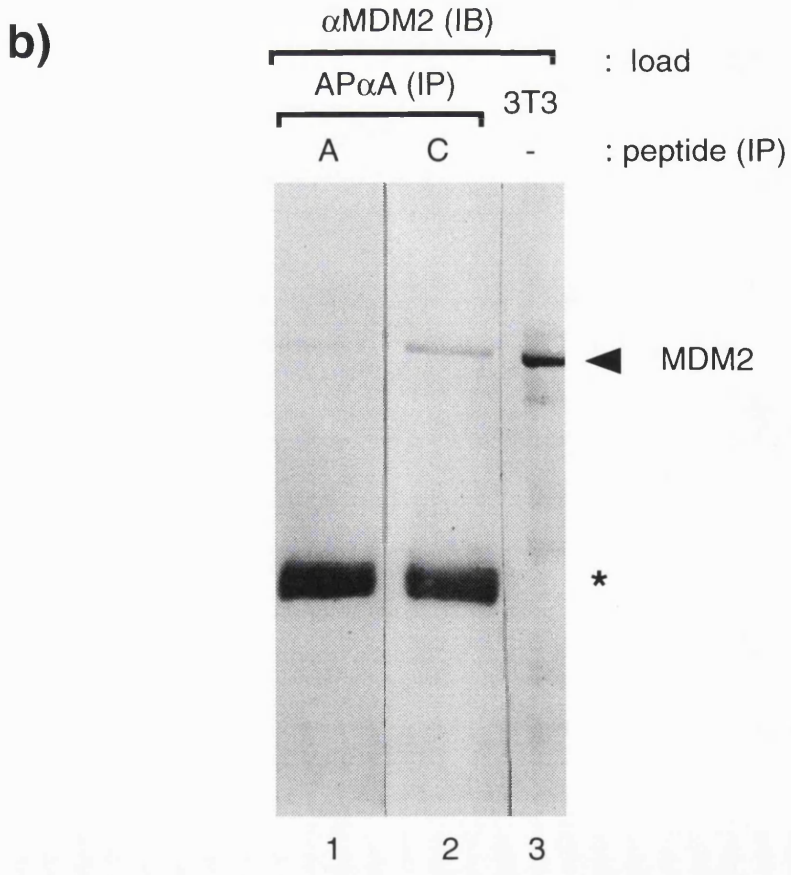
a. *Immunoblot of F9EC cell extract anti-p53-immunoprecipitate probed with APαA.*

Immunoblot (IB) of an immunoprecipitate (IP) from F9EC cell LSL extract using a monoclonal antibody to p53 (421 : lanes 3/4) relative to the extract that the immunoprecipitation was made from (lanes 1/2). Each gel-lane was cut into two and probed with APαA in the presence of peptide A (lanes 1 and 4) or in the presence of peptide C (lanes 2 and 3).

b. *Immunoblot of NIH 3T3 cell extact APαA-immunoprecipitate probed with a monoclonal antibody to MDM2.*

Immunoblot (IB) of the immunoprecipitate (IP) by an APαA fraction containing gel retardation activity from NIH-3T3 cells LSL extract, performed in the presence of peptide A (pep A : lane 1) or peptide C (pep C : lane 2). The extract that the immunoprecipitation was made from was also immunoblotted (lane 3). All lanes were probed with a monoclonal antibody to MDM2. Antibody cross reactions to the large concentration of immunoglobulins employed in the immunoprecipitation step were indicated by *.


a)
APαA (IB)
: load
F9EC
αp53 (IP)
A
C
C
A
: peptide (IB)
p55U
p55L
p55U
1
2
3
4


b)
αMDM2 (IB)
: load
APαA (IP)
3T3
A
C
-
: peptide (IP)
MDM2
*
1
2
3

The interaction between p53 and DP-1 could also be demonstrated by the precipitation of DP-1 with Sepharose-bound bacterially expressed histidine-tagged p53. The p53 fusion protein was extracted from bacteria and purified via an N-terminal histidine "tag" which allows purification from bacterial extracts using nickel ion-chelated Sepharose beads. As opposed to precipitation experiments involving GST tagged protein (as the GST-E2F-1 precipitation experiment in **figure 5.8**), a "tag-only" (analogous to GST protein-only) bead control was not possible for the histidine-tagged p53. Simply to use nickel-Sepharose as a control for a precipitation would have been inadequate since it could be argued that despite the high purity of histidine tagged extractions, the p53-bearing beads, having been through a bacterial extract and the thousands of proteins in it, could have adhered to the bacterial factors ultimately responsible for the observed interaction with DP-1. Histidine tagged purification yields very pure protein, although the highest level of purity is only generated by the imidazole elution of tagged proteins. When elution is not performed and the histidine tagged protein is allowed to remain on the beads, it remains amongst non-histidine protein contaminants which would usually have been left behind at the elution stage. Nickel-Sepharose-p53 preparations were therefore made from two different bacterial extracts. One from a culture that had been IPTG-induced in the normal manner (see Experimental Protocols) and one which had been grown in the absence of IPTG, thus making "induced" and "non-induced" p53-containing Sepharose (**figure 7.3a**). As a result the induced beads contained normal high levels of p53 (lane 2), while the non-induced Sepharose contained only a small amount (lane 3), probably due to low-level "leaking" of the *p53* IPTG-inducible promoter. The beads were mixed with F9EC cell extract, washed and analysed by immunoblotting with APαA to ascertain any DP-1 interaction (**figure 7.3b**). Induced Sepharose was found to precipitate a polypeptide that co-migrates with p55U identified by APαA in the F9EC extract that the precipitate was made from (compare lanes 1 and 2) but which was not recognised in the presence of peptide A (lane 3) and so was likely to be p55U. The polypeptide was not precipitated by the non-induced Sepharose, suggesting that the precipitation of DP-1/p55U from cellular extract was dependent on the presence of high levels of p53 protein, supporting the evidence that DP-1 and p53 can form an interaction.

The association of p53 with DP-1 p55U rather than p55L was supported by numerous failed attempts to shift DRTF1/E2F with antibodies to p53 in gel retardation assays, including the antibody used in the original immunoprecipitation of DP-1. Similar studies also failed to show the MDM2 gene product in association with DRTF1/E2F by including *in vitro* translated or bacterially purified MDM2 in gel retardation assays with DP/E2F heterodimers, DRTF1/E2F from cell extracts or immunoprecipitated DRTF1/E2F from cell extracts. Attempts to supershift or

Figure 7.3
Bacterially produced purified p53 will precipitate DP-1 p55U.

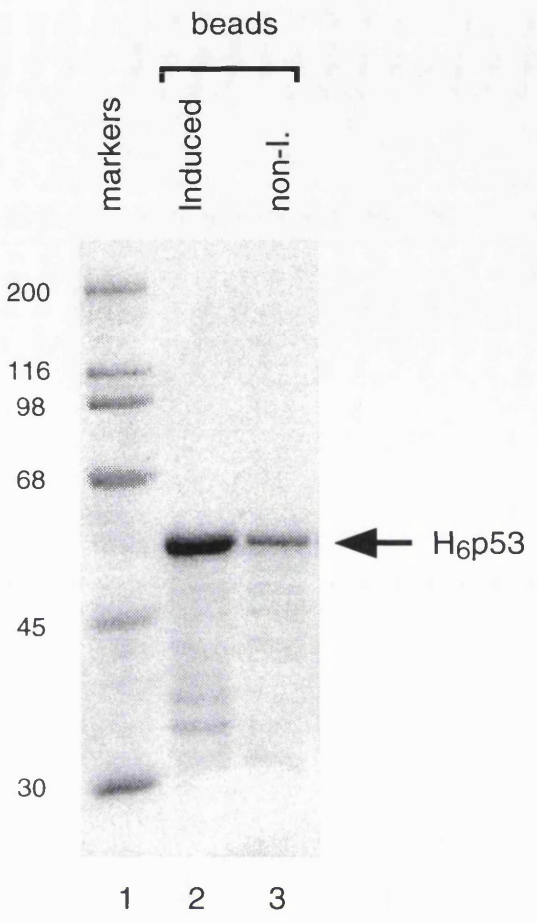
a. *Coomassie stained SDS PAGE of bacterially purified histidine-tagged p53 attached to nickel-ion beads.*

An equal solid volume (15µl) of histidine tagged p53 (H_6p53) attached beads, from IPTG-induced bacterial culture (Induced : lane 2) and non-induced culture (non-I. : lane 3), was assayed by SDS PAGE and coomassie staining. The samples were run next to standard molecular weight protein markers (lane1) whose sizes are indicated in KDal.

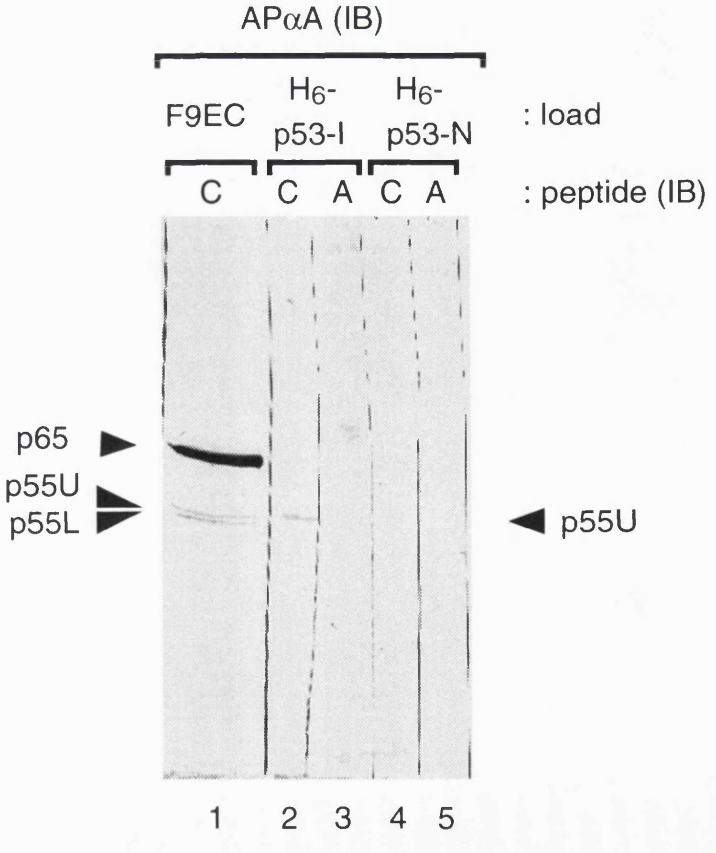
b. *Immunoblot of F9EC cell extract histidine-tagged p53-precipitate probed with APαA.*

Immunoblot (IB) of histidine-tagged, and bead attached, affinity purified p53-precipitations from F9EC LSL extracts. p53-associated nickel-chelated beads were prepared from bacteria containing the histidine-p53 plasmid, either induced for expression with IPTG (H_6p53-I - lanes 2/3) or not (H_6p53-N - lane 4/5). H_6p53-I bead-aliquots contained approximately 500ng p53 and H_6p53-N only trace amounts. Precipitates using the two types of beads were loaded next to the extract that the precipitations took place from (lane 1). All lanes were probed with APαA in the presence of peptide C (C : lanes 1, 2 and 4) or in the presence of peptide A (A : lanes 3 and 5).

a)


beads
markers
Induced
non-I.
200
116
98
68
45
30
H6p53
1
2
3

b)


APαA (IB)
F9EC
H6-p53-I
H6-p53-N
: load
C
C
A
C
A
: peptide (IB)
p65
p55U
p55L
p55U
1
2
3
4
5

immunoprecipitate DRTF1/E2F in gel retardation assays with antibodies to MDM2 also failed. Immunoprecipitation with antibodies to MDM2 was also attempted in the hope of determining which form of DP-1 it interacts with. MDM2 has been shown to be able to form a direct interaction with DP-1 *in vitro* (Martin *et al.*, 1995) but failure to detect it in DRTF1/E2F could indicate that it interacts with the p55U-form of DP-1. Alternatively it may interact with DP-1 via p53. These possibilities are considered in the Discussion Chapter 8.

p53, DP-1 and E2F-1

The functional consequences of the interaction between DP-1 and p53 were investigated by transfection studies to monitor the effect of p53 on E2F site driven transcription. This demonstrated that p53 reduces E2F-dependent transcription and that *MDM2* transfection increases the activity from the E2F promoter (Sørensen *et al.*, 1996). The interaction of p53 with the non-DNA binding form of DP-1, p55U, suggested that the effect of p53 on E2F-driven transcription might not involve direct effects on the DNA-bound DRTF1/E2F complex. However, it was demonstrated through a mutational analysis of DP-1 that p53 interacts with a region of DP-1 which incudes its E2F-heterodimerisation domain (Sørensen *et al.*, 1996 ; see Discussion, Chapter 8, for a full report and consideration of these results). This prompted a study to ask whether p53 is able to modulate the DNA binding activity of DRTF1/E2F despite an apparent preference for interacting with the non- or low-DNA binding p55U-form of DP-1.

To investigate this possibility gel retardation assays were employed to probe the effect of p53 proteins on the E2F-site DNA binding activity of DP-1/E2F-1 heterodimers or the DRTF1/E2F from cell extracts. This was not an easy assay and much time was taken up pursuing very weak effects. Both bacterially produced and *in vitro* translated p53 was employed, but the latter turned out not to be concentrated enough to get clear effects. This was partially due to an inherent E2F DNA binding activity in reticulolysates that is revealed at higher loadings in gel retardation assays. Co-*in vitro* translating p53 with DP-1 and E2F-1 was unsuccessful because the translation of one plasmid would usually quench that of another. Effects of adding p53 to cellular DRTF1/E2F, either in an extract or as the immunoprecipitated form, had unclear effects and GST-E2F-1/GST-DP-1 heterodimers were not affected at all. The clearest results were provided by the heterodimer activity of *in vitro* translated DP-1 and E2F-1 to which was added bacterially purified histidine tagged p53 (**figure 7.4a**).

Figure 7.4

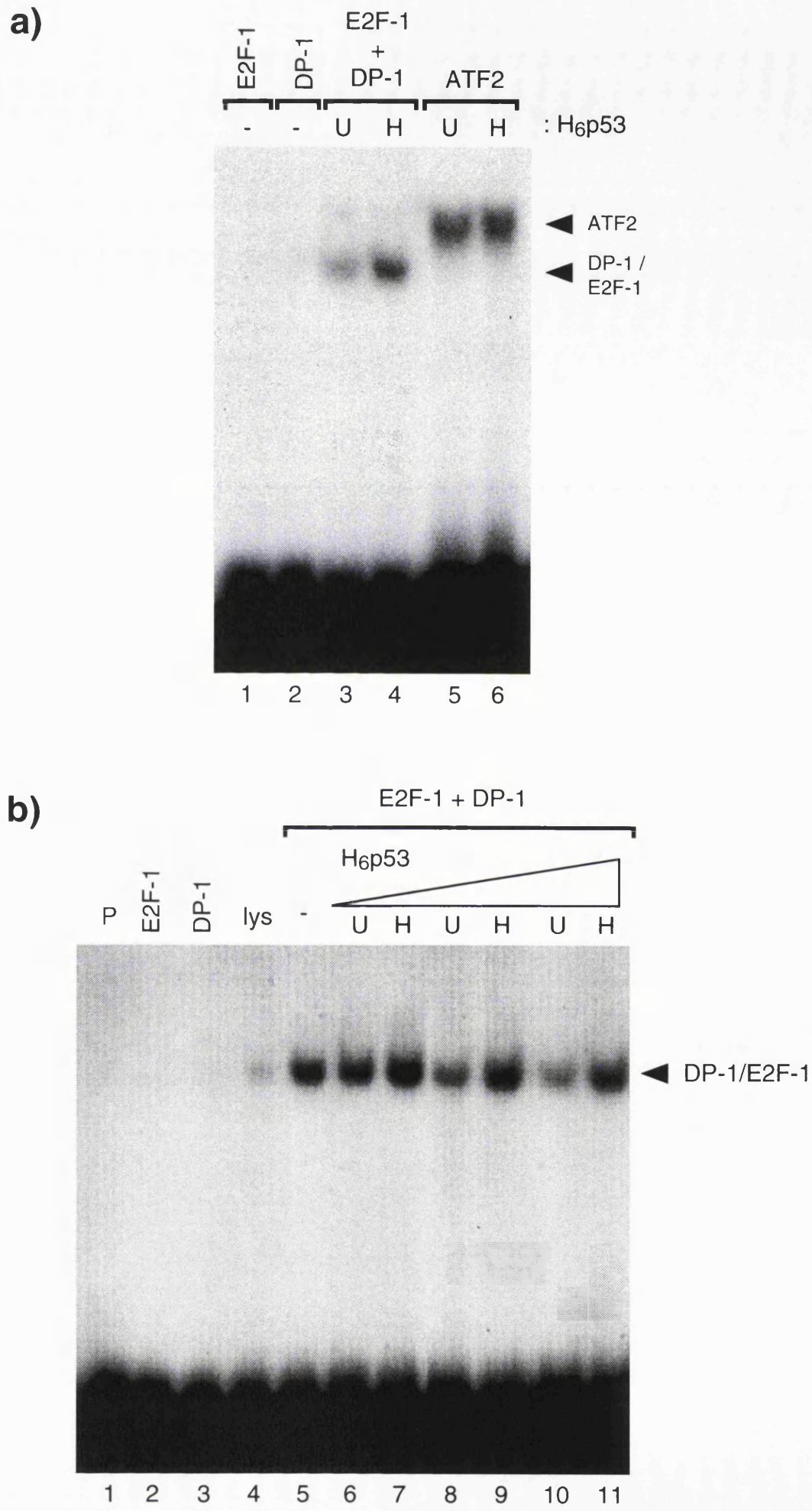
p53 inhibits DP-1/E2F-1 heterodimer DNA binding activity to the E2F site.

a. *Gel retardation analysis of E2F-1/DP-1 heterodimer exposed to p53 protein.*

Gel retardation assay of reticulolysate *in vitro* translated transcription factors in the presence of functional or de-natured p53. In lane 1 and 2, E2F-1 and DP-1 respectively, are separately loaded (1µl and 3µl respectively). In lanes 3 and 4 they are loaded together. Lanes 1-4 are probed with labelled E2F DNA binding site. The *in vitro* translated transcription factor ATF2 is loaded in lanes 5 and 6 (1µl each) and is probed with labelled ATF DNA binding site. Added to the reaction mixtures in lanes 3-6 is approximately 400ng of histidine tagged, bacterially expressed and purified p53. In lanes 4 and 6 this has been heat denatured at 100°C for five minutes prior to addition (H) as opposed to non-heat treated (U) in lanes 3 and 5. In contrast to other gel retardation assays presented in this thesis, here the primary incubation was performed entirely on wet ice for 50-60 minutes prior to addition of probe, which was incubated in the usual manner - this optimises the inhibitory effect of p53.

b. *Gel retardation analysis of E2F-1/DP-1 heterodimer exposed to increasing concentrations of p53 protein.*

Gel retardation assay probed with labelled E2F site, alone in lane 1 (P). Reticulolysate *in vitro* translated E2F-1 and DP-1 are loaded alone in lanes 2 and 3 (1µl and 3µl respectively), and together in lanes 5 to 11, with the equivalent amount of reticulolysate reaction mixture-only (4µl) added to lane 4. Bacterially expressed and purified histidine tagged p53 is included in lanes 6 and 7, 8 and 9, and, 10 and 11 at 50, 150 and 450ng respectively. In lanes 7, 9 and 11, p53 was heat denatured at 100°C for five minutes prior to addition (H) as opposed to un-treated p53 (U : lanes 6, 8 and 10). In contrast to other gel retardation assays, here the primary incubation was performed entirely on wet ice for 50-60 minutes prior to addition of probe, which was incubated in the usual manner - this optimises the inhibitory effect of p53.


a)
E2F-1
DP-1
E2F-1 + DP-1
ATF2
- - U H U H : H6p53
ATF2
DP-1 / E2F-1
1 2 3 4 5 6
b)
E2F-1 + DP-1
H6p53
P E2F-1 DP-1 lys - U H U H U H
DP-1/E2F-1
1 2 3 4 5 6 7 8 9 10 11

E2F-1 (lane 1) or DP-1 (lane 2) on their own had little or no DNA binding activity (see Introduction, Chapter 1). Adding these two components together in the same reaction mixture in the presence of heat-denatured histidine tagged p53 (lane 4) resulted in the well characterised increase in E2F site DNA binding due to cooperative heterodimerisation (see Introduction Chapter 1). If however the p53 was not heat-treated, there was a marked reduction of the DNA binding activity of the heterodimer (lane 3). This effect was clarified after much experimenting and modulation of the normal incubation procedure of the gel retardation assay (see legend). Normally histidine tagged bacterially expressed protein is eluted off Nickel-ion beads using an imidazole buffer containing Tris-HCL and sodium chloride. Due to the quite large amounts of p53 required in this assay (see legend) it was necessary to dialyse out the salt or to contend with a slightly lowered elution efficiency of imidazole buffer minus salt (see Experimental Protocols) so as not to comprimise heterodimer DNA binding. The effect was specific to the E2F-heterodimer, and not a general non-specific effect on the DNA binding activity of transcription factors since p53, heat treated or not, had no effect upon the DNA binding of *in vitro* translated ATF2 to the ATF binding site (lanes 5 and 6). Titrating p53 into the heterodimer mixture (**figure 7.4b**) was found to reduce the DNA binding activity of the DP-1/E2F-1 heterodimer in a p53-concentration- and integrity- dependent manner.

The bacterially expressed p53 used in these assays was histidine-tagged, harvested under 6 molar salt conditions and eluted with imidazole to provide a pure preparation (**figure7.5a**). The p53 protein was specifically eluted at 150mM imidazole (lane 2) in a very pure form, and not at the lower 75mM imidazole level (lane 1), indicating that the eluted protein indeed contained several sequential histidines (Quiagen) and was thus the expressed protein, in this case p53. The purified p53 was also identified by immunoblotting with two different monoclonal antibodies to p53 (**figure 7.5b**) including one (421) which previously was used to immunoprecipitate DP-1 from cell extract (**figure 7.2a**). Thus positive antibody identification, correct predicted mobility (compare to protein markers in **figures 7.5a** and **7.5b**) and specific elution served to assure of the correct identity of p53 and thus to control for the specific nature of the DP-1/E2F-1 DNA binding inhibitory effect (**figure 7.4**).

In addition to reducing the DNA binding activity of DP-1/E2F-1 heterodimers, p53 also had inhibitory effects on other DP-family members in association with E2F-1 (**figure 7.6**). The greatest observed effects were on DP-1, DP-3α (lanes 1-3), and DP-3γ (lanes 7–9), with a lesser effect on DP-3β (lanes 4–6), and no apparent effects on DP-3δ (lanes 10-12) and DP-2 (lanes 13-15). This indicates that p53 may target different populations of DRTF1/E2F. Analysis of the amino acid sequences of these different DP-family members showed there to be great homology amongst them

Figure 7.5

The p53 fusion protein employed was pure and positively immunogenically identified.

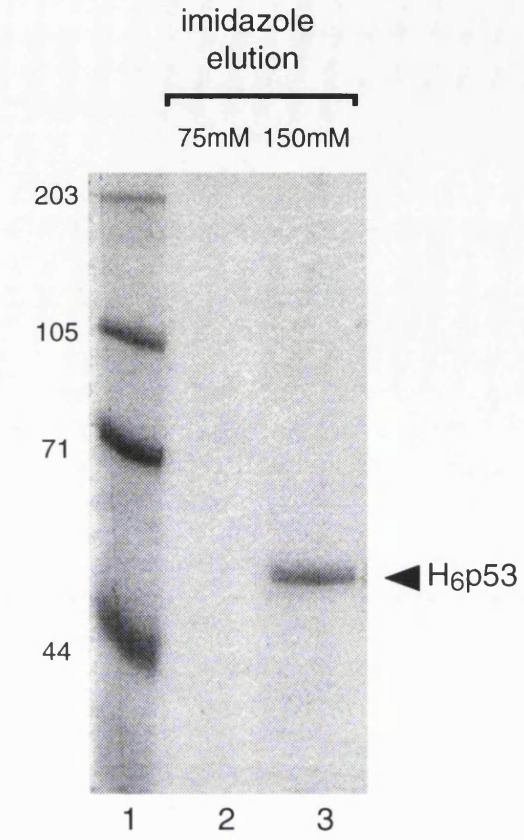
a. *SDS PAGE/coomassie stain of purified bacterially expressed histidine-tagged p53.*

Histidine tagged p53 was prepared as described in the Experimental Protocols and finally eluted from nickel-chelated using 150mM imidazole (lane 3) and analysed by SDS-PAGE and coomassie staining. Washes were performed at lower concentrations of imidazole (lane 2) to remove any bacterial multi-histidine-containing polypeptides. Molecular weight markers were run in lane 1, sizes in KDal are indicated.

b. *Immunoblot of histidine tagged p53 probed with two different antibodies to p53.*

Immunoblot of approximately 50ng per lane (lanes 2 and 3) of histidine tagged p53 using two different monoclonal antibodies to p53. Molecular weight markers were run in lane 1 and visualised with ponceau stain. Sizes in KDal are indicated.

a)


imidazole
elution
75mM 150mM
203
105
71
44
H6p53
1 2 3

b)

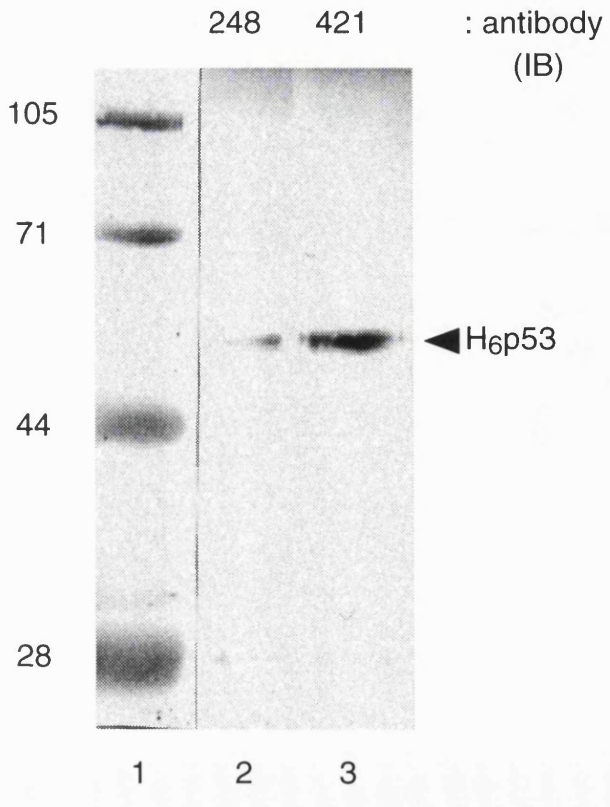
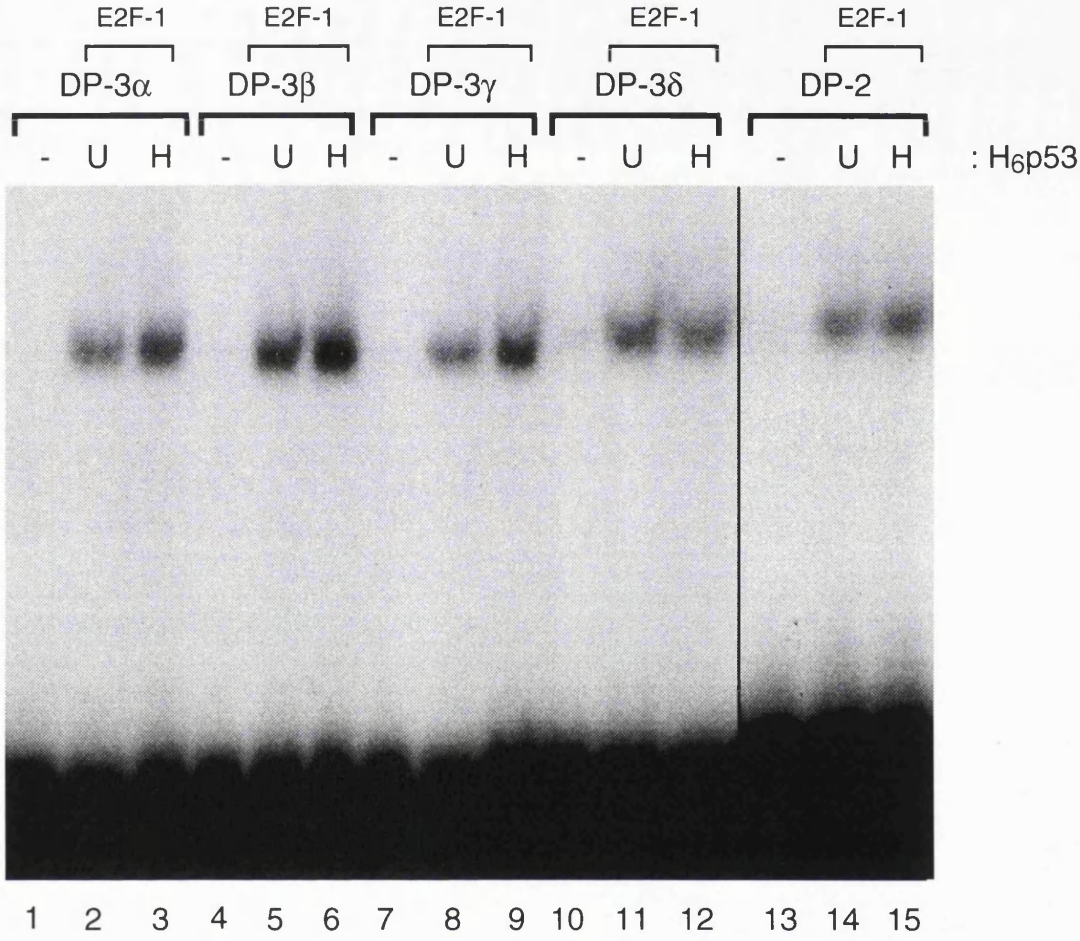

248 421
: antibody
(IB)
105
71
44
28
H6p53
1 2 3

Figure 7.6

Different DP-family members, heterodimerised with E2F-1, are targeted by p53.

Gel retardation assay of different reticulolysate in vitro translated DP-family members exposed to p53.

Gel retardation assay of different reticulolysate *in vitro* translated DP-family members, DP-3α, β, γ and δ, and DP-2, (2-3μl each) with E2F-1 (1μl) probed with labelled E2F DNA binding site. DP-family members were loaded alone in lanes 1, 4, 7, 10 and 13, and with E2F-1 in the remaining lanes. Added to the reaction mixtures in lanes 2 and 3, 5 and 6, 8 and 9, 11 and 12, and, 14 and 15 was approximately 400ng of histidine tagged, bacterially expressed and purified p53. In the latter of each pair p53 was heat denatured at 100°C for five minutes prior to addition (H) as opposed to un-treated (U) in the former. In contrast to other gel retardation assays, here the primary incubation was performed entirely on wet ice for 50-60 minutes prior to addition of probe, which was incubated in the usual manner - this optimises the inhibitory effect of p53.


E2F-1
E2F-1
E2F-1
E2F-1
E2F-1
DP-3α
DP-3β
DP-3γ
DP-3δ
DP-2
- U H - U H - U H - U H - U H
: H6p53
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

within the putative p53-interaction region defined in DP-1 (Sørensen *et al.*, 1996 ; DP-1 residues 171-331), therefore any effect on p53 binding would be likely to come from domains outside this region. DP-3α and δ both contain the E-box, a novel nuclear localisation signal (De la Luna *et al.*, 1996), yet their responses to p53 were quite opposite. They only differ in additional N-terminal region in DP-3α which has some homology to the N-terminal domain of DP-1. Perhaps this region promotes the interaction with p53. DP-3β and DP-3γ both lack the E-box and differ only in the presence of an additional glutamine residue in DP-3γ. DP-3γ appears to be the more sensitive to p53, yet no other DP-family members have this residue. This is clearly an area in need of further investigation and could provide valuable clues to the mode of DP-targeting by p53.

p53, DP-1, E2F-1 and pRb

These are the first data that indicate a direct modulation of DRTF1/E2F DNA binding activity by p53. Since DRTF1/E2F is already known to be regulated by another tumour suppressor gene product, pRb, it was deemed interesting to investigate the outcome if the E2F-heterodimer were exposed to both pRb and p53 at the same time. GST-N-terminally truncated-pRb was bacterially expressed and purified by standard procedures (see Experimental Protocols) and assayed by SDS-PAGE and coomassie staining (**figure 7.7**). The purified fusion protein had the predicted size of approximately 91KDal but some C-terminally degraded GST-pRb was isolated with it.

Surprisingly p53 did not appear to be able to inhibit pRb-associated heterodimer (**figure 7.8a**). Specific inhibition by p53 of the E2F-1/DP-1 heterodimer was clear (lanes 1 and 2), but the more slowly migrating GST-pRb/E2F-1/DP-1 complexes observed in lanes 3 and 4 appeared not to be affected. Intuitively one would have expected that since p53 inhibition possibly involves the sequestering of DP-1 via its heterodimerisation domain (see above and Sørensen *et al.*, 1996), the involvement of pRb in the heterodimerisation complex would not prevent this. pRb interacts with a separate domain on E2F-1, but the results presented here could suggest that it may sterically prevent p53s access to DP-1.

The faster migrating complexes observed in lanes 3 and 4 did not appear to be affected by p53 which would have been expected of the non-complexed E2F-1/DP-1 heterodimer. However a small amount of E2F binding activity in this assay was not E2F-1/DP-1 heterodimer, but rather the endogenous background activity present in the reticulolysate. This activity co-migrated with the heterodimer but titration of GST-pRb (**figure 7.8b**) showed that a proportion of the apparent heterodimer will not

Figure 7.7

Purified GST-pRb fusion protein analysed by SDS PAGE and coomassie staining.

Purified recombinant glutathione S-transferase-pRb protein expressed in bacteria was analysed by SDS PAGE and coomassie staining.

20µl of a primary reduced-glutathione buffer elution volume of 100µl was loaded (lane 2). Standard molecular weight protein markers were loaded in lane 1, their sizes indicated in KDal. GST-pRb is indicated.

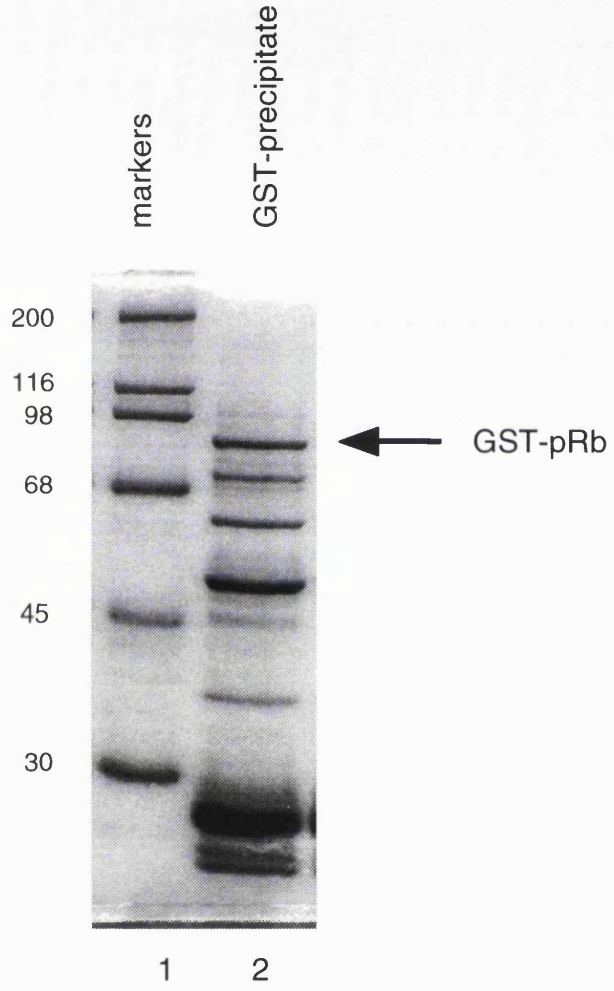

markers
GST-precipitate
200
116
98
68
45
30
GST-pRb
1
2

Figure 7.8

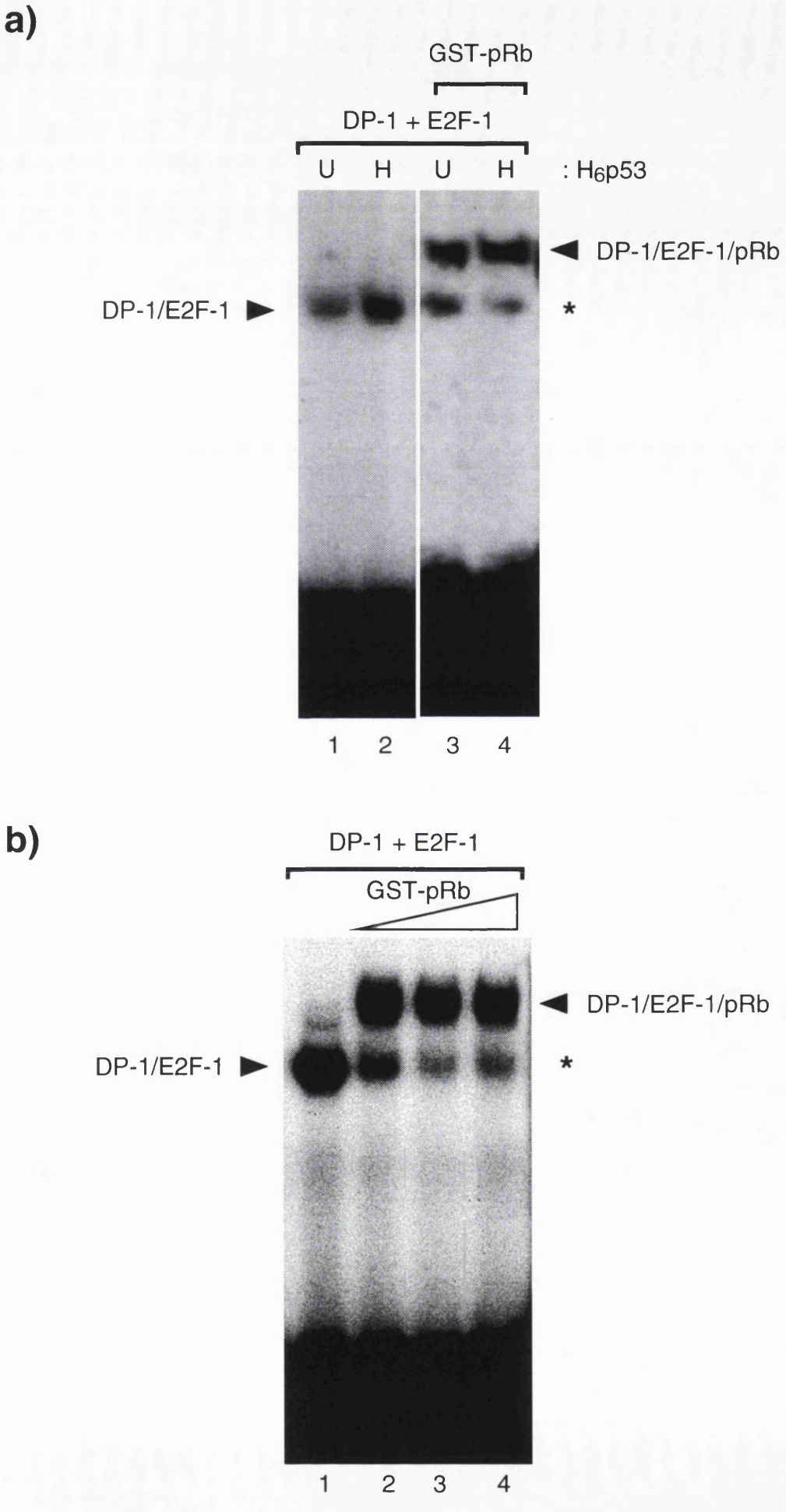
pRb-complexed heterodimer DNA binding activity on the E2F site is not targeted by p53.

a. *Gel retardation to assay p53 inhibition of DP-1/E2F-1 heterodimer in the presence of pRb.*

Gel retardation assay probed with the E2F DNA binding site with *in vitro* translated DP-1 (3µl) and E2F-1 (1µl) together in all lanes, and approximately 20ng of GST-pRb added to lanes 3 and 4 (all lanes are from the same gel retardation assay). Added to the reaction mixtures in all lanes was approximately 400ng of histidine tagged, bacterially expressed and purified p53. In lanes 2 and 4 this was heat denatured at 100°C for five minutes prior to addition (H) as opposed to untreated (U) in lanes 1 and 3. * indicates aberrant E2F site binding activity in lanes 3 and 4. As opposed to other gel retardation assays, here the primary incubation was performed entirely on wet ice for 50-60 minutes prior to addition of probe, which was incubated in the usual manner - this optimises the inhibitory effect of p53.

b. *Gel retardation assay of in vitro translated DP-1/E2F-1 heterodimer exposed to increasing levels of GST-pRb.*

Gel retardation assay probed with labelled E2F DNA binding site. All lanes contain *in vitro* translated DP-1 (3µl) and E2F-1 (1µl) together. In lanes 2, 3 and 4, approximately 10, 30 and 90ng respectively of GST-pRb was added. Aberrant E2F site binding activity detected in lanes 3 and 4 is indicated by *.


a)
GST-pRb
DP-1 + E2F-1
U H U H
: H6p53
DP-1/E2F-1/pRb
DP-1/E2F-1
*
1 2 3 4
b)
DP-1 + E2F-1
GST-pRb
DP-1/E2F-1/pRb
DP-1/E2F-1
*
1 2 3 4

form a complex and so may not be representative of E2F-1/DP-1 (lanes 2-4, marked with *). Some background E2F DNA binding activity was present in reticulolysate-only loadings (can just be detected in **figure 7.4b**, lane 4) which are equivalent in volume, and have undergone the same incubation procedure as the E2F-1/DP-1 mixture (lane 5). This background activity may contain E2F/DP-like factors whose DNA binding activity may be enhanced in the presence of the *in vitro* translated DP-1 and E2F-1, but form active E2F site binding complexes that do not respond to pRb and p53 as the E2F-1/DP-1 heterodimer would. Certainly **figure 7.6** makes it clear that not all heterodimer combinations with E2F-1 respond identically to p53 and different E2F-family members direct alternative pocket protein preferences (see Introduction). It is thus likely that the lack of p53 inhibition of this faster migrating activity is because this activity is not true heterodimer and will interact with neither p53 nor pRb. It was not possible ever to inhibit 100% of all heterodimer with p53, no matter how much was added, and a possible explanation to this is the presence of this non-specific activity. Certainly more work is called for to clarify this issue.

Chapter 8

Discussion

Phosphorylation and DP-1

The transcription factor DRTF1/E2F has aroused considerable interest in the field of cell cycle regulation in the past few years due to its apparent central role in the control of the proliferative state and its provision of the first clear link between the cell cycle and proliferation-inducing events at the level of transcription. The control by the cell cycle apparatus of the phosphorylative status, and hence the activity, of the retinoblastoma gene product has been hailed as a significant link between the cell cycle and the control of downstream proliferation-inducing events through the regulation of the activity of DRTF1/E2F (see Introduction). Experimental results reported in this thesis (Chapters 3-6) provide evidence that the cell cycle is able to target the activity of the DRTF1/E2F in a novel manner which is both direct and independent of pRb.

p55U and p55L and the cell cycle

The DP-1 protein product was demonstrated to exist in at least two different immunogenic forms, referred to as p55U and p55L (Upper and Lower respectively), reflecting their different mobilities in a denaturing polyacrylamide gel (Chapter 3). p55U is in a state of hyper-, and p55L, hypo-phosphorylation, suggesting that they are differentially phosphorylated forms of each other (Chapter 6). The p55L-form appeared to exclusively represent DP-1 DNA-binding contribution to DRTF1/E2F in F9EC cells, while p55U lacked detectable DNA binding activity (Chapter 5). One potentially important site of phosphorylation in DP-1, identified immunochemically, corresponds with a cyclin-dependent kinase consensus site (Chapter 6). The phosphorylation status of DP-1 is cell cycle regulated and could influence the activity of DRTF1/E2F (Chapter 5).

Suspicions that the two phospho-forms of DP-1 might be cell cycle regulated came from early experiments (Bandara *et al.*, 1994) in which serum starved NIH-3T3 cells were used to study cell cycle progression. Cell extracts made at different times after re-stimulation, and assayed by gel retardation for E2F site DNA binding activity, demonstrated that DRTF1/E2F activity was low at G0 and rose progressively to a

peak at the G1/S boundary as observed by others (Mudryj *et al.*, 1991 ; Shirodkar *et al.*, 1992 ; Schwarz *et al.*, 1993). When the same extracts were assayed by immunoblotting using the APαA reagent, p55U was found to be present throughout the cell cycle while p55L on the other hand only became detectable concomitantly with the induction of DRTF1/E2F activity at G1/S (Bandara *et al.*, 1994). This result supports both the perception of p55L as the DNA-binding form of DP-1, and also implicates the p55L, and hence the state of DP-1 phosphorylation, with the cell cycle regulation of the DRTF1/E2F.

One of the big questions arising from this study is what the role of the apparently large amounts of non-E2F site binding p55U is in the cell. Is this a reservoir of hyper-phosphorylated DP-1 from where to rapidly recruit p55L in a non-transcription/translation dependent manner ? - Or are there more specialised roles concerned with p55U ? The next section ("A tale of two tumour suppressors") postulates possible ulterior functions of p55U.

Cyclin-dependent kinases and the DP-1/E2F-1 heterodimer

. The direct phosphorylation and consequent regulation of the E2F/DP heterodimer, represents a novel target for the regulatory kinases of the cell cycle.

Targeting of DP-1

An *in vitro* transcription assay, consisting of reconstituted cloned and purified RNA polymerase II basal transcription factors, which is DRTF1/E2F-responsive, was used to analyse the effects of phosphorylation on DP-1 and E2F-1 (Dynlacht *et al.*, 1994). In this assay cyclin A/CDK2 was able inhibit pRb binding to the E2F-1/DP-1 heterodimer in line with established hypotheses (Whyte, 1995 ; see Introduction). Higher concentrations of cyclin A/CDK2 in this assay additionally reduced the DNA binding activity of the heterodimer. Hence it was demonstrated that cyclin A/CDK2 can have two conflicting activities : One where the activity of DRTF1/E2F was induced and one which would inhibit (Dynlacht *et al.*, 1994). Cyclin E/CDK2 could also inhibit pRb but showed no apparent effect on the DNA binding activity of the heterodimer. Both E2F-1 and DP-1 appeared to be phosphorylated by cyclin A/CDK2 but not by cyclin E/CDK2. The peptide digest maps of *in vitro* phosphorylated DP-1 versus HA-tagged transfected/*in vivo* purified DP-1 showed apparently identical phosphorylation patterns suggesting a degree of *in vivo* relevance to these results (Dynlacht *et al.*, 1994).

Others (Krek *et al.*, 1994) have reported the existence stable cyclin A-kinase/E2F-1 complexes in non-transfected cell lines and similar complexes in transfected cells that did not depend on pRb but on a discrete set of sequences in the N-terminus of E2F-1 which appeared to specifically bind cyclin A (Krek *et al.*, 1994). Cyclin E complexes were not detected in natural cell lines and E2F-1 bound neither cyclin E nor cyclin B1 *in vitro* (Krek *et al.*, 1995). The cyclin A/E2F-1-transfected complexes formed in a cell cycle-dependent manner, and at greatly increased levels as cells progressed into S-phase. Transfected E2F-1 mutants unable to bind cyclin A formed un-complexed DNA binding heterodimers which persisted all the way into G2 as if having escaped DNA binding inhibition. Down regulation of DRTF1/E2F in S/G2 correlated with increased DP-1 phosphorylation and mutation of the cyclin A binding sequence in E2F-1 also prevented this S-phase phosphorylation of DP-1 (Krek *et al.*, 1995).

These transfection data thus draw a connection between the phosphorylation of DP-1 and the cyclin A/E2F-1 dependent down-regulation of DRTF1/E2F. In accordance with this, cyclin A/CDK2 phosphorylated DP-1/E2F-1 heterodimer *in vitro*, which consequently displayed reduced DNA binding activity while treatment with phosphatase reversed this effect (Krek *et al.*, 1995). More *in vivo* evidence was provided by the demonstration of reduced DRTF1/E2F activity when immunoprecipitated by anti-cyclin A in the presence of ATP as opposed to a non-hydrolysable analogue (Krek *et al.*, 1995). The mutant E2F-1 effects on the DNA binding activity of DRTF1/E2F are predictably reflected in transcriptional activation properties as E2F-1 mutants unable to bind cyclin A *trans*-activated more strongly in the presence of cyclin A than wild-type (Krek *et al.*, 1995). Thus, phosphorylation of DP-1 by cyclin A/CDK2 may suppress activation of DRTF1/E2F-dependent genes in S/G2 phase when they are no longer required. The cyclin A binding motif appears to be conserved in E2Fs -2 and -3 but not in E2F-4 and -5 (Krek *et al.*, 1995).

The evidence presented in this thesis is very compatible with the above data. The observed *in vivo* DP-1 doublet which displayed differential affinity to the E2F site as well as a differential state of phosphorylation, is a very good candidate for the product of a cyclin A/E2F-1-mediated phosphorylation. This report also provides a possible explanation for the loss of E2F site DNA binding activity of the hyper-phosphorylated form of DP-1 by the suggestion that this form cannot heterodimerise with at least E2F-1 (Chapter 5). Furthermore, the DP-1 doublet has been observed in all cell lines tested including HeLa, Saos2, NIH-3T3, 3T3-DM, human foreskin keratinocytes, Daudi, (data not shown) primary acute human myeloid-leukaemia (Williams *et al.*, 1995) and F9EC cells suggesting that this level of regulation may be a very fundamental and general aspect of proliferative regulation.

Targeting of transcription factors by phosphorylation

Protein phosphorylation is the modification of choice when rapid modulation of protein activity in response to changes in metabolic activity, environmental conditions and hormonal signals is required (Hunter & Karin, 1992 ; Karin, 1994). As described in the Introduction, the regulation of transcription factors by phosphorylation has been documented to target aspects of their function such as nuclear transport, *trans*-activation, dimerisation and DNA binding (Hunter & Karin, 1992). In several respects, the potential phosphorylative regulatory system described here, concerning DP-1 and the DRTF1/E2F complex, bears resemblance to the c-Jun transcription factor in the AP-1 complex.

The c-Jun and c-Fos protein families each contribute to the dimeric sequence-specific activator AP-1 which, as opposed to the DP-/E2F- families, can take the form of either homo- or hetero-dimers. AP-1 activity is regulated in response to a vast array of extracellular stimuli (Angel & Karin, 1991). Part of this regulation is executed through the induction of c-Fos and c-Jun genes, another is mediated through post-translational modification of AP-1 proteins. In resting cells c-Jun is phosphorylated on five regulatory sites, three of which are clustered next to the carboxy-terminal DNA binding domain (T231, S243, S249). Phosphorylation of the carboxy terminal sites inhibits DNA binding by c-Jun homodimers (Boyle *et al.*, 1991 ; Lin *et al.*, 1992) but has no measurable effect upon c-Jun/c-Fos heterodimers (Karin, 1996). Two of these sites are constituitively phosphorylated by casein kinase II (T231, S249) and microinjection of CKII suppresses induction of AP-1 activity (Lin et al, 1992). The other two phosphorylation sites are located at the amino-terminus (S63, S73) and their phosphorylation stimulates the transcriptional activity of c-Jun without affecting its DNA-binding activity (Smeal, 1992) either as a c-Jun homodimer or as a heterodimer with c-Fos (Karin, 1996). Activation of protein kinase C results in rapid site-specific de-phosphorylation of c-Jun (at the three carboxyterminal sites) and is coincident with increased AP-1 binding activity. Phosphorylation *in vitro* of c-Jun proteins by glycogen synthase kinase 3, decreases their DNA binding activity and mutation of S243 blocks phosphorylation of all three sites *in vivo* and increases the inherent *trans*-activational activity of c-Jun at least 10-fold (Boyle et al 1991). From this it is suggested that c-Jun is present in resting cells in a latent, phosphorylated form that can be activated by site-specific dephosphorylation in response to protein kinase C activation (Boyle et al 1991). A protein kinase which specifically phosphorylates c-Jun at S63 and S73, named JNK (c-Jun N-terminal kinase) has been identified and found to be a member of the MAP kinase group of signal transducing enzymes (Dérijard et al, 1994).

Targeting of E2F-1

There is some evidence that phosphorylation of E2F-family members may also have an effect on the DRTF1/E2F status. *In vivo* phosphorylation on E2F-1 at serine S375 near the pRb binding site has been suggested to be accomplished by cyclin A/CDC2 and to a lesser extent by cyclin A/CDK2 (Peeper *et al.*, 1994). Phosphorylation of E2F-1 on S375 greatly enhanced its affinity for pRb *in vitro* (Peeper *et al.*, 1994). In contrast, phosphorylation of E2F-1 on serine residues 332 and 337 has been reported to prevent its interaction with pRb, while being a pre-requisite for interaction with the adenovirus E4 gene product (Fagan *et al.*, 1994). These residues were phosphorylated *in vivo* around the G1 of cell cycle, and by CDC2 or cyclin D1-CDK4 kinase *in vitro*. CDC2 is however not likely to be the physiological kinase due to cell cycle timing of its activation which is more associated with late S-phase to G2 (Fagan *et al.*, 1994). These latter data are in line with observations made in Saos 2 cells, which are pRb-deficient, where introduction of pRb causes them to arrest in the G1 phase. This arrest can be overcome by the expression of cyclins A, E or D (Hinds *et al.*, 1992 ; Dowdy *et al.*, 1993 ; Ewen *et al.*, 1993). Suppression of pRb-mediated growth arrest by cyclin A or E is accompanied by pRb hyper-phosphorylation and the loss of E2F-pRb complexes (Hinds *et al.*, 1992 and see Introduction). Suppression by cyclin D1, however, has been reported not to be accompanied by pRb hyper-phosphorylation, nevertheless, E2F-pRb complexes are still lost (Dowdy *et al.*, 1993). This could suggest that the mode of action of cyclin D1 associated kinase activity is to target E2F-1, rather than pRb, with the same consequential effect of disrupting the E2F-1/pRb complex. It has also been reported that phosphorylation of E2F-1 by cyclin A/CDK2 *in vitro* during S-phase will reduce its DNA binding activity (Kitagawa *et al.*, 1995). This study was however carried out in disregard/ignorance of DP-family contribution to E2F-DNA binding activity and so its physiological relevance is unclear.

Potential sites of phosphorylation in DP-1

A mutant DP-1 lacking the N-terminal potential CDK phosphorylation sites (Serine 23, 80, 98 and Threonine 66, 83 : - converted to Alanine) has been employed to identify potential phosphorylation sites of cyclin A/CDK2 (Krek *et al.*, 1995). This mutant formed a stable and active heterodimer with E2F-1 and super-retroviral infection with E2F-1 into NIH-3T3 cells caused an accumulation of S-phase cells in a similar manner seen with mutant cyclin A-binding site-E2F-1 but not observed with the wild-type DP-1. The arrest was attributed to the interference with the ongoing

DNA replication process by unscheduled DRTF1/E2F activity and was used by the authors to propose that down-regulation of DRTF1/E2F by phosphorylation on DP-1 N-terminal sites is essential for normal S-phase progression (Krek *et al.*, 1995).

However, the mutant DP-1 employed did not include the only other potential CDK site in DP-1, Threonine-388 (T388) at the C-terminus, which is the very site which has been implicated, by the studies presented in this thesis, as a possible target of a repressive phosphorylative event (see Chapter 6). The monoclonal antibody, 32.3, only recognises the hypo-phosphorylated form of DP-1 (p55L) and the C-terminal DP-1 peptide it was raised to contains the threonine residue at position 388. When T388 was phosphorylated, 32.3 no longer recognised the peptide (**figure 6.4**) suggesting that the T388 residue could be phosphorylated in the p55U but not in the p55L-form of DP-1. An experiment which analysed extracts taken from NIH 3T3 cells at different stages of the cell cycle showed that 32.3 maximal recognition of p55L also coincided with G1/S phase (**figure 5.5**) and suggested that the phosphorylation event on DP-1 may include the C-terminus and thus implicates T388 with G1/S phase phosphorylation (Chapter 6).

The DP-1 protein mutated in all but the C-terminal potential CDK site was still labelled by cyclin A/CDK2 *in vitro* and its phosphorylation level, although reduced, was not abolished in transfections (Krek *et al.*, 1995). While this does not prove that the remaining phosphorylation is due to the T388 site *in vivo*, it still leaves the possibility open and it is certainly encouraging that cyclin A/CDK2 will phosphorylate this mutant *in vitro* (Krek *et al.*, 1995). Krek *et al.* play down the potential contribution of the C-terminus without truly having investigated its role by for example making a DP-1 mutated at T388 and studying its effect in cyclin A co-transfections. Their results certainly implicate N-terminal sites in repressive phosphorylation events but this does not exclude the possibility that the T388 has a contributory role in heterodimer inhibition. It is of course also possible that any differential phosphorylation of the C-terminus may simply be symptomatic of the kinase/phosphatase status of the cell at a particular time of the cell cycle with no functional significance. Alternatively it is possible that phosphorylation of the C-terminus of DP-1 has a different role altogether. It should not be ignored that the T388 site is the *only* CDK consensus site that is conserved in *all* DP-family proteins (**figure 3.5**) while the N-terminal CDK sites are unique to DP-1. Phosphorylation at this site may involve functions of the DP-family that are phosphorylation dependent but common to all, such as p53 association (see next section).

Physiological model for regulation of DRTF1/E2F through cell cycle dependent phosphorylation

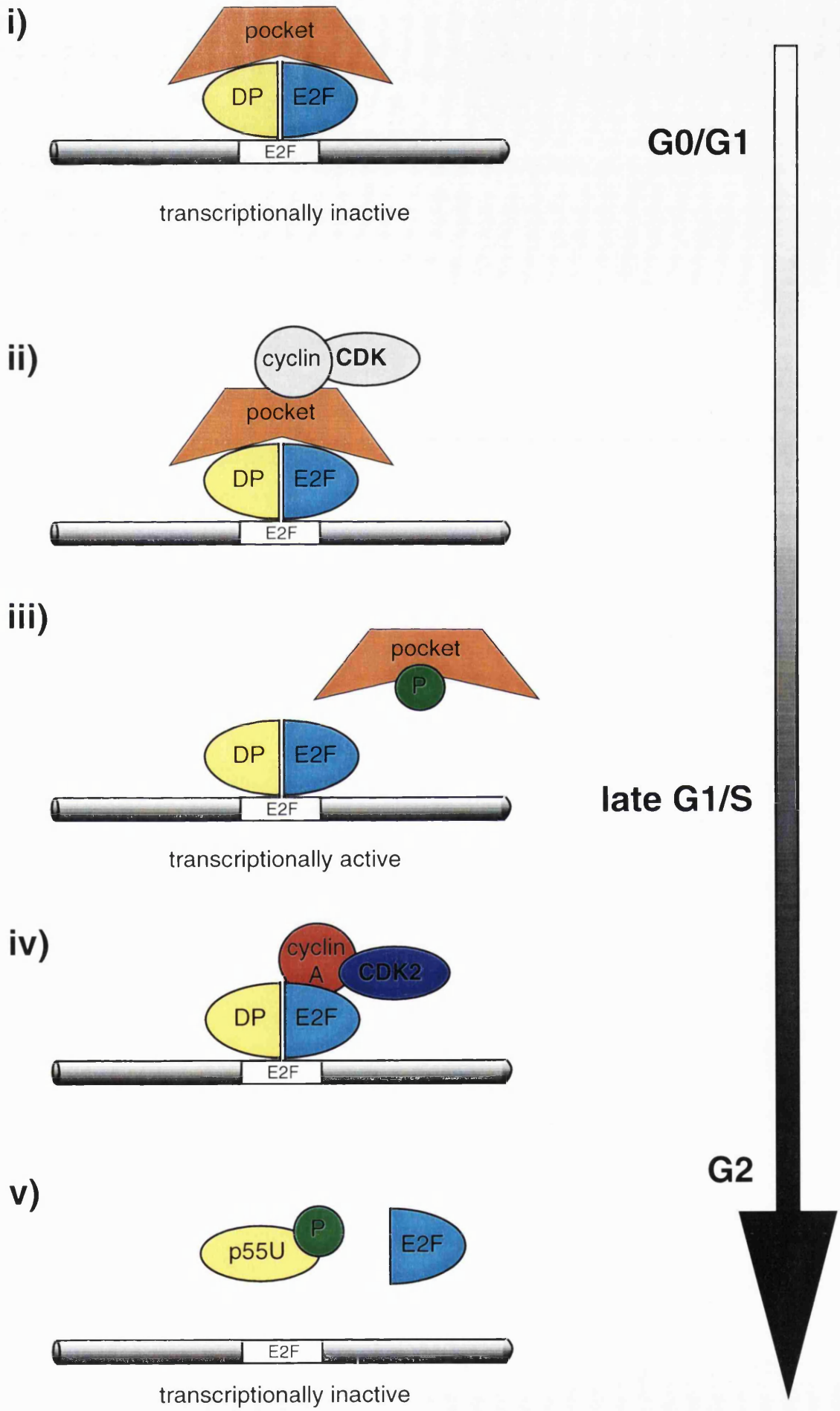
Cyclin A and E are both associated with the DRTF1/E2F complex (Bandara *et al.*, 1991a ; Mudryj *et al.*, 1991 ; Bandara *et al.*, 1992 ; Lees *et al.*, 1992 ; Devoto *et al.*, 1992) although unlike cyclin D (Dowdy *et al.*, 1993 ; Kato *et al.*, 1993), they are unable to make direct interaction with pRb (Hinds *et al.*, 1992). It now seems that a physical basis for the cyclin A DRTF1/E2F interaction via the E2F-1 has been found as well a potential role for it in the regulation of DRTF1/E2F DNA binding activity (Krek *et al.* 1994 & 1995). As yet it seems unclear how cyclin E interacts with the complex and how it may exert a function.

The implication of the cyclin A/CDK2 kinase complex in the down-regulation of DRTF1/E2F during S-phase makes sense in terms of the timing of cyclin A presence during the cell cycle. Thus far we have only been able to explain cell cycle regulation of DRTF1/E2F activation via the potential roles of cyclin A, E and D on pRb in G1 phase (see Introduction and references above). The later S/G2 phase reduction of DRTF1/E2F DNA binding activity can now be tentatively attributed to direct phosphorylation of DP-1 (Chapter 6 and **figure 8.1**) by cyclin A/CDK2 (Krek *et al.* 1994 & 1995).

Suppression of DRTF1/E2F DNA binding activity is essential for the orderly progression of S-phase, disruption of this event results in S-phase delay or even apoptosis (Krek *et al.*, 1995). Cyclin A-kinase function is essential for S-phase progression (Girard *et al.*, 1991 ; Pagano *et al.*, 1992) and so it would seem that an important target of cyclin A-associated kinase could be DP-1. Cyclin A concentration gradually rises from the G1/S transition to the middle of S-phase and its overexpression can override pRb-induced arrest (Hinds *et al.*, 1992). Intriguing *in vitro* observations indicate that *lower* concentrations of cyclin A/CDK2 will disrupt pRb binding to E2F-1/DP-1, while higher concentrations will start to inhibit DNA binding of the heterodimer (Dynlacht *et al.*, 1994). *In vivo* support for this, however, has yet to be demonstrated. This nevertheless raises the possibility that cyclin A is both involved in the up- and down-regulation of DRTF1/E2F - If this is the case, it is little wonder that cyclin A is such a critical contributor to S-phase progression. It is clear that overactivation of DRTF1/E2F can be disastrous for a cell, let alone the host organism (see Introduction and next section), and so it would seem that the down-regulation of this activity is as important as its activation.

Figure 8.1
Hypothesis of the regulation of DRTF1/E2F activity by phosphorylation.

(**i**) The DP-E2F heterodimer is depicted as an oval (Yellow half : DP, Cyan half : E2F) bound to an E2F site on the DNA template (grey bar). Bound to the heterodimer is a pocket protein (Orange hat) which masks the transactivation domain of the E2F-moity of the heterodimer thus preventing transcriptional activation during G0 and the first half of G1. (**ii**) As G1 progresses the pocket protein is targeted by cyclin/CDK complexes which phosphorylates it (**iii**) thus inhibiting its interaction with the DP/E2F heterodimer and promoting transcription from E2F-containing promoters during late G1 and S phases. (**iv**) At late S phase E2F-1, and possibly E2F-2 and -3, are complexed by the cyclin A/CDK2 kinase complex (red circle and blue oval) which phosphorylates the DP-moiety (**v**) (Phosphate : P - Green circle) to generate the p55U isoform of DP-1, which might prevent its interaction with the E2F-family member and hence cause dissociation from the E2F site with concurrent loss of transcriptional activation.


i)
pocket
DP
E2F
E2F
transcriptionally inactive
G0/G1
ii)
cyclin
CDK
pocket
DP
E2F
E2F
iii)
pocket
P
DP
E2F
E2F
transcriptionally active
late G1/S
iv)
cyclin
A
CDK2
DP
E2F
E2F
G2
v)
p55U
P
E2F
E2F
transcriptionally inactive

As the cell progresses through the latter stages of the cell cycle, what DRTF1/E2F binding activity there is left, appears to be predominantly involved in pocket protein complexes (Whyte, 1995). The report that E2F-1 phosphorylation by cyclin A/CDC2 increased its affinity for pRb (Peeper *et al.*, 1994) could potentially mean that such a phosphorylation is responsible for the recreation of pocket complexed heterodimer later in the cell cycle. It will be interesting to elucidate the processes responsible for pRb de-phosphorylation, and for example to determine if E2F-1 phosphorylation will allow repression by phosphorylated pRb.

A tale of two tumour suppressors

The evidence presented in this thesis raise the possibility that the major human tumour suppressor gene product, p53, can directly target and regulate the activity of the proliferation/S-phase promoting transcription factor DRTF1/E2F. The other major human tumour suppressor, pRb, is already known to be a modulator of DRTF1/E2F activity, and previously, any effect of p53 on DRTF1/E2F has been assumed to occur via pRb through the stimulation of the p21 gene (Ko & Prives, 1996 ; see Introduction). It now seems that both of these important tumour suppressors may both target the same transcription factor directly and independently, despite their different roles in the cell cycle and in the control of the tumourigenic state. This raises implications for the currently perceived view of DRTF1/E2F as a transcription factor complex and provides us with further understanding into the workings and co-operativity of pRb and p53 in neoplastic suppression.

p53 binds DP-1 and inhibits DRTF1/E2F

A physiological interaction between DP-1 of the S-phase inducing transcription factor DRTF1/E2F and the tumour suppressor p53 was demonstrated by the immunoprecipitation, using an antibody to p53, of DP-1 from non-transfected F9EC cell extracts (**figure 7.2**). *In vitro* studies demonstrated that bacterially expressed DP-1 fusion protein can make a specific interaction with *in vitro* translated p53 (Sørensen *et al.*, 1996) and that p53 fusion protein complexes with cellular DP-1 (**figure 7.3**), suggesting that a direct protein-protein p53/DP-1 interaction is a possible basis for the complex detected *in vivo*. Confirmatory observations suggesting a DP-1/p53 interaction were made at a similar time (O'Conner *et al.*, 1995) but unresolved and conflicting evidence remain as to whether p53 can form direct contact with E2F-1 or not (O'Conner *et al.*, 1995 ; Sørensen *et al.*, 1996).

In vitro translated DP-1 mutants precipitated using bacterially expressed p53-histidine tagged fusion protein, demonstrated that p53 interacts with a central region of DP-1 (residues 171-331 : Sørensen *et al.*, 1996). This region contains domains conserved between different DP family members (DCB1, DCB2 : Girling *et al.*, 1994 ; Ormondroyd *et al.*, 1995), and importantly, the DEF Box which is critical for E2F/DP family heterodimerisation. This suggested that p53 can interfere with this event which is so crucial to high affinity E2F site binding by the DRTF1/E2F transcription factor (see Introduction). Accordingly, it was demonstrated that p53 and E2F-1 will compete for DP-1 binding (Sørensen *et al.*, 1996) suggesting that p53

would be unable to interact with the DP/E2F family DNA binding heterodimer. This hypothesis was supported by the repeated failure to demonstrate the presence of p53 in cellular DRTF1/E2F complexes using antibodies to p53 (*including* the one used for the *in vivo* immunoprecipitation of DP-1) on the DRTF1/E2F complex in gel retardation assays, by attempts to immunoprecipitate E2F site DNA binding activity using antibodies to p53, and also by the observation that p53 interacts with the non-DNA binding form of DP-1, p55U (**figure 7.2**).

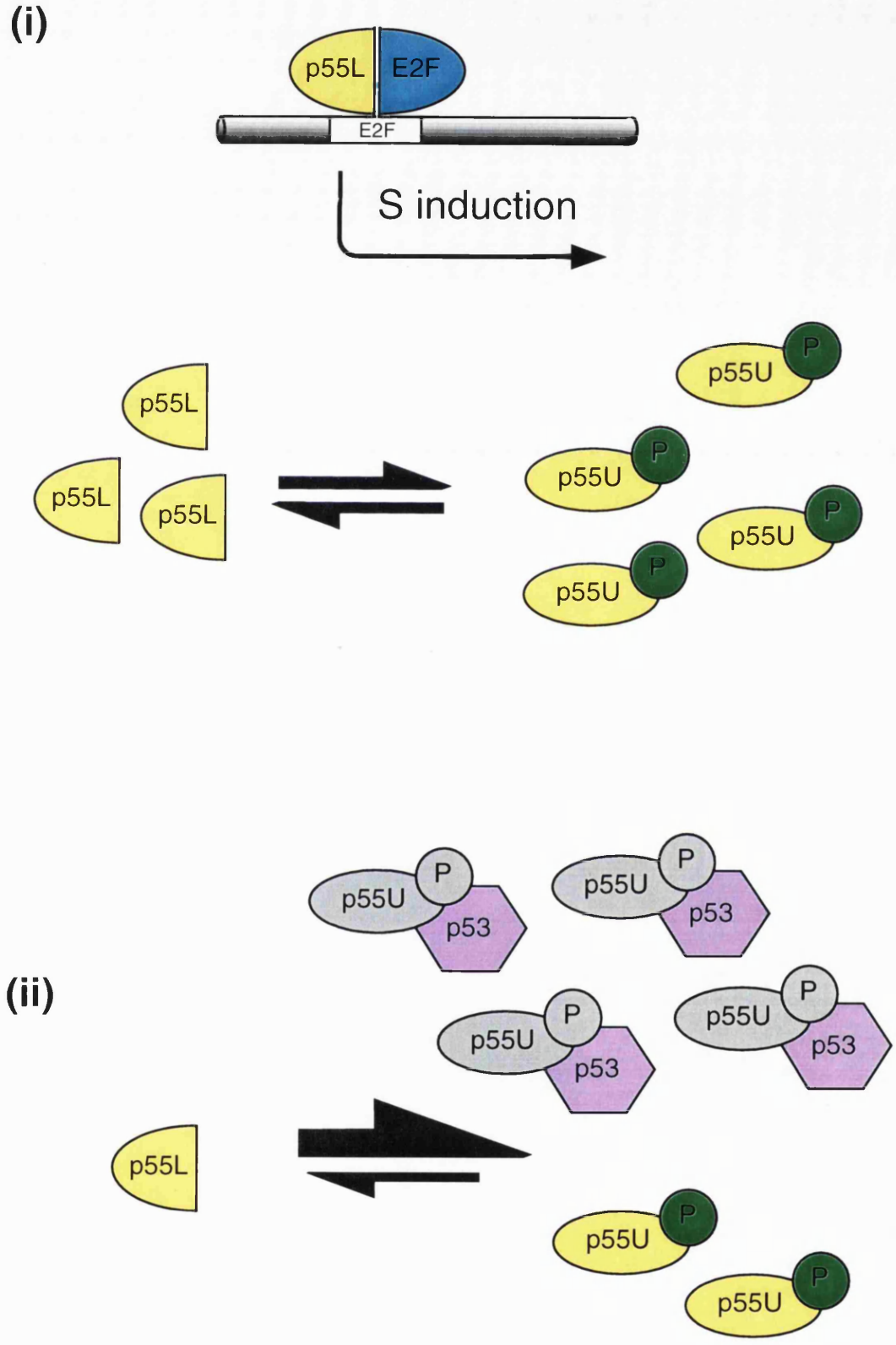
If p53 can remove E2F-1 from DP-1, then the prediction would be that p53 will interfere with DP-1/E2F-1:E2F-site DNA binding activity. This was specifically demonstrated by reduction in the E2F site DNA binding activity of the heterodimer when exposed to p53, in a manner dependent on p53 concentration and integrity (**figure 7.4**). This suggests that p53 binding to DP-1 creates a non-E2F-site binding complex that excludes E2F-1. Inhibition of the DNA binding component of DRTF1/E2F by p53 would be expected to have an effect on the DRTF1/E2F transcriptional activation properties and this was demonstrated by transfection studies in an E2F-site dependent manner in NIH-3T3 and Saos2 cell lines (Sørensen *et al.*, 1996 ; O'Conner *et al.*, 1995). The DRTF1/E2F-inhibition properties of p53 by the direct targeting of the heterodimer, defines a novel activity for p53 and importantly suggests that it can regulate the activity of DRTF1/E2F in a manner that is independent of the retinoblastoma gene product since Saos2 cells lack functional pRb.

Molecular basis of DRTF1/E2F DNA binding inhibition by p53

p53 preferentially interacts with just one phospho-form of DP-1, p55U, which is believed to be a type of DP-1 with low, or no, affinity for both the E2F-site and possibly for at least one E2F family partner (Chapter 5 and 7 and the phosphorylation section of this Chapter, above). It initially seems counter-intuitive that p53 prevents DNA binding by the interaction with a non-DNA binding factor. However, this can be reasoned by the possibility that the p55U and p55L phosphoforms of DP-1 exist in equilibrium with each other. If p53 has a higher affinity for the non-DNA binding form, p55U, then a surge of p53 concentration, as a consequence of for example DNA damage (see Introduction ; Ko & Prives, 1996), may encourage the conversion of p55L to p55U by tilting the equilibrium toward the latter to compensate for its complexing to p53. The consequent depletion of p55L levels would reduce DRTF1/E2F activity (**figure 8.2).** The phosphorylation section deals with the normal cell cycle conversion of one phosphoform to another by the probable action of cyclin A/CDK2. It may not be unreasonable to postulate that p53 may aid the conversion by recruiting a similar activity to DP-1.

Figure 8.2
p53 inhibition of DP-1/E2F by modulation of the equilibrium between DP-1 isoforms.

(**i**) The DP-1 isoforms, p55L (yellow half-ovals) and p55U (yellow ovals) may be in an equilibrium with each other. p55L is a form which can heterodimerise with an E2F-family member to form the DNA-binding component of the transcription factor complex DRTF1/E2F. p55U is a hyper-phosphorylated version of p55L (green circles = phosphorylation) and probably unable to heterodimerise with E2F-family members and more than likely not able to engage in DNA binding. Equilibrium conversion of p55U to p55L is associated with increased activity of DRTF1/E2F which in turn promotes S-phase entry by the enhancement of specific gene expression. (**ii**) A surge in the intracellular concentration of activated p53 tumour suppressor protein results in its complexing with p55U forms of DP-1 which then effectively are removed from the p55U pool (grey complexes). To compensate for this loss, the p55U/p55L equilibrium becomes biased towards the conversion of p55L to p55U, consequentially reducing p55L levels and hence the activity of DRTF1/E2F. Down-regulation of DRTF1/E2F causes G1 arrest.


(i)
p55L
E2F
E2F
S induction
p55L
p55L
p55L
p55U
P
p55U
P
p55U
P
p55U
P
(ii)
p55U
P
p53
p55U
P
p53
p55U
P
p53
p55U
P
p53
p55L
p55U
P
p55U
P

G1 arrest

An equilibrium model does however not easily explain why p53 should inhibit heterodimer formation in the *in vitro* situation of the gel retardation assay. It is possible that p53 has a low affinity for the DNA binding form of DP-1 while preferentially binding to the non-DNA binding form. When DP-1 is exposed to sufficient quantities of p53, then binding to, and inhibition of, the DNA binding form of DP-1 might take place. Certainly, relatively high concentrations of p53 were required to observe the DP-1/E2F-1 inhibition (see **figure 7.4** and accompanying legend). Alternatively, as this experiment employed reticulolysates, which conceivably could contain kinases which phosphorylate DP-1, a p53-mediated inhibitory phosphorylation event could have been responsible for the observed effect. This could explain why I was repeatedly unable to demonstrate p53 inhibition of heterodimers composed of bacterially expressed and purified GST-E2F-1 and -DP-1 fusions, although this could have been due to steric hindrance from the bulky GST-domains involved.

The idea that p53 might directly or indirectly recruit a kinase activity that will phosphorylate DP-1 to form p55U is not entirely unreasonable in the light of recent evidence that a surge of cyclin D1 concentration follows p53 induction, and that this response, in co-operation with p21, is necessary for p53-induced cell cycle arrest (Del Sal *et al.*, 1996). Inappropriate cyclin D1 expression has been shown by many to cause cell cycle arrest at S-phase (see Introduction/cell cycle section **:** Quelle *et al.*, 1993 ; Baldin *et al.*, 1993 ; Atadja *et al.*, 1995) and so raises the possibility that a cyclin D1 kinase is responsible for phosphorylating DP-1 in response to increased p53 concentrations. This effect would clearly somehow have to by-pass the inhibitory effects of the concomitant rise in p21 concentration. Any kinase involvement can be tested quite simply experimentally.

The scenarios can thus be envisaged whereby a surge in active p53 concentration causes sequestration of DP-1 non-DNA binding form (p55U). This has an effect on the equilibrium between the two forms of DP-1, due to the effective removal of p55U from the p55 pool, such that p55L is converted to the p55U form, either actively or passively, to compensate. Alternatively the surge in the concentration of p53 is sufficiently high to promote a lower affinity binding to the p55L form and hence inhibit directly. Either way, DRTF1/E2F DNA binding activity is lowered as a consequence of p55L reduction. Experiments need to be carried out to investigate whether p53-induced DRTF1/E2F inhibition *in vivo* is accompanied by conversion of p55L to p55U or whether the ratio of phosphoforms remains unchanged (**figure 8.2**).

It could be argued that the reason the p55U is unable to bind the E2F-site is *because* of the bound p53. It has yet to be determined whether all p55U is associated with p53. It could be conceived that phosphorylation of DP-1 by cyclin A/CDK2 does not in itself induce heterodimer dissociation but that it encourages the association of p53 and thus a consequent heterodimer dissociation. This is however unlikely in the light of the experiments (Dynlacht *et al.*, 1994) demonstrating DP-1 phosphorylation and consequent loss of DNA binding activity in an *in vitro* transcription reconstitution assay that does not involve, and unlikely to hold any significant p53. Hence it is likely that cyclin A/CDK2-associated reduction of DNA binding activity is due to the phosphorylation event itself and that inhibition by p53 is a separate activity. This will be easily tested by assaying the DNA binding properties of the two forms of DP-1 in Saos2 cells, where p53 is absent.

Transcription factors targeted by p53

The p53 protein product appears to be able to exert its tumour-suppressive activity via both sequence-specific DNA binding, to promote or repress gene expression (Liu *et al.*, 1993 ; Ragimov *et al.*, 1993), and via specific protein-protein interactions (Ko and Prives, 1996 ; see Introduction). The cellular proteins that interact with p53 appear to possess a wide range of different functions. One category involve proteins associated with DNA repair and replication such as RPA (Dutta *et al.*, 1993) and TFIIH components (Xiao *et al.*, 1994), another involves kinases such as casein kinase II (Filhol *et al.*, 1992), calcium binding proteins such as S100b (Baudier *et al.*, 1992) and proteins associated with the ubiquitin degradation pathways (Huibregtse *et al.*, 1993).

One group of p53-associating polypeptides comprise factors that themselves associate with DNA. The TBP component of the basal transcription factor TFIID can bind p53 which through this interaction can mediate transcriptional repression or activation (Horikoshi *et al.*, 1995). TBP and p53 co-operatively interact on DNA containing a p53 binding site, both in the absence and presence of a TATA-box. p53 strongly inhibits TBP interaction with the TATA box in promotors that lack a p53 DNA binding site (Horikoshi *et al.*, 1995). p53 represses transcription of a number of cellular and viral genes with promoters lacking p53 binding sites including *c-Fos*, *c-Jun*, *IL-6*, *RB*, and *Bcl-2* (Donehower and Bradley, 1993 ; Jackson *et al.*, 1993 ; Miyashita *et al.*, 1994) but only those promoters containing TATA boxes, not Inr elements, are inhibited by p53 (Mack *et al.*, 1993). The human $TAF_{II}31$ also binds p53 and is a critical protein required for p53-mediated transcriptional activation (Lu and Levine, 1995).

Binding of p53 to TAFs and TBP concerns the modulation of the basal transcription machinery in an apparently very general manner since these transcription factors are involved in the regulation of a very wide range of genes (see Introduction). The potency of p53 as a broad-range repressor may be an important component of its tumour repressor function.

The interaction of p53 with DP-1 suggests that p53 may also be able to target specific gene expression. There are some examples of p53 interaction with other transcription factors, although the exact mechanisms involved have yet to be elucidated.

p53 has been reported to bind to the transcription factor Sp-1 (Borellini & Glazer, 1993). The DNA-binding protein Sp1 functions as an important trans-activator during cell development and differentiation, and plays a key role in the activation of many cellular and viral gene promoters, including many that are regulated during the cell cycle. Sp-1 DNA binding complexes in human erythroleukaemia cells also contain p53 (Borellini & Glazer, 1993) and p53/Sp-1 complexes can bind both p53 and Sp-1 DNA sequences. Thus it seems possible that p53 can interact with some transcription factors while bound to DNA as opposed to the apparent situation with DP-1 where DNA binding is disrupted. However there is also some evidence that as with DP-1, p53 can repress transcription by disrupting DNA/protein complexes involving Sp1 in an SV40 promoter (Perrem *et al.*, 1995). Another transcription factor whose activity appears to be specifically repressed by p53 is the CCAAT binding factor (CBF), a transcriptional activator of the hsp70 gene (Agoff *et al.*, 1994). p53 represses the heat shock protein-70 promoter whose expression is normally mediated by CBF (Agoff et al 1994). p53 also interacts with the Wilms tumour predisposing gene product (WT1 ; Maheswaran *et al.*, 1993). WT1 is a zinc finger transcription factor that can act both as an activator or a repressor. Deletions of the WT1 gene, or point mutations which destroy the DNA binding activity of the protein, are associated with the development of Wilms tumour, a paediatric kidney cancer. When bound to the early growth response gene consensus sequence (EGR1), WT1 mediates transcriptional repression. p53 and WT1 were demonstrated to physically associate in transfected cells and the interaction to modulate their ability to *trans*-activate their respective targets (Maheswaran *et al.*, 1993). In the absence of p53, WT1 acts as a potent transcriptional activator of early growth response gene 1 (EGR1) rather than as a transcriptional repressor. In contrast WT1 exerts a co-operative effect on p53, enhancing its ability to *trans*-activate a specific promoter. Expression of WT1 resulted in increased steady state levels of p53 through its stabilisation (Maheswaran *et al.*, 1993). WT1 also enhanced p53 binding

to its target DNA sequence, increasing its *trans*-activation properties. WT1 inhibited p53-mediated apoptosis triggered by UV radiation but did not affect p53-mediated cell cycle arrest (Maheswaran *et al.*, 1995).

Thus there are other examples of transcription factors which are modulated by p53. The p53/WT1 interaction suggests that it is important to investigate how DP-1 might affect p53 activity.

DRTF1/E2F and p53 activity

The retinoblastoma gene product and p53 are probably the best characterised human tumour suppressor polypeptides and are believed to occupy pivotal roles in the prevention of cancer (see Introduction). Their functions in the healthy cell, however, appear quite different. pRb seems to have an integrated role in the normal running of the cell cycle (see Introduction ; Whyte, 1995) while p53 probably takes on a sideline role as a cellular "ombudsman", believed only to become involved in the cell cycle at times of stress in connection with DNA damage (see Introduction ; Ko & Prives, 1996). This is illustrated most clearly by the strikingly different phenotypes acquired when these genes are homozygously inactivated in mice. *RB*-minus mice are unviable and die long before birth (Clarke *et al.*, 1992 ; Jacks *et al.*, 1992 ; Lee *et al.*, 1992) while *p53*-minus mice are apparently perfectly viable, bar a susceptibility towards developing neoplasia (see below ; Donehower *et al.*, 1992 ; Jacks *et al.*, 1994 ; Williams *et al.*, 1994).

Despite these quite different contributions to the regulation of the cell cycle, it has become clear that the roles of these two factors in the prevention of the tumourigenic state are quite interlinked.

Viral targeting of tumour suppressors

As ever in elucidation of cellular signal transduction events, major clues to the activity of tumour suppressors have been acquired through the study of the means employed by DNA tumour viruses to infiltrate the prolific pathways of the cell. It seems significant that these viruses often target both p53 and pRb in their quest to subvert the cell, suggesting that both proteins occupy rate limiting roles in tumour prevention.

Viral oncoproteins encoded by the genomes of the Adenovirus (E1a), human papilloma virus (E7) and the simian virus-40 (Large T antigen : T.agn), all bind the large pocket of the hypo-phosphorylated form of pRb (see Introduction ; Vousden, 1995). Some forms of these same viruses also display p53-targeting properties : Adenovirus E1b as well as T.agn will bind p53 and prevent aspects of its function while the human papilloma virus E6 protein will promote the cellular ubiquitin-dependent degradation of p53 (Levine *et al.*, 1991). In a similar vein, a regular feature of human tumours is the inactivation of both *p53* and *RB* (Hamel *et al.*, 1993 ; Williams *et al.*, 1994).

Various experiments have taken advantage of the selective pRb and p53 targeting powers of viral oncogenic components to shed light on their differential, yet apparently co-operative, roles in the prevention of the tumourigenic state. Transgenic mice expressing wild type T.agn in B and T cells in the choroid plexus epithelium (CPE) developed tumours in those tissues. In contrast, mice expressing a mutant T.agn that binds pRb but not p53, developed only CPE tumours that formed slowly and showed morphological signs of apoptosis (McCarthy *et al.*, 1994 ; Saenzrobles *et al.*, 1994). Mice, wild-type for *p53*, expressing the HPV-16 E7 gene in photoreceptor cells exhibited retinal degeneration as a result of apoptosis while the same expression in mice heterozygously mutant for *p53*, initiated retinal tumour formation (Howes *et al.*, 1994). Transgenic mice expressing HPV E7 in the developing ocular lens exhibited apoptosis in lens cells and this was inhibited by co-expression of HPV E6 (Pan & Griep, 1994). Expression of E1a in BRK or REF52 cells induced *p53*-dependent apoptosis (Debbas & White, 1993 ; Lowe & Ruley, 1993c) and *RB*-knockout mice died at day 13.5 of development, showing signs of excessive apoptosis in neural tissues (Jacks *et al.*, 1992 ; Lee *et al.*, 1992 ; Clarke *et al.*, 1992).

There is thus a perceived connection between the loss of pRb function and death by apoptosis that appears to occur in a p53-dependent manner. Loss of pRb function alone, in the presence of p53, is clearly not always enough to trigger cell death since for example, $RB^{-/-}$ embryos do survive some time and many cell lines that are $RB^{-/-}$ are stable (for example Saos2 and H1299 cells). It is possible that the effect of a rise in p53 concentration, triggered by for example DNA damage, will depend on the status of pRb. This notion is supported by observations in mouse embryonic fibroblasts where anti-neoplastic drugs caused accumulation of p53 in $RB^{+/+}$, $RB^{+/-}$ and $RB^{-/-}$ cells, but only caused apoptosis in $RB^{-/-}$ cells with cell cycle arrest in $RB^{+/+}$ and $RB^{+/-}$ (Almasan *et al.*, 1995).

DRTF1/E2F activity and p53-dependent Apoptosis

One interpretation of the viral data is that p53 is sensitive to the activity of DRTF1/E2F, an important target of pRb and deregulated in cells when pRb is dis-functional. Hence it could be postulated that the deregulation of DRTF1/E2F could promote p53-mediated apoptosis. This idea is supported by the observation that when E2F-1-encoding DNA was introduced into quiescent cells by microinjection, they proceeded to enter S-phase but then promptly died by apoptosis through a mechanism that was at least part p53 dependent (Qin *et al.*, 1994 ; Wu & Levine, 1994). Viral transfection of E2F-1 into NIH-3T3 cells led to cell cycle arrest, but if mutant E2F-1 whose product is unable to bind cyclin A was transfected, DRTF1/E2F inhibition in S-phase was abolished and the cells apoptosed (Krek *et al.*, 1995). It is not known if this apoptosis was p53-dependent, however it supports the notion that cells are targeted by apoptosis upon DRTF1/E2F deregulation.

There is some evidence that endogenous p53 protein is induced as a function of DRTF1/E2F activity (Hiebert *et al.*, 1995). This could be indirect in that pre-mature S-phase entry leads to DNA damage which could promote p53 accumulation. Alternatively p53 accumulation could be a direct response to DRTF1/E2F by an as yet unresolved mechanism. The increase of p53 was not observed by other proliferative signals such as enforced c-Myc expression in the same cell type (Hiebert *et al.*, 1995) suggesting that a specific DRTF1/E2F-dependent p53-response pathway may exist. But either directly or indirectly, p53 could function as a sensor of DRTF1/E2F as illustrated by observations that during the normal cell cycle, the peak p53 protein level (Bischoff *et al.*, 1990) temporally follows the peak of DRTF1/E2F transcriptional activity (Mudryj *et al.*, 1990 ; Johnson *et al.*, 1994). The cyclical variations in p53 concentration probably have limited or no effect on the cell cycle, as illustrated by the viability of the *p53*-null mice (see above). But the effect may be a symptomatic reflection of what occurs in a pre-neoplastic cell where DRTF1/E2F activity is over-activated to levels higher than during a normal cell cycle and may thus have a mimicking response from the cellular levels of p53. Thus deregulated DRTF1/E2F may cause an accumulation of p53 sufficiently high to trigger its tumour-suppressive effects on the cell. It can be speculated that MDM2 could have a role in the normal cell cycle of preventing *p53*-mediated apoptosis in response to the normal cyclical activities of DRTF1/E2F.

When DRTF1/E2F is not deregulated, p53-accumulation will often cause just cell cycle arrest (see above), but in a situation of compromised pRb function, and hence increased DRTF1/E2F activity, p53 accumulation will guard against potential

tumourigenesis by promoting apoptosis. Thus p53 can potentially be seen as a monitor of aberrant DRTF1/E2F activity which may help to explain why DNA tumour viruses seek to functionally inactivate p53 when activating DRTF1/E2F (Moran, 1993).

A cautionary note, however, must accompany the appealing model above due to evidence that *p53* is not always as efficient in inducing an apoptotic pathway when the DRTF1/E2F activity is directly induced. For example E6 fails to protect keratinocytes from cytotoxic effects of E2F-1 (Melillo *et al.*, 1994) and mutant p53 only partially protects fibroblasts overexpressing E2F-1 from apoptosis in low serum conditions (Qin *et al.*, 1994). The *E2F-1* induced apoptosis observed following withdrawal of survival factors is likely independent of p53 function as p53 levels diminish (Hiebert *et al.*, 1995). It thus seems that under some circumstances, unregulated DRTF1/E2F can induce apoptosis in a p53-independent manner. It would thus seem that p53 is only one monitor of DRTF1/E2F, albeit most likely an important one.

The exertion of p53-mediated functions *in vivo*

As described in the Introduction, there are two sides to p53 activity : Cell cycle arrest and apoptosis. This is clearly illustrated from the inter-relationship between DRTF1/E2F and p53 as described in the above section. There is currently some debate about the relative contributions, and roles, of these two activities in tumour suppression and also concerning how p53 exerts these functions, be they via stimulation of transcription from p53-site bearing promoters, or via protein-protein post-translational effects of p53. These issues are discussed below followed by the next section which deals with how the p53 interaction with DP-1 might concern these processes.

Apoptosis, cell cycle arrest and cancer

p53 knockout mice develop normally but have a greatly increased susceptibility towards developing cancerous cells (Donehower *et al.*, 1992 ; Jacks *et al.*, 1994 ; Williams *et al.*, 1994). This is one of the many experimental examples that hail *p53* to be a gene involved in tumour suppression with little or no contribution to embryonic development or normal cell growth. One of the targets of p53 is the gene which encodes the p21 protein (*waf1* : El-Deiry *et al.*, 1993). If *waf1* is knocked out in a mouse its cells display some deficiency in cell cycle arrest in response to DNA damage while apoptotic functions appear normal (Deng *et al.*, 1995). In this mouse,

the two activities of p53 thus appear to have been at least partially separated. Intriguingly, the mouse does not have any enhanced susceptibility towards cancer indicating that the mere p21-enhancing aspect of p53 activity is not involved in tumour prevention suggesting that the greater tumour suppressive activity of p53 may reside with apoptosis as opposed to cell cycle arrest.

This observation begs the question why p53 possesses cell cycle arrest activity at all. The generally perceived role of p53-mediated cell cycle arrest is one of arresting the growth of cells that are being exposed to stress which may potentially cause DNA damage, such as UV light, ionising radiation *etceteras* (see Introduction ; Ko & Prives, 1996). This gives the cell an opportunity to repair DNA-damage rather than proceed with cellular division with mutations that may potentially be tumourigenic. In other circumstances, if the DNA damage is too high, or if the cell is being pushed into entering the cell cycle under circumstances where it would normally not, p53-mediated apoptosis occurs instead to remove the proto-neoplastic cell.

The result of the *waf1* knockout mouse could suggest that only the apoptotic function of p53 is tumour-suppressive. This does make some sense in that an animal whose cells have lost the ability to arrest, and hence repair, in response to tumour generating conditions, but which retain the ability to apoptose if any of these were to adopt cancer-like characteristics, then that animal will remain protected from tumour formation. It is however possible that the animal will be disadvantaged due to excessive, and unnecessary, cell death which may not be detectable in laboratory mice. A reverse situation in which the animals cells retain the ability to arrest in response to DNA damage but are unable to apoptose, would make the animal susceptible to cancer as proto-neoplastic cells would not be removed. So even though the apoptosis side of p53 activity is perhaps the more directly tumour-preventing one, it is possible that the cell cycle arrest function is a very necessary accompaniment to this activity in order to avoid the excessive use of cell death and to repair DNA damage whenever possible. This is supported by the observations that p53-induced cell cycle arrest appears to protect cells from apoptosis (Rowan *et al.*, 1996) suggesting that there are mechanisms which allow the cell time to repair itself before having to make the ultimate sacrifice.

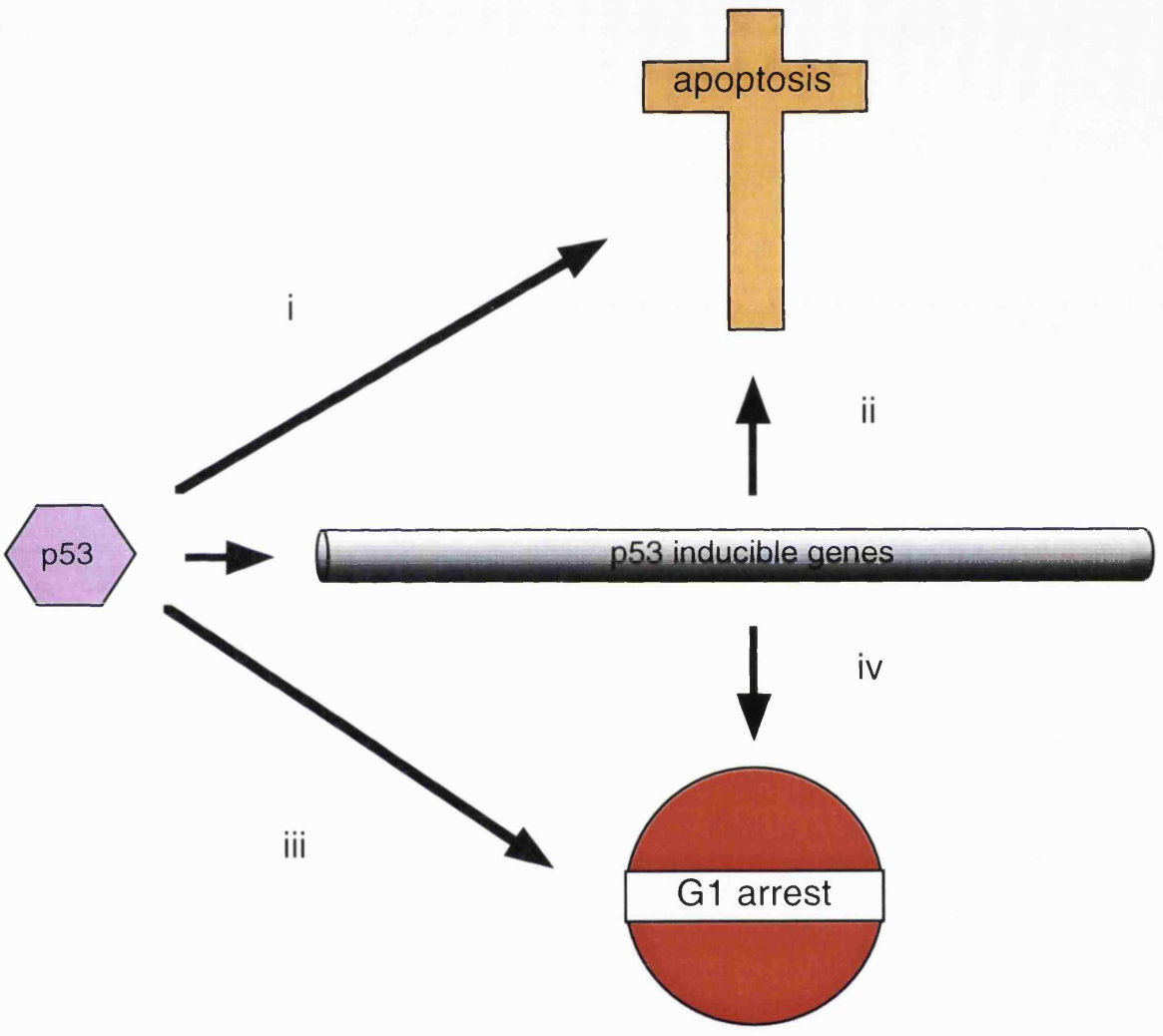
Pre- or Post-translational effects

One of the major questions facing the p53 field at the moment is the relative contributions of transcriptional activation of p53-responsive genes versus direct post-translational effects of the p53 protein on other polypeptides (**figure 8.3**). One of the main observed features of the *p53* mutants discovered in cancer cells is a

Figure 8.3

Induction of apoptosis or G1 arrest by p53 via protein-protein effects and the induction of gene expression.

p53 (Purple hexagon) can promote both G1 arrest (stop sign) and apoptosis (wooden cross). This can possibly be in part mediated by protein-protein effects (**i** and **iii**) and by the induction of specific gene expression (**ii** and **iv**).


apoptosis
i
ii
p53
p53 inducible genes
iv
iii
G1 arrest

predominance of base alterations in the DNA binding domain of the protein or mutations that either affect DNA binding or the *trans*-activational activity (Pietenpol *et al.*, 1994 ; Hainaut, 1995). This has fuelled a widely held belief that the main anti-tumourigenic function of p53 lies in its ability to *trans*-activate, and possibly repress, a range of genes associated with either apoptosis or cell cycle arrest. However at least one activated activity, the p21 gene, as seen above, has no tumour suppressing activity. By the association of post-translational effects with apoptosis, an increasing amount of reports are now suggesting that the gene *trans*-activation activity may not after all be the main source of anti-neoplastic activity in p53 (see below).

While there is generally a good correlation between *trans*-activation activity and the suppression of cell growth (Crook *et al.*, 1994 ; Ory *et al.*, 1994 ; Pietenpol *et al.*, 1994 ; and see Introduction) implicating downstream transcription targets like *waf1*, the connection between p53-mediated transcription with apoptosis is however less so. Although p53 has been implicated with several genes known to be involved in the apoptotic response (see Introduction), apoptosis in HeLa cells can be mediated by p53 mutants which cannot bind DNA nor *trans*-activate genes (Haupt *et al.*, 1995). Furthermore it has been reported that apoptosis can occur in the absence of *de novo* RNA and protein synthesis (Caelles *et al.*, 1994 ; Wagner *et al.*, 1994).

Several studies have dissociated *trans*-activation from the ability of p53 to suppress transformation. Both transcriptionally active mutants which fail to suppress transformation and transcriptionally inactive mutants which retain transformation suppression function have been identified (Unger *et al.*, 1993 ; Crook *et al.*, 1994 ; Zhang *et al.*, 1994). One such mutant, p53175P, from a human papillomavirus positive metastatic cervical carcinoma (Crook *et al.*, 1994), retains transcriptional activation function and can cause G1 arrest in Saos2 cells, while it has lost the ability to suppress transformation in primary rat cells by E7 and ras. Additionally it has been shown to be defective in its ability to induce apoptosis (Rowan *et al.*, 1996) which is in support of the *waf1* knockout implication that the apoptotic side of p53 activity appears to be more tumour suppressive. Certainly some p53-activated genes have been associated with apoptosis but for example increased Bax expression, commonly associated with apoptosis, is not always detected during p53-mediated cell death (Allday *et al.*, 1995 ; Canman *et al.*, 1995).

It thus seems that the putative predominant tumour-suppressive activity of p53, apoptosis, can be achieved in the absence of transcriptional activation. We can however not ignore the wealth of naturally occurring p53 mutations that affect its DNA binding, and hence its *trans*-activational activity (Pietenpol *et al.*, 1994 ;

Hainaut, 1995). The likely explanation is probably that in a physiological context both pre- and post-translational effects are important but it is also possible that the function of the DNA binding domain may not solely be associated with transcriptional modulation but may harbour other functions that have yet to be characterised.

Physiological relevance of p53 and DP-1 interaction

p53 associates with DP-1, this reduces the DNA binding activity of E2F-1/DP-1 heterodimers and compromises *trans*-activation of E2F-site responsive genes by E2F-1/DP-1 co-transfections. Taking the apparent importance of DRTF1/E2F in the control of cellular proliferation into account (see Introduction), it is quite likely that this aspect of p53 activity is associated with a post-translational cell cycle arresting property. This section discusses what role such an interaction might have physiologically, and also the possibilities that the interaction with DP-1 may be of significance in other aspects of p53 properties such as apoptosis.

Molecular basis of G1 inhibition by p53/DP-1

Intuitively, the p53 inhibition of the DP-1/E2F-1 heterodimer is associated with the property of p53 to halt the cell cycle as opposed to involvement with apoptotic pathways. p53 targeting of the DRTF1/E2F is completely in line with it being a G1 repressor as DRTF1/E2F reaches a peak of activity during the G1/S phase of the cell cycle (Mudryj *et al.*, 1991 ; Shirodkar *et al.*, 1992 ; Schwarz *et al.*, 1993). E2F-1 appears to be able to overcome a G1 block induced by gamma irradiation (Degregori *et al.*, 1995) which could suggest that p53 acts to inactivate E2F-1-related activity, hence possibly via the targeting of DP-1. p53 appears to bind not just DP-1, which seems to constitute a very major component in the DRTF1/E2F activity of many cell types tested (Girling *et al.*, 1993a ; Wu *et al.*, 1995), but also DP-2 and -3, as demonstrated *in vitro* (Sørensen *et al.*, 1996). Accordingly, p53 appears to disrupt E2F-1 heterodimers that include most, though not all, of these DP family members (**figure 7.6**). Interestingly it seems that there are differences between the various DP-3 splice-forms in their E2F-1-heterodimer susceptibility to p53 repression. This might indicate that p53 preferentially targets sub-populations of DP-/E2F-family heterodimers.

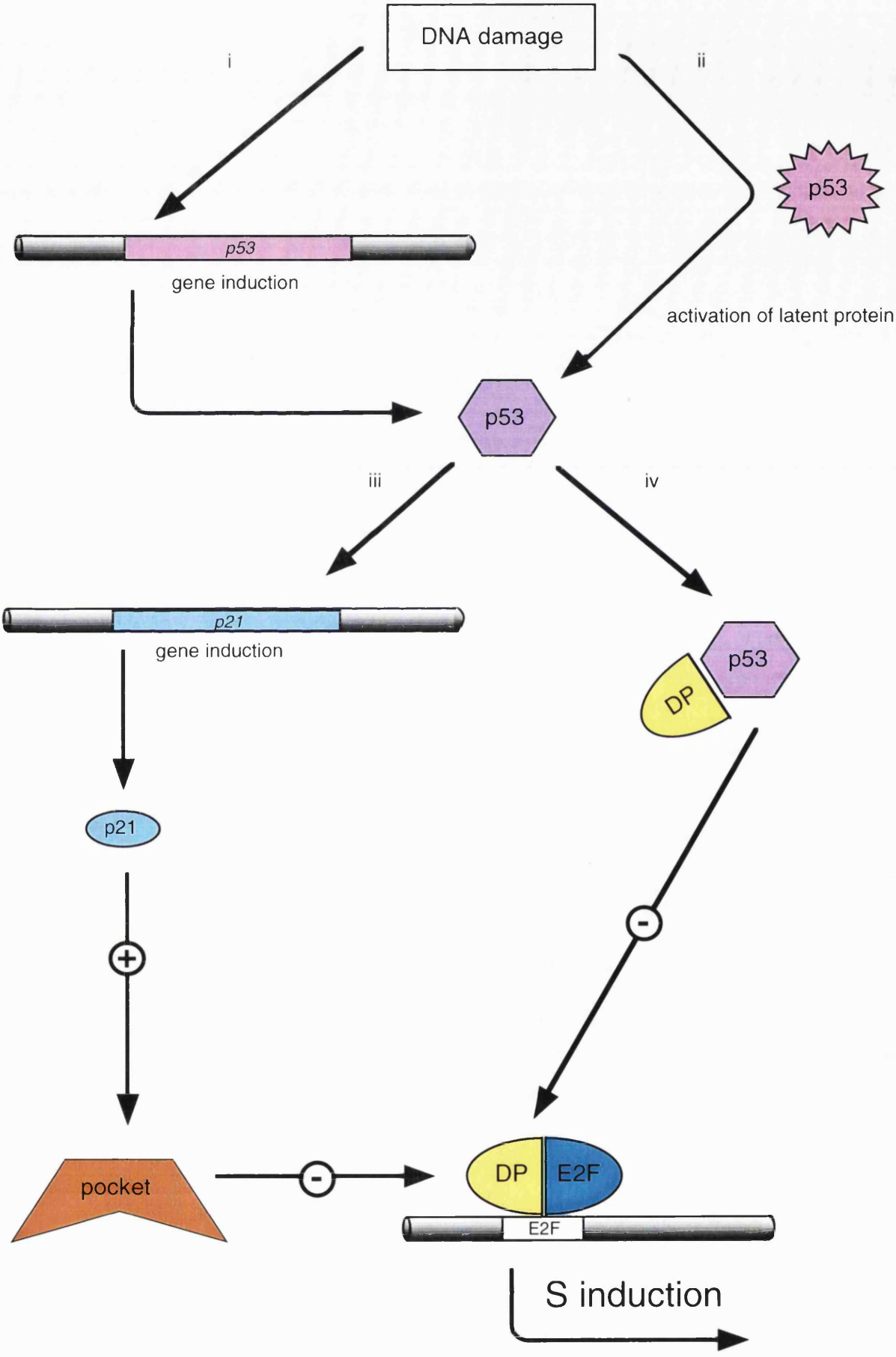
The issue of whether p53 will also target all E2F family member-complexes with susceptible DPs needs to be resolved. This is quite interesting since there are indications that certain E2F-family members are cell cycle phase specific. For example E2F-4 and -5 pocket protein complexes have been associated with the early stages of the G1 phase (see Introduction) so p53 might this way target a specific sub-phase of G1.

Why would there be a need for this p53 direct action against the heterodimer when p53 has already been shown to be likely to regulate DRTF1/E2F via pRb and p21 (see Introduction ; El-Deriry *et al.*, 1993) ? - It is possible that both mechanisms are essential (**figure 8.4**). *In vitro* data indicates that p53 does not target pRb complexed heterodimer (Chapter 7 and **figure 7.8**) and so any free heterodimer might be selectively targeted by p53. If this is the case, then the consequence of raised p53 intracellular concentrations would be two-fold for DRTF1/E2F. Firstly, via the p21 pathway, higher levels of hypo-phosphorylated pRb appears due to inhibition of the mechanisms responsible for its phosphorylation (El-Deriry *et al.*, 1993). Hypo-phosphorylated pRb complexes with free heterodimer to form transcriptionally inactive DNA binding complexes (see Introduction ; Zamanian & La Thangue, 1992 ; Hiebert *et al.*, 1992 ; Dowdy *et al.*, 1993). Secondly, at the same time p53 will also specifically target and disrupt several free DP-family heterodimer forms. This will mean that there will be reduced competition for binding to the E2F site from these free forms, hence pRb complexed heterodimer binding will be favoured. The two DRTF1/E2F inhibitory pathways may be mutually interdependent to achieve cellular arrest. The inhibition of the pRb phosphorylation in particular is not a very direct action on DRTF1/E2F, since hypo-phosphorylated pRb is not actively being generated. The concomitant free heterodimer-disrupting activity of p53 might be important to make full use of the available hypo-phosphorylated pRb.

The promotion of pRb-complexed heterodimers on the E2F sites by this mechanism may be significant if interpreted in the light of the newly perceived role of pRb, and other pocket proteins, as not just repressors of the E2F site but also as *trans*-repressors of the whole gene associated with a E2F site-bearing promoter (see Introduction ; Schulze *et al.*, 1995 ; Qin *et al.*, 1995 ; Sellers *et al.*, 1995 ; Zwicker *et al.*, 1996). Under this interpretation the heterodimer is viewed as not only a transcription factor, but under the right circumstances, as simply a DNA-binding platform for a generally repressing pRb. By encouraging the formation of pRb complexes on E2F sites, p53 would not just promote E2F site repression, but rather a complete repression on all E2F-site bearing genes, something which would not be achieved by merely removing all DRTF1/E2F from E2F sites. Thus any non-

Figure 8.4
Hypothesis of two pathways of DRTF1/E2F repression by p53.

Levels of active p53 (Purple hexagon) in the cell are raised in response to DNA damage by the induction of *p53* gene expression (**i**) and the activation of latent p53 protein (Pink sun) (**ii**). p53 can repress the activity of DRTF1/E2F, and hence the induction S-phase progression, via two pathways : (**iii**) The induction of the expression of the p21 gene whose product inhibits the activity of cyclin/CDK kinase activities, thus promoting the non-phosphorylated state of pocket proteins (orange hat) which inhibit the DP/E2F heterodimer (yellow and cyan oval bound to the DNA E2F site). (**iv**) The direct complexing of p53 with the DP-moiety (Yellow half-oval) of the DP/E2F heterodimer which is essential for heterodimer DNA binding and hence transactivation, both of which are consequently disrupted.


DNA damage
i
ii
p53
p53
gene induction
activation of latent protein
p53
iii
iv
p21
gene induction
p53
DP
p21
pocket
DP
E2F
E2F
S induction

DRTF1/E2F-activated, alternatively activated or basal transcription would also be repressed. In a sense p53 could be encouraging the creation of early-G1-like conditions where little free heterodimer is detected and pRb is in a hypo-phosphorylated, heterodimer-complexed form (Mudryj *et al.*, 1991 ; Shirodkar *et al.*, 1992 ; Schwarz *et al.*, 1993).

It is possible that part of the cell cycle-arresting activity of p53 is due to direct effects on the heterodimer of DRTF1/E2F and that this, in co-operation with the p21 pathway, is a tumour-preventing activity. In support of this comes the observation that the DP-1 binding region of p53 (73-143 : Sørensen *et al.*, 1996) overlaps with the so-called conserved domain II which is frequently mutated in tumours (Harris, 1993 ; Ko & Prives, 1996) suggesting a possible role in the regulation of the tumourigenic state. Thus mutations of p53 which are unable to directly disrupt the DP-/E2F-heterodimer may conceivably have compromised tumour-suppressive powers. Indeed, O'Conner *et al.* (1995) have shown that one of the most common point-mutations of p53 found in tumourigenesis, his175, represses transcription from the E2F-site much less efficiently than wild-type p53. It is thus possible that a cell cycle arresting activity is inherent in the p53 protein alone and independent of transcriptional activation. A very important experiment which needs to be carried out is the assaying of naturally occurring mutants of p53 for *trans*-activational activity versus the ability of the polypeptide product to interact with DP-1. Perhaps many mutants of p53 that are unable to *trans*-activate will also have lost their ability to bind DP-1. The different contributions to cell cycle arrest of p21 inhibition and p53-DP-1 interaction respectively represents an important area in need of further investigation. For instance it will be interesting to investigate whether UV-treatment or p53-overexpression generates a lower proportion of free heterodimer as well as increased proportions of pRb complex in *waf1* $^{(+/+)}$ and *waf1* $^{(-/-)}$ cells.

It remains to be seen whether viral complexes with p53 will interfere with its DP interaction just as they are known to prevent its interaction with its DNA binding site. Disruption of p53-DP-1 complexes is however quite likely in the case of one viral oncoprotein, the human papilloma virus E6 which stimulates the ubiquitin-dependent p53 degradation (Levine *et al.*, 1991).

Molecular basis of induction of apoptosis by p53/DP-1

The aspect of p53 activity which causes apoptosis, is likely to be a major tumour suppressing aspect of p53 function (see above). DP-1 is a transcriptional co-factor for the DRTF1/E2F transcription factor, yet an apparently large proportion of DP-1 appears, throughout the cell cycle, to be in a transcriptionally inactive state (p55U-see phosphorylation section). Studies of DP-1 in Ras co-operation assays has

demonstrated it to posses a proto-oncogenic activity which is independent of the ability of DP-1 to promote transcription from E2F sites (Jooss *et al.*, 1995). It is thus very intriguing that p53 forms a complex with the transcriptionally inactive form of DP-1 and that the area of DP-1 interaction in the p53 protein locates to one of the four "hot spots" of p53 mutation found in naturally occurring human tumours (Harris, 1993 ; Ko & Prives 1996 ; see above). It is thus possible that the interaction of p55U with p53 has a role in DRTF1/E2F-p53 mediated apoptosis.

Speculation could have it that the p53 binding to DP-1 releases E2F-1 and thus triggers apoptosis in a similar manner seen when E2F-1 is overexpressed in cells (Qin *et al.*, 1994 ; Wu & Levine, 1994). The oncogenic potential that overexpressed DP-1 displayed in the Ras-co-operation assay may also possibly be exerted by the sequestering away of p53 : The effect of DP-1 binding on p53 activity is an aspect of the DP-1/p53 interaction that has yet to be investigated. The DP-1 binding domain in p53 (Sørensen *et al.*, 1996) overlaps with its DNA binding domain (Ko & Prives, 1996) and it has been reported that the overexpression of DP-1 can down-regulate p53-dependent transcription independently of MDM2 (see next section ; Sørensen *et al.*, 1996 ; O'Conner *et al.*, 1995). Clearly this area is in need of further investigation, for example into the apoptotic activities of non-DP-1-binding mutants of p53.

p53/DP-1 and the apoptosis/G1-arrest switch

Activation or introduction of p53 in many cell types results in arrest in the G1 stage of the cell cycle (Kastan *et al.*, 1991 ; Kuerbitz *et al.*, 1992 ; Di Leonardo *et al.*, 1994). Other cell types undergo rapid apoptotic death following wild-type p53 expression (Oren, 1994) but the regulation of the decision to undergo cell cycle arrest or programmed cell death is not understood (Bates & Vousden, 1996). Nevertheless there is substantial evidence that apoptosis is exhibited by cells containing wild-type p53 which suffer additional perturbations in normal cell cycle control such as loss of the tumour-suppressor protein pRb (see above).

One exiting aspect of the discovery of the interaction of p53 with DP-1 is in relation to the wealth of data suggesting that p53 is sensitive to DRTF1/E2F status and will cause cell cycle arrest or apoptosis according to the activity of this transcription factor. This raises the possibility that the p53-DP-1 interaction provides a pathway cross-talk, allowing for p53 to monitor the activity of DRTF1/E2F. This could function via the conversion of p55U to p55L, associated with DRTF1/E2F activation, which could release p53 and possibly raise its cellular concentration beyond a threshold. Alternatively the p53-p55U complex may function as an active inhibitor of apoptosis or a stimulator of cell cycle arrest.

MDM2 and the DRTF1/E2F

In vivo evidence suggests that MDM2 is involved in a complex that includes DP-1 (**figure 7.2**) and *in vitro* evidence suggests that this interaction can be direct (Martin *et al.*, 1995). When MDM2 is overexpressed in U2OS cells the DP-1/E2F-1-dependent expression from E2F-driven promoters is enhanced (Xiao *et al.*, 1995 ; Martin *et al.*, 1995) and is dependent on an N-terminus region of MDM2 also required for binding both DP-1 and E2F-1 (Martin *et al.*, 1995). This effect has been suggested to be due to the complexing of MDM2 with pRb which prevents pRb interaction with the E2F-1, -2 or -3, and hence promotes DRTF1/E2F transcription (Xiao *et al.*, 1995). There is however additional, and possibly conflicting data, concerning the effect of MDM2 in cells that lack both p53 and pRb. Some (Martin *et al.*, 1995) claim that stimulation is retained while others (Xiao *et al.*, 1995) that MDM2 has no effect upon E2F-driven transcription in these cells. This question has yet to be resolved.

Thus a situation has arisen whereby three cell cycle regulating factors : MDM2, DP-1 and p53, all appear to be able to directly bind to each other. The *in vivo* detected DP-1-MDM2 complex could therefore potentially be via p53 since the p53-MDM2 interaction region has been fine mapped to a small area of the N-terminus (residues 18-23 ; Picksley *et al.*, 1994), while the interaction with DP-1 lies in the region 73-143. It would thus seem that p53 could potentially bind DP-1 and MDM2 at the same time. This however awaits experimental verification.

It is unclear what function a MDM2-p53-DP-1 heterotrimeric complex would have, but its existence might explain how antibodies to DP-1 can immunoprecipitate MDM2, while no MDM2 has been detected in DNA-binding DRTF1/E2F (Chapter 7 and Martin *et al.*, 1995). Exposing DRTF1/E2F or purified heterodimer to MDM2 in a gel retardation assay has no effect making it unlikely that MDM2 exerts any DRTF1/E2F transcription-promoting effects at the level of the DNA. This would suggest that MDM2 complexes the p55U-non-DNA binding form of DP-1, which is also the form that binds p53 (Chapter 7). Certainly it is possible that p55U forms an *in vivo* direct complex with MDM2, but the significance of such a complex is unclear. It thus remains unresolved whether MDM2 can induce DRTF1/E2F activity independently of pRb, and if it can, by what mechanism. Further experimental investigation is clearly needed, for example by exposing *in vivo* DP-1-MDM2 complexes to adenovirus E1a or E1b oncoproteins to determine if the interaction is dependent upon pRb or p53 respectively.

The other two heterotrimer combinations that can be conceived are potentially impossible since the MDM2 and p53 binding sites in DP-1 overlap (226-375 and 171-331 respectively : Martin *et al.*, 1995 and Sørensen *et al.*, 1996) and so do the DP-1 and p53 binding regions in MDM2 (1-220 and 1-73 respectively : Picksley *et al.*, 1994 and Martin *et al.*, 1995). Most of this mapping, however, has been done quite crudely and so clarification is again awaited from more detailed studies and from experimental analysis of the combinatorial possibilities for binding. What this potentially means, though, is that p53 and MDM2 could compete for binding to DP-1 and hence potentially compete to activate or de-activate it. Furthermore DP-1 and p53 could compete for binding to MDM2, which is perhaps more unlikely as both complexes would potentially promote the proliferative state.

Perspectives

The regulation of DRTF1/E2F by the cell cycle and by the p53-DNA damage/cellular stress response pathway have both been postulated to work via the targeting of the phosphorylation status of pRb through the modulation of the activity of the cyclin dependent kinases (see Introduction). Changing the pRb phosphorylative status affects its interaction with mainly the E2F-family portion of the DNA binding heterodimer of DRTF1/E2F, an interaction that compromises DRTF1/E2F *trans*-activation activity while unaffecting its DNA binding activity.

Antibodies to DP-1 are able to shift, in many cases, all detectable E2F binding activity in gel retardation assays of extracts from a large variety of cell types. This suggests that the DNA binding form of DP-1, or a very related protein, is a near-universal heterodimeric contributor to DNA binding E2F family members (Girling *et al.*, 1993b ; Lees *et al.*, 1993 ; Ivey-Hoyle *et al.*, 1993 ; Wu *et al.*, 1995). The great prevalence of DP-1 contribution to total DRTF1/E2F activity also indicates that E2F-1 rarely, or never, forms homodimers *in vivo* as opposed to what can be observed *in vitro*. DP-1 does not poses its own *trans*-activation domain, this is carried by its E2F-family heterodimerisation partner that in turn depends on DP-1 for high affinity DNA binding and hence efficient *trans*-activation (Bandara *et al.*, 1993 ; Krek *et al.*, 1993 ; Helin *et al.*, 1993b). DP-1 can thus be seen as an essential co-activator to DRTF1/E2F and a potential potent target of regulatory factors. The targeting of DP-1 would be consequential for the DNA binding activity of DRTF1/E2F as a whole and would be likely to have a profound effect on the state of proliferation of the cell (see Introduction).

The results presented in this thesis propose two novel pathways for the regulation of DRTF1/E2F that *both* regulate via DP-1 as opposed to via E2F/pRb. Evidence was presented that the cell cycle and p53 pathways can regulate the activity of DRTF1/E2F in a pRb-independent manner via the direct targeting DP-1. Both of these novel pathways act to modulate the DNA binding activity of DRTF1/E2F as opposed to modulating its *trans*-activational activity. The results illuminate DP-1 as a major target for the regulation of the DRTF1/E2F DNA binding activity by different regulatory pathways.

Clearly more work is needed to clarify this newly perceived role of DP-1. The exact phosphorylation sites on DP-1 need to be mapped and any differences between p55U and p55L determined. Of particular concern is of course the phosphorylation state of the C-terminus of DP-1. It is of great importance to determine if this is phosphorylated *in vivo* and more particularly if this phosphorylation is modulated during the cell cycle. Mutational studies on DP-1 should also be able to strengthen or

discredit the phosphorylation hypothesis. It would be particularly interesting to see the DRTF1/E2F DNA binding pattern during the cell cycle in T388-mutant DP-1 transfected cells and also to determine their response to co-*trans*-activation with E2F-1 and cyclin A. The generation of DP-1 mutated at key phosphorylation sites will allow analysis of their specific roles in the regulation of DP-1 activity. It is possible that there are more than two different phosphoforms of DP-1 which may not be discerned by immunoblotting techniques. Mutational analysis of the phosphorylation sites might reveal such multiple forms. Confirmation of the indication that the hyper-phosphorylated form of DP-1, p55U, cannot interact with E2F-family members needs to be sought. Whether p55U can or cannot interact with an E2F partner, the question still begs what the role of such an apparently large amount of non-DNA-binding DP-1 is doing in the cell. Is it simply a reservoir ready to be activated or does it have a specified role ?

The effects of p53 on DRTF1/E2F predicted *in vitro* need to be confirmed *in vivo*. It will be interesting to study DRTF1/E2F-DNA binding activity and the ratio of p55U and p55L following p53 transfection or UV treatment. It would also be interesting to study the ability of p53 to repress DRTF1/E2F activity in cells that are mutant for the p21 gene. In general it would be useful to try to discern between p21- and DP-1-mediated inhibition of DRTF1/E2F by p53. Mutant p53 from tumour lines and p53 that is mutant in either cell cycle arrest or apoptotic function should be screened for binding activity to DP-1. This might establish what aspect of p53 function DP-1 is concerned with and whether this might have any contribution to tumourigenesis. There is also a need to determine the role of the DP-1/p53 complex with respect to p53, for example what effect DP-1 has on the DNA binding activity of p53. This might also provide some clues as to the function of the observed DP-1/MDM2 complex.

Finally, these two new aspects of DRTF1/E2F regulation via DP-1 might predict mechanisms that are deregulated in the neoplastic state. It is possible that tumourigenic mutants of DP-1 exist that are unresponsive to p53 or unable to be inhibited by the cell cycle kinases by phosphorylation. Identification and understanding of any such aberrations would further our understanding of cancer.

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◊