A thesis entitled

Transcriptional Control During the Cell Cycle and Apoptosis

Presented by

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To my family,

Abstract

Both E2F and p53 are sequence specific transcription factors that play important roles in controlling early cell cycle progression. The pathway of control mediated through E2F governs the transition from G1 into S phase whereas p53 in response to genotoxic stress can facilitate cell cycle arrest or apoptosis. Here, I show that there is a physical and functional interaction between p53 and DP-1. p53, which interacts with a distinct form of DP-1, competes with E2F-1 for DP-1 leading to a reduction in the level of E2F-1/DP-1 heterodimer transcriptional activity. The results establish DP-1 as a common cellular target in two distinct pathways of growth control mediated through the activities of pRb and p53 tumour suppressor proteins.

From previous studies it is known that cells expressing aberrant levels of E2F-1 can undergo p53-dependent apoptosis. However, I demonstrate that E2F-1 can induce apoptosis in $p53^{-/-}$ tumour cells and that DP-1 is able to augment the E2F-1-induced apoptosis, yet by itself it has no ability to induce apoptosis, consistent with the ability of DP-1 to enhance E2F-1-mediated transcriptional activation. Surprisingly, E2F-1-induced apoptosis requires DNA-binding but not *trans* activation, suggesting that this apoptosis is not simply the consequence of the direct activation of genes required for apoptosis.

The mechanisms which influence the outcome of p53 induction are not clear, although transcription of the p53 target gene, encoding the cdk-inhibitor $p21^{Waf1/Cip1}$, correlates with p53-mediated cell cycle arrest. Using a combination of biochemical and functional assays, I identify that p300 as a co-activator required for p53-dependent transcriptional activation of *Waf1/Cip1*. Furthermore, I show that the cdk-inhibitor $p21^{Waf1/Cip1}$ autoregulates in a positive fashion transcription through modulating the activity of the p53/p300 complex, whilst negatively regulating the

activity of E2F by preventing cdk-dependent phosphorylation of pRb. Consistent with a role for p21$^{Waf1/Cip1}$ in the autoregulation of p53-dependent transcription, p300 augments the ability of p53 to cause G1 arrest. In addition, I find that p300, which also functions as a co-activator for E2F/DP heterodimer, enhances the E2F-1-induced apoptotic activity. Thus, a functional interaction between p300 and either p53 or E2F-1 has a profound impact on early cell cycle progression, specifically in regulating the contrasting outcomes of cell cycle arrest and apoptosis.

p300/CBP proteins have been implicated as critical regulators of distinct cellular pathways, such as those leading to differentiation, cell cycle arrest and apoptosis. To elucidate the mechanisms of transcriptional activation by p300, it was considered possible that additional control may be exerted through proteins that physically interact to regulate the activity of p300. I demonstrate that a newly identified protein, termed JMY, makes a physiological complex with p300 in mammalian cells. Also, this new protein functionally co-operates with p300 in the transcriptional activation of p53, and possesses the properties of a co-activator for p53. I find that JMY has potent effects on certain p53-dependent genes, such as *bax*, and physiologically JMY co-operates with p300 in promoting apoptosis. These results reveal a new level of control that is important in dictating the cellular response to p53.

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Abbreviations

ATF	Activating transcription factor
β-gal	β-galactosidase
bp	Base pair
BRG-1	Brabma-related protein
BRK	Baby rat kidney
cAMP	Cyclic adenosine mono-phosphate
CAT	Chloramphenicol acetyltransferase
CBP	CREB-binding protein
CDK	Cyclin dependent kinase
C/EBP	CCAAT-box/enhancer-binding protein
ced-3	Caenor-habditis elegans
CH	Cysteine/histidine-rich domain
CK	Casein kinase
CMV	Cytomegalovirus
CR	Conserved region (in the adenovirus E1A protein)
CRE	cAMP-response element
CREB	CRE-binding protein
Cyc	Cyclin
DAPI	4,6-diamidino-2-phenylindole
DBD	DNA binding domain
DHFR	Dihydofolate reductase
DMEM	Dulbecco's modificatied eagle's medium
DMSO	Dimethylsulphoxide
DNA pol α	DNA polymerase α
DNA-PK	Double-stranded DNA-activated protein kinase

dNTP	Deoxy-nucleoside triphosphate
DTT	Dithiothreitol
dUTP	Deoxy-uracil triphosphate
EDTA	Ethylene diamine tetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
ER	Oestrogen
FACS	Fluorescence activated cell scanning
FCS	Foetal calf serum
F9EC	F9 embryonal carcinoma
FITC	Fluorescein isothiocyanate
GAD	Gal4 activation domain
GR	Glucocorticoid receptor
GST	Glutathione-S-transferase
HA	Hemagglutinin protein (derived from influenza virus)
HAT	Targeted histone acetylation
HBS	HEPES-buffered saline
HBV	Hepatitis B virus
HDAC1	Histone deacetylase 1
HEPES	N-[2-Hydroxethyl]piperazine-N'-[2-ethanesulfonic acid]
HLH	Helix-loop-helix
HPV	Human papilloma virus
IB	Immunoblotting
ICE	Interleukin 1β-converting enzyme
IGF-BP3	Insulin-like growth factor-binding protein 3
IL-2	Interleukin-2
INF α	Interferon α
IP	Immunoprecipitation
IPTG	Isopropyl-ß-D-thiogalactopyranoside
IR	Irradiation

IVT	*In vitro* translated
JMY	Junction-mediating and regulatory protein
KID	Kinase-inducible domain
LOH	Loss of heterozygosity
Luc	Luciferase
MCK	Muscle creatine kinase
MDM 2	Murine double minute 2
MOZ	Monocytic-leukemia zinc-finger
MyoD	Myogenic HLH transcription factor
NLS	Nuclear localization signal
NP-40	Nonidet P-40
p53 BP	p53 binding protein
p55L	Faster migrating half of DP-1 p55 doublet
p55U	Slower migrating half of DP-1 p55 doublet
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline tween-20
P/CAF	p300/CBP-associated factor
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PKC	Protein kinase C
PMSF	Phenylmethysulfonyl fluoride
Pol II	RNA polymerase II
PR	Progesterone
pRb	Retinoblastoma gene product
RAR	Retinoic acid receptor
REF	Rat embryo fibroblast
RGC	Ribosomal gene cluster
RTS	Rubinstein-Taybi syndrome

RXR	Retinoid X receptor
SAP	Stress-activated protein
SDS	Sodium dodecyl sulphate
SRC-1	Steroid receptor co-activator 1
SREBP	Sterol regulatory element binding protein
STAT	Signal transducer and activator of transcription
SV40-LT	Simian virus 40 large T antigen
TAD	*Trans*activation domain
TAF	TBP-associated factor
TBP	TATA binding protein
TdT	Terminal deoxy-transferase
TFs	Transcription factors
TK	Thymidine kinase
Topo.1	Topoisomerase 1
TR	Thyroid hormone
TRITC	Tetramethylrhodamine isothiocyanate
Tris	Tris(hydroxymethyl)methylamine
TS	Thymidylate synthetase
TUNEL	TdT-mediated dUTP nick end labelling
Tween 20	Polyoxyethylene sorbitan monolaurate
UV	Ultraviolet light
VP16	Virion protein 16 *trans* activation domain
v/v	Volume per volume
wt	Wild type
WT 1	Wilm's tumour-1
w/v	Weight per volume
YY1	Ying yang 1
Zip	Leucine zipper

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

Declaration

All work presented within this thesis was performed entirely by myself and in no way forms part of any other thesis. Any material supported by other persons is acknowledged. The work was carried out while I was a postgraduate student at the Division of Biochemistry and Molecular Biology, Institute of Biomedical and Life Science, University of Glasgow, UK, from March 1995. I was under the supervision of Professor Nicholas B. La Thangue.

Chang-Woo Lee

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Publications

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

Lee, C.W. and N.B. La Thangue. 1998. Promoter specificity and co-activator control of the p53-related protein p73. Submitted for publication.

Shikama, N.*, **C.W. Lee***, L. Delavaine, J. Lyon, W. Cairns, M. Krstic-Demonacos, and N.B. La Thangue. 1998. A new component of the p300/CBP co-activator complex that regulates E2F and the p53 response. Submitted for publication.

Cairns, W., N. Shikama, M. Krstic-Demonacos, **C.W. Lee**, J. Lyon, and N.B. La Thangue. 1998. Regulation of nucleosome assembly proteins by p300/CBP co-activators. Submitted for publication.

Lee, C.W., T. Sorensen, N. Shikama, and N.B. La Thangue. 1998. Functional interplay between p53 and E2F through co-activator p300. Oncogene 16:2695-2710.

Sorensen, T.*, R. Girling*, **C.W. Lee***, J. Gannon, L. Bandara, and N.B. La Thangue. 1996. Functional interaction between DP-1 and p53. Molecular and Cellular Biology 16:5888-5895.

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

*** denotes joint first authorship.**

Chapter 1.
Introduction

In all eukaryotes, cell cycle progression is regulated not only through the activity of transcription factors, but also through the activity of activators and repressors which might modulate their activity (La Thangue, 1994; Hartwell and Kastan, 1994; Dynlacht, 1997). Co-ordination of these complex processes is thought to be important for growth-related decisions which will influence whether the cell proliferates, enters quiescence, or differentiates (Nevins, 1992; La Thangue, 1994). A critical period for integrating growth regulating signals occurs during early cell cycle progression where, in the appropriate conditions, cells become committed to the division cycle (Nevins, 1992; La Thangue, 1994). The transition from G1 into S phase is governed by a number of cell cycle regulatory proteins with established roles in controlling proliferation, such as the tumour suppressor protein p53, the E2F transcription factors, and the retinoblastoma tumour suppressor protein pRb (Nevins, 1992; La Thangue, 1994; Lam and La Thangue, 1994; Weinberg, 1995).

In addition, pathways that act positively and negatively are exerted during physiological processes such as development, differentiation, senescence, and apoptosis (Hunter and Karin, 1992; Lam and La Thangue, 1994; Raff, 1996; Vousden, 1996; White, 1996). In many cases, arrest of cell proliferation takes place under circumstances in which the integrity of the genome has been compromised, and failure to arrest proliferation would release cells with highly unstable genomes that could evolve into tumour cells or trigger the apoptotic signals (Hartwell and Kastan, 1994; Weinberg, 1995; White, 1996).

Apoptosis plays an indispensable role during embryogenesis, in adult tissue homeostasis, in the regulation of the immune system and in the development of the nervous system so as to eliminate unwanted, potentially dangerous cells or superfluous cells from the organism (Fisher, 1994; Hoffman and Liebermann, 1994;

Steller, 1995; Vaus and Strasser, 1996). The mechanisms which control apoptosis are thought to be highly conserved in all mammalian cells, and must therefore be tightly regulated. The genes regulating and executing the apoptotic programme are just beginning to be identified and are currently an area of intense investigation (Ko and Prives, 1996; Hansen and Oren, 1997). However, the events which determine whether a particular cell should arrest the cell cycle or activate the apoptosis pathway are not fully understood.

1-1. p53, the cellular mediator for growth control and cell death

The human tumour suppresor gene *p53* encodes a 393 amino acid phosphoprotein that exhibits sequence-specific DNA binding and directly interacts with various cellular and viral proteins (Ko and Prives, 1996; Levine, 1997) (Table 1-1 and Figure 1-1). The p53 protein is a critical regulator of tumourigenesis, as evidenced by the fact that *p53* is lost through mutation or inactivation by viral oncoproteins in more than half of human cancer. Moreover, individuals with Li-Fraumeni syndrome, who inherit a mutant *p53* allele, are highly predisposed to various cancers (Malkin, 1993). Homozygous disruption of *p53* alleles in the mouse does not result in developmental defects but leads to a dramatically increased incidence of diverse tumours (Donehower *et al.*, 1992), this being consistent with the postulated role of p53 as a guardian of genomic integrity (Lane, 1992; Pietenpol and Vogelstein, 1993).

At the physiological level, p53 has been implicated as a key mediator of the cellular response to DNA damage. DNA damage induces accumulation of active

p53, which can eventually lead to a G1 arrest or to apoptosis (Kastan *et al.*, 1992; Lu and Lane, 1993; Pietenpol and Vogelstein, 1993; Crook *et al.*, 1994; Macleod *et al.*, 1995; Polyak *et al.*, 1996; Chen *et al.*, 1996; Ko and Prives, 1996). It has been shown that p53 senses DNA damage and arrests the cell cycle in either the G1/S or G2/M checkpoints to allow DNA repair to take place. If repair is not successful, p53 initiates apoptosis thus preventing the propagation of genetic defects to successive generations of cells. In addition, there is evidence that p53 can affect the initiation of apoptosis in a dose-dependent manner when induced by agents that cause DNA-strand breakage (Ko and Prives, 1996). However, p53 appears to be necessary for some, but not all forms of apoptosis (Clarke *et al.*, 1993; Allday *et al.*, 1995; Macleod *et al.*, 1996).

The choice between cell cycle arrest and apoptosis mediated by p53 is not completely understood. Therefore, the factors governing how a cell responds to p53 activation is a subject therefore of great interest.

1-1.1. Cell cycle arrest mediated by p53 is dependent on its transcriptional activity.

The wild-type p53 protein is a sequence-specific transcription factor which can activate genes possessing p53 consensus sites. The biochemical activity of p53 most tightly associated with tumour suppression and growth arrest involves a damage-dependent activation of sequence-specific transcriptional activity. p53 induces the transcription of a number of genes (Table 1-1), the products of which directly mediate growth arrest as is the case of $p21^{Waf1/Cip1}$ (Pietenpol *et al.*, 1994; Crook *et al.*, 1994).

Table 1-1 Proteins that have a functional relationship with p53.

Viral and cellular proteins interacting with p53	
SV40 T antigen	Blocks p53 DNA binding domain
Ad E1B 55kDa	Blocks p53 transcriptional activation domain
HPV E6	Promotes the degradation of p53
c-Abl	p53-mediated cell cycle arrest
TFIID/TBP	Binds amino and carboxyl termini of p53
TFIIH (XPB, XPD)	Helicase modulated by wild type p53, not mutant p53
WT1 (Wilms tumour-1)	Stabilizes p53 protein, and inhibits apoptosis although permitting growth arrest
The products of p53-responsive genes.	
p21*Waf1/Cip1*	Inhibit several cyclin-dependent kinases; bind cdk's, cyclins, and PCNA; arrest the cell cycle
MDM2	Product of an oncogene; inactivates p53-mediated transcription and so forms an autoregulatory loop with p53 activity
GADD45	Induced upon DNA damage; binds to PCNA and can arrest the cell cycle; involved directly in DNA nucleotide excision repair
Cyclin G	A novel cyclin (it does not cycle with cell division) of unknown function and no known cyclin-dependent kinase
Bax	A member of the Bcl2 family that promotes apoptosis; not induced by p53 in all cell types
IGF-BP3	The insulin-like growth factor binding protein-3; blocks signalling of a mitogenic growth factor

Data in this table are compiled together with data from Hall *et al.* (1996), Ko and Prives (1996), Hansen and Oren (1997), and Levine (1997).

1) Sequence-specific DNA binding activity.

p53 binds to promoters containing TGCCT repeats, the so-called RGC sequence (Kern *et al.*, 1991; Maheswaran *et al.*, 1995), present in the promoter of the muscle creatine kinase (MCK) gene (Weintraub *et al.*, 1991; Zambetti *et al.*, 1992). Analysis of multiple genomic p53 target sites has also led to a consensus binding site, defined as two copies of the motif 5'-PuPuPuC(A/T)(T/A)GPyPyPy-3' separated by 13 bp (El-Deiry *et al.*, 1992; Sturzbecher and Deppert, 1994). The central region of p53 (comprising amino acid residues from 102 to 292) contains the DNA-binding (core) domain (Pavletich and Pabo, 1993; Prives, 1994) (Figure 1-1). It has a protease-resistant core and independently folded domain containing a Zn^{2+} ion that is required for its sequence-specific DNA-binding activity (Cho *et al.*, 1994; Vogelstein and Kinzler, 1994; Prives, 1994). More than 90% of the missense mutations in *p53* reside in this core domain, and fall into two classes. The mutations in amino acid residues such as R248 and R273, the two most frequently altered residues in the protein, result in defective contacts with the DNA and loss of the ability of p53 to act as a transcription factor (Ko and Prives, 1996). A second class of p53 mutations disrupts the structural basis of the β sheet and the loop-sheet helix motif that acts as a scaffold in the domain (Hupp *et al.*, 1992; Cho *et al.*, 1994; Prives, 1994; Levine, 1997). Interestingly, the carboxy-terminal domain of p53 recognizes certain types of damaged DNA including short single strands which also activate the sequence-specific DNA binding function of p53 (Lee *et al.*, 1995; Jayaraman *et al.*, 1995).

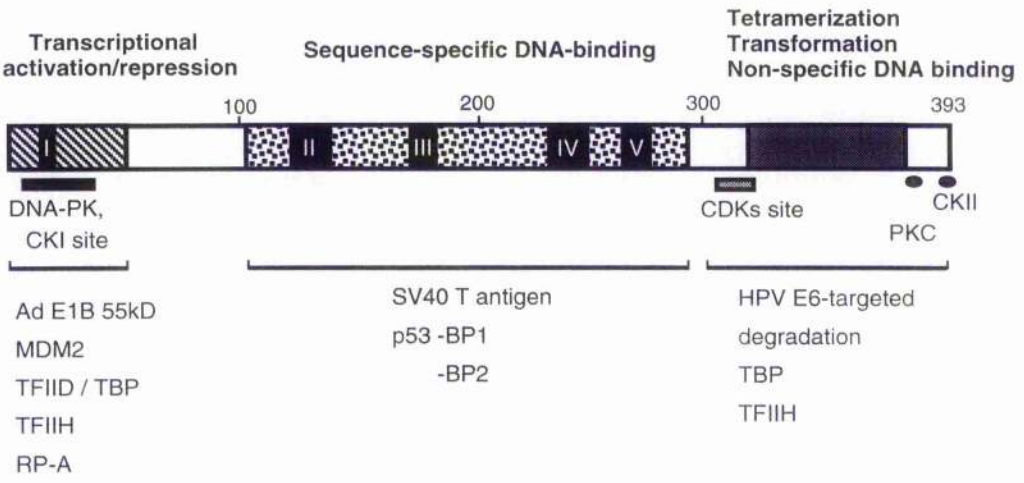


Figure 1-1

Structural organization of the p53 protein.

Hatched boxes I to V represent evolutionarily conserved regions. Indicated above are the positions of defined phophorylation sites by DNA-PK, cyclin-dependent kinases (CDKs), CKI, CKII, and PKC. Some of the proteins which interact with particular regions of p53 are shown at the bottom (see Table 1-1 for further details).

2) Transcriptional suppression / transcriptional activation of specific target genes.

The first 42 amino acids at the amino-terminus constitute a transcriptional activation domain that interacts with the basal transcriptional machinery and positively regulates gene expression (Fields and Jang, 1990; Lin *et al.*, 1995). This region makes contacts the TATA-associated factors TAFII70 and TAFII31 (Lu and Levine, 1995), TAFII40 and TAFII60 (Thut *et al.*, 1995), which are subunits of TFIID, and also the TATA-binding protein, TBP (Chen *et al.*, 1993; Lin *et al.*, 1994). These interactions are thought to contribute to the ability of p53 to repress general transcription in the absence of a cognate recognition site.

Various downstream target genes of p53 have been identified. A summary of them is given in Table 1-1. As mentioned previously, the *p53* gene is induced in response to DNA damage or under conditions unfavourable for DNA synthesis, under certain circumstances this will result in a halt in the cell cycle which allows the cell to repair its DNA or to await for more favourable conditions (El-Deiry *et al.*, 1993; Harper *et al.*, 1993; Dulic *et al.*, 1994; Waldman *et al.*, 1995; Chen *et al.*, 1996). The main player in the cell cycle arrest is the cyclin dependent kinases (CDKs) inhibitor $p21^{Waf1/Cip1}$. Upregulation of $p21^{Waf1/Cip1}$ expression by p53 in response to radiation can inhibit cyclin-dependent kinase activities (Dulic *et al.*, 1994). As result of this inhibition, hypophosphorylated pRb accumulates (Sherr, 1993; Pines, 1995; Weinberg, 1995), and a G1 arrest is induced by the consequent repression of E2F responsive genes (Koh *et al.*, 1995; Lukas *et al.*, 1995) (Figure 1-2). It is known that the phosphorylation of pRb is an important step for growth stimulation of quiescent cells (Nevins, 1992; Lam and La Thangue, 1994). Members of the CDK family are responsible for phosphorylating pRb (Figure 1-2).

$p21^{Waf1/Cip1}$ inhibits not only the G1-acting cyclinE-CDK2 complex but also G1/S-acting cyclinA-CDK2, cyclinA-cdc2 that acts in S and early G2, and it also showns inhibitory activity against cyclinD-CDK4/6 complex (Hinds *et al.*, 1992; Dowdy *et al.*, 1993; Ewen *et al.*, 1993; Cox and Lane, 1995; Sherr and

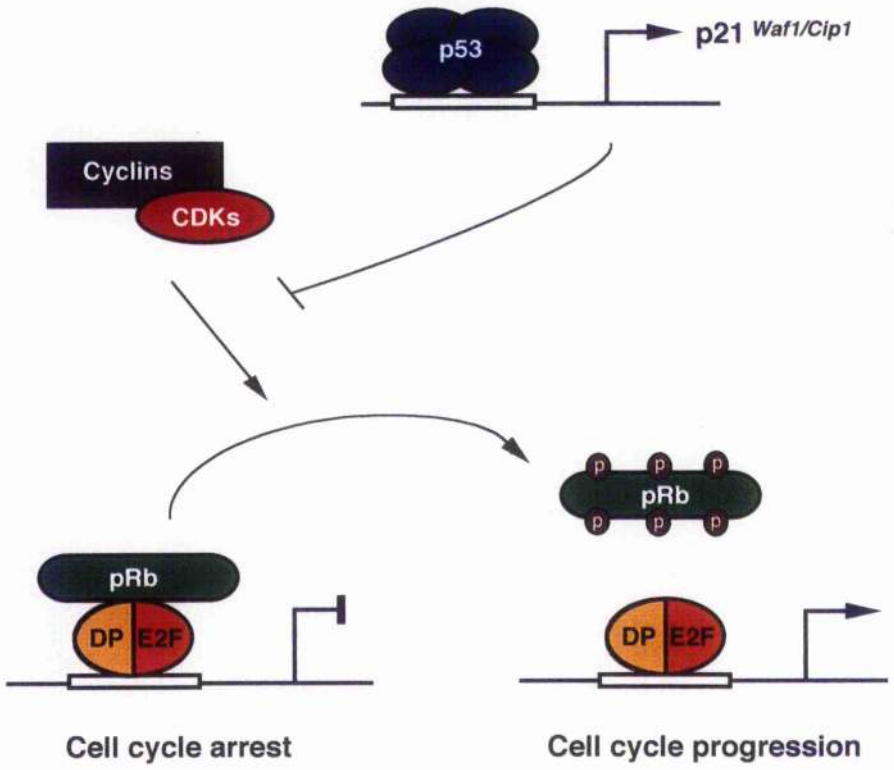


Figure 1-2

Model for activation of cell cycle arrest in G1 by p53.

p53 protein can transcriptionally induce p21 expression, which inhibits cyclin/CDK phosphorylation of pRb. Hypophosphorylated pRb sequesters the transcription factor E2F (complex E2F/DP), and thereby blocks progress through G1 by inhibiting expression of E2F-responsive genes (see Figure 1-5 for further details).

Roberts, 1995). The two known pRb-related proteins p107 (Ewen *et al.*, 1991) and p130 (Hannon *et al.*, 1993; Li *et al.*, 1993) are also substrates of cyclin/CDK. Thus, $p21^{Waf1/Cip1}$ inhibition of cyclinD1-CDK4 and cyclinE-CDK2 can affect the activities of p107 and p130, which regulate the other E2F family members E2F-4 and -5 (Beijersberger *et al.*, 1994; Ginsberg *et al.*, 1994; La Thangue, 1994). The repressive hypophosphorylated form of pRb is targeted by DNA viral oncoproteins such as adenovirus E1A, simian virus 40 large T antigen, and human papillomavirus E7, which bind directly to and inactivate pRb (La Thangue and Rigby, 1987; Nevins, 1992; Tommasino and Crawford, 1995; Levine, 1997). In that way, the DNA tumour viruses stimulate quiescent cells to undergo DNA replication, thereby providing an environment permissive for viral replication.

Interestingly, $p21^{Waf1/Cip1}$ also binds to PCNA (proliferating cell nuclear antigen) (Waga *et al.*, 1994; Gulbis *et al.*, 1996) and these complexes block the role of PCNA as a DNA polymerase processivity factor in DNA replication, but not its role in DNA repair (Figure 1-3). Thus, $p21^{Waf1/Cip1}$ can act on cyclin-CDK complexes and PCNA to stop DNA replication.

Mice deficient in the *Waf1/Cip1* gene ($Waf1/Cip1^{-/-}$) develop normally, and mouse embryo fibroblasts derived from these mice are partially deficient in their ability to arrest cells in G1 in response to DNA damage (Deng *et al.*, 1995).
By contrast, the removal of both *Waf1/Cip1* alleles from a cancerous cell line in culture that contained a wild-type *p53* allele completely eliminated the DNA damage-induced G1 arrest in these cells, indicating that $p21^{Waf1/Cip1}$ is sufficient to enforce a G1 arrest in this situation (Polyak *et al.*, 1996; Levine, 1997).

mdm2 is a protooncogene, amplified in various tumours, which possesses two p53 response elements in its first intron and can be transcriptionally induced by p53 (Momand *et al.*, 1992; Juven *et al.*, 1993). The MDM2 protein directly interacts with p53 (in amino acid residues 18 to 23) (Figure 1-1), and thereby abolishing p53's transcritpional activity (Momand *et al.*, 1992; Lin *et al.*, 1994; Thut *et al.*, 1997).

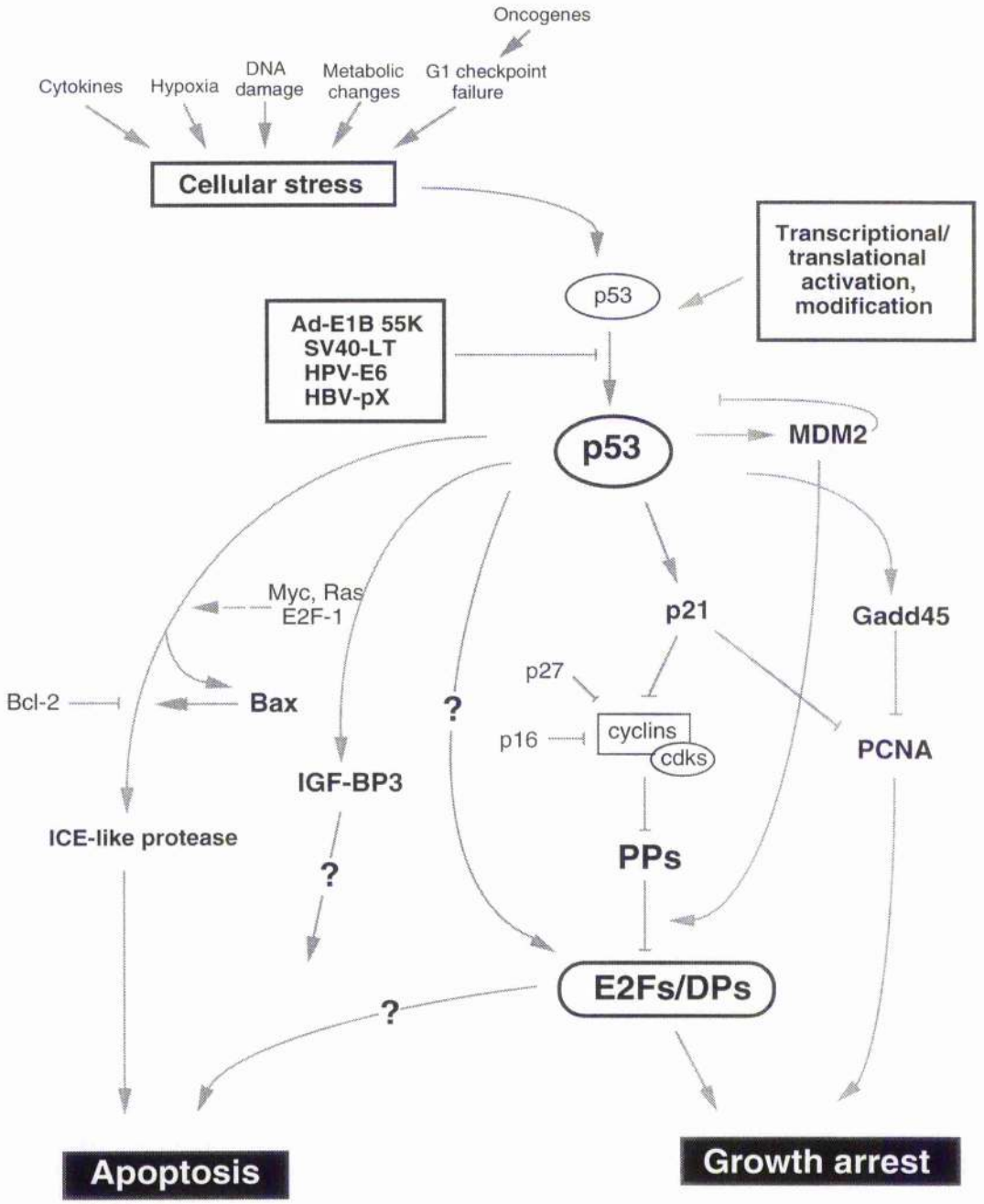


Figure 1-3

p53-dependent pathways for cell cycle arrest and apoptosis.

(Data in this figure are compiled together with data from Cox and Lane (1995), Hall *et al.* (1996), Ko and Prives (1996), Hansen and Oren (1997), and Levine (1997))

This interaction participates in a negative feedback loop in which both the activity of p53 and the expression of MDM2 are regulated in the cells (Chen *et al.*, 1994) (Figure 1-3). In addition, MDM2 regulates the stability of p53 protein, possibly through a mechanism that involves export from the nucleus and thereafter ubiquitin-dependent degradation of p53 (Haupt *et al.*, 1997; Kubbutat *et al.*, 1997; Roth *et al.*, 1998). Although induced by UV light in a p53-dependent manner, the time of *mdm2* induction actually correlates with the recovery of cellular DNA synthesis (Perry *et al.*, 1993). Thus MDM2 may be involved in cell recovery after damage, rather than repair. The over-expression of MDM2 in tumours reduces the p53-dependent G1 arrest (Chen *et al.*, 1994; Cox and Lane 1995). Moreover, the interaction between p53 and MDM2 is influenced by phosphorylation of the p53 activation domain which has been suggested to be mediated by kinases induced by genotoxic stress (Shieh *et al.*, 1997), thus enabling the negative growth regulating effects of p53 in response, for example, to irradiation to be overcome.

In addition, *gadd45* also contains a p53 binding site in its intron 3 and is induced by DNA damaging a p53-dependent manner (Kastan *et al.*, 1992). Although the function of GADD45 in the cell cycle is not completely clear, recent evidence suggests that it can directly interact with the replication and repair factor PCNA and to inhibit the entry of cells into S phase (Smith *et al.*, 1994; Kastan *et al.*, 1992). The over-expression of the product of the *gadd45* gene in some cell types promotes G1 arrest (Figure 1-3). Perhaps, it is possible that GADD45 plays the backup role for $p21^{Waf1/Cip1}$.

1-1.2. p53 is not the only determinant of the cell cycle checkpoints.

Cells could be arrested by p53-independent mechanisms, as has been suggested by different lines of evidence (Strasser *et al.*, 1994). Apoptosis in proliferating $p53^{-/-}$ T lymphoma cells is accompanied by cell cycle arrest.

Dexamethasone-treated cells accumulated only in G1, but irradiated cells accumulated in G2, as anticipated from studies on $p53^{-/-}$ fibroblasts (Kastan *et al.*, 1992). It seems likely therefore that p53 is not the only determinant of the cell cycle checkpoint triggered by DNA damage. Furthermore, it has been found that $p21^{Waf1/Cip1}$ expression during development and in the adult mouse can induced by DNA-damaging agents through p53-independent mechanism and that the gene can be induced by serum or upon differentiation in the absence of p53 function (Macleod *et al.*, 1995). So, it is possible that the p53-independent G1 arrest might be mediated by $p21^{Waf1/Cip1}$, or by other cyclin-CDKs inhibitors such as $p16^{INK4a}$ or $p27^{Kip1}$ (Serrano *et al.*, 1993; Sherr and Roberts, 1995; Kamb, 1995).

1-1.3. Apoptosis induced by p53.

The question of whether transcriptional activation is important for p53-dependent apoptosis has been addressed in a number of elegant studies (Lowe *et al.*, 1994; Symonds *et al.*, 1994; Bates and Vousden, 1996; Ko and Prives, 1996; Hansen and Oren, 1997; Levine, 1997). Two of the genes that are regulated by p53 may influence the decision to commit to an apoptotic pathway : *bax* and *IGF-BP3* (insulin-like growth factor-binding protein 3) (Miyashita and Reed, 1995; Buckbinder *et al.*, 1995).

The Bax gene has been implicated as a direct target for p53 (Miyashita and Reed, 1995) , and the over-expression of Bax can efficiently induce apoptosis (Ludwig *et al.*, 1996) (Figure 1-3 and Table 1-1). Furthermore, it has been reported that Bax binds to Bcl-2 and antigonizes its ability to block apoptosis, so p53-dependent synthesis of Bax has been suggested to be a stimulus for cell death (Oltavi *et al.*, 1993; Miyashita and Reed, 1995). The over-expression of anti-apoptotic proteins, Bcl-2 or the adenovirus E1B 19kDa protein, abrogates p53-mediated apoptosis without impairing p53-mediated G1 arrest and prevents the

apoptosis seen with deregulated c-Myc (Fanidi *et al.*, 1992; Debbas and White, 1993; Chiou *et al.*, 1994; Wagner *et al.*, 1994; Han *et al.*, 1996; Ko and Prives, 1996). The balance of Bcl-2 to Bax may therefore be a critical factor in determining proliferation or apoptosis. At high levels, Bcl-2 protein may compete for the p53-induced Bax to protect cells from p53-induced apoptosis. Induction of Bax in response to irradiation (IR) appears to correlate with p53 status in human cells and *p53*$^{-/-}$ mice exhibit increase levels of Bcl-2 and decreases in Bax protein levels in several cells and tissues (Ko and Prives, 1996). Moreover, tumour growth is accelerated and apoptosis drops dramatically in *bax*-deficient mice, indicating that it is probably required for a full p53-mediated response (Yin *et al.*, 1997).

The *IGF-BP3* also has been identified as a p53 responsive gene induced in cells after DNA damage (Figure 1-3 and Table 1-1). IGF-BP3 inhibits the insulin-like growth factor (IGF) mitotic signalling by binding to IGF and preventing its interaction with its receptor (Buckbinder *et al.*, 1995). IGF has been shown to function as an efficient survival factor, protecting cells from p53-dependent, myc-induced apoptosis (Harrington *et al.*, 1994). Thus, it is possible that the effect of activation of IGF-BP3 by p53 is to abrogate survival signals, rather than growth signals, to make the cell more sensitive to other apoptotic functions of p53.

A lot of evidence supports a correlation between apoptosis and transcriptional activity by p53. Tumour-derived mutant alleles of p53 (with the mutation Ala143, His175, or Trp248) encode proteins that fail to induce apoptosis in transiently transfected H1299 cells (Friedlander *et al.*, 1996). In addition BRK cell lines transformed by E1A and expressing a temperature-sensitive p53 Val135 mutant undergo apoptosis after shift to 32°C (the permissive temperature to form the wild-type conformation of p53), whereas cells expressing a transcriptionally defective p53 Gln22, Ser23 ($p53^{22/23}$) were dramatically impaired for the ability to mediated E1A-induced apoptosis in the same conditions (Sabbatini *et al.*, 1995). Moreover, experiments in transgenic mice expressing a p53 mutant harboring only the carboxy-terminal domain p53 (amino acid residues 302 to 390) results in mice

developing aggressive brain tumours by the inactivation of endogenous p53 (Bowman *et al.*, 1996). Thymocytes from transgenic mice expressing a T antigen capable of binding to p53 have also been shown to be resistant to radiation-induced apoptosis (McCarthy *et al.*, 1994).

1-1.4. p53 induction is not necessary for apoptosis in some circumstances.

It has been found that p53 induction is not the only trigger for cell death following DNA damage. Thymocytes, cells which enter apoptosis in response to DNA damage through a p53-dependent pathway, also undergo p53-independent cell death during the normal process of lymphoid development (Clarke *et al.*, 1993; Lowe *et al.*, 1993), providing evidence that p53 is not the sole contribution to apoptosis. Cells from T lymphomas derived from $p53^{-/-}$ mice are killed rapidly and efficiently by irradiation and DNA-damaging agents (Strasser *et al.*, 1994). Furthermore, it was shown that apoptosis is p53-dependent in the central nervous system but p53-independent in the peripheral nervous system (Macleod *et al.*, 1996). Experiments using human B cell lines treated with cisplatin resulted in DNA damage that induced apoptosis, during the G1/S boundary of cell cycle, when p53 is transcriptionally active, and G2/M phase, when p53 is transcriptionally inactive (Allday *et al.*, 1995), suggesting the existence of another cell cycle checkpoint at G2/M phase. More clearly, the mutant p53 Gln22, Ser23 ($p53^{22/23}$), which abrogates the transcriptional activating function of p53, triggered apoptosis in HeLa cells (Haupt *et al.*, 1995b), and p53 dl214, which contains only the first 214 amino acid residues of murine p53, can induce apoptosis, despite lacking the major portion of the DNA-binding domain (Haupt *et al.*, 1995b). Interestingly, the over-expression of MDM2 failed to abolish p53-mediated apoptosis in HeLa cells, despite a complete abrogation of its *trans* activation ability, while MDM2 dramatically inhibited p53-mediated apoptosis in H1299 cells (Haupt *et al.*, 1996). Thus, taken

together it appears that the transcriptional activity of p53 is not necessary for the induction of apoptosis in all cells. Nevertheless, this does not exclude the possibility that several pathways co-operate to induce cell death. The evidence suggests that different p53 functions, both transcriptionally-dependent and -independent, play a role in activating these pathways.

1-1.5. Cell cycle arrest and apoptosis by p53 may be differentially regulated functions.

Numerous studies have shown that growth arrest and apoptosis are independent functions of p53. Murine hematopoietic cell lines undergo G1 arrest in response to irradiation in the presence of survival factors or growth factors, but when they are absent, p53-dependent apoptosis ensues (Gottlieb *et al.*, 1994; Canman *et al.*, 1995). Uncoupling growth arrest and apoptosis has also been demonstrated through the use of several mutants forms of p53 (Haupt *et al.*, 1995b; Rowan *et al.*, 1996; Friedlander *et al.*, 1996). In particular, p53 175P, a mutant which was originally isolated from a human papilloma virus type 16 (HPV 16) positive metastasis of a cervical carcinoma, can induce a cell cycle arrest in some cell lines but shows loss of apoptotic function (Rowan *et al.*, 1996; Ludwig *et al.*, 1996), indicating that the loss of p53 apoptotic function without concomitant loss of growth arrest can suffice to relieve p53 dependent tumour suppression and thereby contribute to tumour development. Furthermore, Bcl-2 has been shown to prevent p53-mediated apoptosis without impairing p53-mediated growth arrest (Chiou *et al.*, 1994; Debbas and White, 1994). In addition, it also has been documented that the activation of p53 Val135 in rat REF52 cells caused the growth arrest at G2/M phase, without inducing apoptosis (Stewart *et al.*, 1995), further highlighting the complexity of p53 functions in different cell types and tissues (Zhan *et al.*, 1994; Allday *et al.*, 1995).

Various studies suggest that apoptosis often occurs as a default mechanism when cells fail cell cycle checkpoints in the presence of p53 function. Whereas loss of pRb function and over-expression of E2F are known to disrupt the G1 arrest and favour apoptosis (Wu and Levine, 1994; Qin *et al.*, 1994; Shan *et al.*, 1996; Macleod *et al.*, 1996; Lee at al., 1998), a similar effect has also been shown for p21$^{Waf1/Cip1}$ deficiency (Macleod *et al.*, 1995; Chen *et al.*, 1996; Donatella *et al.*, 1996; Polyak *et al.*, 1996). In particular, experiments on fibroblasts from *Waf1/Cip1*$^{-/-}$ mice have shown them to be defective in G1 arrest in response to DNA damage (Deng *et al.*, 1995). However, thymocytes from the same mice showed no defect in response to DNA damage-induced apoptosis, in marked contrast to *p53*$^{-/-}$ thymocytes. Recently, hybrid assay studies using p53 expressing cancer cell lines, which yielded growth arrest in some lines or apoptosis in others suggest, however, that some cells can experience a dominant apoptotic signal which renders them refractory to rescue by growth-inhibitory proteins (Polyak *et al.*, 1996).

1-1.6. Accumulation or modulation of p53.

In normal circumstances, the p53 protein remains in a latent state but, following genotoxic, irradiation with either ionizing radiation or UV light, cytokines, hypoxia, metabolic changes, or other forms of stress (Figure 1-3), post-transcriptional mechanisms result in an accumulation of p53 and/or increased half-life, and thereafter cell cycle arrest or apoptosis, effects that contribute to the suppression of malignant disease (Ko and Prives, 1996).

In addition to increasing p53 protein levels, cells may respond to DNA damage by modulating the function of the protein, at least in part by phosphorylation as p53 is multiply phosphorylated at serines and threonines within its amino- and carboxy-terminal regions *in vivo* and *in vitro* (Cox and Lane, 1995; Hall *et al.*, 1996; Ko and Prives, 1996).

The following protein kinases have been shown to phosphorylates p53; CDKs, casein kinase I and II (CKI and CKII), double-stranded DNA-activated protein kinase (DNA-PK), and protein kinase C (PKC) (Hall *et al.*, 1996; Ko and Prives, 1996) (Figure 1-1). For example, recently it was shown that phosphorylation of human p53 at serine 15 (by DNA-PK) occurs after DNA damage and that this leads to reduced interaction of p53 with its negative regulator, MDM2 (Shieh *et al.*, 1997). In contrast with E2F, phosphorylation dramatically stimulates the binding of p53 to its target sites in the *Waf1/Cip1* and *gadd45* genes (Wang and Prives, 1995). Phosphorylation thus induces a conformational change, conferring sequence selectivity on a relatively nonspecific DNA-binding protein (Hupp *et al.*, 1992; Wang and Prives, 1995; Ko and Prives, 1996). The transcriptional potential of E2F and p53 can thus be modified by cell cycle specific cyclin/CDK combinations. Furthermore, p53 function can be activated by some cellular proteins, such as c-Abl tyrosine kinase (Yuan *et al.*, 1996; Liu *et al.*, 1996). Ionizing radiation stimulates the phosphorylation of DNA-PK by c-Abl inhibiting the ability of DNA-PK to form a complex with DNA (Kharbanda *et al.*, 1997), and c-Abl binds to p53 and enhances its transcriptional activity (Goga *et al.*, 1995). Moreover, the stabilization of p53 has been observed with the expression of either adenovirus E1A or E7 protein of oncogenic forms of HPV, both which bind pRb but not p53 (Lowe and Ruley, 1993; Demers *et al.*, 1994). Also, the cellular product of the Wilms' tumour gene, WT1, another tumour suppressor, also stabilizes p53 (Maheswaran *et al.*, 1995).

1-2. The E2F pathway

A variety of data suggest that the ability to activate a G1 cell cycle checkpoint in transformed cells is more important than the ability to influence the expression of apoptosis mediators. Therefore, it seems more likely that p53-mediated growth arrest and apoptosis are tightly inter-related at the cellular level. In particular, members of the E2F/DP transcription factor family and of the pRb family appear to play an important role in the decision as to whether a cell will respond to wild-type p53 activation by executing a viable G1 arrest or undergoing apoptosis.

1-2.1. Cell cycle progression is coupled to the regulation of E2F.

Studies on the mechanisms of how the cell cycle is co-ordinated have provided important information on how physiological processes influence proliferation. The transcription factor E2F is believed to play a crucial role in the growth-promoting and growth-arresting processes (Nevins, 1992; Girling *et al.*, 1993; Bandara *et al.*, 1993; La Thangue, 1994; Muller, 1995; Buck *et al.*, 1995) (Figure 1-4). E2F is a heterodimeric factor which results from the interaction of two families of proteins: E2F (1 to 5) and DP (1 to 3), where each E2F protein requires a DP protein as an obligate heterodimeric partner, heterodimerization producing a significant increase in DNA binding activity (Girling *et al.*, 1993; Bandara *et al.*, 1993).

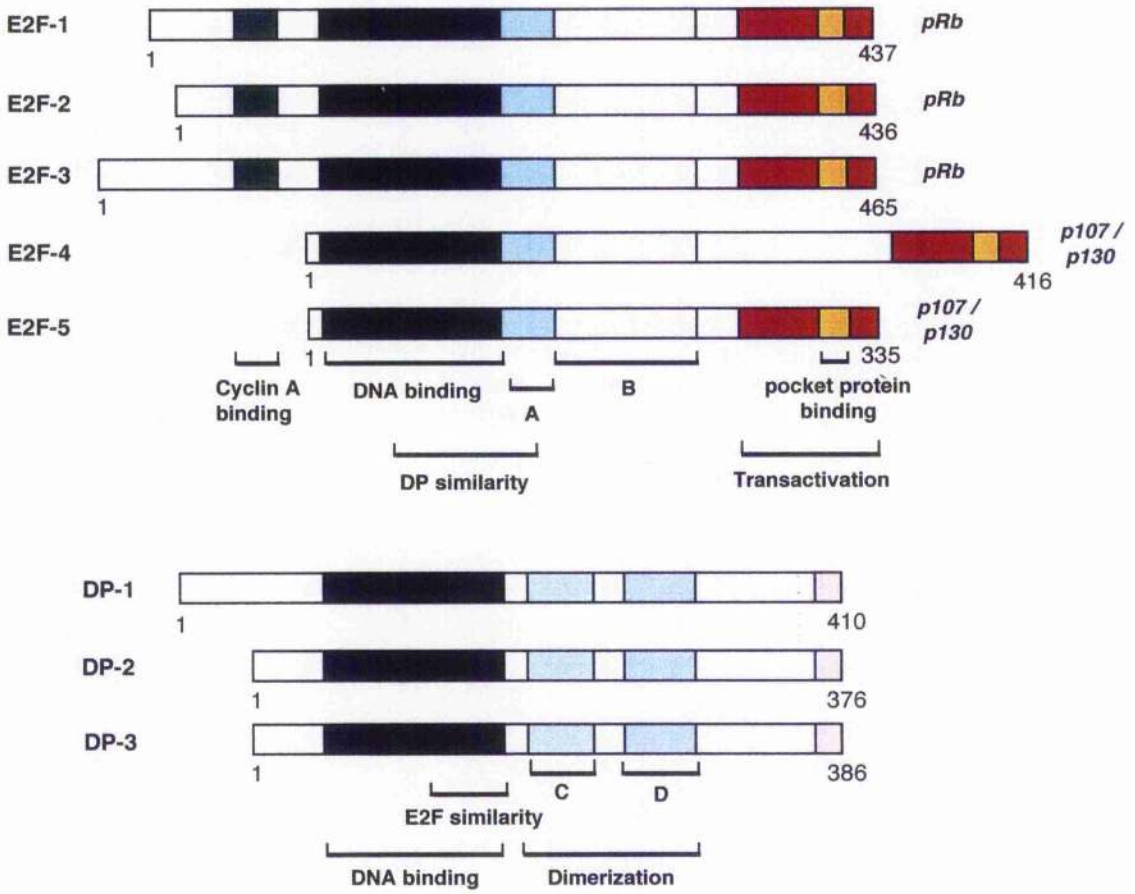


Figure 1-4.

Sequence comparison of E2F and DP proteins.

Shaded regions or hatched boxes correspond to sequences conserved in E2F family or DP family, respectively. Note that E2F-1, -2, and -3 preferentially bind to pRb, and E2F-4 and -5 to p107 and p130. E2F-1, -2, and -3 include a cyclin A binding site. 'A' and 'B' are both areas of sequence similarity which as yet have no clear function. DP proteins also contain a region of homology to E2F-1 to -5. 'C' and 'D' are regions of sequence similarity in all DP proteins. The heterodimerization domain of E2Fs and DPs is located in DNA binding.

Many of the genes which are believed to play an important role in regulating early cell cycle progression contain binding sites, TTTCGCGC, for the E2F transcription factor in their transcriptional control sequences. Among these are the proto-oncogenes *c-myc*, *N-myc* and *B-myb*, genes encoding enzymes required for DNA synthesis such as DHFR, thymidine kinase, thymidylate synthase and DNA polymerase α, and genes encoding cell cycle regulators such as cdc-2, cyclinA, E and D1 (La Thangue, 1994; Adams and Kaelin, 1995; Muller, 1995) (Figure 1-5). By deletion or mutation of E2F sites in many of these promoters, it has been demonstrated that E2F sites are important for the correct temporal expression pattern of the target gene. However, given the different times at which these target genes are expressed, it is clear that distinct mechanisms must be responsible for the timing of their activation (Figure 1-5). One of these mechanisms may be based on the fact that five E2F and three DP subunits heterodimerize in various combinations to form functionally active DNA-binding complexes (Bandara *et al.*, 1993; La Thangue, 1994; Muller, 1995; de la Luna *et al.*, 1996; Allen *et al.*, 1997).

The potential role of E2F and DP proteins in cell cycle control is underscored by the properties of the proteins which are known to influence the transcriptional activity of E2F (Figure 1-6). For example, a group of proteins which negatively regulate the cell cycle, including the retinoblastoma tumour suppressor protein (pRb) and its relatives p107 and p130 bind to and inactivate the transcriptional activity of E2F (Bandara and La Thangue, 1991; Chellappan *et al.*, 1991; Zamanian and La Thangue, 1992; Nevins, 1992; Schwarz *et al.*, 1993; Cobrinik *et al.*, 1993) (Figure 1-4).

It has been known that several viral oncoproteins, such as the adenovirus E1A protein, SV40 large T antigen and the E7 proteins of certain human papilloma viruses, bind to the pocket which mediates binding to the E2F binding region in pRb, relieving the transcriptional inactivation and subsequently releasing the E2F transcriptionally active form (La Thangue and Rigby, 1987; Bandara and La Thangue, 1991; Nevins, 1992; Lam and La Thangue, 1994; Vousden, 1995;

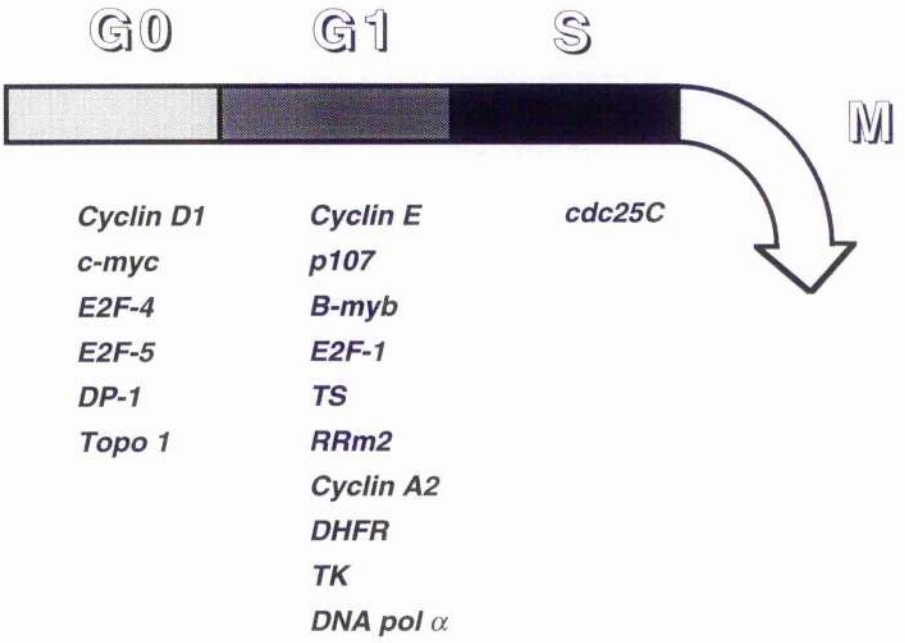


Figure 1-5

Genes regulated by E2F.

(Data in this figure was obtained from Hartwell and Kastan (1994), La Thangue (1994), and Muller (1995))

Mymryk, 1996). Thus, the DNA tumour viruses deregulate E2F activity so as to stimulate quiescent cells to undergo DNA replication.

Another group of these regulators which control cell cycle progression are the cyclins and CDKs which affect both basal and activated transcription (Bandara *et al.*, 1991; Sherr, 1993; Pines, 1995; Dynlacht, 1997). As described previously, cyclin/CDKs complexes are believed not only to influence the activity of the pocket proteins but also to form complexes directly at least with the E2F-1, -2, and -3 subfamily members. This results in the phosphorylation of the DP subunit (Dynlacht *et al.*, 1994; Krek *et al.*, 1994 and 1995) and a subsequently inactivation of E2F/DP function which allows exit from S phase.

Ectopic expression of E2F-1 results in the expression of genes necessary for DNA synthesis and progression into S phase as described (Figure 1-5) (Kowalik *et al.*, 1995; Almasan *et al.*, 1995). Further evidence that E2F promotes entry into S phase derives from studies with *Drosophila melanogaster* lacking E2F-1 function in which cells are arrested in the G1 phase of the cell cycle after the seventeenth embryonic division (Duronio *et al.*, 1995).

1-2.2. E2F and DP family possess proto-oncogenic activity.

Considerable evidence suggests that E2F-1 can be oncogenic. E2F-1 transformed rat embryo fibroblast (REF) cells display anchorage-independent growth and form colonies in agarose, and injection of the transformed REF-E2F-1 cells into nude mice cause tumours (Singh *et al.*, 1994). In addition, in conjuction with an activated *ras* oncogene, and an E2F-1 chimeric protein, in which pRb binding region replaced with VP16 activation domain, exhibits increased transformation activity (Johnson *et al.*, 1994), suggesting that regulation of E2F activity by pRb may be critical in maintaing normal cellular growth control. Furthermore, DP-1 or DP-2 can co-operate with activated Ha-*ras* in the

transformation of rat embryo fibroblasts, and this oncogenic activity is unlikely to be due to the regulation of E2F site transcription, suggesting an E2F-independent effector function for DP (Jooss *et al.*, 1995). More recently, it has been shown that Ras and Myc can co-operate in inducing accumulation of E2F and resulting in cell cycle progression (Leone *et al.*, 1997). The inhibition of Ras activity blocks the normal growth-dependent activation of G1 CDK, and prevents activation of the target genes of E2F. However, although expression of Ras gene alone is not sufficient to induce S phase, co-expression of Ras with Myc allows the generation of cyclinE-dependent kinase activity and the induction of S phase (Leone *et al.*, 1997), demonstrating a functional relationship between Ras/Myc and E2F-1 in oncogenic transformation.

1-2.3. E2F-1, a possible link between the loss of pRb and the activation of p53.

Apart from the role of E2F-1 in stimulating proliferation, over-expression of E2F-1 in some tissue culture cells can induce apoptosis that is p53-dependent (Wu and Levine, 1994; Qin *et al.*, 1994; Kowalik *et al.*, 1995; Almansan *et al.*, 1995; Shan *et al.*, 1996). The E2F-1/DP-1-induced cell death correlates with an accumulation of the p53 protein, suggesting that E2F may activate p53 to induce death (Almansan *et al.*, 1995; Kowalik *et al.*, 1995). Interestingly, an E2F-1 mutant that is defective in binding pRb but active in *trans* activation is a more potent activator of apoptosis (Shan *et al.*, 1996). The combined over-expression of E2F-1 and its heterodimeric DP-1 partner drives apoptosis in 32D cells even in the presence of survival factors such as IL-3 (Hiebert *et al.*, 1995; Almasan *et al.*, 1995). Moreover, E2F-1 mediated apoptosis is suppressed by co-expression of wild-type pRb or a dominant negative mutant of p53 (DeGregori *et al.*, 1997). In contrast, co-expression of a natural occuring pRb mutant or wild-type p53 did not suppress the induction of apoptosis (Qin *et al.*, 1994; Wu and Levine, 1994; DeGregori *et al.*,

1997). Therefore, the inhibition of E2F-1-dependent apoptosis may explain the anti-apoptotic activity of pRb.

The co-operation between E2F-1 and p53 has lead to the suggestion that apoptosis may be mediated by a conflict of growth stimulatory signals activated by E2F-1 and the growth inhibitory functions of p53 (Qin *et al.*, 1994; Wu and Levine, 1994; Shan *et al.*, 1996; Lee *et al.*, 1998). In addition, in experiments using *Drosophila melanogaster* imaginal discs, ectopic E2F-1 expression induces S phase and apoptosis, suggesting that the down-regulation of E2F-1 upon entry into S phase may be necessary to prevent the induction of apoptosis (Asano *et al.*, 1996). Although p53 can augment E2F-1-induced apoptosis in several systems, recent studies have suggested that the pro-apoptotic effects of E2F-1 are not entirely dependent on p53 and pRb (Qin *et al.*, 1994; Hass-Kogan *et al.*, 1995; Asano *et al.*, 1996; Lee *et al.*, 1998). In particular, the introduction of E2F-1 in SAOS-2 cells cause a significant levels of apoptosis (Lee *et al.*, 1998). Thus, it may be possible that the E2F-1-mediated induction of apoptosis appears to result from the activation of a cell death pathway.

1-2.4. The physiological role of E2F-1.

Despite considerable progress in elucidating the multiple functions of E2F-1, it is uncertain whether the predominant function of E2F-1 is to promote entry into S phase, suppress G1 arrest, and/or regulate apoptosis.

E2F-1$^{-/-}$ mice develop and reproduce normally, although they display a defect in thymocyte apoptosis indicating that E2F-1 may function to suppress cell proliferation (Field *et al.*, 1996). In addition, *E2F-1*$^{-/-}$ mice develop a broad and unusual spectrum of tumours including reproductive tract sarcoma, lung adenocarcinoma, and lymphomas (Yamasaki *et al.*, 1996). Although over-expression of E2F-1 in tissue culture cells can stimulate cell proliferation and

oncogenic transformation, that loss of E2F-1 in mice results in tumourigenesis suggests that E2F-1 also functions as a tumour suppressor (Johnson *et al.*, 1994; Field *et al.*, 1996; Yamasaki *et al.*, 1996). However, under normal circumstances E2F-1 may have a dual role as a suppressor of cell proliferation in G0/G1 and an activator of proliferation in G1/S.

The fact that a deletion of E2F-1 leads to a decrease in apoptosis and enhanced proliferation suggests that *in vivo* the negative effects of E2F-1 on cell cycle progression may be more important than the positive effects. Nevertheless, the fact that E2F-1 is a member of a large family of related genes leaves open the possibility that E2F-1 also plays an important role in promoting S phase entry in many cell types but that in its absence this role is compensated for by other family members.

1-2.5. Cell cycle regulation of pRb and its relatives by phosphorylation.

The retinoblastoma protein (pRb) is a 110 kDa nuclear phosphoprotein that is inactivated in retinoblastomas and a variety of other tumour types (Lee *et al.*, 1987; Hu *et al.*, 1990). pRb has the ability to suppress cell proliferation, and this activity is controlled by its cell cycle dependent phosphorylation (Weinberg, 1992; Whyte, 1995; Wang, 1997). A variety of studies shown that pRb phosphorylation begins in late G1 and continues until M phase (Dowdy, 1993) (Figure 1-6). In G0 and early G1, pRb is maintained in a state of low phosphorylation. Late in G1, pRb becomes extensively phosphorylated on a number of serine and thereonine residues (Lees *et al.*, 1991). During mitosis, pRb is rapidly dephosphorylated by an anaphase-specific phosphatase (Ludlow *et al.*, 1993). It has also been suggested that the growth suppressing activity of pRb is mainly mediated by binding and inhibiting the transcription factor E2F (Chellappan *et al.*, 1991; La Thangue, 1994; Bandara *et al.*, 1994; Whyte, 1995).

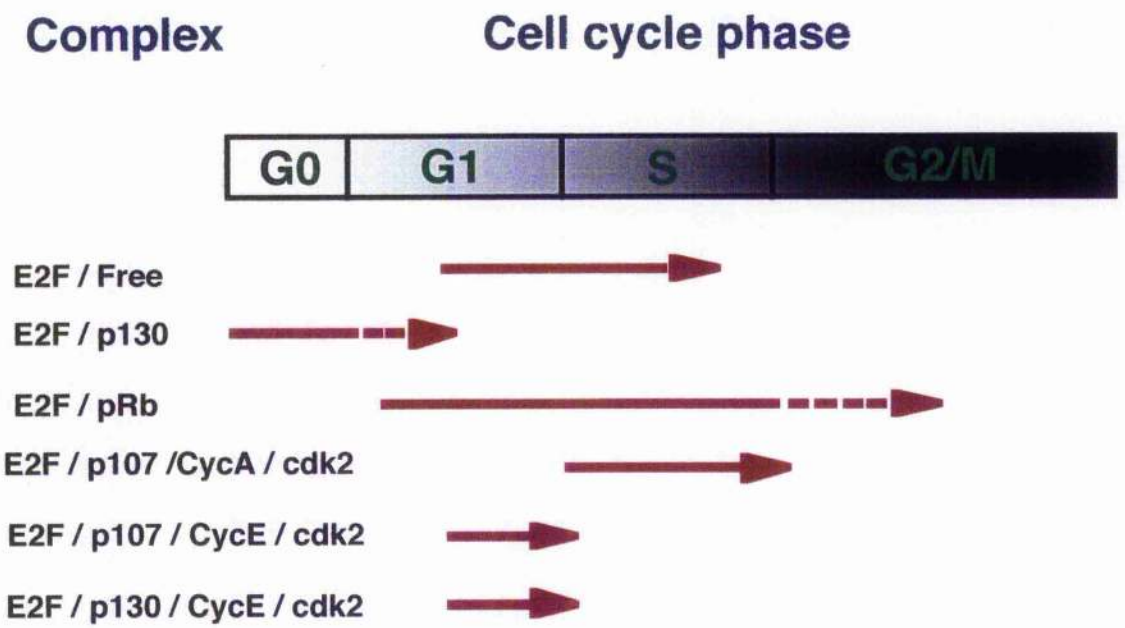


Figure 1-6

Cell cycle dependence of E2F containing complexes.

The diagram, which presents a summary of E2F control during the cell cycle, shows free E2F (transcriptionally active) accumulating towards the end of G1 to peak during S phase. The regulation of pRb, p107, and p130 to E2F are indicated.

The interaction of pRb with E2F-1 alone or DP-1 alone is weak, whereas interaction with the E2F-1/DP-1 heterodimer is highly stable (Bandara *et al.*, 1993; Helin *et al.*, 1993).

Interestingly, pRb and two of its homologs, p107 and p130, seem to interact with specific E2F proteins : pRb with E2F-1, -2, -3 / DP-1, -2, -3 ; p107 with E2F-4, -5 / DP-1, -2, -3 ; and p130 with E2F-4, -5 / DP-1, -2, -3 (La Thangue, 1994; Bandara *et al.*, 1994; Muller, 1995; Allen *et al.*, 1997) (Figure 1-4 and Table 1-2). This interaction forms a ternary complex that prevents E2F-mediated transcription by RNA polymerase II (Chellappan *et al.*, 1991; Lam and La Thangue, 1994; Weintraub *et al.*, 1995). The significance of the different interactions between pRb family and E2F family member is unclear. But, p130/E2F complexes are abundant in quiescent or differentiated cells but are less common in populations of cycling cells (Cobrinik *et al.*, 1993; Smith *et al.*, 1996) (Figure 1-6). Conversely, p107/E2F complexes are most readily detected in cycling cells, together with a cyclin/CDK containing complex during S-phase (Cobrinik *et al.*, 1993; Ginsberg *et al.*, 1994) (Figure 1-6). pRb/E2F complexes have been found in extracts of differentiated cells, quiescent cells, G1 phase cells, and possibly even in S-phase cells (Schwarz *et al.*, 1993). Recently, it has been shown that pRb and p107/p130 provide different functions in E2F regulation by identifing target genes that are dependent on pRb family proteins for their normal expression in mouse knockout studies (Hurford *et al.*, 1997).

The inhibition in E2F activity by pRb binding may be the result of (1) a block of the E2F *trans* activation domain, (2) a block in the interaction with the basal transcriptional machinery and (3) the decrease in histone acetylation promoted by the binding of pRb to histone deacetylase 1 (HDAC1) (Weintraub *et al.*, 1995; Chow and Dean, 1996; Luo *et al.*, 1998). The pRb and E2F family interaction is normally controlled during the cell cycle by altering the phosphorylated status of pRb: the hypophosphorylated forms bind E2F, whereas the hyperphosphorylated forms do not (La Thangue, 1994; Bandara *et al.*, 1994; Whyte, 1995) (Figure 1-2).

The phosphorylation of pRb family of proteins is carried out by cyclinA/CDK2, cyclinE/CDK2, or cyclinD/CDK4/6, thus co-ordinating transcription regulation and cell cycle control (Bandara *et al.*, 1991; Sherr and Roberts, 1995; White, 1995). Progression through the G1/S phase involves the activation of cyclinD/CDK4, cyclinD/CDK6, cyclinE/CDK2 and cyclinA/CDK2. CyclinD (D1, D2 and D3) complexed with CDK4 or 6 are involved in the phosphorylation of pRb during G1 and in response to mitotic agents which in turn accelerate progression through the G1 phase. CyclinE peaks at the G1/S transition indicating a role in entry into S-phase (Sherr and Roberts, 1995; Pines, 1995; Whyte, 1995). Thus, the different cyclin-CDK complexes may exert distinct effects on pRb family function.

The activity of the CDKs is negatively regulated by CDK inhibitors such as $p15^{INK4b}$, $p16^{INK4a}$, $p21^{Waf1/Cip1}$, and $p27^{kip1}$ (Serrano *et al.*, 1993; Harper *et al.*, 1993; Xiong *et al.*, 1993; Hannon and Beach, 1994; Sherr and Robert, 1995; Weinberg, 1995) (Figure 1-3). The genes encoding $p15^{INK4b}$ and $p16^{INK4a}$ are mutated in certain tumour cell lines, such as those derived from oesophageal, squamous cell carcinomas, glioblastomas, lung and bladder carcinomas (Schmidt *et al.*, 1994; Kamb *et al.*, 1994; Nobori *et al.*, 1994; Zhou *et al.*, 1994; Jen *et al.*, 1994). Mutant alleles in the $p16^{INK4a}$ gene fail to inhibit kinase activity and arrest growth and, furthermore, arrest by wild-type $p16^{INK4a}$ correlates with the expression of wild-type pRb (Serrano *et al.*, 1995; Lukas *et al.*, 1995; Koh *et al.*, 1995). Thus, the effect of these kinase inhibitors is likely to mediated by modulating the activity of pRb.

Numerous experiments have demonstrated a critical role for both Myc and Ras activities in allowing cell cycle progression (Alevizopoulos *et al.*, 1997; Leone *et al.*, 1997). Recently, it was shown that the inhibition of Ras activity blocks the normal growth dependent activation of CDK, prevents the activation of the target genes of E2F, and results in the cell cycle arrest in G1 (Alevizopoulos *et al.*, 1997; Leone *et al.*, 1997). Furthermore, co-expression of Ras and Myc induce the accumulation of active cyclin/CDKs and E2F, coincident with the loss of the

$p27^{kip1}$, which allows the induction of S phase (Alevizopoulos *et al.*, 1997; Leone *et al.*, 1997). These results suggest that pRb is a crucial link between Ras / Myc and E2F pathway in tumourigenesis. Therefore, pRb represents an important intermediary between the cell cycle proteins and the transcriptional machinery that regulates growth progression.

1-2.6. Regulation of pocket proteins by cellular and viral oncoprotein.

pRb requires a domain termed the pocket domain from amino acid 395 to 876, in order to interact with many of its cellular binding proteins and exert growth suppression (Figure 1-7 and Table 1-2). Indeed, most of the retinoblastoma tumours involve mutation in this pocket region disrupting the ability of pRb to bind to proteins such as E2F (Hu *et al.*, 1990; Bandara and La Thangue, 1991; Chellappan *et al.*, 1991; Zamanian and La Thangue, 1992) (Figure 1-7). Further, viral oncoproteins such as SV40 large T antigen, adenovirus E1A, and HPV E7, proteins which contains the LXCXE motif involved in pRb binding (Whyte, 1995; Vousden, 1995; Wang, 1997), may transform cells in part by binding to this pocket, rendering pRb incapable of binding to E2Fs and promoting their growth progression (Bandara and La Thangue, 1991; Zamanian and La Thangue, 1992; Chellappan *et al.*, 1992; Kaelin *et al.*, 1992; Vousden, 1995). The role of hypophosphorylated pRb in the negative regulation of cell growth is supported by the observation that these viral oncoproteins preferentially bind to this form of pRb (Vousden, 1995; Whyte, 1995). Therefore, either the sequestration of pRb by viral oncoproteins or mutations in the pocket region can eliminate normal pRb function and contribute to the development of many different types of human cancers (Hu *et al.*, 1990; Vousden, 1995).

Two pRb homologs, p107 and p130, also contain a pocket region that allows binding to the same cellular and viral oncoproteins, suggesting these relatives of pRb are involved in a similar mechanism of pRb function.

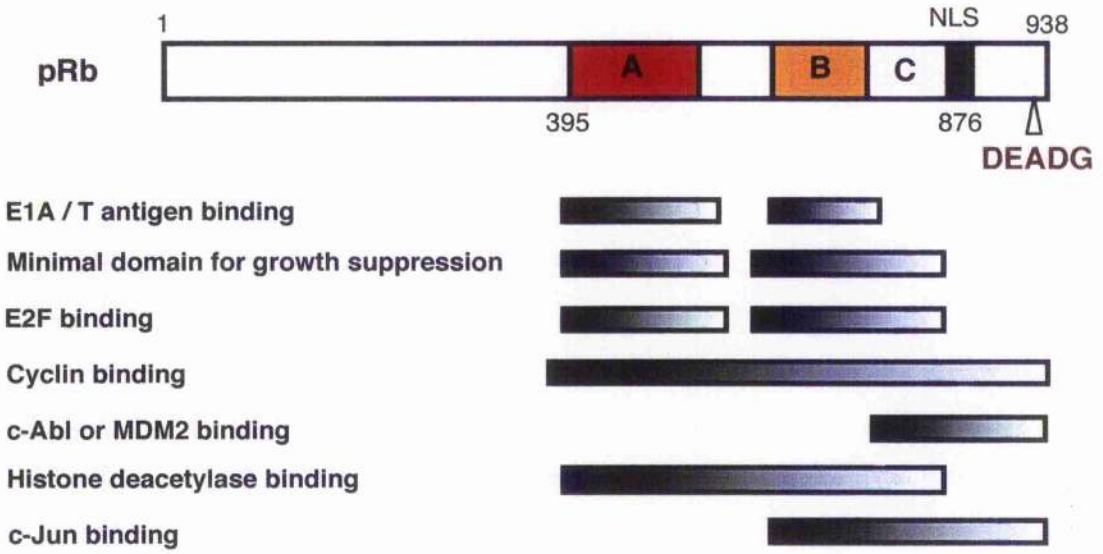


Figure 1-7.

Functional domain in pRb.

The domain for growth suppression from amino acid residue 395 to 876 contains for viral oncoproteins, E2F, D-type cyclin, and histone deacetylase binding. NLS and c-Abl or MDM-2 binding region are within the C region. The binding of c-Jun involves in the B pocket as well as a carboxy-terminal domain. Amino acids, DEADG, are the consensus cleavage site for Ced-3/ICE proteases.

Table 1-2 Cellular proteins interacting with pocket proteins.

pRb	E2F-1, -2, -3 / DP-1, -2, -3	Repression of transcription via E2F binding sites
	ATF2	Increased transactivation by ATF2 dimers
	MyoD (myogenic HLH transcription factor)	Co-operate in the induction of myogenic differention and cell cycle arrest
	c-Abl	Repression of tyrosine kinase activity and transcriptional activation; c-Abl induces cell cycle arrest
	Elf-1	T-cell specific transcription factor: Elf-1 regulates the activity of T-cell genes
	MDM-2	Inhibition of pRb growth regulatory function
	PU.1	Transcription factor
	UBF	Transcription factor (RNA polymerase I)
	BRG-1 (*Brabma*-related protein)	Disruption of nucleosome structure; co-operate in the induction of cell cycle arrest
	Cyclins D1, D2, D3	Regulatory subunits for CDKs
	HDAC (histone deacetylase)	Deacetylating histones and disrupting nucleosome structure
	c-Jun	Stimulates c-Jun transcriptional activity
p107	E2F-4, -5 / DP-1, -2, -3	Repression of transcription via E2F binding sites
	c-Myc	Repression of transactivation by c-Myc; proto-oncogene
	Cyclins A, D1, D2, D3, E	Regulatory subunits for CDKs
p130	E2F-4, -5 / DP-1, -2, -3	Repression of transcription via E2F binding sites
	Cyclins A, D1, D2, D3, E	Regulatory subunits for CDKs

Data in this table are obtained from Muller (1995), Whyte (1995), La Thangue (1997), Wang (1997), Luo *et al.* (1998), and Nead *et al.* (1998).

But, despite the ability of p107 and p130 to restrict cell cycle progression, neither p107 or p130 have been shown to act as tumour suppressor or be mutated in natural occuring tumours.

The pocket region of pRb contains at least three distinct protein binding sites, for the LXCXE motif, for E2F, and for c-Abl or MDM2 (Wang, 1997) (Figure 1-7 and Table 1-2). A domain in the carboxy-terminus of pRb (residues 768 to 928) binds to the ATP-binding lobe of the c-Abl tyrosine kinase, abrogating pRb-induced growth arrest. Phosphorylation of pRb at G1/S releases c-Abl, leading to the activation of this kinase (Welch and Wang, 1993 and 1995). The carboxy-terminal region of pRb (residues 792 to 928) also binds MDM2, resulting in the inhibition of pRb growth regulatory function (Xiao *et al.*, 1995). Interestingly, this carboxy-terminal region of pRb is required for the growth suppression function of pRb and for a stable interaction with E2F-1. Thus, MDM2 neutralizes the G1 arrest activity of two important tumour suppressor proteins p53 and pRb.

Many other proteins have been reported to bind pRb, included MyoD, ATF-2, Sp1, BRG1, and c-Myc (Wang, 1997) (Table 1-2). But, in most of these cases, the significance of pRb binding is not yet clear. However it has been demonstrated that pRb through its pocket region also interact with histone deacetylase (Luo *et al.*, 1998). Therefore, pRb may repress transcription of endogenous cell cycle genes containing E2F sites through recruitment of histone deacetylase, which deacetylates histones on the promoter, thereby promoting formation of nucleosomes that inhibit transcription (Luo *et al.*, 1998). Interestingly, it has been recently reported that the B pocket and carboxy-terminal domain of pRb directly interact with the leucine zipper region of c-Jun, and thus this interaction stimulates c-Jun transcriptional activity, providing evidence of a new role for pRb as a transcriptional activator (Nead *et al.*, 1998). But, this complexes are only observed in terminally differentiating keratinocytes and cells entering the G1 phase of cell cycle after release from serum starvation (Nead *et al.*, 1998).

However, given the fact that many cellular transcription factors bind to the pRb pocket region, it may assemble into a number of different transcription complexes to regulate the growth control. Surprisingly, all natural pRb mutations in cell lines derived from retinoblastomas, osteosarcomas, small cell lung carcinoma, bladder carcinomas and prostate carcinomas are occurred in this pocket region (Hu *et al.*, 1990), suggesting that pRb pathway in tumour cells is deregulated through the pocket.

1-2.7. The role of pRb in apoptosis and differentiation.

Even though the function of pRb is dispensable in tumour cells, in addition to suppressing growth, pRb also appears to inhibit apoptosis and facilitate differentiation. Mice carrying a targeted disruption of the *Rb* gene die between days 13.5 and 14.5 of gestation, exhibiting defects in fetal liver hematopoiesis as well as in lens and nervous system development with increased levels of apoptosis (Clarke *et al.*, 1992; Jacks *et al.*, 1992; Morgenbesser *et al.*, 1994). Similarly, in tissue culture cells inactivation of pRb through the E1A protein induces apoptosis (Debbas and White, 1993; Sabbatini *et al.*, 1995), and targeting the E7 protein to lens fiber cells promotes an apoptotic outcome (Pan and Griep, 1994). Subsequent inactivation of p53, either by co-expression of the E6 protein (Pan and Griep, 1994) or targeted disruption of the *p53* gene (Morgenbesser *et al.*, 1994), overcomes the apoptosis evident in conditions of *Rb* loss.

Neuronal differentiation correlates with elevated levels of pRb in embryonal carcinoma cells (Slack *et al.*, 1993) and is impaired in $Rb^{-/-}$ embryos (Lee *et al.*, 1994). These results underscore the delicate balance that pRb may help maintain between proliferation, apoptosis, and differentiation. Moreover, recent work has suggested that loss of *Rb* activates both p53-dependent and independent cell death pathways in the developing mouse nervous system (Macleod *et al.*, 1996). Other

interesting results have shown that the treatment of anti-neoplastic drugs caused the accumulation of p53 in the nuclei of both $Rb^{+/+}$ and $Rb^{-/-}$ mouse embryonic fibroblasts, and while p53 induction led to apoptosis in $Rb^{-/-}$ cells, $Rb^{+/-}$ and $Rb^{+/+}$ cells underwent cell cycle arrest without apoptosis (Almason *et al.*, 1995).

It was shown that activated E2F-1 and p53 can act to induce apoptosis (Wu and Levine, 1994), and this may explain how mutations in *Rb* and *p53* co-operate in the development of certain tumour types (Williams *et al.*, 1994; Macleod *et al.*, 1996). Death resulting from the loss of *Rb* can be blocked by the elimination of *p53* (Weinberg, 1995). Therefore, the combined loss of *Rb* and *p53* is highly tumourigenic in mice (Williams *et al.*, 1994). Interestingly, pRb contains a consensus Ced-3/ICE cleavage site at the carboxy-terminus (Wang, 1997) (Figure 1-7). Programmed cell death results from the activation of a family of cysteine proteases, as exemplified by the *C. elegans* Ced-3 and the mammalian ICE proteins (Martin and Green, 1995; White, 1996). Cleavage of pRb closely paralles death and could be blocked by Ced-3/ICE inhibitors (Bing and Don, 1996), suggesting that pRb is a target of the death-effector proteases and is cleaved during apoptosis. However, no functional significance has been attributed yet to this pRb cleavage.

1-3. Co-activator p300/CBP, an integrating signal with cell cycle regulatory transcription factors

The oncogenic products of DNA tumour viruses are involved in a variety of cellular processes including transcriptional activation and repression, growth control, cell differentiation, and transformation (Moran, 1993; Vousden, 1995).

Recently, it has become apparent that these activities are the result of interactions between viral oncoproteins and various cellular proteins involved in the control of cell growth and the regulation of transcription as described previously. Among these targets are p53, pRb and its relatives p107 and p130, all of which exhibit properties of negative regulators of cellular proliferation (Lam and La Thangue, 1994; Cobrinik *et al.*, 1996; Vousden, 1995). In addition, another targets of more recent interest is the p300/CBP family.

The p300 protein and the related CBP, referred to below as p300/CBP, are highly homologous nuclear proteins that were originally identified by their ability to interact with adenovirus E1A and with the transcription factor CREB, respectively (Kwok *et al.*, 1994; Eckner *et al.*, 1994). Comparison of the p300 and CBP sequences indicates that they have 75% similarity and 63% identity across their entire length (Arany *et al.*, 1994). In addition to sequence-specific DNA binding factors and the RNA polymerase II transcriptional machinery, co-activators are involved in the process of transcriptional activation (Roeder, 1996; Ptashne and Gann, 1997). It is thought that some co-activators, but not all, facilitate transcriptional activation by promoting interactions between sequence-specific activators and the RNA polymerase II transcriptional machinery (Roeder, 1996; Ptashne and Gann, 1997). This function has been well characterized for the p300/CBP proteins.

1-3.1. p300/CBP are transcriptional co-activators involved in growth control and signal transduction pathways.

Many studies have demonstrated that p300/CBP proteins act as transcriptional co-activators. The over-expression of p300 or CBP results in transcriptional activation by viral as well as cellular enhancers/promoters (Eckner *et al.*, 1994; Arany *et al.*, 1995; Lundblad *et al.*, 1995). Furthermore, p300/CBP

proteins can directly interact with a variety of viral and cellular proteins to physically bridge sequence specific binding factors and the basal transcription machinery, resulting in transcriptional activation (Shikama *et al.*, 1997) (Figures 1-8 and 1-9). In addition, a number of cellular enhancers and promoters found to be subject to E1A-mediated inactivation (Braun *et al.*, 1992; Hen *et al.*, 1985; Stein and Ziff, 1987), correlate well with the ability of the incumbent E1A protein to bind to p300/CBP (Stein *et al.*, 1990). Moreover, the transcriptional activation by p300/CBP can be abolished directly and specifically by E1A binding (Arany *et al.*, 1995; Yang *et al.*, 1996; Cook *et al.*, 1996). E1A mutants which lack the p300 binding site have lost the properties of E1A-driven cell cycle progression and its transforming potential (Moran, 1993). Thus, it makes good sense for a viral oncoproteins to possess such a mechanism since the suppression of differentiation favours the retention of cells in a proliferative state contributes to the oncogenic state (Figure 1-10).

Studies on another viral oncoprotein, simian virus 40 (SV 40) T antigen, demonstrated that the deletion of its amino-terminal domain impaired its transforming potential, albeit without affecting p53 and pRb binding (Manfredi and Prives, 1990; Marsilio *et al.*, 1990). Moreover, this T antigen mutant can restore the transforming activity lost by E1A mutants which have lost the ability to interact with p300 (Yacink and Moran, 1991). These studies implicated p300/CBP as a target for T antigen, and subsequent studies have found that T antigen can associate with a hypophosphorylated but ubiquitinated form of p300 (Avantaggiati *et al.*, 1996).

A variety of genes induced by cAMP share a conserved motif in their transcriptional control sequences, referred to as the cAMP-response element (CRE). The CRE binds the CRE-binding protein, CREB, which is directly phosphorylated by activated protein kinase A (Gonzalez and Montminy, 1989). Phosphorylation at Ser 133 stimulates the transcriptional activity of CRE-dependent genes in part by enabling the kinase-inducible domain (KID) in CREB to interact with a co-activator

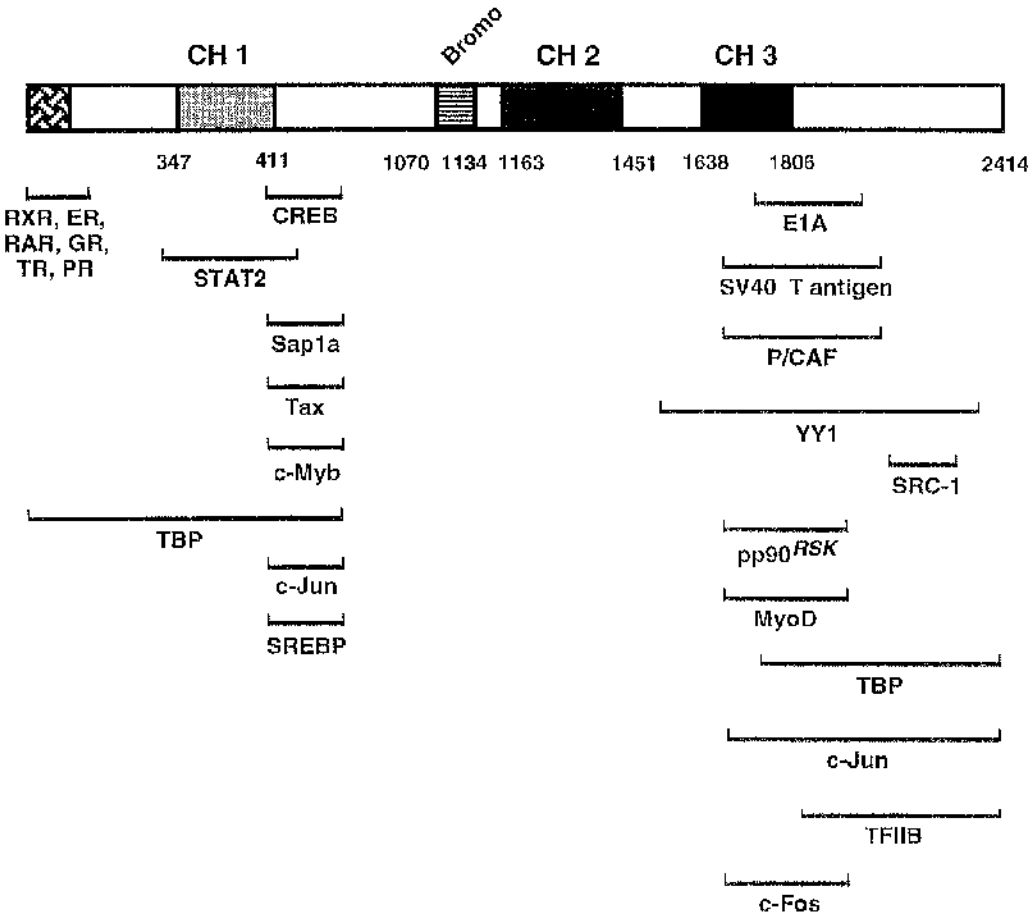


Figure 1-8

Schematic representation of p300/CBP.

The diagram represents p300/CBP together with recognized cysteine/histidine-rich domains, CH1, CH2 and CH3, and bromodomain. Note that the numbers indicate the amino acid residues in human p300. The region in p300/CBP required for binding to target proteins are indicated.

(Data in this figure are compiled together with data from Shikama *et al.* (1997) and Janknecht and Hunter (1996))

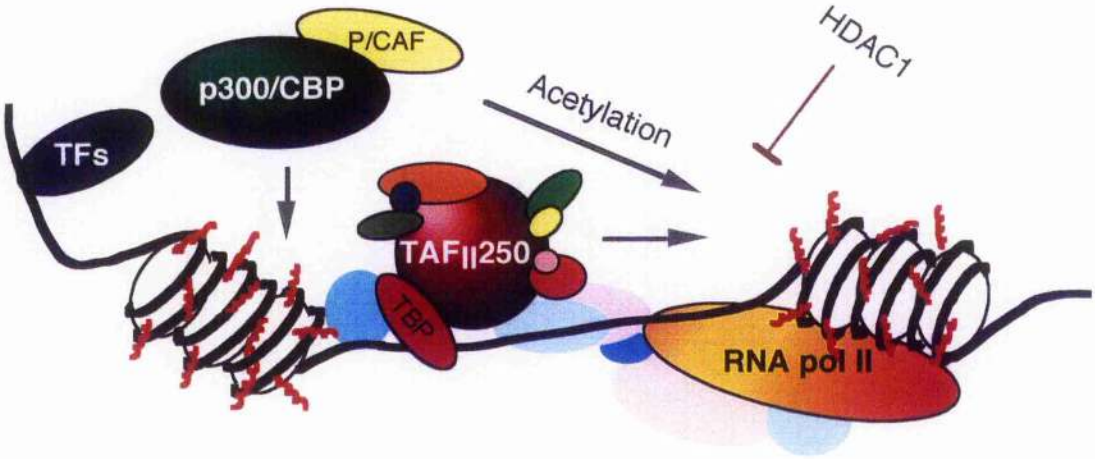


Figure 1-9

p300/CBP co-ordinate transcription with chromatin remodeling.

Transcription factors (TFs) recruit the co-activators p300/CBP, which are able to acetylate all histone components of the nucleosome. Thereby, altered chromatin integrity may facilitate the assembly of general transcription factors, such as TBP and TFIIB, which can associate with p300/CBP. In addition, $TAF_{II}250$ also functions as a histone acetyltransferase. The histone deacetylase (HDAC1) continually deacetylates histones in chromatin, therefore, persistent activity of the co-activator/histone acetyltransferase is required to maintain gene activity.

CBP (Chrivia *et al.*, 1993). Therefore, it is clear that p300/CBP are intimately involved in growth control, progression of cell cycle, and cell differentiation. These two co-activators make contact with, and connect the functions of many well-studied proteins such as c-Jun, c-Myb, c-Fos, CREB, MyoD, Stat 2, YY1, and nuclear hormone receptors (Shikama *et al.*, 1997) (Figure 1-8). Previous studies suggested that CBP can associate with c-Jun, specifically with the form of c-Jun phosphorylated by Jun N-terminal kinase (Arias *et al.*, 1994), and similarly with a carboxy-terminal region in the c-Fos transcription factor (Bannister and Kouzarides, 1995). Fos and Jun proteins heterodimerize to form a group of mitogen-regulated transcription factors known as AP-1. Because Fos and Jun proteins, by virtue of their role in mitogenesis, are implicated in proliferation control, it is somewhat surprising that E1A, which confers oncogenic properties on cells, counteracts the interaction between Fos and Jun with CBP (Arany *et al.*, 1995; Lundblad *et al.*, 1995).

The c-Myb proto-oncogene product also utilizes CBP as a co-activator, and again this interaction compromised by E1A (Oelgeschlager *et al.*, 1996; Dai *et al.*, 1996). Recent data found that p300/CBP not only stimulates the activity of c-Myb and C/EBPβ individually, but establishes synergy between them, presumably by simultaneously binding both transactivators (Mink *et al.*, 1997). Furthermore, C/EBP associates with other p300/CBP targets in addition to c-Myb, such as CREB/ATF family of transcription factors (Vallejo *et al.*, 1993), NF-*k*B (Stein *et al.*, 1993), and TBP (Nerlov and Ziff, 1995).

The basic helix-loop-helix protein MyoD also appears to interact with p300 through the region that is bound by E1A, and this interaction is critical for muscle and B cell differentiation (Eckner *et al.*, 1996; Puri *et al.*, 1997).

Signal transduction mediated by the JAK-STAT pathway involves p300/CBP (Bhattacharya *et al.*, 1996). Interferon α (INF-α) induces the transcription of a variety of genes required to produce antiviral effects, many of which require a transcription factor called ISGF 3/STAT 1 and STAT 2 (Darnell *et al.*, 1994). E1A

impedes transcriptional activation mediated by INF-α by inactivating p300/CBP, which binds through its amino-terminal region to STAT 2 (Bhattacharya *et al.*, 1996). Interestingly, it is known that interferons cause cell cycle arrest; thus the inactivation of ISGF 3 may contribute to the observed stimulation of DNA synthesis that correlates with the integrity of the amino-terminal region of E1A.

The E1A protein acts positively in combination with the transcription factor YY1 (Lee *et al.*, 1995). No direct interaction between E1A and YY1 has been reported, but rather p300 can bind to both proteins at the same time, resulting in a tripartite complex. Although YY1-binding sites are frequently present in transcriptional control regions, some sites function to repress transcription and, in these cases, p300 is believed to be responsible for the repressive activity that can be relieved by E1A (Lee *et al.*, 1995).

There is also a connection between p300/CBP and another class of sequence-specific transcription factors, namely the ligand-dependent nuclear receptors including the retinonic acid (RAR and RXR), oestrogen (ER), progesterone (PR), thyroid hormone (TR) and glucocorticoid (GR) receptors (Kamei *et al.*, 1996; Chakravarti *et al.*, 1996; Janknecht and Hunter, 1996). These nuclear receptors depend upon the p300/CBP for their ability to stimulate transcription (Kamei *et al.*, 1996; Chakravarti *et al.*, 1996). Several other co-activators have been implicated in the hormone-dependent activation of nuclear receptors which, interestingly, may interact with receptor bound to p300/CBP. For example, SRC-1 (steroid receptor co-activator 1) recognizes a carboxy-terminal p300/CBP region while nuclear receptors occupy the amino-terminal (Kamei *et al.*, 1996) establishing the important idea that multiple interfaces within p300/CBP may be occupied stimultaneously by different factors. Consistent with this idea, co-expression of CBP with SRC-1 stimulates the activities of oestrogen and progesterone receptor in co-operative manner (Smith *et al.*, 1996), raising the possibility that p300/CBP may function together with diverse co-activators in a common pathway of transcriptional activation.

1-3.2 The phosphorylation of p300/CBP by cell cycle dependent kinase.

p300/CBP phosphorylation levels change during the cell cycle, and during mitosis, hyperphosphorylation has been observed (Yacink and Moran, 1991). Another study showed that not only is p300 phosphorylated in a cell cycle dependent manner but is a substrate for cyclin dependent kinase *in vivo*, and is required for the induction of p53-independent *Waf1/Cip1* gene expression during cellular differentiation (Wang *et al.*, 1993). Interestingly, p300 mediates a biochemical and functional interaction between the Rel A subunit of NF-*k*B and cyclinE-CDK2, with the NF-*k*B-dependent gene expression being enhanced by expression of p300 in the presence of $p21^{Waf1/Cip1}$ (Perkins *et al.*, 1997). Thus, this result suggests that the interaction of NF-*k*B and cyclin dependent kinase through p300/CBP provides a mechanism for the co-ordination of transcriptional activation with cell cycle progression.

1-3.3. p300/CBP as modulators of chromatin.

The DNA in the cell is organised into chomatin. The principle repeat element of chromatin is the nucleosome core, which consists of an octamer of histones, H2A, H2B, H3, and H4, supporting 1.65 turns of the DNA. Nucleosomes are potent repressors of transcription, presumably by physically interfering with the binding of transcription factors. A central question in eukaryotic transcription has been how transcription factors gain access to the DNA tightly "wrapped" or "wound up" in chromatin. Targeted histone acetylation (HAT) is thought to neutralize the positive charge of the histone tails and affect internucleosomal geometry by relaxing the interaction between histones and negatively charged DNA. Thus, the acetylation of histones is involved in the destabilization and restructuring of nucleosomes,

which is probably a crucial event in the control of accessibility of DNA templates to transcription factors (Wade *et al.*, 1997; Shikama *et al.*, 1997) (Figure 1-9).

However, only a few proteins with HAT activity have been found to date; p300/CBP comprise two of the seven known human HAT's (Ogryzko *et al.*, 1996; Bannister and Kouzarides, 1996) (Figure 1-9). Interestingly, p300/CBP proteins are also the only HAT capable of acetylating all four core histones *in vitro*. In addition to their own intrinsic HAT activity, p300/CBP may also indirectly influence chromatin remodeling by its ability to recruit additional co-activators containing HAT activity. Three of the other HAT's, the P/CAF (p300/CBP-associated factor), SRC-1 (steroid receptor co-actovator-1), and ACTR co-factors, bind to each other as well as with p300/CBP (Yang *et al.*, 1996; Jenster *et al.*, 1997; Chen *et al.*, 1997; Spencer *et al.*, 1997) (Figure 1-9). One possible scenario is that transcription factors need to recruit multiple HAT's in order to overcome the repressive effects of deacetylation on promoter activity.

Furthermore, recent research into the enzymatic activities of p300/CBP, P/CAF, and TAFII250 show that they are all able to directly acetylate the basal transcription machinery components TFIIEβ and TFIIF (Imhof *et al.*, 1997) (Figure 1-9), but the physiological significance of these acetylation events are unclear yet. These results suggest that our current understanding of the molecular mechanisms underlying transcriptional regulation underrate the role of co-factors such as p300/CBP.

1-3.4. p300/CBP in cancer and other human diseases.

The autosomal dominant Rubinstein-Taybi Syndrome (RTS) is associated with the mutation of one *CBP* allele, and exhibits a complex phenotype typically including mental retardation, physical abnormalities and increased incidence of neoplasia (Petrij *et al.*, 1995) (Figure 1-10).

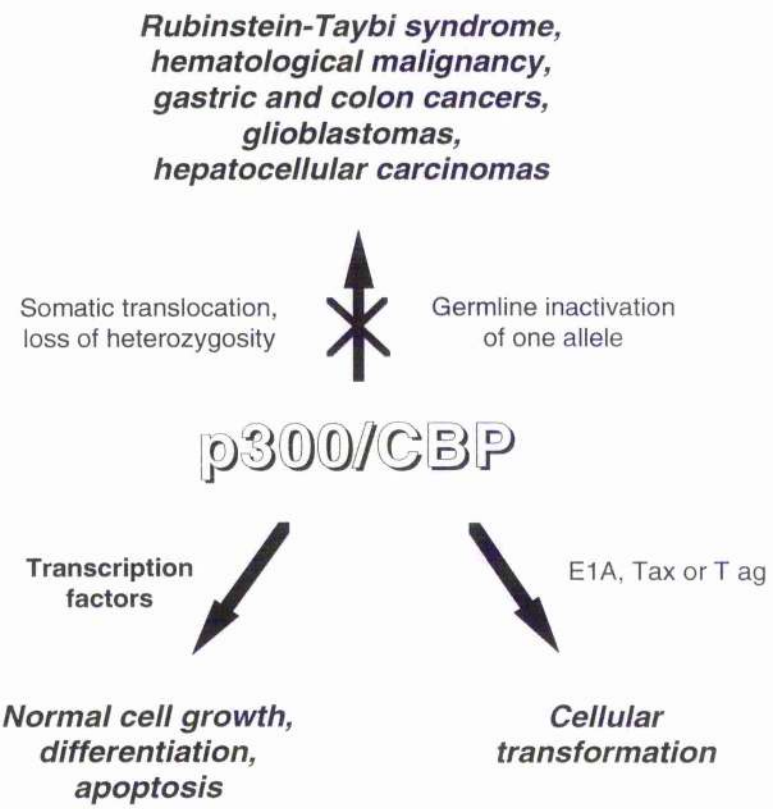


Figure 1-10

Schematic diagram of p300/CBP functions.

Mutations of the *p300/CBP* genes or inactivation of p300/CBP by viral oncoproteins results in a variety of diseases and malignancies. Whereas p300/CBP plays a positive role in differentiation, it may also be involved in negative regulation of cellular growth such as growth arrest and apoptosis.

In addition, bi-allelic inactivating somatic mutations of the *p300* gene have been observed in gastric and colon cancers (Muraoka *et al.*, 1996) (Figure 1-10). Furthermore, the loss of heterozygosity (LOH) of informative markers at chromosome band 22q13, which corresponds with the *p300* locus (Eckner *et al.*, 1994), has been observed in 80% of informative glioblastomas. Loss of *p300/CBP* might therefore allow uncontrolled cell growth. Likewise, LOH around the *CBP* locus has been observed in hepatocellular carcinomas (Sakai *et al.*, 1992) (Figure 1-10). As opposed to the germline chromosomal rearrangement reported in RTS patients, the *CBP* and *p300* genes are also involved in somatic translocations associated with various types of hematological malignancies (Giles *et al.*, 1995; Ida *et al.*, 1997). The fact that *p300/CBP* is prone to translocations, inversions, and deletions suggests the presence of elements confering genomic instability. Therefore, how altered p300/CBP function, resulting from a chromosomal rearrangement, could result in uncontrolled cell growth in myelomonocytic cell lineages can be partly explained by studying at p300/CBP targets.

1-4. Objectives

Little is known about the function of the tumour suppressor p53 in the control of normal cell cycle progression. The balance between the activities of E2F and tumour suppressors such as pRb and p53 is crucial to ensure the normal progression of the cell cycle. In cells, the transcriptional activity of p53 and E2F are believed to be regulated mainly at the protein level. However, other cellular factors might also regulate the transcriptional avtivity of p53 and E2F.

Recent studies have shown that the over-expression of E2F-1 can induce apoptosis in co-operation with p53, as described previously. In addition, studies on the physiological outcomes of deregulating the E2F pathway suggest that the unscheduled cell cycle progression induced by E2F-1 itself leads to apoptosis. Therefore, an important aim of this study is to understand the mechanisms through which E2F-1 induces apoptosis.

Different transcription factors activated by diverse signalling pathways may interfere with one another by competing for a limiting common factor, namely p300/CBP. This regulatory mechanism may more often than anticipated, provide an important level of control in transcription. Furthermore, it is also possible that competition for p300/CBP may allow cross-talk between different cell cycle control pathways. Another aspect of this research investigated the role of p300 in p53 and E2F dependent transcriptional activation. It was found that both E2F and p53 utilise p300 as a transcriptional co-activator. Furthermore, the interaction of p300 with either p53 or E2F-1 had a profound impact on early cell cycle progression, possibly through the activation domains of p53 and E2F-1 competing for p300.

Finally, a question of considerable importance relates to the mechanistic and physiological role of p300/CBP in regulating the p53 response. In this respect, I have studied the role of a novel p300 target protein, JMY, which physically and functionally co-operates with p300 in the p53 response. The data suggest that JMY

has potent effects on certain p53-dependent genes, such as *bax* and, physiologically, co-operates with p300 in promoting p53-dependent apoptosis.

Chapter 2.
Materials and Methods

2-1. Plasmids

The following plasmids have been previously described; pCMV-pRb wt (Zamanian *et al.*, 1992), pCMV-p107 (Beijersbergen *et al.*, 1994), pCMV-HAp130 (Vairo *et al.*, 1995), pCMV-p300 (Eckner *et al.*, 1996), pCMV-HA-JMY (Shikama *et al.*, 1998), pCMV-E2F-1 (Kaelin *et al.*, 1992), pCMV-E2F-4 (Beijersbergen *et al.*, 1994), pCMV-E2F-5 (Buck *et al.*, 1995), pCMV-p21$^{Waf1/Cip1}$ (El-Deiry *et al.*, 1993), pCMV-βgal (Zamanian *et al.*, 1992), and pGal4-p53 (Fields and Jang, 1990). pCDK2-MSPE and pCDK4-MSPE were kindly provided by R. Muller.

To construct pTG13-GL, first, the Bam HI-Bgl II fragment of the HSV1-tk minimal promoter element (-81 to +52) was inserted to the Bgl II site of pXP2 vector (Nordeen, 1988) resulting in pT-luc. Then, the 13 copies of the consensus p53-binding site from pG13-CAT (Kern *et al.*, 1992) were subcloned into the Hind III and Sma I sites of pT-luc. For the Gal4 reporter construct, the Gal4 DNA binding sites and E1b TATA minimal elements from pG5E1b-CAT (Fields and Jang, 1990) were excised by Xho I and Bam HI digestion, and cloned into the pGL3 (Promega). pDHFR-luc has been described previously (Sørensen *et al.*, 1996). pBax-luc and pMDM2-luc, pWWP-luc, and pGADD45-luc were a gift from M. Oren (Haupt *et al.*, 1955b ; Friedlander *et al.*, 1996), B. Vogelstein (El-Deiry *et al.*, 1993), and C. Prives (Chen *et al.*, 1995) respectively.

To construct pCMV-HA-DP1, three copies of HA1 epitope sequences from pGTEP-1 (a gift from B. Futcher) were amplified by PCR and cloned into the pCDNA3 (Invitrogen) with a consensus Kozak sequence introduced immediately 5' of the initiating ATG codon to create pCMV-HA1. The 5' primer used was (5' to 3') GCTGGAATTCTTTTACCCATACGATGTTC and the 3' primer (5' to 3')

CATGCTCGAGAGCAGCGTAATCTGGAAC. PCR amplication was carried out using PWO polymerase (Boehringer Mannheim) and the manufacturer's incubation buffer for 31 cycles as follows : 5min denaturation at 94°C as first step, followed by 31 cycles of denaturation at 92°C for 1min; annealing at 55°C for 1min; extension at 72°C for 1.5min (last cycle 5min). The DP-1 cDNA sequence (Girling *et al.*, 1993) in frame was introduced into pCMV-HA to generate an expression plasmid encoding an amino-terminal HA-tagged full-length DP-1. pCMV-DP-1/NLS was made by inserting a fragment containing the Bell bi-partite nuclear localization signal (amino acid residues 194 to 227) of human foamy virus (Lee *et al.*, 1994) into Kpn I site (residue 327) of the DP-1 in pCMV-DP-1.

For the mammalian two-hybrid assay, several plasmids were constructed as follows: the PCR fragments of VP16 *trans* activation domain, amino acid residues 423 to 490, from Gal4-VP16 (Fields and Jang, 1990) were subcloned in-frame to the pCMV-HA to generate pCMV-VP16. The 5' primer used was (5' to 3') GCTCGGATCCGAGCTCCACTTAGACGGCGA and 3' primer (5' to 3') GCTCCTCGAGCTACCCACCGTACTCGTCAAT. Two p53 PCR fragments encoding amino acids 1 to 393 and 159 to 393 from pCMV-p53, and two E2F-1 PCR fragments encoding amino acids 1 to 437 and 1 to 413 from pCMV-E2F-1 were fused to the VP16 *trans* activation domain in pCMV-VP16 to generate pCMV-p53/VP16, pCMV-p53Δ159/VP16, pCMV-E2F1/VP16, and pCMV-E2F1Δ413/VP16, respectively. pCMV-GAD was made by the insertion of Hind III and Eco RI fragment encoding Gal4 *trans* activation domain, amino acid residues 768 to 881, from pACT II (Clontech) into the pCDNA3. The Nde I and Xba I fragments from pCMV-E2F1 were fused in-frame into pCMV-GAD to make the pCMV-GAD/E2F1. To construct pG4-p300$^{611-2284}$, the Nde I fragments from pCMV-p300 were fused in-frame to the Gal4 DNA-binding domain sequence of pG4m-polyII vector (Bandara *et al.*, 1993). pCMV-E2F-1Δ413 was made by internal deletion from pCMV-E2F1. To construct the Gal4-p300 derivatives, pG4-p300$^{611-1257}$, -p300$^{1302-1572}$, -p300$^{1572-2284}$, and -p300$^{1572-1903}$ were made by

internal detection from pG4-p300$^{611-2284}$, respectively. Also, to generate pCMV-HA-p300$^{611-1257}$ and pCMV-HA-p300$^{1257-2284}$, Afl III and Xba I, and Bam HI and Xho I fragments from pG4-p300$^{611-2284}$, and Sma I and Xba I fragment from pG4-p300$^{1572-2284}$, were inserted into the pCMV-HA1, respectively. pVP16-JMY$^{469-558}$ has been described previously (Shikama *et al.*, 1998).

Two E1A expression vectors, pCMV-E1A and pCMV-E1AΔ2-36 were a gift from J.R. Nevins (Kraus *et al.*, 1992). pCMV-p53 was made by the insertion of p53 cDNA from php53 (Zakut-Houri *et al.*, 1985) into the pCDNA3. pCMV-p53$^{22/23}$ was kindly provided by A.J. Levine (Lin *et al.*, 1994). In order to map the region in p53 to which DP-1 binds a panel of p53 mutants (Figure 4-3), GST-p53 1-73, 1-143, 1-235 and WT, were made by PCR using human p53 cDNA as template (php53c-1 ; Zakut-Houri *et al.*, 1985). PCR products were cloned in frame into a pGEX-KG (Pharmacia). The protein expression and stability of each construct was verified by western blotting.

2-2. Transfection

For transfection (including flow cytometry), U2OS and SAOS-2 cells were grown in Dulbecco's modified eagles medium (DMEM) supplemented with 10% foetal calf serum (FCS). Cells were plated out 24h before transfection at 2 x 10^5 cells per 6-cm dish (for luciferase and β-galactosidase assays) or 1 x 10^6 cells per 10-cm dish (for immunoprecipitation and flow cytometric analyses). Three hours prior to the transfection, the medium from the cells was removed and replaced it with the fresh growth medium. The calcium phosphate-DNA precipitates, the indicated amounts of each plasmid DNA and 2M $CaCl_2$ (for 6-cm dish ; 70μl, for 10-cm dish ; 160μl), dropwise added to 2xHBS solution (50mM Hepes pH7.1, 280mM NaCl, and 1.5mM Na_2HPO_4). Whenever required, pCDNA3, pCMV-HA1, or pSG5 was used to maintain a constant amount of DNA in each sample. All

transfections were included an internal control pCMV-βgal. Cells were harvested 34-40h post transfection.

2-3. Luciferase and β-galactosidase assays

For luciferase assay, the cell culture supernatant was aspirated, and the cells were rinsed twice with phosphate-buffered saline (PBS). Cell extracts were prepared in reporter lysis buffer (25mM Tris-H_3PO_4 pH7.8, 2mM 1,2-diaminocyclohexane tetra acetic acid, 2mM DTT, 10% glycerol and 1% Triton X-100). The solubilized cells transferred to a microcentrifuge tube and incubated at room temperature for 15min. After removing cell debris, extracts were assayed for luciferase activity by mixing 300μl luciferase assay reagent (1:4, Promega) with 60μl cell extracts and measuring the activity with a luminometer (Berthold Lumat).

To measure β-galactosidase activity, 60μl cell extracts were mixed in 6μl 100xMg solution (0.1M $MgCl_2$ and 4.5M β-mercaptoethanol), 70μl O-nitrophenyl-β-D-galactopyranoside (4mg/ml) and 200μl 0.1M sodium phosphate (pH7.5). The reaction mixtures were incubated at 37°C until a faint yellow color has developed. The reaction was stopped by adding 500μl of 1M Na_2CO_3 to each sample, and the activity was measured at 420nm.

2-4. Immunofluorescence

Transfected cells were fixed in 4% paraformaldehyde at 4°C for 20min, rinsed and permeabilised in PBS containing 0.2% Triton X-100 at 4°C for 10min. Fixed cells were washed three times with cold-PBS and incubated in primary antibody diluted in Tween buffer (0.5M NaCl, 1% BSA, 5mM $Na(PO_4)$ pH6.5 and 0.5% Tween 20) at 4°C for 2hr. Subsequently, cells were washed three times with

cold-Triton buffer (0.3% Triton in PBS) and further incubated in secondary antibody, goat anti-mouse or rabbit IgG conjugated to fluorescein isothiocyanate (FITC) or tetramethylrhodamine isothiocyanate (TRITC) (Southern Biotechnology Associates Inc.) for 1h at room temperature. And then, cover slips were washed three times in PBS, mounted, and viewed under the fluorescence microscope (Olympus).

2-5. Flow cytometry

For flow cytometry analysis about 10μg of an expression vector for the cell surface protein CD20 was co-transfected into cells (grown in 10% FCS) together with 15μg of the each expression vector for p53 or p300 (details given in Figure 5-7). Cells were washed and refed after 16h and harvested 40h later by washing in PBS and thereafter treating with cell dissociation medium (Sigma) for 15min. Cells were washed in DMEM by centrifuging at 2000rpm and resuspended in a small volume of DMEM containing the anti-CD20 antibody leu16 (Becton Dickinson) coupled to fluorescein isothiocyanate (FITC). Cells were incubated upon ice for 45min, further washed twice in PBS and then resuspended in 50% PBS in ethanol at 20°C for 30min. Cells were collected by centrifugation and treated with RNase (125U/ml) for 30min, harvested by centrifugation and suspended in propidium iodide (20μg/ml) in PBS at 4°C for 1h. Flow cytometry was performed on a Becton Dickinson fluorescence activated cell sorter. The intensity of propidium iodine staining was analysed in cell populations that were positive for FITC staining to determine the cell cycle profile of the transfected population using the Consort 30 software. The data presented show a representative example from multiple assays.

2-6. Immunoprecipitation

U2OS cells were transfected with plasmids encoding Gal4-p300, HA-DP1, E2F-1 or p53 (as indicated in Figures 5-2 and 5-3) by the calcium phosphate procedure. After 48h, cells were washed twice in PBS and harvested by scraping into cold TNN buffer (50mM Tris-HCl pH7.4, 120 mM NaCl, 5mM EDTA, 0.5% NP-40, 50mM NaF, 1mM DTT, 1mM PMSF, 0.2mM sodium orthovanedate, leupeptin (0.5μg/ml), protease inhibitor (0.5μg/ml), trypsin inhibitor (1.0μg/ml), aprotinin (0.5μg/ml) and bestatin (40μg/ml)) and incubated on ice for 30min. The cell extract was centifuged for 10 min at 12,000g and pre-cleared by incubating with protein-G agarose for 1h at 4°C with constant rotation. The supernatant was harvested and 2μl of anti-mouse Gal4 monoclonal antibody (100μg/ml, Santa-Cruz) added for 1h at 4°C. Protein-A agarose (50% v/v) (Boehringer Mannheim) was added and the incubation continued for another 1h at 4°C. The agarose beads were collected by centrifugation for 30sec at 5,000g, the supernatant was removed and the pellet was washed three times in TNN buffer. 2xSDS loading buffer (250mM Tris-HCl, pH6.8, 20% glycerol (v/v), 4% SDS (w/v), 0.1% bromophenol blue (w/v), 200mM DTT and 5% β-mercaptoethanol) was added to the final pellet, the sample denatured and loaded onto a 8~10% SDS-polyacrylamide gel. Western blotting was subsequently performed with either anti-mouse p53 (421, kindly supplied by Julian Gannon), anti-mouse HA1 (12CA5, Boehringer Mannheim), or anti-mouse E2F-1 (KH95, Santa-Cruz) monoclonal antibody.

As indicated in Figure 5-2d, SAOS-2 cells were transfected with plasmids encoding p53, pCMV-HA-p300$^{611\text{-}1257}$ or pCMV-HA-p300$^{1572\text{-}2284}$ by the calcium phosphate procedure. Cells were harvested in TNN buffer and extracts were prepared as described above. The supernatant immunoprecipitated with anti-mouse p53 (421) which was harvested with protein-A agarose. Western blotting was subsequently performed with anti-mouse HA monoclonal antibody (12CA5).

In Figure 6-2a, U2OS cells were transfected with expression vectors pG4-p300$^{611-2283}$, pG4, or pCMV-JMY (containing the HA epitopes). After 48h, cells were harvested in TNN buffer and incubated on ice for 30min. The cell extracts were pre-cleared by incubating with protein-G agarose (Pharmacia) for 30min at 4°C, and the supernatant immunoprecipitated with anti-mouse Gal4 monoclonal antibody (Santa-Cruz) which was harvested with protein-A agarose. The agarose beads were collected and washed three times in the extraction buffer before denaturation and SDS-polyacrylamide (7.5%) gel electrophoresis. Immunoblotting was subsequently performed with an anti-mouse HA monoclonal antibody or an anti-peptide rabbit antibody against a peptide taken from JMY (kindly supplied by N. Shikama).

To immunoprecipitate from non-transfected cells (Figure 6-2b), HeLa cells were harvested in nuclear extraction buffer (20mM Hepes pH7.6, 20% glycerol, 250mM NaCl, 1.5mM $MgCl_2$, 1mM EDTA, 0.1% Triton X-100, 1mM DTT, 1mM PMSF, leupeptin (10μg/ml), pepstatin (10μg/ml), aprotinin (10μg/ml) and trypsin inhibitor (10μg/ml)) and immunoprecipitations performed as described above. An anti-p300 monoclonal antibody (Ab-1; Calbiochem) was used in the immunoprecipitation and, after SDS-polyacrylamide gel electrophoresis, immunoblotting was performed with an anti-peptide JMY antibody.

To measure the levels of p53 protein (Figure 6-5), SAOS-2 cells were transfected with the indicated expression vectors and extracts thereafter immunoblotted with the anti-p53 monoclonal antibody DO-1 (Santa-Cruz). The effect of p300 on the level of p53 and E2F-1 was assessed by performing the transfection as indicated and monitoring the level of exogenous protein by immunoblotting with either 421 or KH95.

As a secondary antibodies in immunoblotting, the blots were incubated with alkaline phosphatase-conjugated goat-rabbit or -mouse IgG (1:5,000, Promega), or horseradish peroxidase-conjugated goat anti-rabbit or -mouse IgG (1:4,000) for ECL reagents system (Amersham) as recommended by the manufacturer.

2-7. Metabolic *in vivo* labelling

At 24~30h post-transfection, the tissue culture medium was aspirated, and the cells were washed twice with methionine-free medium, replaced with serum-free and methionine-free medium for 1h. Cells were metabolically labelled with ^{35}S-methionine (0.2mci for 6-cm dish ; 0.5mci for 10-cm dish, Amersham) medium containing 5% dialyzed foetal calf serum for 1hr, washed three times, and chased for 0, 1.5, 3.0, or 4.5hr in normal medium. Cells were harvested in TNN buffer and immunoprecipitated with the 421 monoclonal antibody.

2-8. Apoptosis assays

For apoptosis assays, SAOS-2 and U2OS cells were grown in serum starvation conditions on 3cm-diameter dish either with (for TUNEL assay) or without (for cell death detection ELISA) coverslips and transfected with the each expression plasmid as indicated in figure legends. After 16h incubation with the precipitates, the cells were washed twice with DMEM and further incubated for 24h in DMEM supplemented with 0.2% foetal calf serum . For TUNEL (TdT-mediated dUTP nick end labelling) analysis, cells were washed and fixed in 4% paraformaldehyde for 15min at room temperature, rinsed and permeabilized in PBS cantaining 0.1% Triton X-100 and 0.1% sodium citrate for 2min on ice. Subseqently, cells were incubated in a Ca^{2+} reaction buffer containing fluorescein-dUTP and dNTP, and terminal deoxynucleotidyl transferase (Bochringer Mannheim) at 37°C for 1h in a humid chamber. And then, cells were further incubated in DAPI (2 μg/ml, Sigma) for DNA staining. Cover slips were washed three times in PBS, mounted, and viewed under the fluorescence microscope.

The quantative measurement of mono- and oligo-nucleosomes in the cytoplasm of the apoptotic cells was performed by cell death detection ELISA

(Boehringer Mannheim) using anti-histon-biotin-antibody and anti-DNA-POD-antibody as recommended by the manufacturer.

In Figure 6-9, SAOS-2 cells, grown in 10% foetal calf serum, were transfected with pCMV-p53 (3μg) either alone or together with pCMV-JMY (5μg), pCMV-p300 (5μg) or both. After 14h incubation, cells were washed and further incubated for 24h in the presence of 0.2% serum. Cells were fixed in 4% paraformaldehyde at 4°C for 20min, rinsed and permeabilised in PBS containing 0.2% Triton X-100 at 4°C for 10min. Subsequently, cells were treated with the anti-p53 monoclonal antibody 421, washed and further incubated in tetramethylrhodamine-conjugated goat anti-mouse (Southern Biotechnology Associates Inc.) for 2h at room temperature. And then, the TUNEL assay was performed as described above.

2-9. Purification of p53 protein from insect cells

Spodoptera frugiperda cells (Sf9 cells) were cultured at 27°C in EX-CELL 400 medium (JRH Biosciences) supplemented with 5% foetal calf serum. Cells were subcultured and infected with recombinant baculovirus pVL1393p53 (kindly provided by C. Midgley and D. Lane). Cells were scraped in PBS after 4 days and collected by centrifugation at 1500rpm. Cell lysis was carried out at 4°C for 30min in lysis buffer (0.15M NaCl, 1% Nonidet P-40, 5mM EDTA, 1mM PMSF, 50mM Tris-HCl pH7.5, 5mM DTT, 10mM NaF and 1mM benzamidine). After centrifugation of the lysate, the supernatant was diluted 3-fold in 15% (v/v) glycerol, 25mM Hepes, 0.1% Triton X-100, 5mM DTT and 1mM PMSF, and p53 protein was purified from the lysate on Hi-Trap heparin sepharose (Pharmacia) in a linear KCl gradient from 0.1 to 0.9M KCl (Hansen *et al.*, 1996).

2-10. Glutathione S-transferase recombinant protein

A 500ml mid-logarithmic culture (the transformed DH5α in LB-broth) was induced by the addition of 1mM (a final concentration) isopropyl-β-D-thiogalactopyranoside (IPTG) and incubated for 6hr at 30°C. Bacterial pellets were resuspended in 10ml PBS containing lysozyme (10mg/ml), 5mM PMSF, 50mM DTT, leupeptin (0.5μg/ml), apotinin (0.5μg/ml), bestatin (40μg/ml) and trypsin inhibitor (1.0μg/ml) while kept cool on ice, and then sonicated briefly (double 5 second burst) on ice. Bacterial debris were pelletted by centrifugation and re-centrifugation at 12,000rpm for 20min (at 4°C) each. 300μl of glutathione-agarose beads (33% v/v) in suspension (0.05% NaN_3 in PBS) were added to the supernatant and mixed with constant rotation at 4°C for 1hr. The suspension was placed in a 50ml-Falcon tube and washed once with 50ml PBS containing 0.5% NP-40 (or Igepal) and twice with PBS only, by spinning in a bench-top centrifuge at 5,000rpm for 5min (at 4°C) each time. The washed beads were placed in an eppendorf tube at 4°C.

2-11. *In vitro* binding assay

For the *in vitro* binding reaction shown in Figure 4-3, approximately 10μg of GST or GST-fusion protein bound to glutathione-agarose beads were added to 15μl of *in vitro*-translated DP-1 in reaction buffer (50mM Tris-HCl pH 8.0, 150mM NaCl, 0.5% NP-40, 1mM PMSF, 1mM DTT and aprotinin (2μg/ml)). *In vitro*-transcription and translation was carried out in a TNT T7/SP6 coupled reticulocyte lysate system (Promega) as recommended by manufacturer. After incubation for 2.5hr at 4°C, the beads were collected and washed four times in reaction buffer. Proteins were released in SDS-sample buffer, electrophoresed, and immunoblotted

with an anti-DP-1 (A) or an anti-DP-1 (D) polyclonal antibody (Sørensen *et al.*, 1996).

Using the baculovirus expressed p53 protein, 50µl of protein-A agarose (50% v/v) was incubated either with or without anti-p53 monoclonal antibody 421 in reaction buffer at 4°C for 2hr. This immuno-complex was mixed with *in vitro*-translated / ^{35}S-methionine labelled DP-1, E2F-1 or luciferase and 100µg of cell extracts derived from NIH3T3 cells for a further 1hr incubation at 4°C with constant rotation. The beads were prepared and electrophoresed on 10% SDS-polyacrylamide gel, and the assay was monitored by autoradiography.

2-12. Histidine tagged protein

His-tagged proteins were purified from the pellet of IPTG-induced 500ml-bacterial culture. The bacterial pellets were resuspended in 10ml of denaturing buffer (100mM Sodium phosphate, 10mM Tris base, 6M Guanidine hydrochloride and 30mM imidazole (pH8.0)) and gently stirred for 2hr at room temperature. $MgCl_2$ was added to the final concentration of 5mM, and cellular debris were cleared by repeated centrifugation at 4°C. 400µl of nickel-agarose (solid, Qiagen) was added to the supernatant, which was then rotated for 1hr at room temperature. The resin was washed stepwise with two 50ml volumes each of denaturing buffer (pH8.0), denaturing buffer (pH6.4), and renaturing buffer (25mM Sodium phosphate pH7.0, 300mM NaCl and 10mM β-mercaptoethanol) containing 1M, then 0.1M, and lastly no guanidine hydrochloride. Protein was eluted off the resin by sequential washes with imidazole buffer (150mM Imidazole, 100mM NaCl and 50mM Tris-HCl (pH7.9)).

2-13. Gel retardation assays

To supershift the E2F binding activity, the binding sequence for E2F derived from the adenovirus (ad5) E2a promoter from -71 to -50, 5'-GATCTAGTTTTCGCG CTTAAATTTGA-3' and 3'-ATCAAAAGCGCGAATTTAAACTCTAG-5', was radio-labelled with ^{32}P-α-GTP (Amersham) as described previously (La Thangue *et al.*, 1990). Each gel shift reaction was made up to a final volume of 20μl and reaction mixtures generally contained reaction buffer (4x; 200mM Tris-HCl pH7.9, 24mM $MgCl_2$, 0.8mM EDTA, 4mM DTT and 60% glycerol (v/v)), *in vitro*-translated DP and E2F proteins, and additional 2μg of sonicated-salmon sperm DNA (Sigma) to remove the non-specific DNA binding activity. The mixture was incubated for 10min at 30°C, and added with approximately 6ng of ^{32}P-α-GTP-labelled oligonucleotide probe for a further 10min incubation at 30°C. Reaction mixtures were loaded on to a non-denaturing 4% polyacrylamide Tris-acetate EDTA (TAE) gel at 4°C.

Chapter 3.
Nuclear accumulation is necessary for E2F-dependent apoptosis.

3-1. Introduction.

The function of individual E2F/DP heterodimers is not known. While all E2F/DP complexes stimulate transcription of reporter genes from consensus E2F-binding sites, different E2F complexes may regulate E2F-dependent cellular promoters through different E2F-binding sites (La Thangue, 1994; Muller, 1995). Functional distinctions between heterodimeric E2F/DP transcription factor complexes include tissue-specific patterns of expression (Bandara *et al.*, 1994; La Thangue, 1994; Wu *et al.*, 1995), differences in the time of expression during the cell cycle (Schwarz *et al.*, 1993; Cobrinik *et al.*, 1993; Hartwell and Kastan, 1994), and preferential association with different members of the pocket proteins or other cell cycle regulators (Chellappan *et al.*, 1991; Cobrinik *et al.*, 1993; Bandara *et al.*, 1994, Lam and La Thangue, 1994; Vairo *et al.*, 1995; Lee *et al.*, 1998). Thus, there are distinct specificities in the activation of genes by individual E2F family members (DeGregori *et al.*, 1997).

Recent studies have shown that the over-expression of E2F-1 can induce apoptosis in co-operation with p53 (Qin *et al.*, 1994; Wu and Levine, 1994; Kowalik *et al.*, 1995). However, studies on the physiological outcomes of deregulating the E2F pathway suggest that the unscheduled cell cycle progression induced by E2F-1 itself leads to apoptosis (Krek et al., 1995; Hass-Kogan et al., 1995; Shan et al., 1996; Berry et al., 1996). Moreover, the observation that *E2F-1*$^{-/-}$ mice show an impaired apoptotic function and increased incidence of cancer development (Field et al., 1996; Yamasaki et al., 1996), however, suggests that E2F-1 may play a more direct role as a tumour suppressor, independent of E2F-induced cell proliferation. In

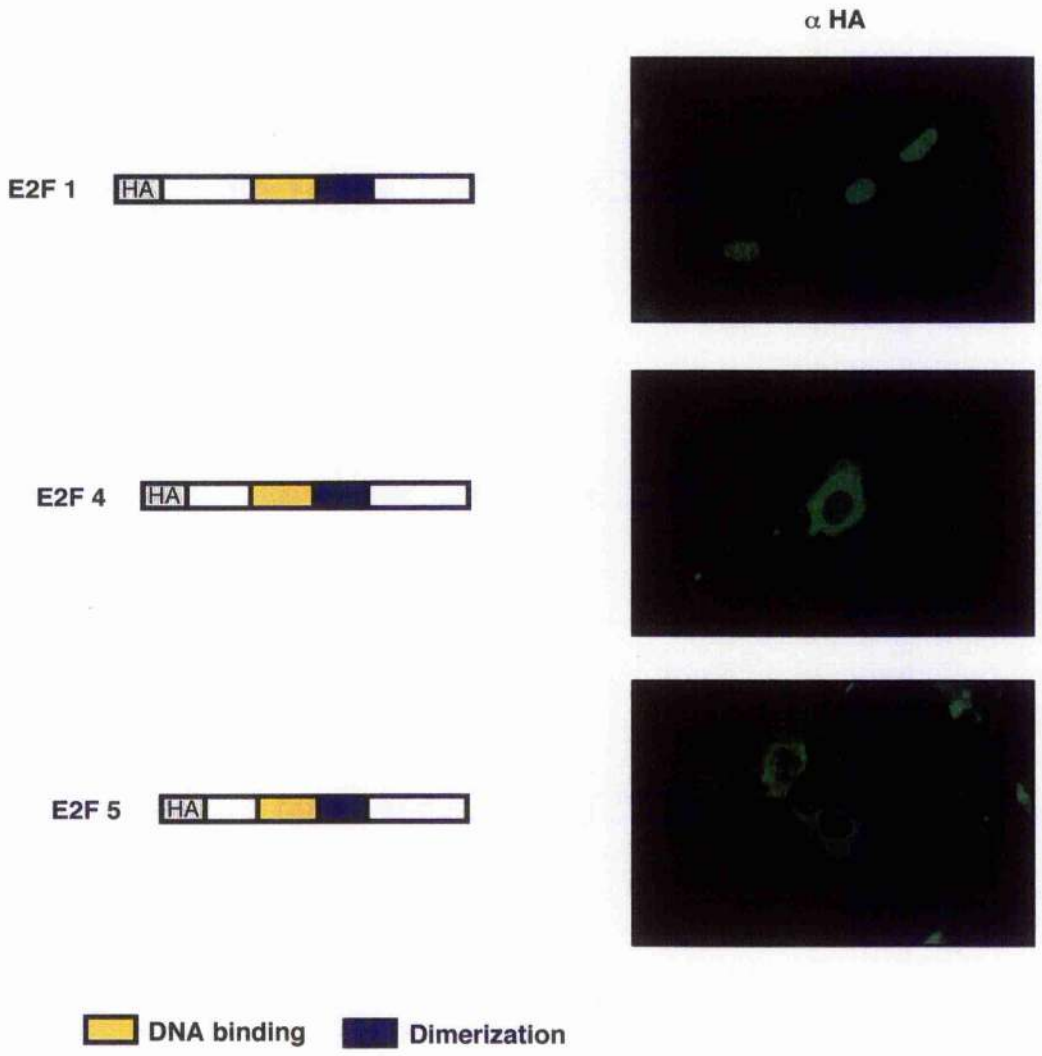
this study, I have investigated the nuclear accumulation of E2F family protein, and the mechanisms through which E2F-1 induces apoptosis.

3-2. Results.

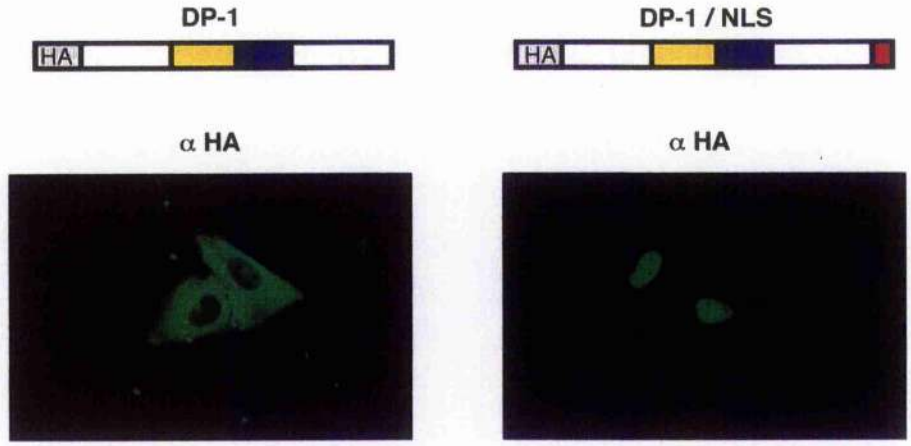
3-2.1 Intracellular localization of E2F/DP proteins.

To address the role of intracellular localization in E2F function, hemagglutinin (HA)-tagged E2F-1, -4, -5 and DP-1 were expressed by transient transfection in U2OS cells and their cellular distribution determined by indirect immunofluorescence. In these conditions, E2F-1 was found exclusively in nuclei (Figure 3-1a). In contrast the majority of E2F-4 and -5 were distributed throughout the cytoplasm, whilst a small percentage of cells expressing E2F-4 and -5 exhibited some nuclear staining (Figure 3-1a). Thus, these results imply that protein in the E2F family can be divided into two functional subgroups, as suggested from their primary sequence and functional properties (Buck *et al.*, 1995; de la Luna *et al.*, 1996; Allen *et al.*, 1997). E2F-1 possesses an intrinsic nuclear localization signal (NLS) (Krek *et al.*, 1994) and, consequently, non NLS-containing E2F family members are likely to rely on other proteins to provide NLS activity. Furthermore, immunostaining also showed that the intracellular distribution of the exogenous DP-1 failed to be localized in nucleus (Figure 3-1b). The absence of DP-1 in nuclei was probably due to the lack of a NLS since the exogenous DP-1 could efficiently accumulate in nuclei after attaching a foreign NLS (Figure 3-1b), the bi-partite NLS taken from the Bel1 protein of human foamy virus (Lee *et al.*, 1994). These data suggest that DP-1 is not actively retained in the cytoplasm but rather its cytoplasmic location is passive.

a)



b)



c)

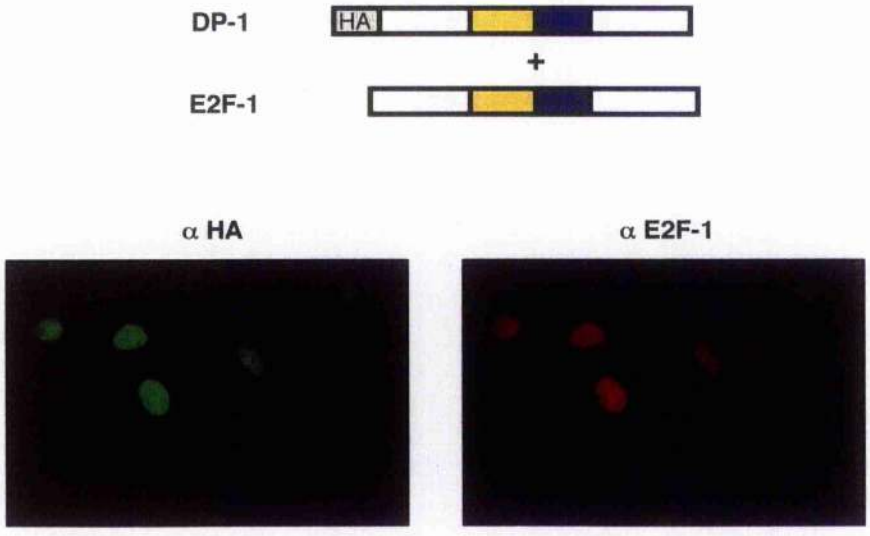


Figure 3-1

The intracellular localization of E2F proteins.

a) Cartoon representation of the HA-tagged E2F family members E2F-1, 4 and 5. DNA binding and dimerization domains are indicated by yellow and blue, respectively. U2OS cells were transfected with 4μg of the relevant expression plasmid and the intracellular location of exogenously expressed HA-tagged E2F-1, 4 and 5 was assessed by immunofluorescence with an anti-HA monoclonal antibody.

b) The intracellular distribution of exogenous DP-1 (left panel) or DP-1/NLS (right panel), which DP-1 had been attached a foreign NLS taken from the Bel 1 protein in U2OS cells was assessed by immunofluorescence using anti-HA monoclonal antibody . The position of the NLS is indicated by red.

c) The cellular localization of exogenous E2F-1 and DP-1 in U2OS cells expressing both proteins was assessed by immunofluorescence using either anti-HA monoclonal antibody or the anti-E2F-1 polyclonal antibody. Anti-HA immunoreactivity was visualized with fluorescein-conjugated anti-mouse immunoglobulin (left panel) and anti-E2F-1 immunoreactivity with rhodamine-conjugated anti-rabbit immunoglobulin (right panel). Note that co-expression of both E2F-1 and DP-1 within the same cell caused DP-1 to efficiently accumulate in the nucleus.

3-2.2. Co-expression of DP-1 with E2F-1 causes DP-1 to efficiently accumulate in nucleus.

It was reasoned that in the absence of an autonomous NLS a possible mechanism to promote the nuclear accumulation of DP-1 may involve an interaction with one of its physiological partners, the E2F-1 protein. To test this idea, I studied the location of the E2F-1 protein in U2OS cells and thereafter the effect of co-expressing E2F-1 and DP-1 in the same cells. A HA-tagged E2F-1 protein visualised by immunostaining with an anti-HA antibody was exclusively nuclear (Figure 3-1a). To assess the influence of E2F-1 on DP-1, both proteins were co-expressed and their intracellular distribution determined by double immunostaining with anti-HA monoclonal antibody for DP-1 and anti-E2F-1 polyclonal antibody for E2F-1 (Figure 3-1c). Neither the fluorescein-conjugated anti-mouse immunoglobulin or rhodamine-conjugated anti-rabbit immunoglobulin cross-reacted with the anti-E2F-1 polyclonal or anti-HA monoclonal antibody (de la Luna *et al.*, 1996). There was a striking difference in the distribution of DP-1 upon co-expression of E2F-1; cells expressing the E2F-1 protein contained nuclear DP-1 (Figure 3-1c), in contrast to its cytoplasmic location in the absence of E2F-1 (Figure 3-1b). These data strongly suggest that upon forming a DP-1/E2F-1 heterodimer, E2F-1 has a dominant influence on recruiting DP-1 to a nuclear location, and nuclear localization may provide an additional level of control over E2F activity.

3-2.3. E2F-1 can induce apoptosis independent of p53.

Although E2F-1 over-expression can induce apoptosis in co-operation with p53 (Qin *et al.*, 1994; Wu and Levine 1994; Kowalik *et al.*, 1995), recent studies have suggested that the growth inhibitory effects of E2F-1 are not entirely dependent on p53 (Melillo *et al.*, 1994; Qin *et al.*, 1994; Berry *et al.*, 1996) and pRb has been

shown to protect *p53*$^{-/-}$ cells from apoptosis in an E2F-1 binding dependent manner (Hass-Kogan *et al.*, 1995). Furthermore, a p53-independent apoptotic activity of E2F is strongly supported by a recent study, showing that deletion of *Rb* in mice and therefore deregulation of E2F result in apoptosis in both *p53* positive and negative embryos (Macleod *et al.*, 1996).

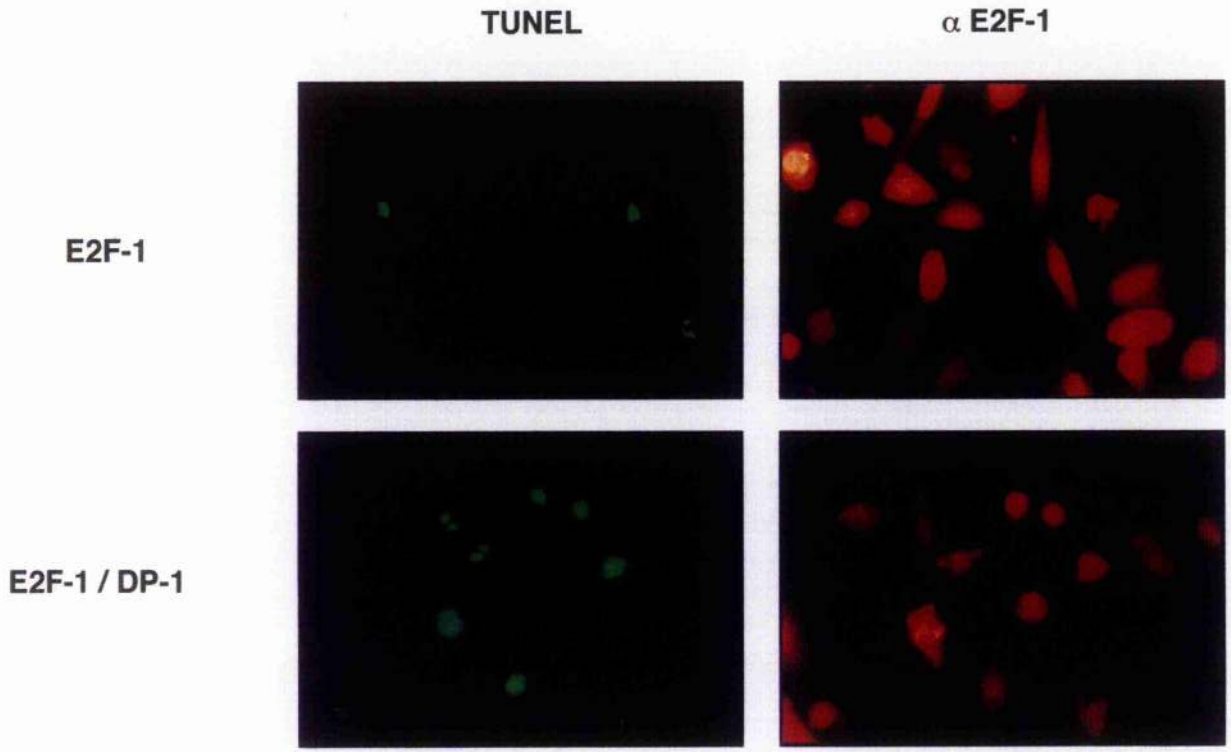
Here, I was interested to understand the role of E2F in apoptosis in *p53*$^{-/-}$ cells. SAOS-2 cells, *p53* null (*p53*$^{-/-}$) human tumour cell line expressing a carboxy-terminal truncated pRb that is defective for E2F binding (Hass-Kogan *et al.*, 1995), were transiently transfected with E2F-1 expression plasmid and cultured in serum starvation conditions, conditions favour that apoptosis (White, 1996). The transfected cells were determined by immunoflorescence labelling of the cells expressing exogenous E2F-1 (Figure 3-2a right panel), and the level of apoptosis measured by TdT-mediated dUTP nick end labelling (TUNEL) assay (Figure 3-2a left panel). Over-expression of E2F-1 alone, in the absence of p53, was sufficient to induce apoptosis (Figure 3-2a). There was a three- to five-fold increase in apoptotic cells in E2F-1 transfected SAOS-2 cells compared to cells transfected with a control vector (data from TUNEL assay not shown).

3-2.4. Properties of E2F required for apoptosis.

E2F-1 and DP-1 have been shown to form a tight complex when both genes are over-expressed by co-transfection in the same cells (Bandara *et al.*, 1994), and this heterodimer has been suggested to be the physiologically significant form of E2F activity (Bandara *et al.*, 1994; Wu *et al.*, 1995; Shan *et al.*, 1996).

To investigate the effect of DP-1 expression on E2F-1-induced apoptosis, SAOS-2 cells were co-transfected with DP-1 and E2F-1 expression plasmids. Co-expression of DP-1 and E2F-1 dramatically increased the level of apoptosis compared to E2F-1 alone (Figure 3-2a).

a)



b)

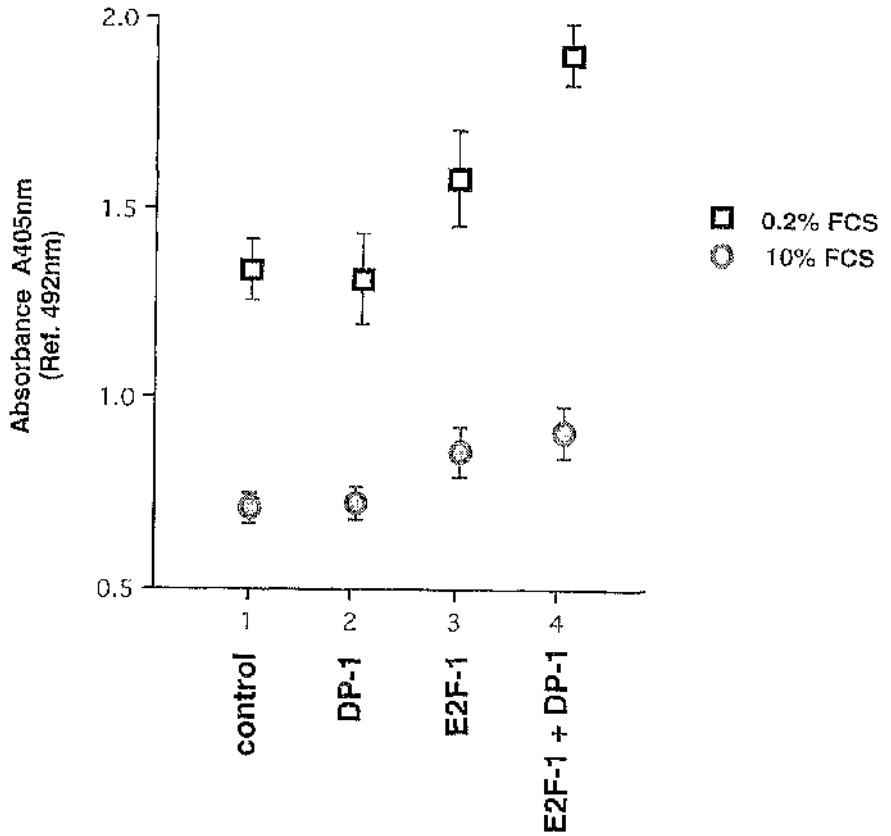

Absorbance A405nm
(Ref. 492nm)
2.0
1.5
1.0
0.5
1
2
3
4
control
DP-1
E2F-1
E2F-1 + DP-1
0.2% FCS
10% FCS

Figure 3-2

E2F-1 induces p53-independent apoptosis in SAOS-2 cells.

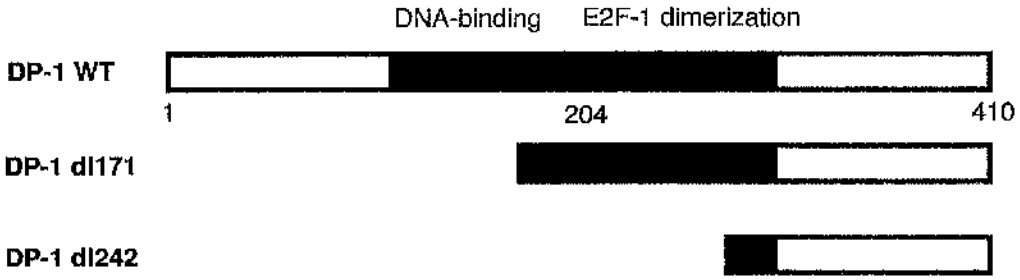
a) Cells were transiently transfected with 3μg of each expression plasmid for E2F-1 and DP-1 as indicated, and grown in conditions of serum starvation (0.2% FCS). Cells were fixed and treated with the anti-E2F-1 antibody (right panel) or assayed for the level of apoptosis by TUNEL (left panel).

b) To quantify the apoptotic effect of E2F-1 and/or DP-1, SAOS-2 cells were grown in 3cm-diameter dishes and transfected with 2μg of each expression plasmids as indicated. 16h after post-transfection, cells were further incubated for 12h in 0.2% or 10% FCS. Nucleosomes in the cytoplasm of the apoptotic cells were assayed by cell death detection ELISA. The values indicated represent the average of three different readings. Error bars indicate standard deviations of triplicate transfections.

To quantify this effect, SAOS-2 cells were transfected as described above. Mono- and oligo-nucleosomes in the cytoplasm of the apoptotic cells were prepared and measured by cell death dection ELISA (see Materials and Methods). As shown previously, E2F-1 alone clearly induced apoptosis, whereas combined expression of DP-1 and E2F-1, in agreement with the morphological data, significantly increased the level of apoptosis (Figure 3-2b). In contrast DP-1 alone did not show any significant apoptosis in the absence of E2F-1 (Figure 3-2b, track 2). Furthermore, to confirm the ability of DP-1 to enhance E2F-1-induced apoptosis, I tested two DP-1 mutants in which the DNA-binding domain (DP-1 dl171) or both the DNA-binding and the E2F-1 dimerization domains (DP-1 dl242) were deleted (Figure 3-3a). As shown in Figure 3-3b, it was clear that two mutants failed to augment the E2F-1-induced apoptosis. Therefore, these results may suggest that the increase in apoptosis seen with E2F-1 and DP-1 was occured as the result of an increase in the DNA binding and transcriptional activation ability of E2F-1.

In an attempt to understand the machanism of E2F-1-mediated p53-independent apoptosis, I tested the apoptotic function of a panel of E2F-1 mutants, E2F-1(1-413), E2F-1(1-380), E2F-1Y411C, E2F-1Δ24, and E2F-1(152-438) (Figure 3-4a). Because the ability of E2F-1 to drive cells through cycle has been shown to require an intact DNA-binding and *trans* activation domain (Bandara *et al.*, 1993; Helin *et al.*, 1993), it was necessary to determine the importance of E2F-1 *trans* activation activity to the apoptotic function. Using the TUNEL assay, it was clear that the *trans* activation defective mutants, E2F-1(1-413) and E2F-1(1-380), were both competent to induce apoptosis in transfected SAOS-2 cells (Figure 3-4b and 3-4c). Their ability to induce apoptosis was much more than that of the wild-type E2F-1. But, interestingly, the DNA-binding defective mutant, E2F-1(152-438), failed to induce apoptosis. These results indicate that E2F-1-induced apoptosis requires DNA-binding but not *trans* activation, results that are consistent with recently published data from others (Phillips *et al.*, 1997; Hsieh *et al.*, 1997).

a)


DNA-binding
E2F-1 dimerization
DP-1 WT
1
204
410
DP-1 dl171
DP-1 dl242

b)

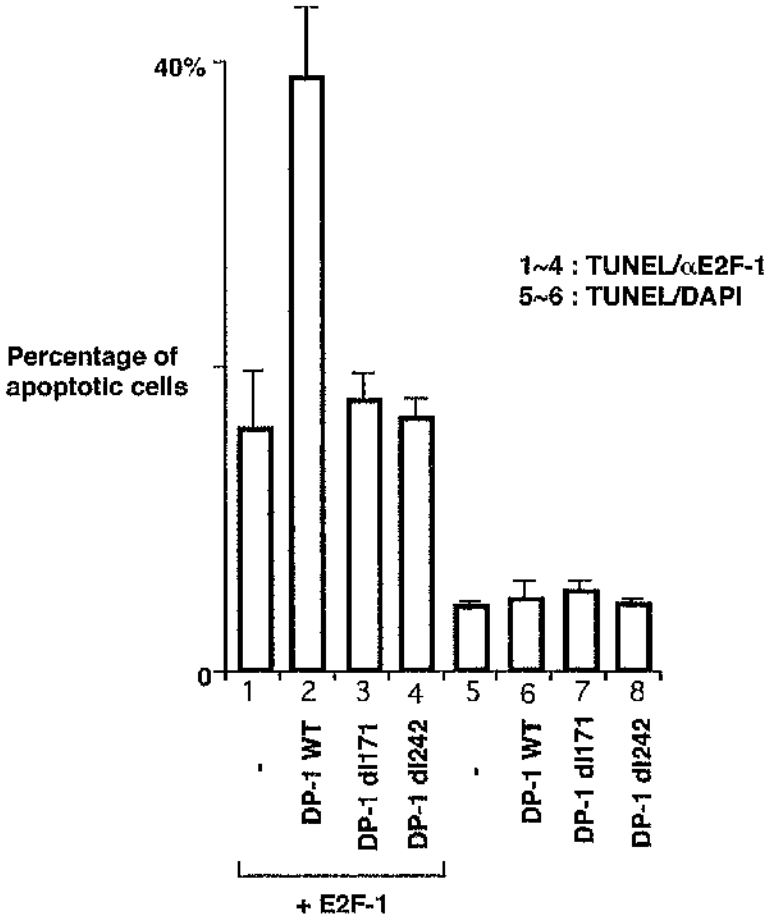

40%
0
Percentage of apoptotic cells
1~4 : TUNEL/αE2F-1
5~6 : TUNEL/DAPI
1
2
3
4
5
6
7
8
-
DP-1 WT
DP-1 dl171
DP-1 dl242
-
DP-1 WT
DP-1 dl171
DP-1 dl242
+ E2F-1

Figure 3-3

The dimerization of E2F-1 and DP-1 augments apoptosis.

a) A diagram of DP-1 mutants used in the apoptosis.

b) Quantitative comparison the effect on apoptosis in SAOS-2 cells caused by E2F-1 with DP-1 wild-type or mutants are shown. DP-1 dl171 and dl242 are a carboxy-terminal truncated mutants. DP-1 dl171 lacks the DNA-binding region, and DP-1 dl242 is deleted of both DNA-binding and E2F-1 heterodimerization region. Cells were transiently transfected with 3μg of E2F-1 and DP-1 expression plasmid. The percentages of E2F-1 positive cells were determined by polyclonal anti-E2F-1 antibody, and further assayed for the level of apoptosis by TUNEL (tracks 1 to 4). The apoptotic effects by DP-1 wild-type or mutant in the absence of E2F-1 was determined by the comparison of TUNEL-positive population to the number of DAPI-positive cells (tracks 5 to 8). The level of apoptosing cells represents the average of three independent readings.

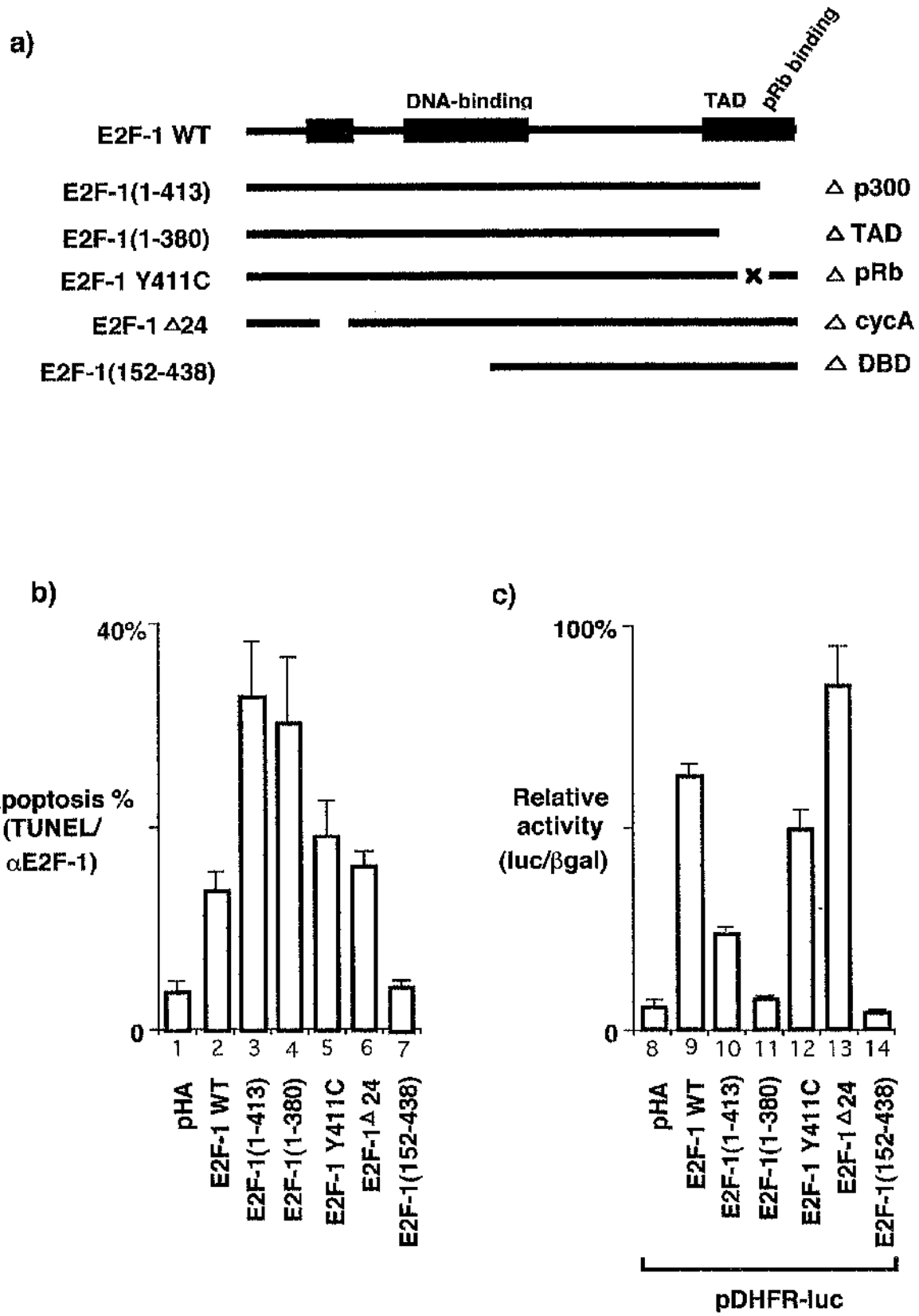

a)
DNA-binding
TAD
pRb binding
E2F-1 WT
E2F-1(1-413)
E2F-1(1-380)
E2F-1 Y411C
E2F-1 Δ24
E2F-1(152-438)
Δ p300
Δ TAD
Δ pRb
Δ cycA
Δ DBD
b)
40%
Apoptosis %
(TUNEL/
αE2F-1)
0
1 2 3 4 5 6 7
pHA
E2F-1 WT
E2F-1(1-413)
E2F-1(1-380)
E2F-1 Y411C
E2F-1Δ 24
E2F-1(152-438)
c)
100%
Relative
activity
(luc/βgal)
0
8 9 10 11 12 13 14
pHA
E2F-1 WT
E2F-1(1-413)
E2F-1(1-380)
E2F-1 Y411C
E2F-1 Δ24
E2F-1(152-438)
pDHFR-luc

Figure 3-4

***Trans* activation is not necessary for E2F-1 induced apoptosis.**

a) A diagram of E2F-1 mutants used in the apoptosis and the transcriptional activation assays. E2F-1(1-413) is a carboxy-terminal truncated E2F-1 mutant. It lacks the p300-binding domain and part of the *trans* activation domain. E2F-1(1-380) is a *trans* activation domain truncated mutant, and is competent for DNA-binding. E2F-1Y411C is a point mutant of E2F-1 that is defective for pRb binding. E2F-1Δ24 is an internal deletion mutant which deleted of cyclinA-binding site. E2F-1(152-438) is an amino-terminal truncated mutant that is defective for DNA-binding.

b) 3μg of each expression vector for E2F-1 wild-type or mutant was introduced in SAOS-2 cells as indicated. Cells were fixed and treated with the anti-E2F-1 polyclonal antibody and assayed for the absolute percentage level of apoptosis by TUNEL assay.

c) The E2F reporter pDHFR-luc (1μg) together with expression vectors for wild-type or mutant E2F-1 (1μg) were transfected into SAOS-2 cells as indicated. The value shown are the average of duplicate readings and represent the relative level of luciferase to the β-galactosidase activity from the internal control.

Moreover, as expected, the E2F-1Y411C, which is defective for pRb binding, also retained the ability to induce apoptosis (Figure 3-4b).

Previous studies have shown that loss of negative regulation of E2F-1 DNA binding activity by cyclinA-associated kinase results in the inability of cells to leave S-phase and eventual apoptosis (Krek *et al.*, 1995). Conversely, the analysis of an E2F-1 mutant deleted of the cyclin A-binding site (E2F-1Δ24) was indistinguishable from the activity of the wild-type E2F-1 protein in the apoptosis assay (Figure 3-4b), suggesting that cyclinA-binding is not necessary for this fuction.

Because it was shown that E2F-1-induced apoptosis required DNA-binding but not *trans* activation (Figure 3-4), it is unlikely that the inhibition of E2F-1-induced apoptosis by pRb occurs through its suppression of E2F-1 *trans* activation. Nevertheless, existing data imply that pRb appears to be involved in apoptotic function (Clarke *et al.*, 1992; Jacks *et al.*, 1992; Morgenbesser *et al.*, 1994; Haupt *et al.*, 1995a; Shan *et al.*, 1996; Macleod *et al.*, 1996). For instance, p53-mediated apoptosis could be overcome by excess pRb, whereas co-expression of p53 appeared to enhance the ability of E2F-1 to induce apoptosis (Qin *et al.*, 1994; Haupt *et al.*, 1995a). Moreover, the loss of pRb function and over-expression of E2F are known to disrupt the G1 arrest and favour apoptosis (Shan *et al.*, 1996; Macleod *et al.*, 1996). Additional studies have provided convincing evidence that SAOS-2 derivatives expressing wild-type pRb exhibited increased viability and decreased apoptosis following treatment at a variety of radiation doses (Hass-Kogan *et al.*, 1995). Therefore, the effect of pRb on E2F-1 was investigated using TUNEL and luciferase assays. As shown in Figure 3-5, pRb significantly inhibited E2F-1-induced apoptosis (compare tracks 1 and 3) in consistent with the ability of pRb to repress E2F-1-mediated transcriptional activation (compare tracks 7 and 9).

In contrast, p300 functions as a transcriptional co-activator for the E2F/DP heterodimer (in this thesis and Lee *et al.*, 1998). Thus, it is possible that cells expressing p300 can promote E2F-1-induced apoptosis, as it has been shown to

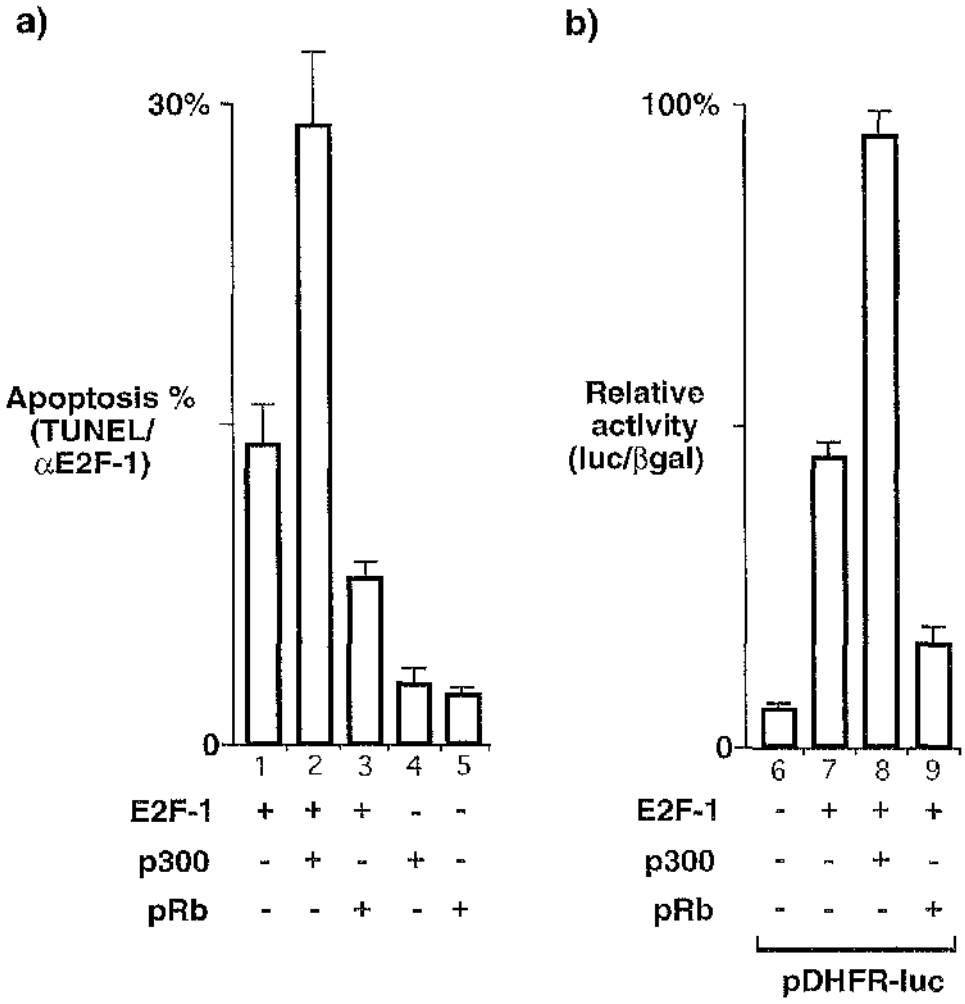

a)
30%
Apoptosis %
(TUNEL/
αE2F-1)
0
1 2 3 4 5
E2F-1 + + + - -
p300 - + - + -
pRb - - + - +
b)
100%
Relative
actlvity
(luc/βgal)
0
6 7 8 9
E2F-1 - + + +
p300 - - + -
pRb - - - +
pDHFR-luc

Figure 3-5

p300 and pRb have different effects on E2F-1 mediated apoptosis.

a) 2μg of E2F-1 expression plasmid together with 4μg of pRb or p300 expression plasmid were transfected into SAOS-2 cells. Cells were fixed and treated with the anti-E2F-1 polyclonal antibody and further assayed for the absolute percentage level of apoptosis by TUNEL assay (tracks 1 to 3). The apoptotic effects by p300 or pRb in the absence of E2F-1 was determined by the comparison of TUNEL-positive population to the number of DAPI-positive cells (tracks 4 and 5)

b) The E2F reporter pDHFR-luc (1μg) together with expression vectors for E2F-1 (0.1μg) and DP-1 (1μg) either alone or together with p300 (6μg) or pRb (6μg) were transfected into SAOS-2 cells as indicated. The value shown are the average of duplicate readings and represent the relative level of luciferase to the β-galactosidase activity from the internal control.

stimulate the *trans* activation of E2F-1 (figures 3-5b and 5-3). When p300 was co-expressed with E2F-1 there was a significant increase in the proportion of apoptotic cells, compared to E2F-1 alone (Figure 3-5, compare tracks 1 and 2), in contrast to the effect of pRb on E2F-1.

3-3. Conclusion.

E2F DNA-binding activity arises when a member of two families of proteins, E2F and DP, form heterodimeric complexes, an interaction which results in co-operative transcriptional and DNA-binding activity (La Thangue, 1994). From the data presented here, I conclude that the activity of E2F is mediated at the level of intracellular location through a dependence on heterodimer formation for nuclear translocation. Therefore, these data define a new level of control in the E2F transcription factor whereby interplay between E2F heterodimers dictates the levels of nuclear DNA-binding activity.

In addition, I demonstrated that E2F-1 can induce apoptosis in $p53^{-/-}$ tumour cells and that DP-1 is able to augment the E2F-1-induced apoptosis, yet by itself it has no ability to induce apoptosis, consistent with the ability of DP-1 to enhance E2F-1-mediated transcriptional activation, while inducing little *trans* activation by itself. Although it was clear that the apoptotic function of E2F-1 requires DNA-binding but not *trans* activation, the over-expression of p300 dramatically enhanced the E2F-1-induced apoptotic activity, in contrast to pRb which suppressed the E2F-1-induced apoptosis. These results suggest that E2F-1-induced apoptosis is not simply the consequence of apoptotic gene activation by E2F-1, but is regulated by several pathways. However, it clearly appears that E2F-1 provides an apoptosis-promoting signal independent of p53.

Chapter 4.

Functional relationship between p53 and E2F-1/DP-1.

4-1. Introduction.

The cellular transcription factors E2F/DP and the tumour suppressor protein p53 play important roles in controlling early cell cycle events (Nevins, 1992; La Thangue, 1994; Weinberg, 1995). E2F/DP are believed to co-ordinate and integrate the transcription of cell cycle regulating genes, for example, those involved in DNA synthesis (La Thangue, 1994; Muller, 1995). In contrast, p53 is thought to monitor the integrity of chromosomal DNA and when appropriate interfere with cell cycle progression, for example, in response to DNA damage (Ko and Prives, 1996). However, little is known about the function of the p53 in the control of normal cell cycle progression, although transcription of the p53 target gene, encoding the CDK-inhibitor $p21^{Waf1/Cip1}$, correlates with p53-mediated cell cycle arrest (Sherr, 1993; Dulic *et al.*, 1994; Koh *et al.*, 1995; Lukas *et al.*, 1995; Weinberg, 1995). Nevertheless, p53 has been shown to be able to repress the transcriptional activity of the promoters of thymidine kinase, c-myc and DNA polymerase α(Lin *et al.*, 1992; Moberg *et al.*, 1992; Yuan *et al.*, 1993). Interestingly, these are also the genes which can be *trans* activated by E2F. This suggests that p53 may be able to interfere with E2F function in the normal cell cycle. Thus, I have the possibility that p53 can suppress E2F transcriptional activity, and further that E2F can associate with p53.

4-2. Results.

4-2.1. DP-1 associates with p53.

Cell extracts were prepared from SAOS-2 cells expressing wild-type p53 and the HA-tagged DP-1, and immunoprecipated with the anti-p53 monoclonal antibody 421 followed by immunoblotting with the anti-HA antibody (Figure 4-1). DP-1 was specifically detected in the p53 immunocomplex (Figure 4-1, compare tracks 1 and 2). Furthermore, immunoblotting analysis from the same cell extract, was performed with the anti-HA antibody to confirm the HA-DP-1 expression level. The level of HA-DP-1 was constant in these experimental condition (Figure 4-1, compare tracks 3 to 4 and 5). Further evidence was obtained by using F9EC cell extracts which were incubated with the anti-p53 antibody, and where DP-1 was also present in this immunoprecipitates (Sørensen *et al.*, 1996). However, the data do not address whether the interaction is direct or indirect but, nevertheless, do indicate that the association occurs in physiological conditions.

4-2.2 An immunochemically distinct form of DP-1 associates with p53.

The minimal region in DP-1 capable of effciently binding to p53 occurs within residues 171 to 331 (Sørensen *et al.*, 1996). This region of DP-1 contains several domains which are conserved between other members of the DP family, and a critical region involved in heterodimerization between DP-1 and E2F family members (Figure 1-4).

Similarly, to define the region in p53 required for the association with DP-1, an *in vitro* binding assay was developed in which the ability of p53 to interact with *in vitro* translated DP-1 was monitored. Sørensen *et al.* (1996) characterized the two forms of DP-1 polypeptides, referred to as p55L (lower) and p55U (upper), from

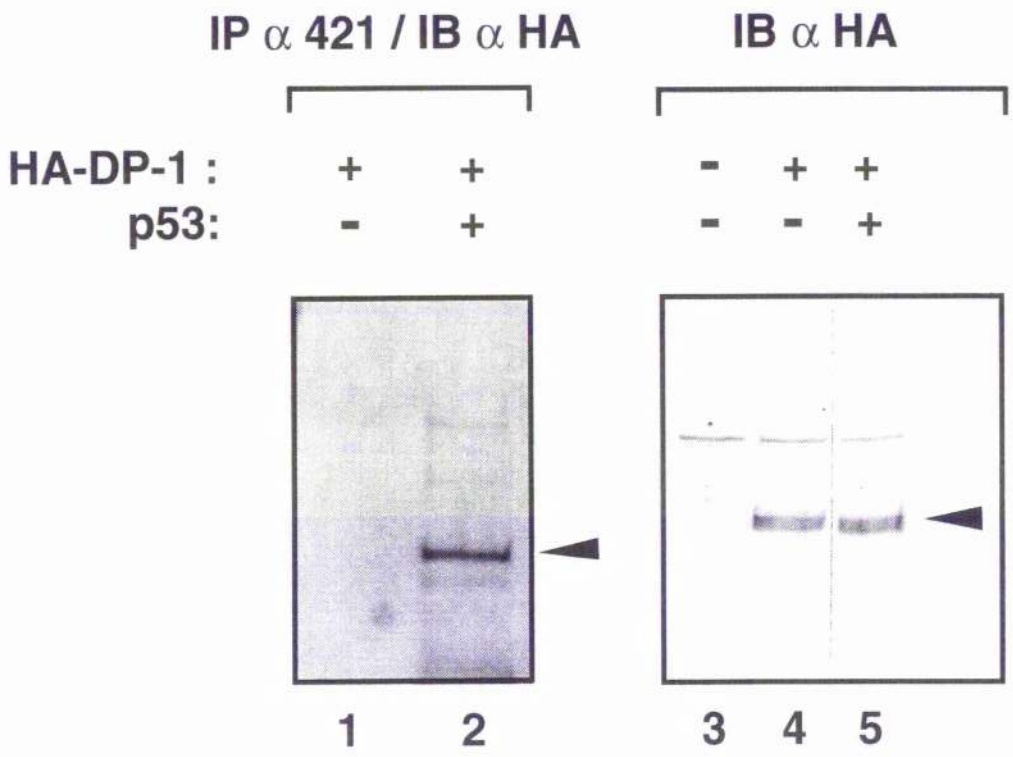

IP α 421 / IB α HA
IB α HA
HA-DP-1 :
p53:
+ +
- +
- + +
- - +
1 2
3 4 5

Figure 4-1

p53 associates with DP-1.

The expression vectors encoding p53 and HA-tagged DP-1 were transfected into SAOS-2 cells and after 36hr immunoprecipitation performed with the anti-p53 monoclonal antibody 421 (tracks 1 and 2) followed by gel electrophoresis. Immunoblotting was performed with the anti-HA monoclonal antibody 12CA5; cell extracts alone shown in tracks 3, 4 and 5. Note that the common band present across tracks 3, 4 and 5 results from non-specific activity with 12CA5. The HA-tagged DP-1 polypeptides are indicated.

extracts of asynchronous cultured 3T3 and F9EC cells. In that study, it was shown that polyclonal anti-DP-1 (D) recognises p55L, in contrast to anti-DP-1 (A) which revealed both forms of p55. Specifically, p55L appeared towards the end of G1 as cells begin to enter S phase, and evidence was presented that p55L is a significant component of E2F DNA-binding activity and that p55U is a form of DP-1 which binds less efficiently to E2F site (Bandara *et al.*, 1994; Sørensen *et al.*, 1996).

The results shown in Figure 4-2a confirm these conclusions as p55U is a form of DP-1 recognised by anti-DP-1 (A) but not anti-DP-1 (D). In a similar fashion, two immunochemically distinct forms of DP-1 could be defined after *in vitro* translation using same two antisera. Specifically, in the absence of translated exogeneous DP-1, anti-DP-1 (A), but not anti-DP-1 (D), recognised the endogenous DP-1 protein (Figure 4-2a, compare tracks 4 and 3 respectively; indicated by ◘). After translation, both antisera recognised the *in vitro* translated DP-1 protein, the exogeneous polypeptide being resolved with marginally faster mobility (Figure 4-2a, tracks 1 and 2; indicated by arrow head).

Evidence that at least two immunochemically distinct forms of DP-1 were present after *in vitro* translation was obtained upon studying the interaction with p53. When p53 was added to the *in vitro* translate, the DP-1 form recognised by anti-DP-1 (A), but not anti-DP-1 (D), was retained by p53 (Figure 4-2b, compare tracks 3 and 4) although DP-1 immunoreactive with both antisera was present in the input translate (Figure 4-2b, compare tracks 5 and 6); the GST portion failed to interact with DP-1 (Figure 4-2b, compare tracks 1 and 2). These data indicate that two distinct forms of DP-1 are present after *in vitro* translation and further that p53 preferentially interacts with the form defined by anti-DP-1 (A). Importantly, this result reflects tha data derived by immunoprecipitation from mammalian cell extracts where p53 co-immunoprecipitated with p55U, a form of DP-1 recognised by anti-DP-1 (A) but not anti-DP-1 (D) (Sørensen *et al.*, 1996). The specificity of p53 for DP-1 in the *in vitro* binding assay therefore possesses some similarity

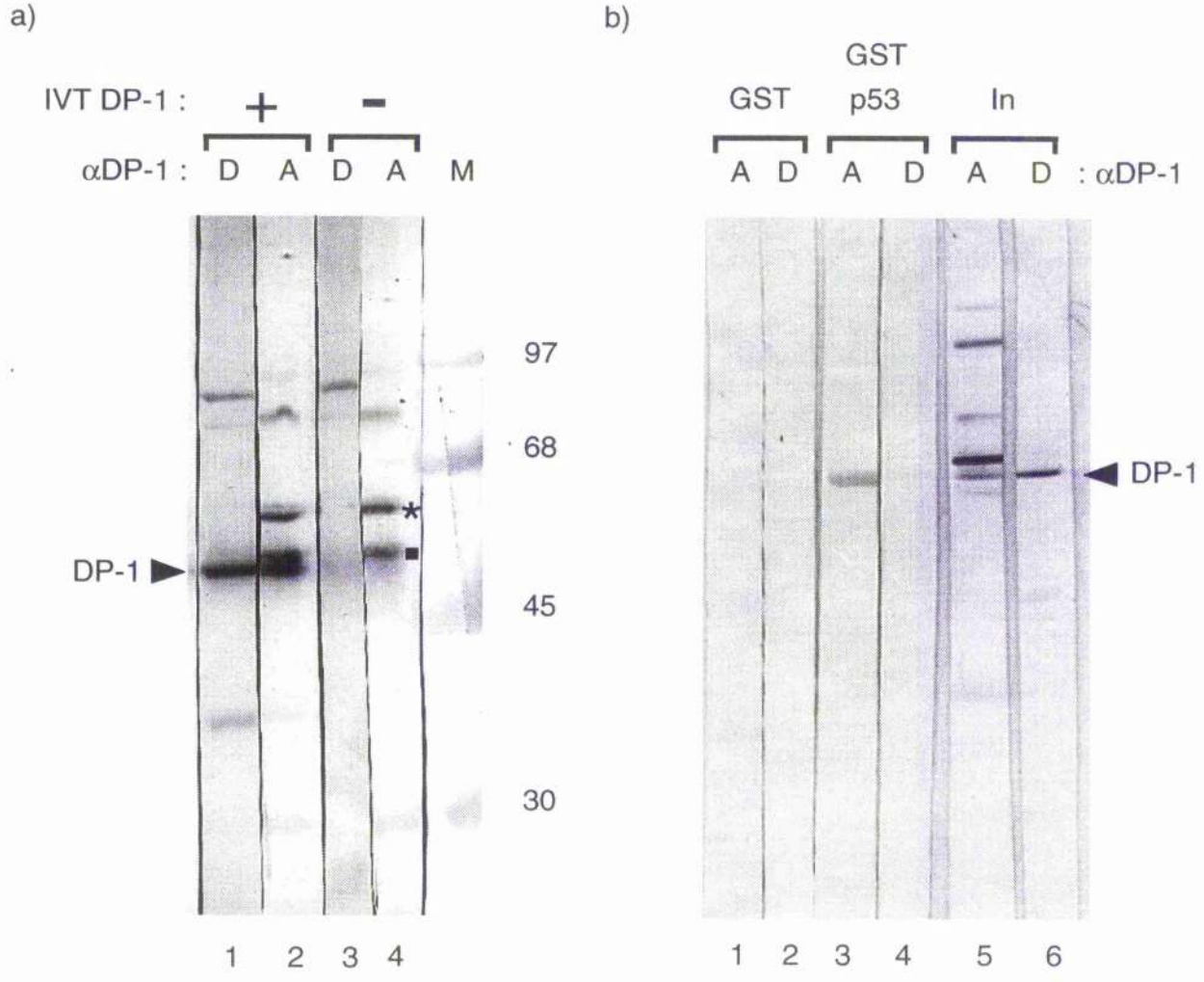

a)
IVT DP-1 : + -
αDP-1 : D A D A M
97
68
45
30
DP-1
*
1 2 3 4
b)
GST
GST p53 In
A D A D A D : αDP-1
DP-1
1 2 3 4 5 6

Figure 4-2

p53 binds to an immunochemically distinct form of DP-1.

a) Reticulocyte lysate programmed either with (tracks 1 and 2) or without (tracks 3 and 4) DP-1 was probed with and anti-DP-1 (αDP-1) antibody, either anti-DP-1(D) (D; tracks 1 and 3) or anti-DP-1(A) (A; tracks 2 and 4). The *in vitro* translated (IVT) DP-1 polypeptide is indicated by the arrowhead; note that the antisera were affinity purified. The polypeptide indicated by the box and recognized by anti-DP-1(A) in track 4 is probably endogenous DP-1; the asterisk indicates a non-specific polypeptide. Lane M contains molecular weight markers, the positions of which (in thousands) are indicated on the right.

b) Either GST-p53 (tracks 3 and 4) or GST alone (tracks 1 and 2) was incubated with *in vitro* translated wild-type DP-1, and the bound DP-1 was assessed by immunoblotting with either anti-DP-1(A) (tracks 1 and 3) or anti-DP-1(D) (tracks 2 and 4). Each track was cut in half along the middle, probed with either antiserum, and realigned. In tracks 5 and 6, the input (In) reticulocyte containing *in vitro* translated DP-1 was probed with anti-DP-1(A) and anti-DP-1(D), respectively; DP-1 is indicated by the arrowhead. Note that GST-p53 binds a form of DP-1 recognized by anti-DP-1(A) but not anti-DP-1(D).

with the interaction in mammalian cells, and supports the conclusion that p53 interacts with an immunochemically distinct form of DP-1.

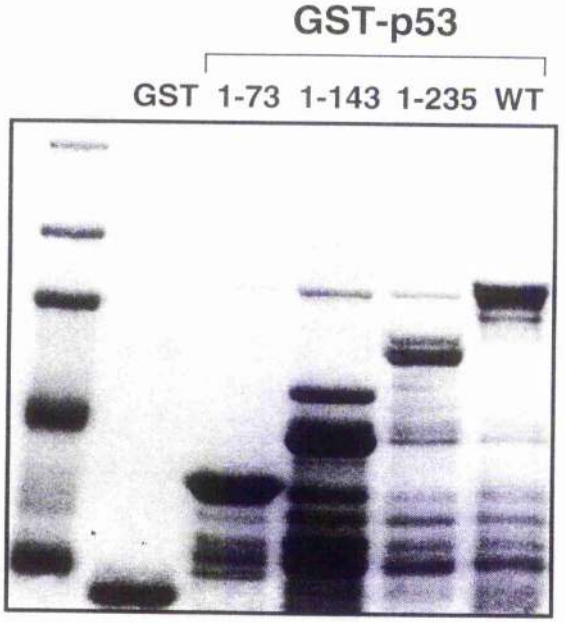
4-2.3 An amino-terminal region in p53 is required for binding to DP-1.

I used GST-pull down assays to define the domain in p53 required for the association with DP-1. As much as 250 amino acid residues could be deleted from carboxy-terminus of p53 without any detrimental effect on the interaction with DP-1 (Figure 4-3b, compare tracks 3, 4 and 5). A further deletion from residues 143 to 73 abolished the interaction (Figure 4-3a and b, compare tracks 2 and 3), thus defining a region in p53 required to bind DP-1. Since the amino-terminal region of p53 contains the MDM2 binding domain (Momand *et al.*, 1992; Oliner *et al.*, 1993; Chen *et al.*, 1994), a domain in p53 necessary for the interaction with DP-1 can therefore be distinguished from that required for MDM2 binding.

4-2.4. p53 can modulate E2F site-dependent transcription.

As DP-1 is a frequent component of E2F (Girling *et al.*, 1993; La Thangue, 1994), I assessed the functional consequence of the interaction of p53 with DP-1 by studying the effects on E2F site-dependent transcription driven by DP-1 and E2F-1, a situation in which it is known that both proteins co-operate in transcriptional activation as a DNA binding heterodimer (Figure 4-4, compare lanes 1 to 6 and 11). In these assay conditions DP-1 alone possesses insignificant transcriptional activity (Figure 4-4, compare lanes 1 to 16). When a wild-type p53 expression vector was co-transfected into SAOS-2 cells the level of *trans* activation mediated by either E2F-1 alone, or DP-1 together with E2F-1, was compromised in a p53 concentration-dependent fashion (Figure 4-4, compare lanes 6 through 10 and 11

a)


GST-p53
GST
1-73
1-143
1-235
WT

b)

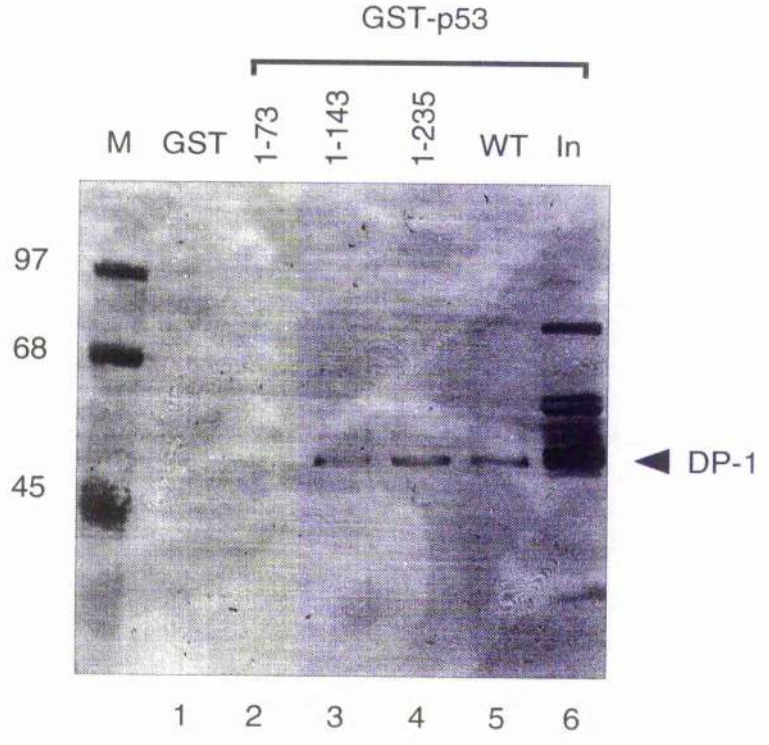

GST-p53
M
GST
1-73
1-143
1-235
WT
In
97
68
45
DP-1
1
2
3
4
5
6

Figure 4-3

Domains in p53 required for the interaction with DP-1.

a) A panel of GST-p53 fusion proteins were used in an *in vitro* binding assay with DP-1.

b) The indicated p53 fusion proteins (tracks 2 to 5) or control GST (track 1) was incubated with *in vitro* translated wild-type DP-1 (track 6), and the bound DP-1 was assessed by immunoblotting with anti-DP-1(A). Lane M contains molecular weight markers ($x10^{-3}$) the positions of which are indicated on the left. In, input

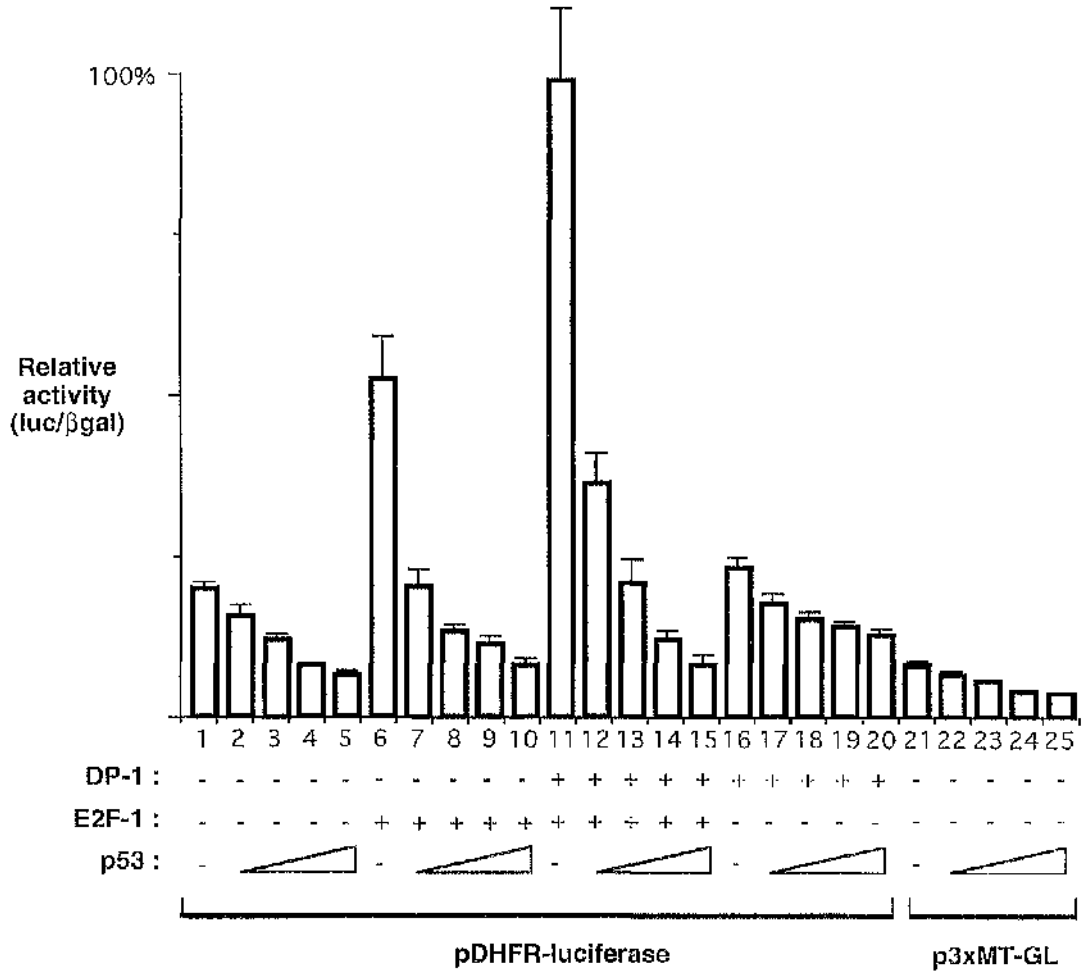

100%
Relative activity (luc/βgal)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25
DP-1 :
E2F-1 :
p53 :
pDHFR-luciferase
p3xMT-GL

Figure 4-4

p53 modulates E2F binding site-depedent transcription.

SAOS-2 cells were transfected with expression vectors encoding E2F-1 (bars 6 to 15; 0.2μg) and DP-1 (bars 11 to 20; 2.0μg), together with an increasing amount of the p53 expression vector (bars 2, 7, 12, and 17, 0.5μg; bars 3, 8, 13, and 18, 1.25μg; bars 4, 9, 14, and 19, 3.75μg; bars 5, 10, 15, and 20, 7.5μg). The reporter constructs were pDHFR-luciferase, containing positions -270 to +20 from the *DHFR* promoter, or p3xMT-GL, and pCMV-βgal was included in each transfection as an internal control; in values shown are based on the activity of pDHFR-luc or p3xMT-GL (luciferase) relative to that of pCMV-βgal (β-galactosidase). The p53 expression vector suppressed the activity of pCMV-βgal, which was taken into consideration in determining the effect of p53 on DP-1 and E2F-1. The values shown represent the means of two readings.

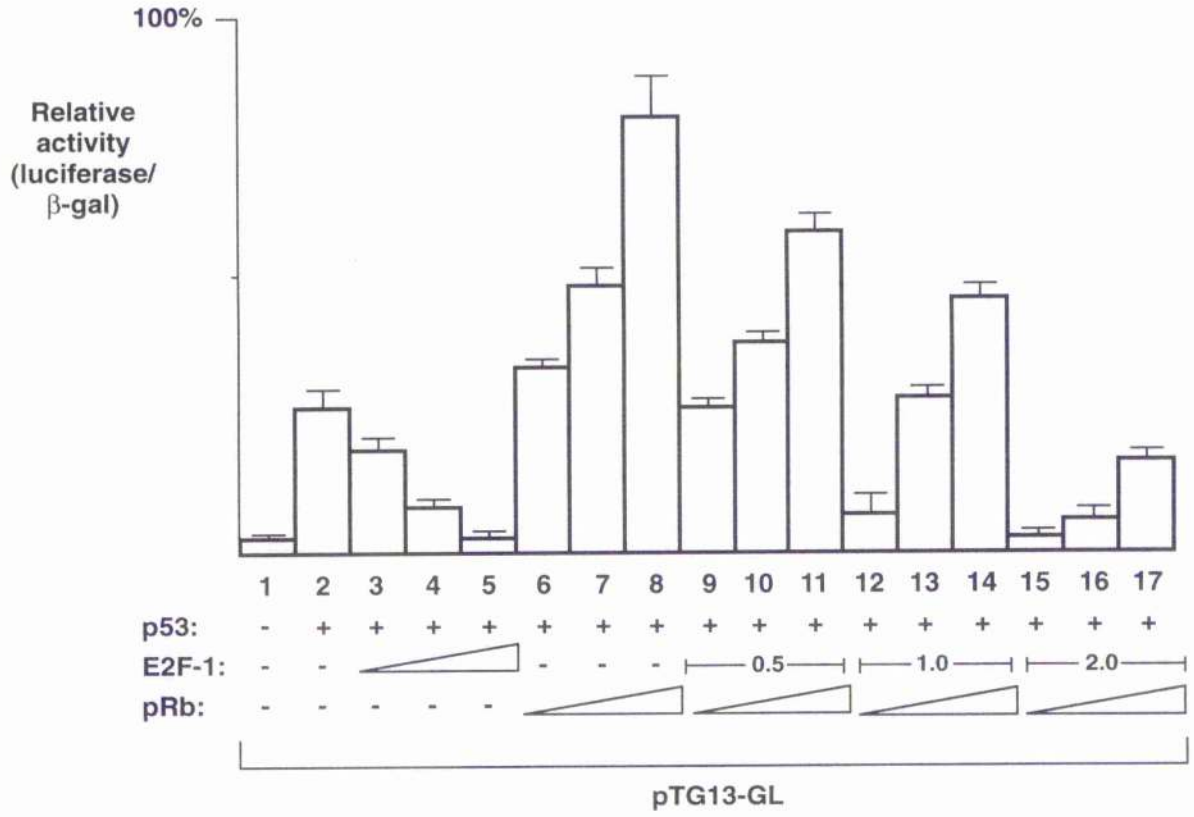
through 15). The activity of a comparable promoter construct deriven by mutant E2F binding sites, p3xMT-GL, was not significantly affected by p53 (Figure 4-4, compare lanes 21 through 25). Furthermore, since this inactivating effect of p53 is calculated as a ratio of the reporter to the transcriptional activity of an internal control (pCMV-βgal) within the same transfection treatment, it is a specific effect of transcriptional activation through the E2F site.

4-2.5. Regulation of p53-dependent transcription by E2F and pRb.

To address the interplay between E2F and p53, it was considered that insight may be gained into the mechanisms involved by evaluating the effects of E2F on p53-dependent transcription. Thus, I introduced wild-type p53 into the SAOS-2 cells, and thereafter monitored the activity of a p53 reporter which was dependent upon p53 for transcriptional activity. The reporter of choice, pTG13-GL, contains p53 binding sites immediately upstream of the herpes virus minimal thymidine kinase (*tk*) promoter together with the SV40 enhancer driving the *luciferase*, and in SAOS-2 possessed minimal activity in the absence of p53 but responded favourably to exogenous wild-type p53 (for example Figure 4-5a, compare tracks 1 and 2); similar data were obtained from transfection studies in U2OS cells.

To establish the influence of the E2F pathway on p53, I introduced increasing levels of E2F-1 or pRb and thereafter assayed p53 activity. Increased levels of E2F-1 caused a concomitant decline in the transcriptional activity of p53 (Figure 4-5a, compare track 2 to 3, 4 and 5) whereas, in contrast, increased levels of pRb stimulated p53-dependent transcription (Figure 4-5a, compare track 2 to 6, 7 and 8). Further support for this idea was provided by co-expressing E2F-1 in the presence of pRb. In these conditions, pRb antagonised the effects of E2F-1 so that a reduction in p53-dependent transcription was no longer apparent (Figure 4-5a, compare track 3 to 9, 10 and 11).

a)


100%
Relative activity (luciferase/ β-gal)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
p53: - + + + + + + + + + + + + + + + +
E2F-1: - - - - - 0.5 1.0 2.0
pRb: - - - - -
pTG13-GL

b)

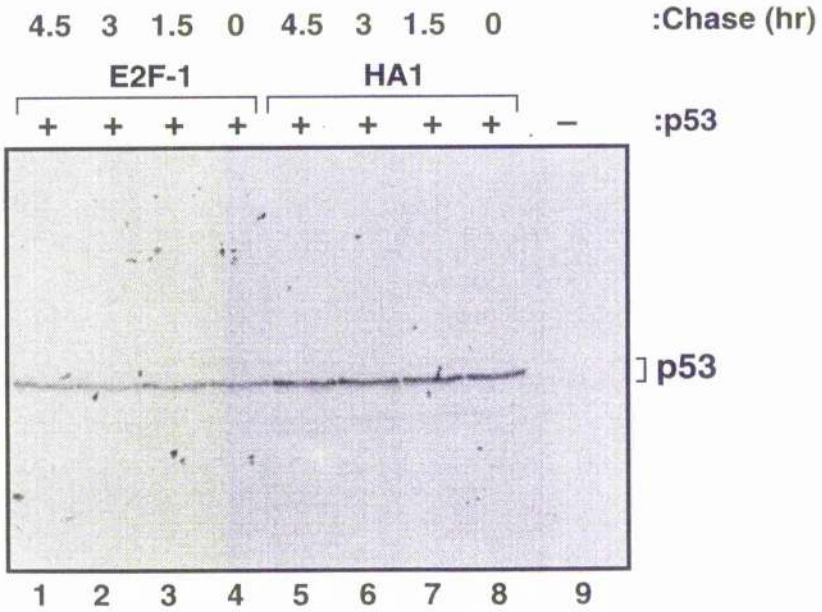

4.5 3 1.5 0 4.5 3 1.5 0 :Chase (hr)
E2F-1
HA1
+ + + + + + + + − :p53
] p53
1 2 3 4 5 6 7 8 9

Figure 4-5

Regulation of p53-dependent transcription by E2F-1 and pRb.

a) The p53 reporter pTG13-GL (containing 13 copies of a synthetic p53 consensus binding sites derived from the promoter of *Waf1/Cip1*) was introduced into SAOS-2 cells together with expression vectors encoding p53 (1 μg; tracks 2 to 17), in the presence of E2F-1 (0.5μg in tracks 3, 9, 10 and 11; 1.0 μg in tracks 4, 12, 13 and 14 or 2.0 μg in tracks 5, 15, 16 and 17), and/or pRb (1.0μg in tracks 6, 9, 12 and 15, 6μg in tracks 7, 10, 13 and 16, and 12μg in tracks 8, 11, 14 and 17). The data presented reflect averages from two sets of values. Throughout pCMV-βgal was included in each transfection as an internal control. The relative activity of luciferase to β-galactosidase is presented.

b) The influence of E2F-1 on p53 protein level was assessed in SAOS-2 cells transfected with expression vectors encoding p53 (3μg; tracks 1 to 8), E2F-1 (3μg; tracks 1 to 4), or the control vector HA1 (3μg; tracks 5 to 8), metabolically labelled with ^{35}S-methionine as described and thereafter chased for the indicated times. The levels of p53 were resolved by immunoprecipitation. A non-transfected treatment is shown in track 9.

At the same time, the stimulation of p53 activity by pRb was less when co-expressed with E2F-1 (Figure 4-5a, compare tracks 6, 7 and 8 to 9, 10 and 11), and a titration of E2F-1 levels in the presence of pRb provided additional evidence for this view, since upon increased levels of E2F-1 there was a proportional decline in the transcriptional activity of p53 (Figure 4-5a, compare tracks 9, 10 and 11 to 12, 13 and 14, and 15, 16 and 17). Thus, the transcriptional activity of p53 is influenced by the levels of E2F-1 and, moreover, the presence of pRb overcomes this effect, suggesting that the p53 and pRb/E2F pathways of growth control are functionally integrated.

Certain control treatments were performed to establish the specificity of E2F-1 on p53. For example, the effects on p53 transcriptional activity were dependent upon exogenous p53 since without p53 no changes in activity on the pTG13-GL reporter in the presence of E2F-1 or pRb were apparent. The half life of exogenous p53 protein was not affected by co-expression of E2F-1 (Figure 4-5b, compare tracks 1 to 4 with 5 to 8) and, further, each transfection treatment was internally controlled with pCMV-βgal to rule out general effects of p53, E2F-1 or pRb on the transcription apparatus. Having established the specificity of the E2F-1 effect upon p53-dependent transcription, I went on to investigate the mechanism involved.

4-2.6. The regulation of p53 activity requires the E2F-1 and p53 *trans* activation domains.

An outcome of the interaction between pRb and E2F-1 is the inactivation of the carboxy-terminal transcription activating domain, which is integrated with the domain recognised by pRb (Flemington *et al.*, 1993; Helin *et al.*, 1993; Kaelin *et al.*, 1992). Since the regulation of p53 activity by E2F-1 was overcome by pRb (Figure 4-5a), we reasoned that the E2F-1 activation domain may be influential in regulating p53 activity. To assess this possibility, a mutant E2F-1 protein which lacks the

carboxy-terminal activation domain, due to truncation up to amino acid residue 413 (E2F-1Δ413), was studied. As expected E2F-1Δ413 had greatly reduced ability to stimulate the transcription of the E2F-responsive *DHFR* promoter in the absence or presence of DP-1 (Figure 4-6a, compare tracks 1 through 5). When assessed for its effect upon p53 activity, and in contrast to wild-type E2F-1, E2F-1Δ413 failed to cause a reduction but rather increased p53-dependent transcription (Figure 4-6b, compare tracks 3 and 4 with 5 and 6). Thus, the integrity of the transcriptional activation domain is necessary for the reduction in p53 activity by E2F-1.

Since the regulation of p53 required the E2F-1 transcriptional activation domain, it was possible that the activation domain in p53 may be involved. Therefore, I tested whether a fusion protein containing the amino-terminal transcription activation domain (amino acid residues 1 to 73) of p53 was sensitive to the effects of E2F-1. In a similar fashion to wild-type p53, the activity of Gal4-p53 was reduced by E2F-1 (Figure 4-6c, compare tracks 2 and 3).

It was important to determine the specificity of the effect of E2F-1 on p53. To this end, I studied whether E2F-1 caused a reduction in the activity of other transcription activation domains. A variety of activation domains were assessed as Gal4 fusion proteins including, of which the p53 activation domain was by far the most sensitive. Further, the inactivation of p53 was not caused through general effects, such as protein stability, because a hybrid protein in which the VP16 activation domain was fused to the wild-type p53 protein became far less sensitive to the effects of E2F-1 (Figure 4-6c, compare tracks 5 and 6 with 8 and 9).

4-3. Conclusion.

These data allow a number of important conclusions to be made. Thus, DP-1 can complex with p53 both *in vitro* and *in vivo*, and because both proteins are key regulators of cell cycle progression, these functional interactions are of potential

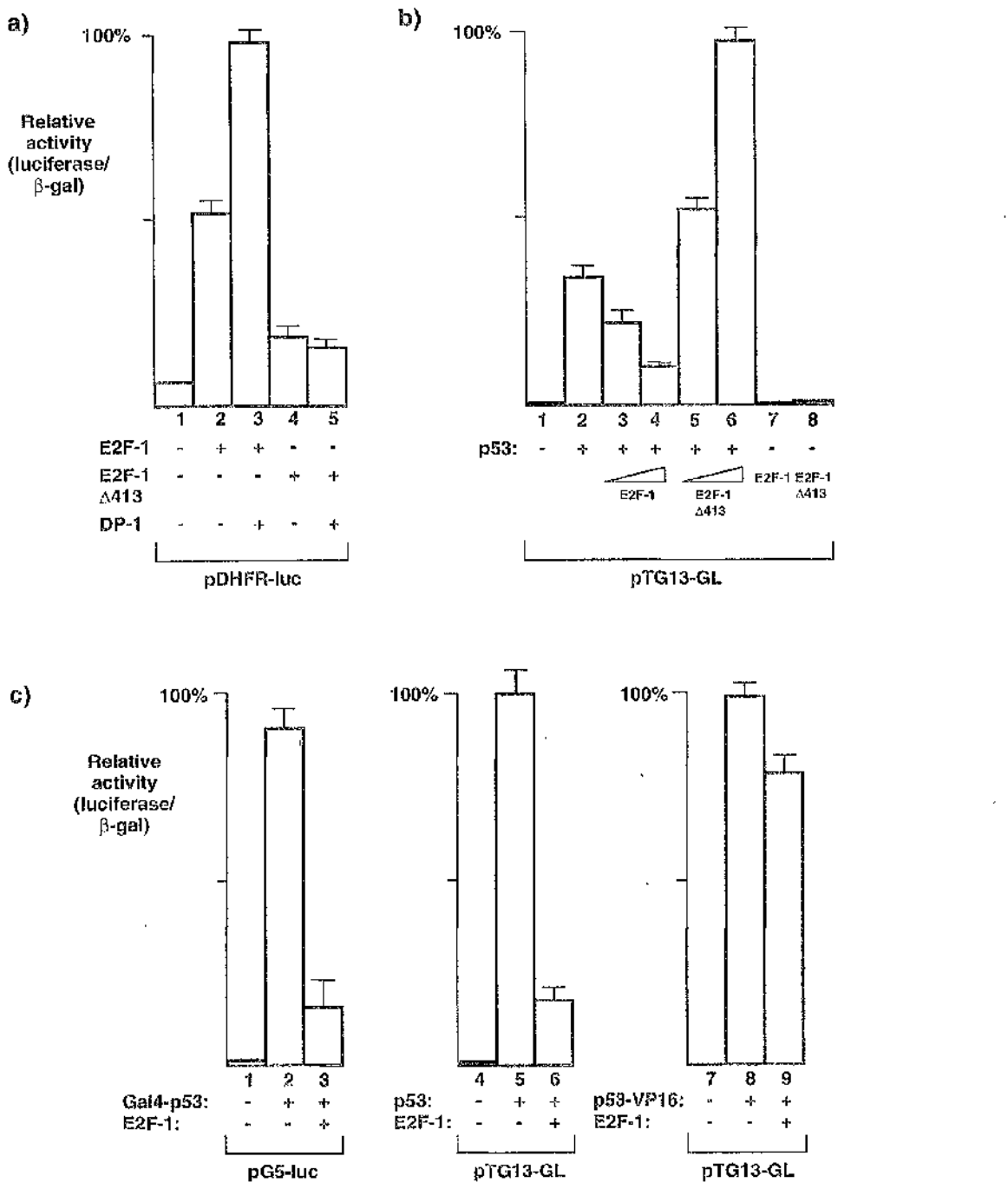

a)
100%
Relative activity (luciferase/ β-gal)
1 2 3 4 5
E2F-1 - + + - -
E2F-1 Δ413 - - - + +
DP-1 - - + - +
pDHFR-luc
b)
100%
1 2 3 4 5 6 7 8
p53: - + + + + + - -
E2F-1
E2F-1 Δ413
E2F-1
E2F-1 Δ413
pTG13-GL
c)
100%
Relative activity (luciferase/ β-gal)
1 2 3
Gal4-p53: - + +
E2F-1: - - +
pG5-luc
100%
4 5 6
p53: - + +
E2F-1: - - +
pTG13-GL
100%
7 8 9
p53-VP16: - + +
E2F-1: - - +
pTG13-GL

Figure 4-6

Regulation of p53 activity requires the E2F-1 and p53 activation domains.

a) The E2F reporter pDIIFR-luc was introduced into SAOS-2 cells together with expression vectors encoding E2F-1 (0.2 μg; tracks 2 and 3) or E2F-1Δ413 (0.2 μg; tracks 4 and 5) together with DP-1 (2 μg; tracks 3 and 5). The data presented reflect averages from two sets of values. Throughout pCMV-βgal was included in each transfection as an internal control, the relative activity of luciferase to β-galactosidase being presented.

b) The p53 reporter pTG13-GL was introduced into SAOS-2 cells together with expression vectors encoding E2F-1 (1 μg in track 3, and 3 μg in 4 and 7) or E2F-1 Δ413 (1 μg in tracks 5, and 3 μg in tracks 4, 6 and 8). Values were calculated as described in (a).

c) Either the Gal4 reporter pG5-luc (1 μg; tracks 1 to 3) or pTG13-GL (1 μg; tracks 4 to 9) was introduced into SAOS-2 cells together with expression vectors encoding Gal4-p53 (1 μg; tracks 2 and 3), p53 (1 μg; tracks 5 and 6) or p53-VP16 (1 μg; tracks 8 and 9) in the presence of E2F-1 (2 μg; tracks 3, 6, and 9). The data presented reflect averages from two sets of values. pCMV-βgal was included in each transfection as an internal control, and the relative activity of luciferase to β-galactosidase activity is presented.

importance in the overall control of the cell cycle. Furthermore, p53 interacts with and immunochemically distinct form of DP-1 and, further, this form of DP-1 is an infrequent component of E2F DNA-binding activity (Bandara *et al.*, 1994; Sørensen *et al.*, 1996), suggesting that p53 competes with E2F-1 for DP-1 with a consequent reduction in DNA-binding activity. These results suggest that p53 plays a regulatory role in the normal cell cycle by limiting E2F/DP transcriptional activity, possibly through pRb-dependent and -independent pathways. Conversely, p53-dependent transcriptional activity is also inhibited by the expression of E2F-1 and, moreover, the presence of pRb overcomes this effect. Interestingly, the regulation of p53 activity requires the E2F-1 and p53 *trans* activation domains. In normal cells, the transcriptional activity of p53 is believed to be regulated mainly at the protein level through its short half life. However, these results, combined with the evidence that the *trans* activation domains of E2F-1 and p53 were involved, imply that other cellular factors might be also involved in the regulation of p53 activity by competing with E2F-1 for a rate limiting transcription target.

Chapter 5.

Cell cycle regulation and apoptosis by p53 and E2F-1/DP-1 through co-activator p300

5-1. Introduction.

A number of different lines of evidence indicated that the p300/CBP family of proteins function as pleiotrophic co-activators that facilitate activation by a wide variety of sequence-specific transcription factors that are involved in the cell cycle, differentiation, and signal transduction (Eckner *et al.*, 1994; Arany *et al.*, 1995; Lundblad *et al.*, 1995; Shikama *et al.*, 1997). Recent studies have shown that p300 is phosphorylated in a cell cycle dependent manner and is a substrate for cyclin dependent kinase (Yacink *et al.*, 1991; Wang *et al.*, 1993; Perkins *et al.*, 1997), suggesting that p300 is critical regulator of cell cycle control. In addition, it is known that p300/CBP proteins are targets for the adenovirus E1A and SV40 large T antigen oncoproteins (Eckner *et al.*, 1994; Arany *et al.*, 1995; Avantaggiati *et al.*, 1996). Interestingly, a common region of the T antigen is critical for binding both p300/CBP and p53 (Lill *et al.*, 1997), suggesting a link between the functions of p53 and p300.

The results presented in chapter 4 raise the possibility that competition for a shared transcription target is responsible for the effect of E2F-1 on p53-dependent transcription. The potential identity of such a molecule was addressed by determining which proteins, when co-expressed with E2F-1 and p53, relieved the repression of p53 activity. Therefore, I focused on the understanding of functional interplay between p53 and E2F/DP through p300.

5-2. Results.

5-2.1. p300 relieves the inactivation of p53 by E2F-1.

Expression of the co-activator p300 (Shikama *et al.*, 1997), a molecule previously implicated in E2F-1 mediated transcriptional activation (Trouche *et al.*, 1996), effectively relieved the repression of p53 (Figure 5-1a, compare tracks 3, 4 and 5) and enhanced in p53-dependent transcription in the absence of E2F-1 (Figure 5-1a, compare tracks 3, 4, 5, 6 and 7). These data argue that p300 functions as a transcriptional co-activator for p53 and, further, suggest that a limiting level of p300 is in part responsible for the inactivating effect of E2F-1 on p53.

To confirm that p300 can provide a co-activator function for E2F-1, the effect of p300 on E2F-1-dependent activity of *DHFR* promoter was assessed. As expected, co-expression of p300 enhanced transcriptional activity in an E2F-1-dependent fashion (Figure 5-1b, compare tracks 2 through 6). Thus, the combined conclusion from these studies suggested that p300 is a shared co-activator utilised by p53 and E2F-1 and therefore a likely target involved in mediating the effect on p53 by E2F-1. That co-expression of pRb relieved the effect of E2F-1 on p53 (Figure 4-5a) is consistent with this result since pRb inactivates the transcriptional activity of E2F-1 (Flemington *et al.*, 1993; Helin *et al.*, 1993), presumably causing the release of p300.

5-2.2. p300 physically associates with p53 and E2F-1.

Having established a functional relationship between p53 and p300, and E2F-1 and p300, I wished to determine if this was due to the physical interaction between each pair of proteins. To test this possibility, I used a p300 derivative, pG4-p300$^{611-2284}$, in which the region between amino acid residue 611 and 2284 is

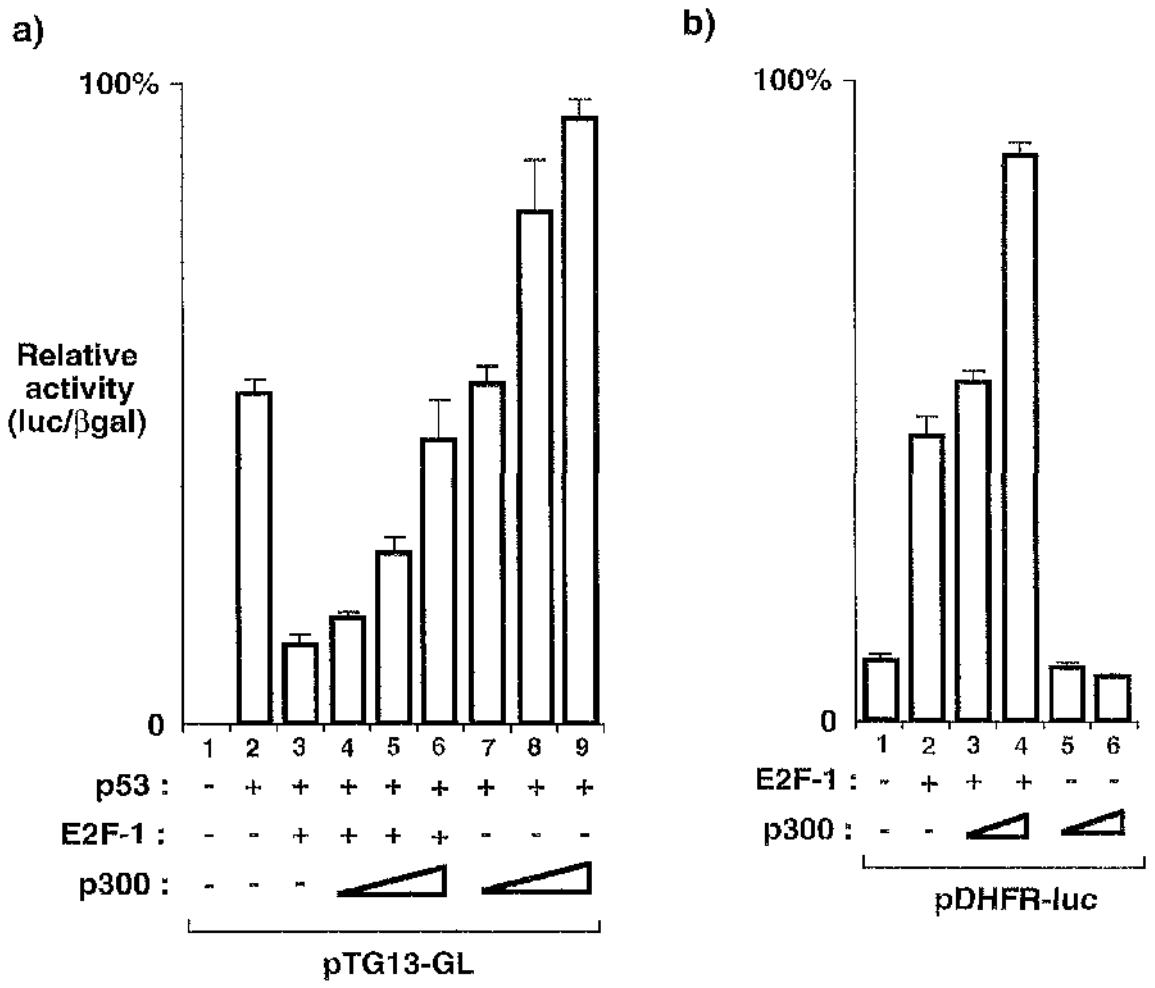

a)
100%
Relative activity (luc/βgal)
0
1 2 3 4 5 6 7 8 9
p53 : - + + + + + + + +
E2F-1 : - - + + + + - - -
p300 : - - -
pTG13-GL
b)
100%
0
1 2 3 4 5 6
E2F-1 : - + + + - -
p300 : - -
pDHFR-luc

Figure 5-1

p300 rescues E2F-1 inactivated p53 and enhances the transcriptional activity of p53 and E2F-1.

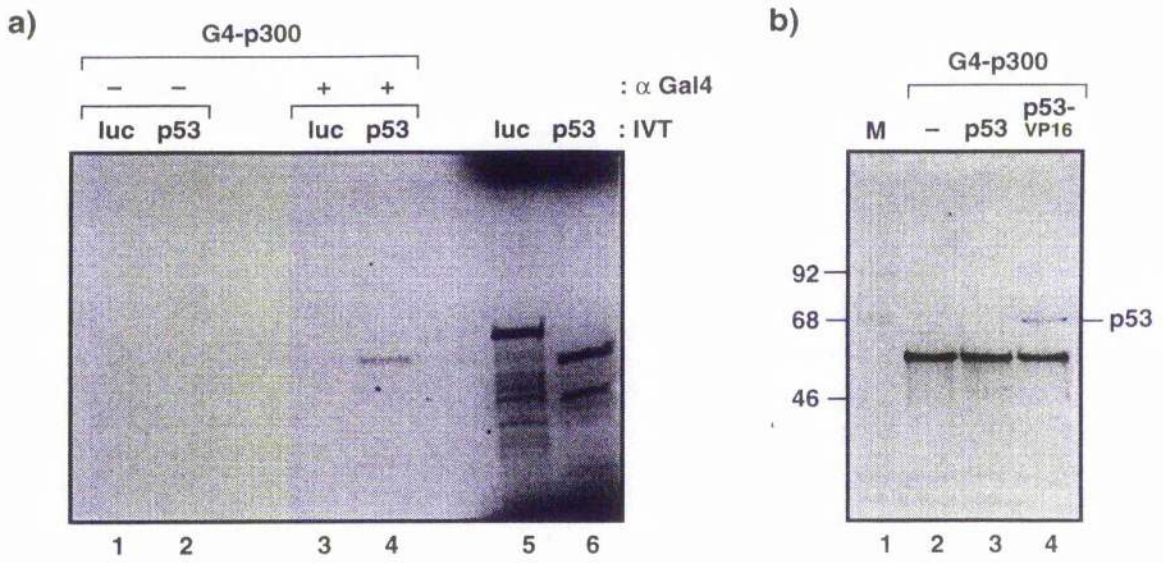
a) The p53 reporter pTG13-GL was introduced into SAOS-2 cells together with expression vectors encoding p53 (1 μg; tracks 2 to 9) in the presence of E2F-1 (2 μg; tracks 3, 4 and 5) with p300 (2, 8 and 16 μg in tracks 4, 5 and 6, and 7, 8 and 9 respectively). The data presented reflect averages from two sets of values. Throughout pCMV-βgal was included within each transfection as an internal control, and the relative activity of luciferase to β-galactosidase presented.

b) The E2F reporter pDHFR-luc was introduced into SAOS-2 cells together with expression vectors encoding E2F-1 (0.2 μg; tracks 2, 3 and 4) together with p300 (2 and 10 μg in tracks 3 and 4, and 5 and 6 respectively). Values were calculated as described in (a).

fused to the Gal4 DNA-binding domain. I chose pG4-p300$^{611-2284}$ for these studies because it is efficiently expressed in a variety of mammalian cells. Extracts were prepared from U2OS cells expressing pG4-p300$^{611-2284}$ and the interaction between p53 and pG4-p300$^{611-2284}$ assessed using a binding assay where *in vitro* translated and radiolabelled p53 or the control luciferase was added to the U2OS cell extract and the association with p300 determined by immunoprecipitation using an anti-Gal4 antiserum (Figure 5-2a, compare tracks 5 and 6). An interaction between p53 and pG4-p300$^{611-2284}$ was detected but not between luciferase (Figure 5-2a, compare tracks 3 and 4), suggesting a specific association between p53 and p300. The detection of p53 in the immunoprecipitate was dependent upon immunoprecipitation since in the absence of the anti-Gal4 antiserum p53 was no longer apparent (Figure 5-2a, compare tracks 2 and 4).

To confirm that p300 and p53 can physically associate *in vivo*, I co-expressed G4-p300$^{611-2284}$ and p53 in U2OS cells, immunoprecipitated with anti-Gal4 followed by immunoblotting with anti-p53 monoclonal antibody 421. Again, p53 was specifically detected in the p300 immunocomplex (Figure 5-2b, compare tracks 2, 3 and 4) establishing that p300 and p53 can physically associate in mammalian cells. Furthermore, the immunoprecipitation was confirmed with anti-p53 antibody using the SAOS-2 cell extracts expressing wild-type p53 and either HA-tagged p300$^{611-1572}$ or HA-tagged p300$^{1572-2284}$ (Figure 5-2c).

Further evidence was obtained by taking a two-hybrid approach in U2OS cells in which G4-p300$^{611-2284}$, which is transcriptionally inactive in these conditions, in contrast to full-length p300 as a Gal4 hybrid which is a very potent transcriptional activator, was used as the "bait" (Figure 5-2d, compare track 2 to 7). For the "prey", I fused wild-type p53 to VP16 which, when expressed with the p300 hybrid, caused a significant increase in the transcriptional activity of the reporter pG5-luc (Figure 5-2d, compare track 7 to 9). Transcription was dependent upon the hybrid p53-VP16 protein since neither p53 nor VP16 caused a similar increase (Figure 5-2d, compare tracks 8, 9 and 10).


a)
G4-p300
– – + + : α Gal4
luc p53 luc p53 luc p53 : IVT
1 2 3 4 5 6
b)
G4-p300
M – p53 p53-VP16
92
68
46
p53
1 2 3 4
c)
IPα421/IBαHA
IBαHA
IBα421
HA-p300C – – + – – + – – +
HA-p300N – + – – + – – + +
p53 + + + + + + + + +

d)

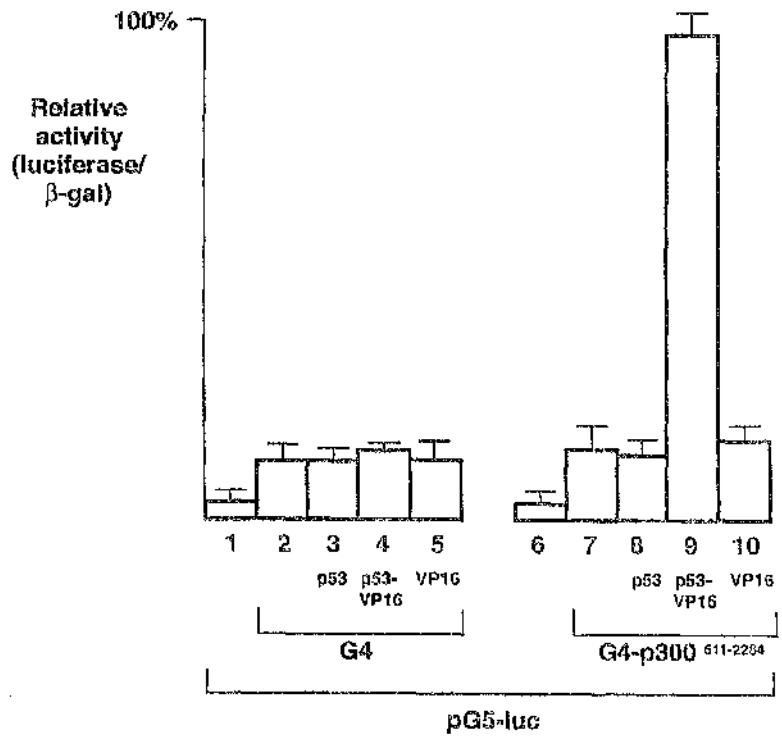

100%
Relative activity (luciferase/ β-gal)
1 2 3 4 5 6 7 8 9 10
p53 p53-VP16 VP16
p53 p53-VP16 VP16
G4
G4-p300 611-2284
pG5-luc

Figure 5-2

p300 physically associates with p53.

a) The expression vector encoding G4-p300$^{611-2284}$ was introduced into U2OS cells and after 48h harvested as described. Either *in vitro* translated ^{35}S methionine-labelled luciferase (tracks 1 and 3) or p53 (tracks 2 and 4) was added to the extract, thereafter immunoprecipitated with the anti-Gal4 antiserum and further resolved by gel electrophoresis. Tracks 5 and 6 show the input *in vitro* translates for luciferase (track 5) or p53 (track 6).

b) The expression vector encoding G4-p300$^{611-2284}$ (tracks 2, 3 and 4) together with either p53 (track 3) or p53-VP16 (track 4) was introduced into U2OS cells and after 48h immunoprecipitation performed with the anti-Gal4 antiserum (tracks 2, 3 and 4) followed by gel electrophoresis. Immunoblotting was performed with the p53 monoclonal antibody 421. Note that the common band present across tracks 2, 3 and 4 results from non-specific activity for the immunoglobulin used in the first immunoprecipitation which obscures the wild-type p53 polypeptide in track 3. Track 1 shows the standard molecular weight polypeptides. The p53-VP16 polypeptide is indicated.

c) The wild-type p53 expression vector together with either HA-tagged p300$^{611-1572}$ or HA-tagged p300$^{1572-2284}$, designated as HA-p300 N or HA-p300 C respectively, was transfected into SAOS-2 cells and after 40h immunoprecipation performed with the antibody 421 followed by gel electrophoresis. Immunoblotting was performed with the anti-HA monoclonal antibody. Furthermore, immunoblotting analysis from same cell extracts, which were used in immunoprecipitation, was performed with either the anti-HA antibody or the antibody 421 to confirm the expression levels of each transfected plasmid.

d) Two-hybrid assay in U2OS cells where the Gal4 reporter pG5-luc was introduced together with expression vectors pG4 encoding either the Gal4 DNA binding domain (1μg; tracks 2, 3, 4 and 5) or pG4-p300$^{611-2284}$ (1μg; tracks 7, 8, 9 and 10) together with either wild-type p53 (5μg; tracks 3 and 8), p53-VP16 (5μg;

tracks 4 and 9) or VP16 (5μg; tracks 5 and 10). The data presented reflect averages from two sets of values. Throughout pCMV-βgal was included within each transfection as an internal control, and the relative activity of luciferase to β-galactosidase presented.

Importantly, the effect of p53-VP16 required p300 protein sequence as there was no apparent effect on the Gal4 DNA-binding domain alone (Figure 5-2d, compare tracks 2 to 5). Similar data from the two-hybrid assay were observed in SAOS-2 cells.

An immunoprecipitation strategy was used to determine if E2F-1 and DP-1 associate with p300. After immunoprecipitating G4-p300$^{611-2284}$, immunoblotting was performed with antisera against either E2F-1 or the haemagglutin (HA) epitope to detect the DP-1 protein. Both E2F-1 and DP-1 could associate with p300 since either protein co-immunoprecipitated with G4-p300$^{611-2284}$ (Figure 5-3a, compare tracks 1 through 3 and 5 through 7).

I confirmed the interaction by taking the two-hybrid approach in which G4-p300$^{611-2284}$ was assessed for an interaction with GAD-E2F-1, a construct in which the Gal4 *trans* activation domain was fused to E2F-1. There was a significant increase in activity when pG4-p300$^{611-2284}$ and GAD-E2F-1 were expressed together (Figure 5-3b, compare tracks 6 and 8). The enhanced transcription was specific for the p300 (Figure 24b, compare tracks 4 and 8) and E2F-1 (Figure 5-3b, compare tracks 8 and 9) sequence in each hybrid protein. The overall conclusion from the biochemical and two-hybrid based assays strongly suggests that p53 and E2F-1 can form a stable physical complex with p300.

To confirm the interpretation from the earlier experiments, namely, that the *trans* activation domain of p53 and E2F-1 is a target for p300, I studied in the two-hybrid assay the interaction between a mutant p53 protein which lacked the amino-terminal *trans* activation domain, p53Δ159-VP16, and a mutant E2F-1 protein which lacked the carboxy-terminal *trans* activation domain, E2F-1Δ413-VP16. Neither of the mutant hybrid proteins were capable of interacting with pG4-p300$^{611-2284}$ compared to the wild-type hybrid protein (Figure 5-3c, compare tracks 3 and 4, and 5 and 6); similar two-hybrid assay results were obtained from SAOS-2 cells. Thus, I conclude that the interaction of p300 with either p53 or E2F-1 is mediated through binding to the *trans* activation domain.

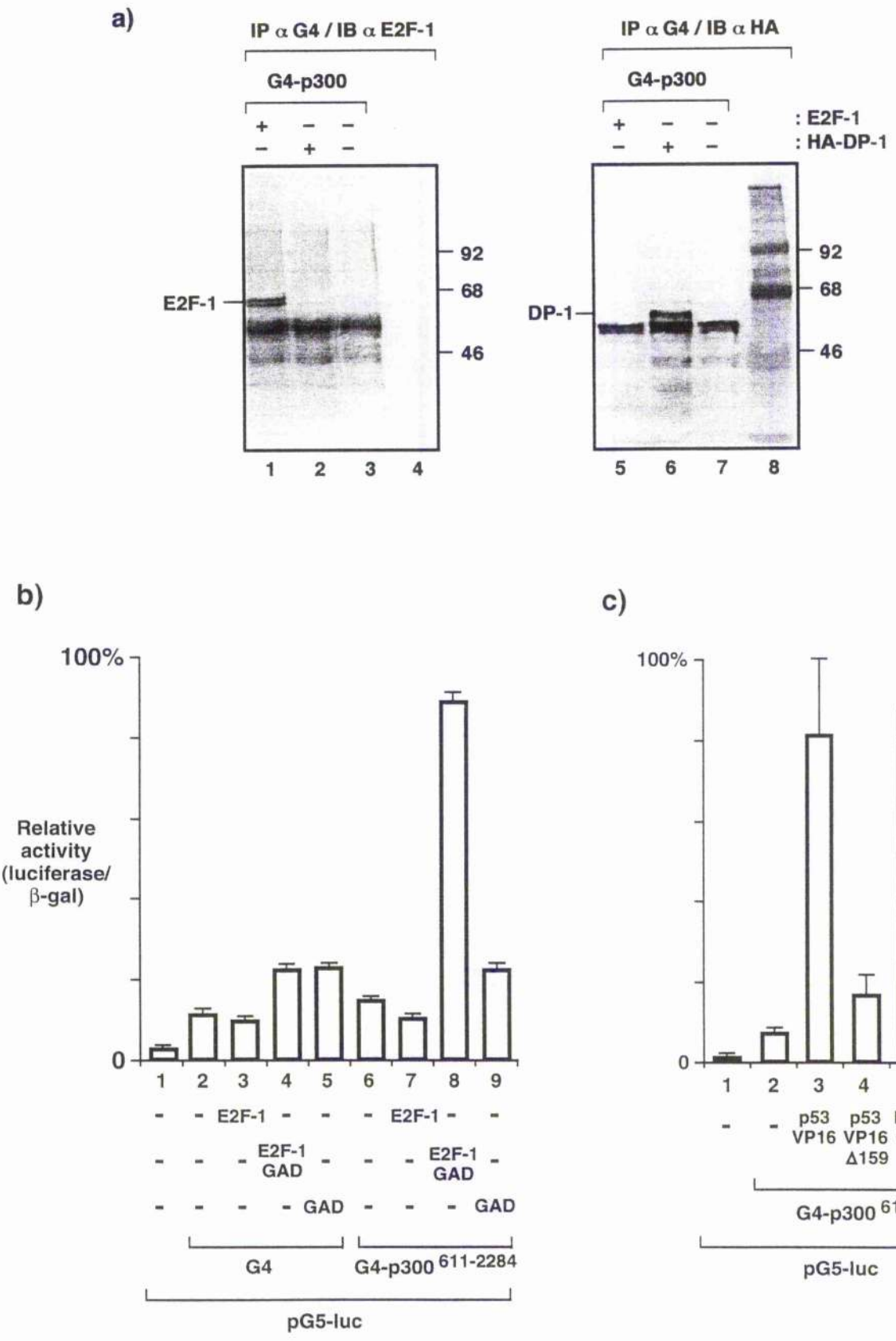

a)
IP α G4 / IB α E2F-1
G4-p300
+ – –
– + –
E2F-1
92
68
46
1 2 3 4
IP α G4 / IB α HA
G4-p300
+ – –
– + –
: E2F-1
: HA-DP-1
DP-1
92
68
46
5 6 7 8
b)
100%
Relative activity (luciferase/ β-gal)
0
1 2 3 4 5 6 7 8 9
E2F-1
E2F-1 GAD
GAD
G4
G4-p300 611-2284
pG5-luc
c)
100%
0
1 2 3 4 5 6
p53 VP16
p53 VP16 Δ159
E2F-1 VP16
E2F-1 VP16 Δ413
G4-p300 611-2284
pG5-luc

Figure 5-3

p300 physically associates with E2F-1 and DP-1.

a) The expression vector encoding G4-p300$^{611-2284}$ (20 μg; tracks 1, 2, 3, 5, 6 and 7) together with either E2F-1 (20 μg; tracks 1 and 5) or HA-DP-1 (20 μg; tracks 2 and 6) were introduced into U2OS cells and after 48h immunoprecipitation performed with the anti-Gal4 antiserum followed by gel electrophoresis. Immunoblotting was performed with either anti-E2F-1 (tracks 1 through 4) or anti-HA (tracks 5 through 8) as described in Materials and Methods. Note that the common band present across tracks 1 to 3, and 5 to 7, results for non specific activity for the immunoglobulin used in the immunoprecipitation.

b) Two-hybrid assay in U2OS cells where the Gal4 reporter pG5-luc was introduced together with expression vectors pG4 (1 μg; tracks 2, 3,4 and 5) or pG4-p300$^{611-2284}$ (1 μg; tracks 6, 7, 8 and 9) together with either wild-type E2F-1 (5 μg; tracks 3 and 7), pGAD-E2F-1 (5 μg; tracks 4 and 8) or GAD (5 μg; tracks 5 and 9). The data presented reflect averages from two sets of values. Throughout pCMV-βgal was included within each transfection as an internal control, and the relative activity of luciferase to β-galactosidase presented.

c) Two-hybrid assay in U2OS cells where the reporter pG5-luc was introduced together with expression vectors for pG4-p300$^{611-2284}$ (1μg; tracks 2 through 6), p53-VP16 (5μg; track 3), p53Δ159-VP16 (5μg; track 4), E2F-1-VP16 (5μg; track 5) or E2F-1Δ413-VP16 (5μg; track 6). The data presented reflect averages from two sets of values, and throughout pCMV-βgal was included as an internal control.

5-2.3. p21$^{Waf1/Cip1}$ enhances p53-dependent transcription by the regulating the p53/p300 interaction.

The *Waf1/Cip1* gene is transcriptionally activated by p53, containing functional p53 binding sites within its transcriptional control region (El-Deiry *et al.*, 1993; Macleod *et al.*, 1995). It was of interest to test the idea that p21$^{Waf1/Cip1}$ may act in an autoregulatory fashion upon the p53/p300 interaction and thereby stimulate transcription. To assess this possibility, I used the two-hybrid assay between G4-p300$^{611-2284}$ and p53-VP16 (Figure 5-4a). Under conditions in which there was a clear interaction between G4-p300$^{611-2284}$ and p53-VP16 (Figure 5-4a, compare tracks 2 and 3), the levels of p21$^{Waf1/Cip1}$ were titrated and effects on transcription measured. As the level of p21$^{Waf1/Cip1}$ increased, there was a concomitant increase in the transcriptional activity of the pG5-reporter (Figure 5-4a, compare tracks 3, 4, 5 and 6). The effect of p21$^{Waf1/Cip1}$ was dependent upon the co-expression of p300 and p53 because there was only a marginal effect on G4-p300$^{611-2284}$ by p21$^{Waf1/Cip1}$ (Figure 5-4a, compare track 2 with 7, 8 and 9). These data indicate that co-expression of p21$^{Waf1/Cip1}$ enhances the activity of the p53/p300 interaction.

The conclusion that p21$^{Waf1/Cip1}$ enhances the interaction between p53 and p300 rests on the results from the two-hybrid assay. Therefore, it was necessary to demonstrate the effect of p21$^{Waf1/Cip1}$ on the activity of transcriptionally active wild-type p53. Increasing levels of p21$^{Waf1/Cip1}$ were introduced together with wild-type p53 and p53-dependent transcription assayed. In the presence of p21$^{Waf1/Cip1}$ there was a marked stimulation of p53 transcriptional activity (Figure 5-4b, compare tracks 2, 3, 4 and 5), an effect dependent upon the presence of wild-type p53 (Figure 5-4b, compare tracks 1 with 6, 7 and 8). I conclude that the expression of p21$^{Waf1/Cip1}$ causes an increase in the transcriptional activity of wild-type p53, and that a likely mechanism based on the earlier results is through potentiating the activity of the p53/p300 co-activator complex.

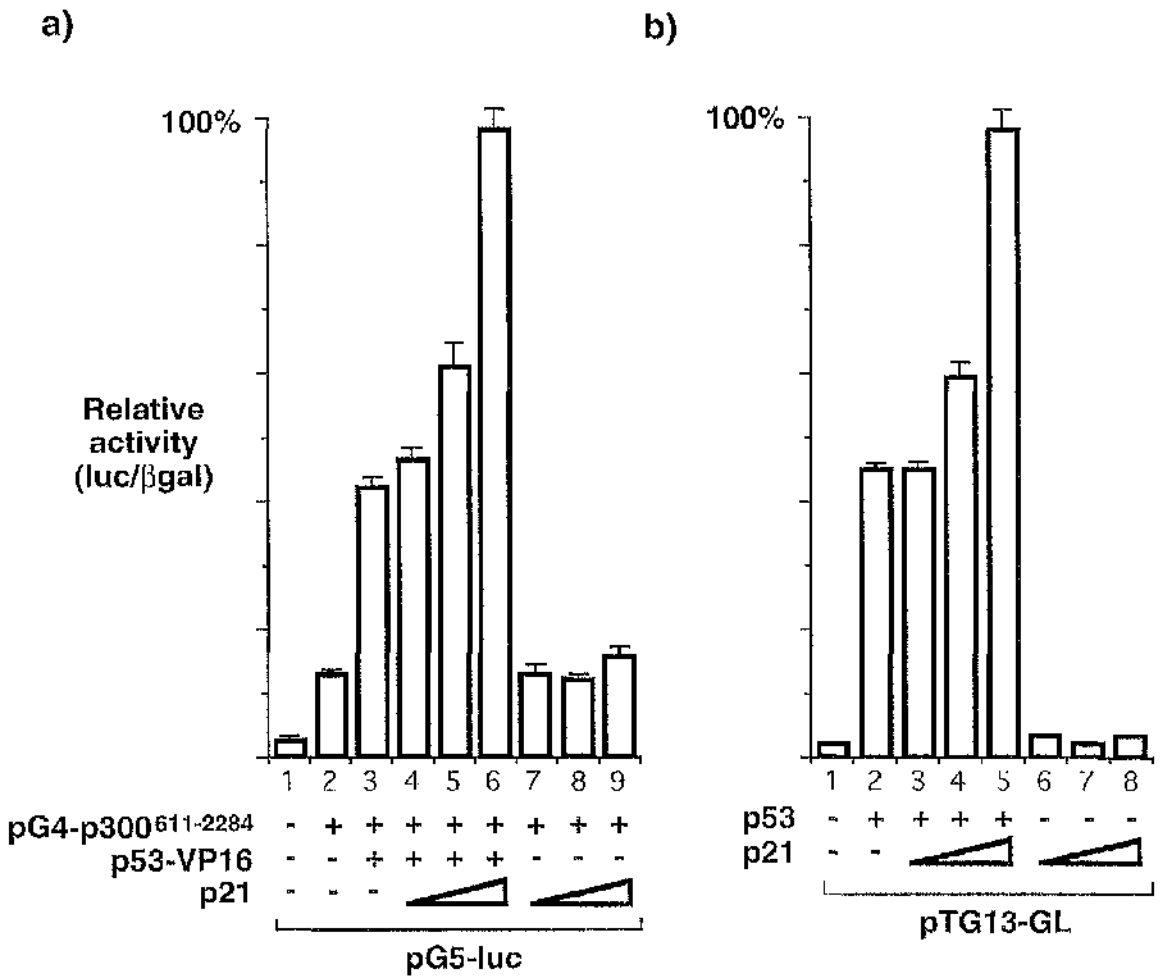

a)
b)
100%
Relative activity (luc/βgal)
1 2 3 4 5 6 7 8 9
pG4-p300611-2284 - + + + + + + + +
p53-VP16 - - + + + + - - -
p21 - - -
pG5-luc
100%
1 2 3 4 5 6 7 8
p53 - + + + + - - -
p21 - -
pTG13-GL

Figure 5-4

p21$^{Waf1/Cip1}$ enhances the activity of the p53/p300 complex and p53-dependent transcription.

a) Two-hybrid assay in SAOS-2 cells where the Gal4 reporter pG5-luc was introduced together with pG4-p300$^{611-2284}$ (1 μg; tracks 2 through 9) and p53-VP16 (5 μg; tracks 3 through 6) in the presence and increasing levels of p21$^{Waf1/Cip1}$ (1 μg in tracks 4 and 7, 5 μg in tracks 5 and 8 and 10 μg in tracks 6 and 9. The data presented reflect averages from two sets of values. Throughout pCMV-βgal was included within each transfection as the internal control, and the relative activity of luciferase to β-galactosidase presented.

b) The p53 reporter pTG13-GL was introduced into SAOS-2 cells together with expression vectors encoding p53 (1 μg; tracks 2 to 5) in the presence of increasing levels of p21$^{Waf1/Cip1}$ (1 μg in tracks 3 and 6, 5 mg in tracks 4 and 7 and 10 μg in tracks 5 and 8). Values were calculated as descibed in a).

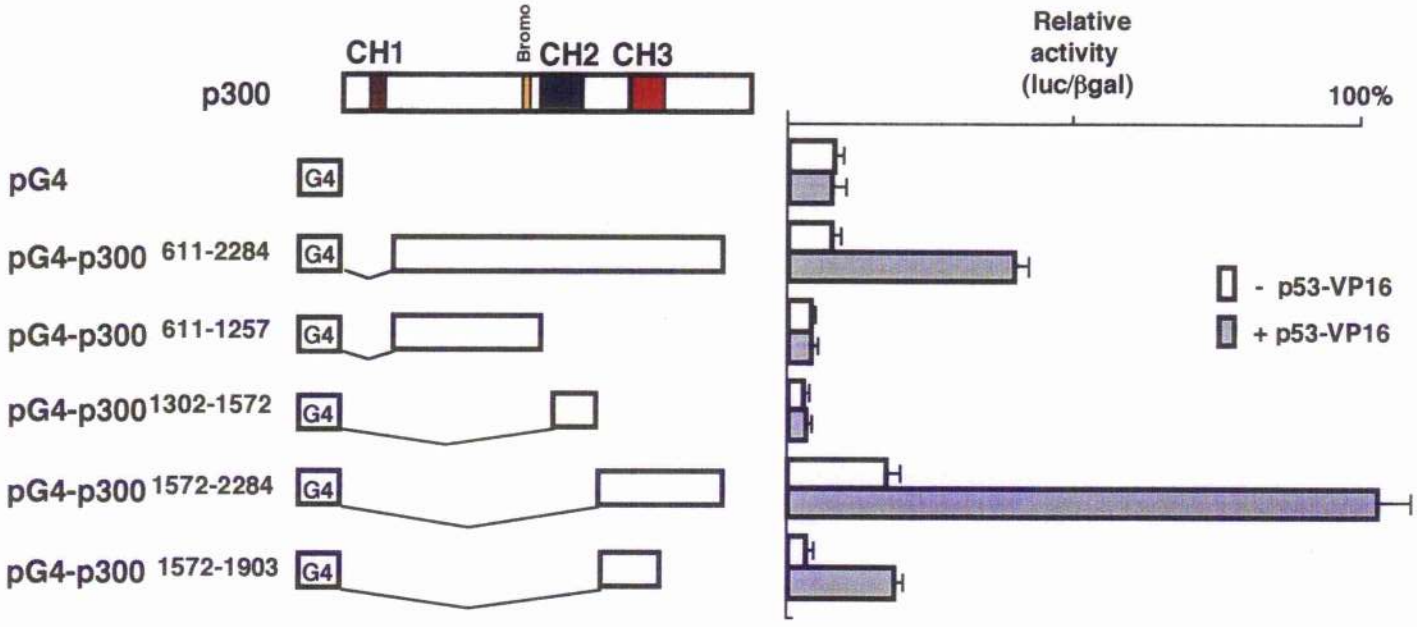
5-2.4. CH3 domain in p300 interact with p53.

To identify the region in p300 that physically interacts with p53, a panel of hybrid proteins were prepared in which different domains of p300 were fused to the Gal4 DNA-binding domain and co-expressed with the hybrid protein p53-VP16 (Figure 5-5a). The p53 interaction domain mapped to the carboxy-terminal region of p300, specifically within the sequence from residue 1572 to 1903, since co-expression of pG4-p300$^{1572-1903}$ with p53-VP16 caused a 6-fold induction of transcription (Figure 5-5a). A similar level of induction was apparent with pG4-p300$^{1572-2284}$ but not for pG4-p300$^{611-1257}$ or pG4-p300$^{1302-1572}$ (Figure 5-5a). Furthermore, in these conditions, all Gal4-p300 hybrid proteins expressed at similar level (Figure 5-5b). Thus, p300 contains a carboxy-terminal domain which is dedicated to a functional interaction with p53. Significantly, this region in p300 is known to bind to the E1A protein (Eckner *et al.*, 1996), suggesting that a competitive mechanisms may account for the inactivation of p53-dependent transcription by E1A through the sequestration of p300.

5-2.5. p300 is required for the activation of *Waf1/Cip1*.

To investigate the role of p300 in the regulation of cellular genes, I used the p53-responsive promoter taken from *Waf1/Cip1* (El-Deiry *et al.*, 1993). The expression of the adenovirus E1A protein in U2OS cells expressing endogenous p53 and p300 caused a significant reduction in the transcriptional activity of p53 (Figure 5-6a, compare tracks 1 and 2), consistent with the ability of E1A to sequester the endogenous of p300 (Arany *et al.*, 1995). In contrast, a mutant E1A protein that lacks the amino-terminal p300 binding domain (Stein *et al.*, 1990; Arany *et al.*, 1995) failed to reduce the transcriptional activity of *Waf1/Cip1* (Figure 5-6a, compare tracks 1 and 3), consistent with a role for p300 in regulating the

a)


CH1
Bromo
CH2
CH3
p300
pG4
pG4-p300 611-2284
pG4-p300 611-1257
pG4-p300 1302-1572
pG4-p300 1572-2284
pG4-p300 1572-1903
G4
Relative activity (luc/βgal)
100%
- p53-VP16
+ p53-VP16

b)

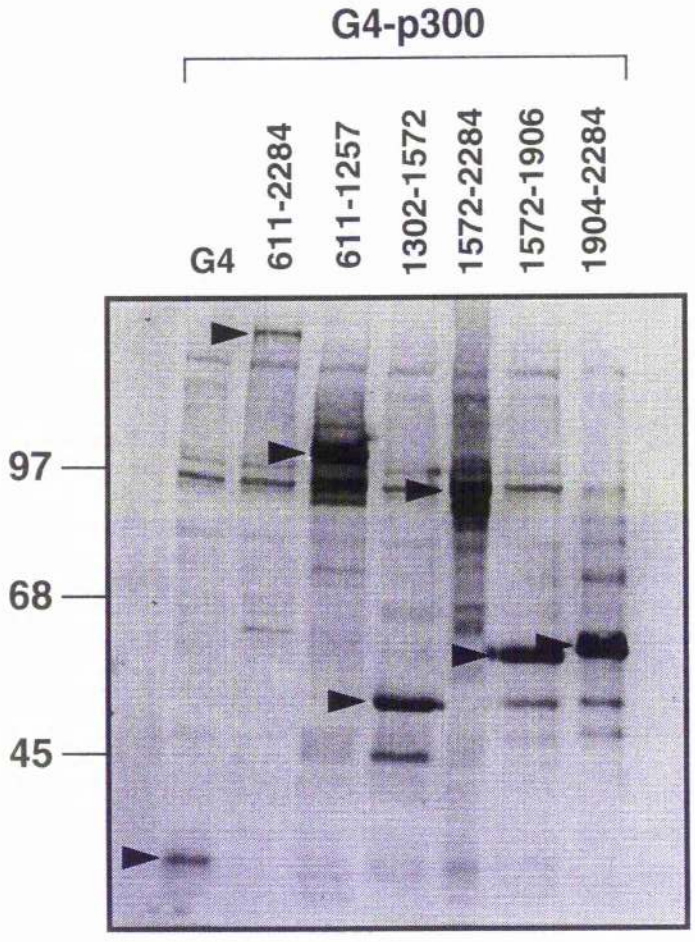

G4-p300
G4
611-2284
611-1257
1302-1572
1572-2284
1572-1906
1904-2284
97
68
45

Figure 5-5

Region of p300 required for the interaction with the *trans* activation domain of p53.

a) Diagrammatic representation of the functional region of p300 is shown in left side. The three cysteine/histidine-rich domain (CH1, CH2 and CH3) were indicated as shaded box. Each Gal4-p300 fusion plasmid (1μg) was transfected with or without p53-VP16 (4μg) into SAOS-2 cells. pCMV-βgal (2μg) was included in each transfection as an internal control. Cells were assayed for luciferase and β-gal activity 32~36h after transfection. The experiment were performed twice, and the results shown represent the average from two sets of value.

b) The expression of Gal4-p300 protein in transiently transfected SAOS-2 cells was determined by immunoblotting with anti-Gal4 monoclonal antibody. The arrows indicate Gal4-p300 hybrid proteins.

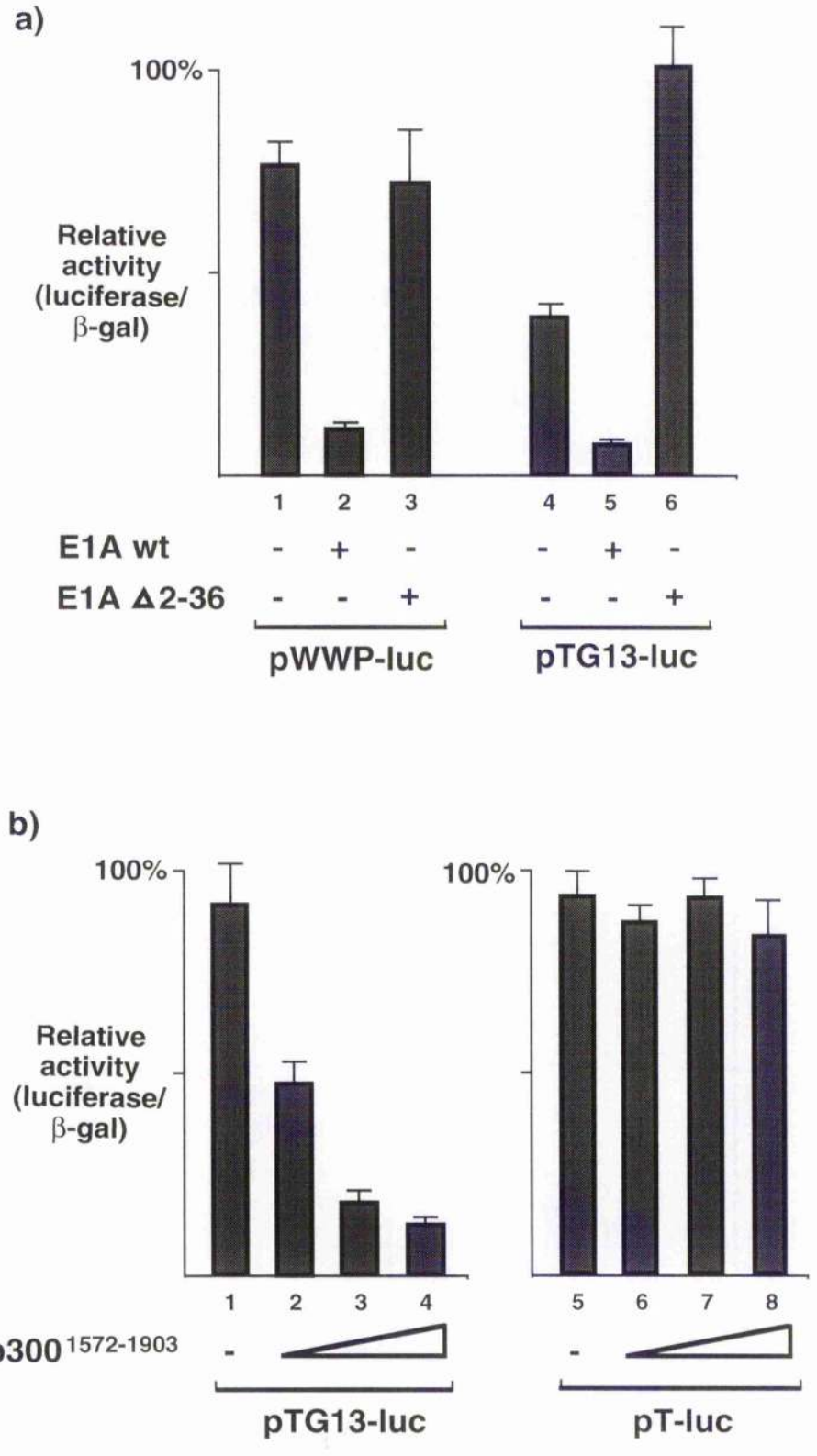

a)
100%
Relative activity (luciferase/ β-gal)
1 2 3 4 5 6
E1A wt - + - - + -
E1A Δ2-36 - - + - - +
pWWP-luc
pTG13-luc
b)
100%
100%
Relative activity (luciferase/ β-gal)
1 2 3 4 5 6 7 8
p300 1572-1903 - - -
pTG13-luc
pT-luc

Figure 5-6

p300 is required for the activation of p53 target genes.

a) **Effect of endogenous p300 on p53 transcriptional activity by adenovirus E1A.** 2μg of each reporter plasmid, pWWP-luc or pTG13-luc, was transfected together with either pCMV-E1A wild-type or pCMV-E1A Δ2-36 (0.5μg) into U2OS cells expressing endogenous p53 and p300. pCMV-βgal (1.5μg) was included in each transfection as an internal control. Cells were assayed for luciferase and β-gal activity 36~40h after transfection. The experiments were performed at least three times, and the results shown represent the average from two sets of value.

b) **Inhibition of p53 transcription by the dominant negative mutant of p300.** Expression plasmid encoding $p300^{1572-1903}$ with increasing amounts (2μg in tracks 2 and 6, 6μg in tracks 3 and 7, and 12μg in tracks 4 and 8) was transfected with either 2μg of pTG13-luc or pT-luc (the backbone reporter plasmid of pTG13-luc) into U2OS cells.

transcription of *Waf1/Cip1*. This result was confirmed with pTG13-GL containing the 13 copies of synthetic p53 binding site originally taken from $p21^{Waf1/Cip1}$ promoter in same experiment condition (Figure 5-6a, compare tracks 4, 5 and 6), suggesting that p300 plays a significant role in regulating the transcriptional activity of *Waf1/Cip1*.

To gain more information on the co-activator properties of p300 for p53-dependent transcription, I used $p300^{1572\text{-}1903}$ which contains the minimal regions of p53 binding and behaves in a fashion expected for a dominant-negative version of p300 (Figure 5-5b). This molecule might prevent the activation of p53-regulated promoters by interfering with complex formation between endogenous p53 and p300. The over-expression of $p300^{1572\text{-}1903}$ clearly inhibited the transcriptional activity of p53 in U2OS cells (Figure 5-6b, compare tracks 1 through 4), since this effect was no longer apparent with pT-luc, which was used as a control reporter containing only a minimal *tk* promoter placed upstream of the *luciferase* (Figure 5-6b, compare tracks 5 through 8). These results suggest that $p300^{1572\text{-}1903}$ likely acts by competing with endogenous p300 for p53, and further that p300 is a crucial component of the p53 response.

5-2.6. Physiological consequences of p300 interacting with p53 and E2F.

The induction of transcription of the gene encoding $p21^{Waf1/Cip1}$ by p53 correlates with cell cycle arrest by p53 (Chen *et al.*, 1996; El-Deiry *et al.*, 1993; Macleod *et al.*, 1995) and the expression of $p21^{Waf1/Cip1}$ is sufficient to prevent cell cycle progression (Dulic *et al.*, 1994; Waldman *et al.*, 1995). It was reasoned that the ability of p300 to interact with and enhance the transcriptional activity of p53 may facilitate cell cycle arrest.

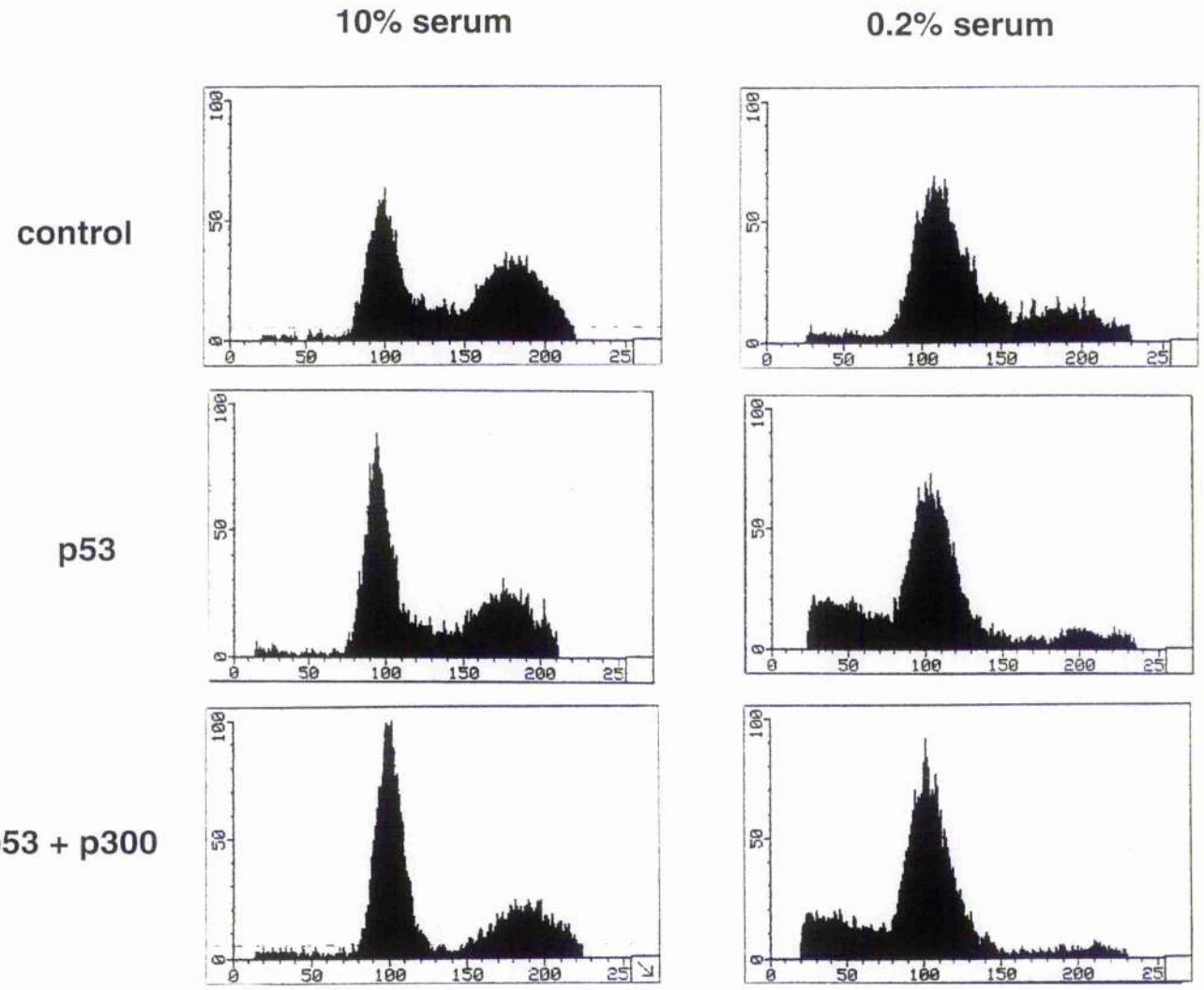
To test this idea, I introduced p53 into asynchronous cultures of SAOS-2 cells and monitored the proportion of transfected cells in G1, S and G2/M, both in

the presence and absence of co-expressed p300 (Figure 5-7a). Transfected cells were identified by the expression of the cell surface protein CD20 and subsequent staining with an anti-CD20 monoclonal antibody, and the percentage of cells in each phase of the cell cycle was measured after treatment with propidium iodide.

I performed the flow cytometry experiments in SAOS-2 where the introduction of p53 caused an accummulation of cells in G1, usually resulting in a significant increase in G1 population (Figure 5-7a); similar results were obtained in U2OS and 3T3 cells. Under these conditions p300 was expressed either alone or together with p53. When p300 was co-expressed with p53 there was a marked increase in the proportion of cells in G1, compared to p53 alone (Figure 5-7a and summarised in Table 5-1); p300 had little effect in the absence of p53. I conclude that p300 can facilitate cell cycle arrest by p53, and that a possible mechanism involves increased transcription of p53 target genes, such as the *Waf1/Cip1* gene, as a result of p300 enhancing the transcriptional activity of p53.

Next, the role of p300 in apoptosis was explored. For this, I used SAOS-2 or U2OS cells that had been cultured in serum starvation conditions, conditions that favour apoptosis (White, 1996). The data presented were derived from SAOS-2 cells, although very similar results were obtained from U2OS cells. Two assays were used to measure the level of apoptotic cells, namely TUNEL and flow cytometry (cells with sub-genomic levels of DNA). Data derived from TUNEL assays are shown (Figure 5-7b and summarised in Table 5-2), similar conclusions being made from the other assay (Figure 5-7a). In SAOS-2 cells, the introduction of wild-type p53 caused a significant level of apoptosis (Figure 5-7b and Table 5-2) consistant with previous reports (Chen *et al.*, 1996; Rowan et al., 1996). When p300 was assessed in the same cells, little difference was apparent from the untreated cells. Unexpectedly, the introduction of p53 together with p300 failed to increase the proportion of apoptosing cells but reduced this effect (Table 5-2). Thus, the binding of p300 to p53 in these experimental conditions appears to favour p53-mediated G1 arrest rather than apoptosis.

a)



b)

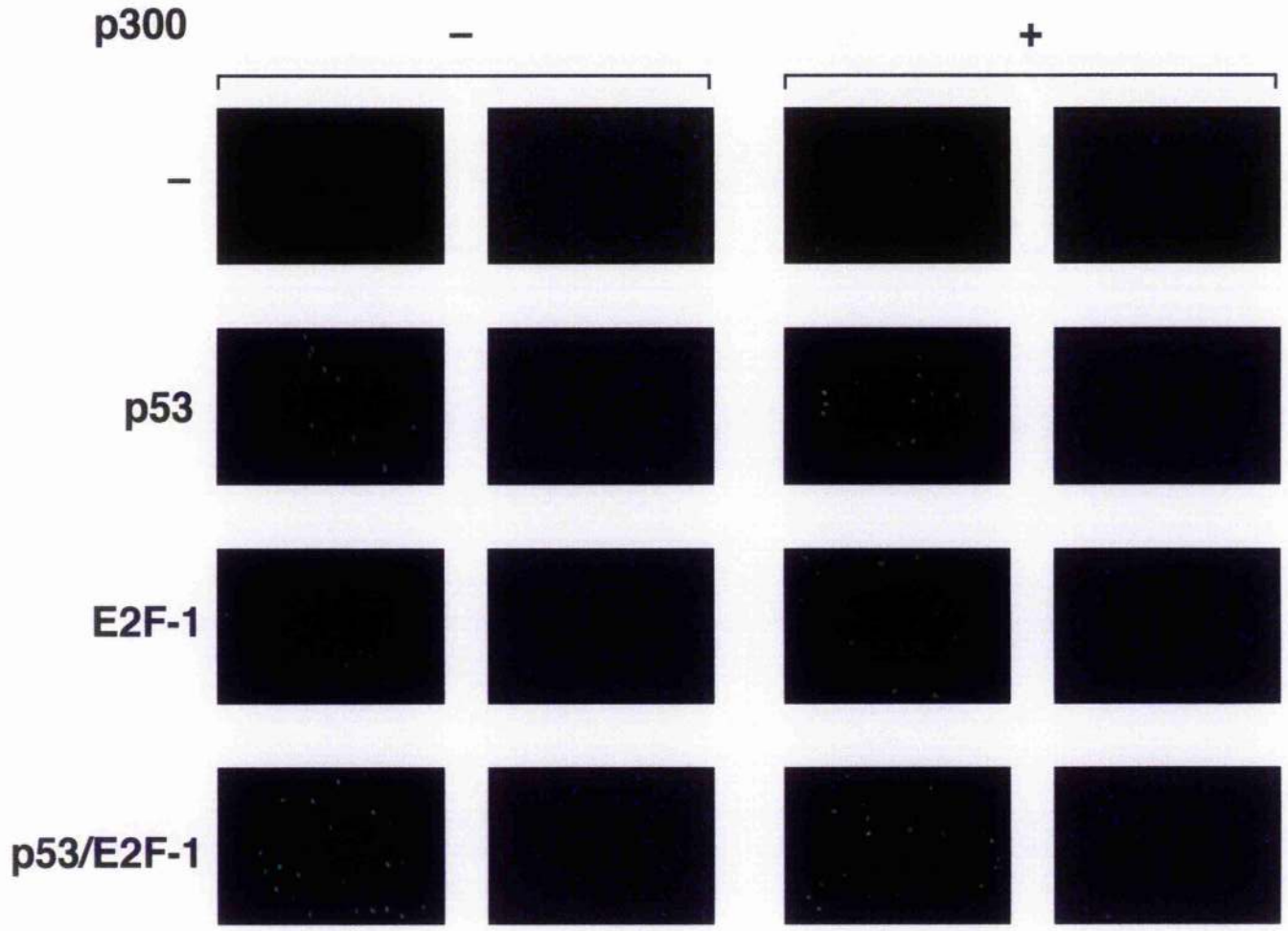


Figure 5-7

Effect of p300 on cell cycle arrest and apoptosis.

a) Flow cytometry was performed on asynchronous cultures of SAOS-2 cells transfected with the CD20 expression vector (10 μg) together with p53 alone or p53 and p300 together. 15 μg of each expression vector was introduced, the total amount of DNA for each treatment being equivalent and made up with empty vector (pcDNA3). Transfected cells were grown in either 10% (left panels) or 0.2% (right panels) foetal bovine serum and identified by staining with anti-CD20 and the cell cycle profile was assessed by staining with propidium iodide. The level of cell cycle arrest for each treatment is summarised in Table 5-1.

b) The effect of p300 on p53-dependent and independent apoptosis was assessed in both SAOS-2 and U2OS cells transfected in conditions of serum starvation (see Materials and Methods) as indicated with 4μg of p53 and/or E2F-1 expression vector in the absence or presence of the p300 expression vector, the total amount being made up with empty vector. Results for SAOS-2 cells are shown when assayed by TUNEL (left hand side of each treatment) and, for comparison, a DAPI stain of the same field of cells. The level of apoptosis for each treatment is summarised in Table 5-2.

Table 5-1. Effect of p300 on p53-mediated cell cycle arrest.

Treatment	% change *		
	G1	S	G2/M
p300	-2.9±0.2	7.3±2.7	4.1±1.1
p53	19.0±3.0	-47.7±2.0	-18.6±1.7
p53 + p300	38.1±4.5	-62.3±5.4	-50.4±7.6

A summary of the level of growth arrest caused by each treatment in SAOS-2 cells. The data shown represent the average of three different readings from the same treatment.

* The percentage change compared to the control treatment (CD 20 expression vector alone) is shown.

Table 5-2. Effect of p300 on apoptosis.

Treatment	Percent apoptotic cells (TUNEL/DAPI)		
	Exp 1	Exp 2	Exp 3
-	1.6(%)	2.8(%)	2.0(%)
p300	2.2	2.9	2.5
p53	17.1	18.5	11.8
p53/p300	11.8	16.6	10.1
E2F-1	7.5	-	6.8
E2F-1/p300	19.6	-	14.2
p53/E2F-1	29.2	-	21.2
p53/E2F-1/p300	23.6	-	-

A summary of the level of apoptosis caused by each treatment in SAOS-2 cells. The percentage of apoptotic cells was determined by counting the number of TUNEL positive cells compared to the total cells examined from DAPI staining. The values indicated represent the average of readings made on three separate coverslips from the same treatment in which about 10 different fields for each coverslip were assessed. The expression of transfected plasmids was confirmed and the transfection efficiency established by indirect immunofluorescence.

I went on to study the effect of E2F-1 in SAOS-2 cells. As it was shown previously (Hass-Kogan *et al.*, 1995; Krek *et al.*, 1995; Shan *et al.*, 1996), E2F-1 when expressed alone caused significant levels of apoptosis (Figures 3-2 and 5-7b and Table 5-2) indicating that the apoptotic activity of E2F-1 is independent of p53. In these conditions, the apoptotic activity of E2F-1 was enhanced by p300 (Figure 5-7b and Table 5-2), in contrast to the effect of p300 on p53 where it increased the efficiency of p53-dependent cell cycle arrest. Further, a clear stimulation in the proportion of apoptotic cells was apparent when E2F-1 and p53 were expressed together (Figure 5-7b and Table 5-2), a result which corroborates earlier reports (Qin *et al.*, 1994; Wu and Levine , 1994). That limiting levels of p300 may inpart be responsible for this enhanced apoptosis was suggested from introducing p300 into cells undergoing E2F-1/p53-dependent apoptosis, upon which the level of apoptosis was significantly reduced (Figure 5-7b and Table 5-2). Interestingly, this observation indicates that the binding of p300 to p53 favours p53-mediated cell cycle arrest whilst the binding of p300 to E2F-1 favours apoptosis.

5-3. Conclusion.

The p300 protein, which possesses the properties of a transcriptional co-activator and is involved in regulating the activity of a variety of sequence specific transcription factors (Shikama *et al* ., 1997), overcame the down-regulation of p53 and, moreover, enhanced the activity of both E2F-1 and p53-dependent transcription, an effect dependent on a physical association between the *trans* activation domain of each protein and p300. Furthermore, I showed that the cdk-inhibitor $p21^{Waf1/Cip1}$ autoregulates in a positive fashion transcription through modulating the activity of the p53/p300 complex, whilst negatively regulating the activity of E2F by preventing cdk-dependent phosphorylation of pRb. My conclusions relating to the biological significance of the interaction between p53 and

p300 suggested that p300 augments the ability of p53 to cause G1 arrest in consistent with a role for p300 as a p53 co-activator in the transcription of *Waf1/Cip1*. In addition, I also demonstrated that p300, which also functions as a co-activator for E2F/DP heterodimer, enhances the E2F-1-induced apoptotic activity. Thus, a functional interaction between p300 either p53 or E2F-1 has a profound impact on early cell cycle progression, specifically in regulating the contrasting outcomes of cell cycle arrest and apoptosis. These results suggest a critical role for p300 in integrating and co-ordinating the functional interplay between the pathways of growth control mediated by E2F and p53.

Chapter 6.

JMY, a new co-activator for p300, regulates the p53 response.

6-1. Introduction.

To elucidate the mechanisms of transcriptional activation by p300/CBP, it is possible that additional control may be exerted through proteins that physically interact with and regulate the activity of p300/CBP. Using yeast two-hybrid with pLexA-p300$^{611-2284}$, Shikama *et al.* (1998) screened and identified a new p300 binding protein, called JMY (junction-mediating and regulatory protein). The sequence of JMY possesses a number of interesting features, such as a cluster of potential phosphorylation sites for S/T-P directed kinases including three consensus sites for cyclin-dependent kinases in the amino-terminal region, a central region which contains a motif that resembles conserved region 2 (CR2) in the adenovirus E1A protein, and a carboxy-terminal domain rich in proline residues (Figure 6-1). In this study, I explored several aspects of JMY biochemistry and function.

6-2. Results.

6-2.1. JMY forms a physical complex with p300.

To determine whether p300 and JMY can interact in mammalian cells, I co-transfected expression plasmids encoding pG-p300$^{611-2284}$ and HA-JMY in U2OS cells, immunoprecipitation with anti-Gal4 monoclonal antibody followed by immunoblotting with anti-HA monoclonal antibody (Figure 6-2a).

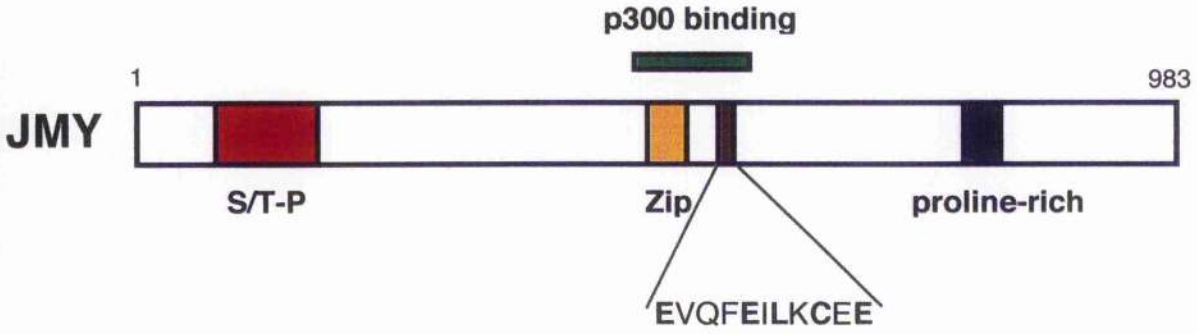

p300 binding
1
983
JMY
S/T-P
Zip
proline-rich
EVQFEILKCEE

Figure 6-1

Diagrammatic summary of functional domains in JMY

The primary amino acid sequence of JMY (983 amino acid residues). The S/T-P motifs in the amino-terminal region are underlined and the central p300 binding domain in JMY (from residue 469 to 558) is highlighted in small box. The hydrophobic residues that conform to a heptad leucine-rich repeat and the adenovirus E1A CR2-like motif, EVQFEILKCEE, are indicated in central region (residues in bold being conserved in E1A CR2). The proline-rich region is highlighted in carboxy-terminal.

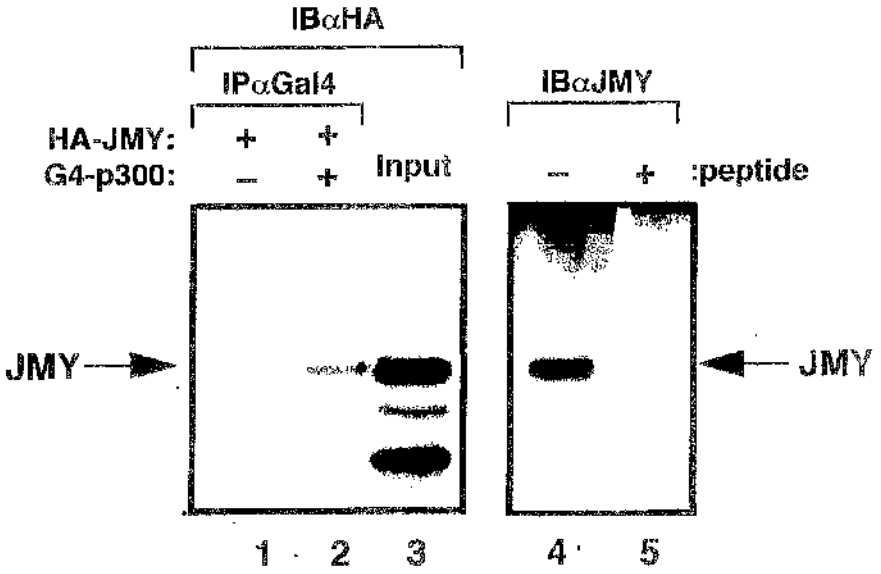
The 110kDa JMY polypeptide was detected in the p300 immunoprecipitation but not in the control treatment (Figure 6-2a, compare tracks 1 and 2), confirming that the interaction between the two proteins can occur in mammalian cells.

As the previous experiments were performed with cells expressing exogenous protein, I assessed whether endogenous JMY and p300 could associate in physiological conditions. For this, I used HeLa cells, which contain significant levels of the JMY polypeptide (Figure 6-2b), and determined whether JMY co-immunoprecipitated with p300. The endogenous JMY polypeptide could be detected in immunoprecipitates with endogenous p300 (Figure 6-2b), supporting the view that JMY and p300 associate in physiological conditions. Thus, JMY and p300 exist as a complex in mammalian cells.

6-2.2. Two domains in p300 interact with JMY.

The region in p300 that is responsible for the interaction with JMY was determined using a mammalian two-hybrid assay and a panel of p300 deletion mutants fused to the Gal4 DNA binding domain (Figure 6-3). The interaction between JMY and p300 occurred with two regions in p300, one encompassed within residues 611 to 1257, and the other within 1572 to 2284, because when JMY-VP16 was co-expressed with either G4-p300$^{611-1257}$ or G4-p300$^{1572-2284}$ the transcriptional activity of the reporter pG5-luc was far more efficient than that observed in the presence of the bait alone (Figure 6-3). Notably, although the intrinsic activity of G4-p300$^{1572-2284}$ was considerably greater than G4-p300$^{611-1257}$, the induction of activity by JMY-VP16 was similar, about 6-fold for either p300 derivative (Figure 6-3). Other regions of p300, such as residues 1302 to 1572, failed to interact with JMY-VP16 (Figure 6-3).

a)


IBαHA
IPαGal4
IBαJMY
HA-JMY: + +
G4-p300: – +
Input
– + :peptide
JMY
JMY
1 2 3 4 5

b)

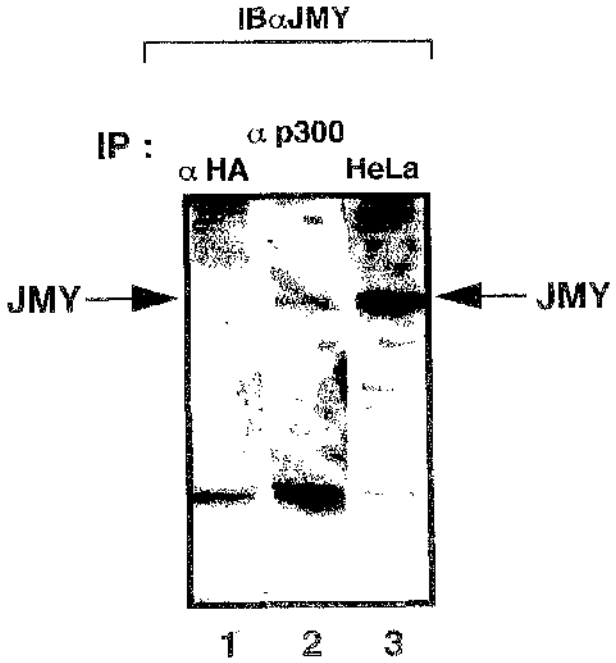
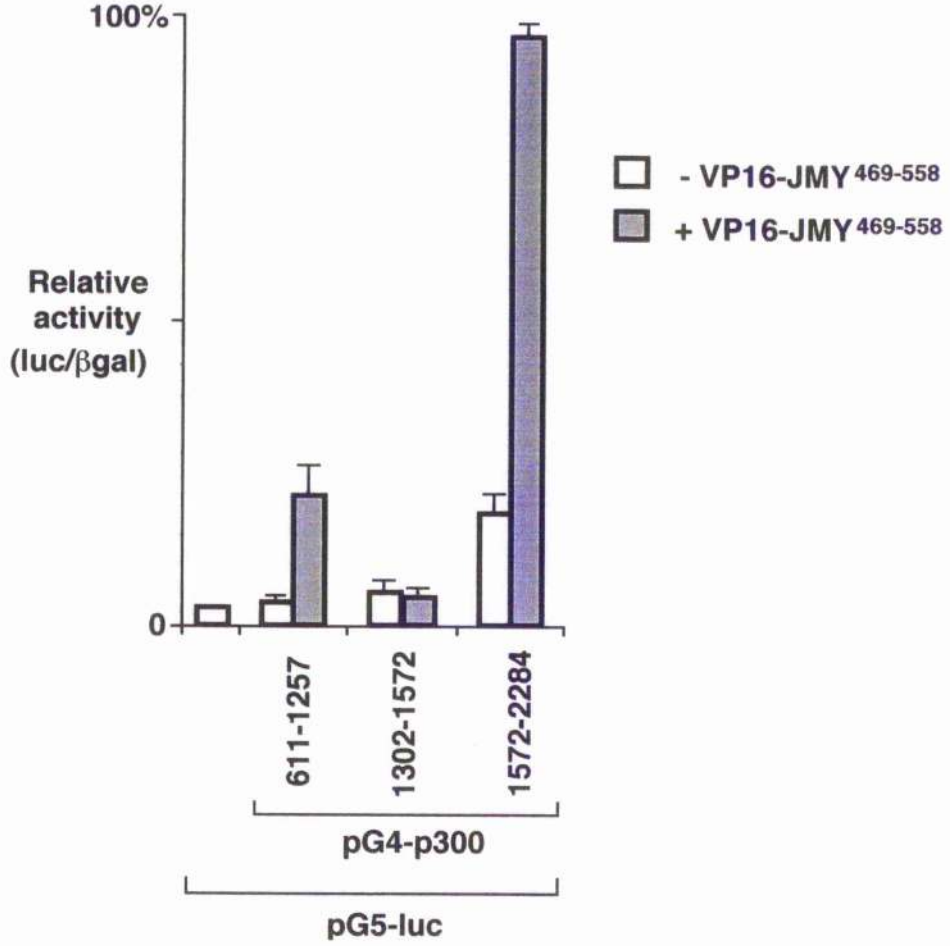

IBαJMY
IP : α p300
α HA
HeLa
JMY
JMY
1 2 3

Figure 6-2

JMY interacts with p300.

a) Co-immunoprecipitation of JMY and p300 from U2OS cells transfected with pG4 (30μg; track 1) or pG4-p300$^{611-2284}$ (30μg; track 2) together with pCMV-HA-JMY (30μg; tracks 1 and 2) as described. After extraction, immunoprecipitation was performed with anti-Gal4 monoclonal antibody followed by immunoblotting with anti-HA monoclonal antibody; the cell extract alone is shown in track 3. In tracks 4 and 5, the cell extract was immunoblotted with an anti-peptide JMY antibody in the absence (track 4) or presence (track 5) of competing homologous peptide. The JMY 110kDa polypeptide is indicated.

b) Immunoprecipitation of JMY with p300 was performed from HeLa cell extracts with the control anti-HA monoclonal antibody 12CA5 (track 1) or the anti-p300 monoclonal antibody Ab-1 (track 2) and thereafter immunoblotted with an anti-peptide JMY antiserum. Track 3 indicates the JMY polypeptide in the HeLa cell nuclear extract.


100%
Relative activity (luc/βgal)
0
- VP16-JMY 469-558
+ VP16-JMY 469-558
611-1257
1302-1572
1572-2284
pG4-p300
pG5-luc

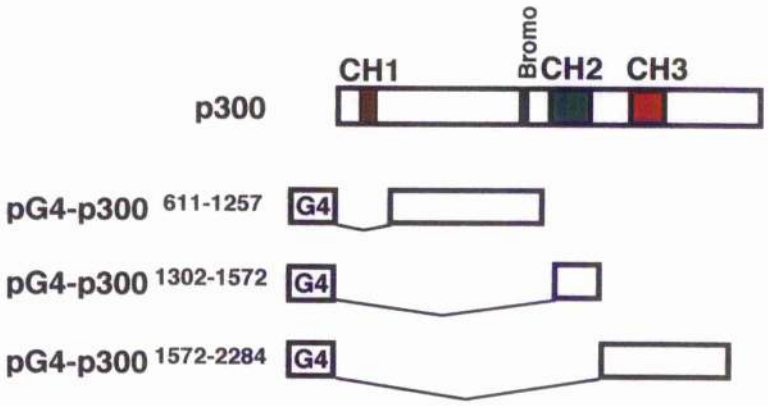

p300
CH1
Bromo
CH2
CH3
pG4-p300 611-1257
G4
pG4-p300 1302-1572
G4
pG4-p300 1572-2284
G4

Figure 6-3

JMY interacts with two domains in p300.

Two-hybrid assay in mammalian cells: the indicated pG4-p300 expression vectors (1μg) were introduced into U2OS cells either alone or together with pVP16-JMY$^{469-558}$ (5μg) and the reporter pG5-luc. The data shown represent the relative activity of luciferase to β-galactosidase and are the average of two treatments. Note that the activity of both pG4-p300$^{611-1257}$ and pG4-p300$^{1572-2284}$ undergoes a 6-fold increase in the presence of pVP16-JMY$^{469-558}$.

6-2.3. JMY possesses the properties of a transcriptional co-activator.

Next, the possibility that JMY possesses the properties of a transcriptional regulator was examined by studying the effect of JMY on p53, a transcription factor which is a known target for p300/CBP proteins (Lill *et al.*, 1997; Gu *et al.*, 1997; Avantaggiati *et al.*, 1997; Lee *et al.*, 1998). The transcriptional activity of p53 was assayed on the promoter taken from *bax* which encodes a protein that facilitates apoptosis and responds to p53 (Miyashita and Reed, 1995; Friedlander *et al.*, 1996). The *bax* promoter was efficiently induced in the presence of exogenous p53 in SAOS-2 cells (Figure 6-4a). p53-dependent transcription was increased by the co-expression of JMY, and a further enhancement occurred when p300 was co-expressed with JMY (Figure 6-4a). The stimulation of transcription by JMY and p300 was dependent upon the integrity of the amino terminal *trans* activation domain, as a p53 derivative containing an inactive *trans* activation domain, $p53^{22/23}$ (Lin *et al.*, 1994), was unresponsive to p300 and JMY (Figure 6-4a).

I obtained additional information on the co-activator properties of JMY by studying the effects of a truncated p300 molecule, namely $p300^{1572\text{-}1903}$, which behaves in a fashion expected for a dominant-negative effect (Figures 5-5, 5-6b and 6-4b). To study the influence of $p300^{1572\text{-}1903}$ on JMY, I employed the *bax* promoter where $p300^{1572\text{-}1903}$ caused a significant reduction in activity (Figure 6-4b, compare tracks 2 to 6 and 7). In conditions where JMY stimulated p53-dependent transcription the introduction of $p300^{1572\text{-}1903}$ caused a significant reduction in p53-dependent transcription (Figure 6-4b), thus supporting a requirement for p300 in transcriptional activation for JMY.

The data shown in Figure 6-4a support the idea that p300 and JMY increase the transcriptional activity of p53. However, it was possible that this occurred through altered protein levels. To assess this possibility, I measured p53, p300 and JMY in conditions where all three proteins were co-expressed.

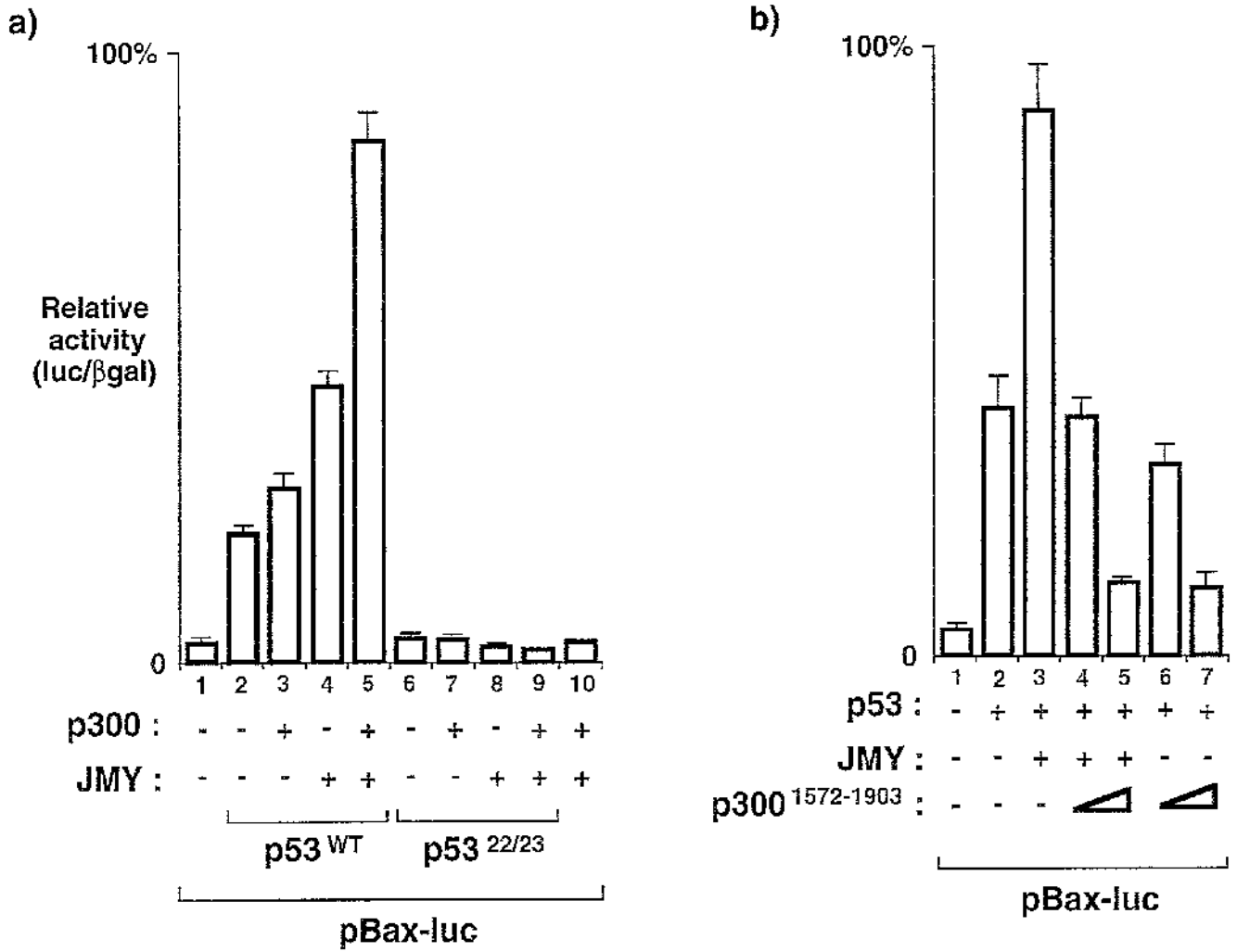

a)
100%
Relative activity (luc/βgal)
0
1 2 3 4 5 6 7 8 9 10
p300 : - - + - + - + - + +
JMY : - - - + + - - + + +
p53 WT
p53 22/23
pBax-luc
b)
100%
0
1 2 3 4 5 6 7
p53 : - + + + + + +
JMY : - - + + + - -
p300 1572-1903 : - - -
pBax-luc

Figure 6-4

JMY possesses the properties of a co-activator for p53.

a) The p53 reporter pBax-luc (0.5μg) together with expression vectors for wild-type p53 (0.05μg) or $p53^{22/23}$ (0.05μg), together with JMY (5μg) or p300 (2.5μg) were transfected into SAOS-2 cells as indicated. The values shown are the average of duplicate readings and represent the relative level of luciferase to the β-galactosidase activity from the internal control.

b) The p53 reporter pBax-luc (1μg) together with expression vectors for wild-type p53 (0.1μg), JMY (6μg) or $p300^{1572-1903}$ (2 or 6μg) were transfected into SAOS-2 cells as indicated. The values shown represent the average of duplicate readings and represent the relative level of luciferase to the β-galactosidase activity from the internal control.

Significantly, co-expression of either p300 or JMY failed to affect the level of p53 protein since by immunoblotting p53 was constant in conditions of increasing JMY or p300 concentration (Figure 6-5). A similar analysis on the levels of both p300 and JMY proteins indicated that, like p53, p300 and JMY levels were not affected by the co-expression of each other or p53 (Figure 6-6). These data therefore indicate that JMY possesses the properties of a co-activator and suggest that it acts together with p300/CBP proteins in facilitating transcriptional activation by p53.

Since in the conditions of the assay co-expression of p300 and JMY efficiently stimulated the activity of *bax* promoter, I tried to detect the levels of endogenous Bax protein. Importantly, the expression of JMY and p300 was sufficient to increase the activity of endogenous genes as co-expression of JMY and p300 with p53 in SAOS-2 cells caused an increase in the level of the Bax protein (Figure 6-7), which was not apparent when p300 was expressed alone.

The co-activator properties of JMY were assessed on other p53-responsive promoters, namely those taken from the *gadd45* (Kastan *et al.*, 1992; Chen *et al.*, 1995), *mdm2* (Momand *et al.*, 1992; Juven *et al.*, 1993) and *Waf1/Cip1* genes (El-Deiry *et al.*, 1993). In SAOS-2 cells, co-expression of JMY and p300 increased the activity of the *gadd45* and *Waf1/Cip1* promoters, in a similar fashion to JMY and p300 on the *bax* promoter, the effect being greater when they were expressed together (Figure 6-8). In contrast, neither exogenous JMY nor p300 could augment the activity of the *mdm2* promoter (Figure 6-8). These results strengthen the conclusion that JMY possesses the properties of a co-activator and, furthermore, imply that JMY preferentially activates some p53 target genes.

6-2.4. JMY regulates p53-dependent apoptosis.

To explore the biological consequence of co-activation by JMY, I evaluated the effect of JMY on wild-type p53 activity which, when induced by genotoxic

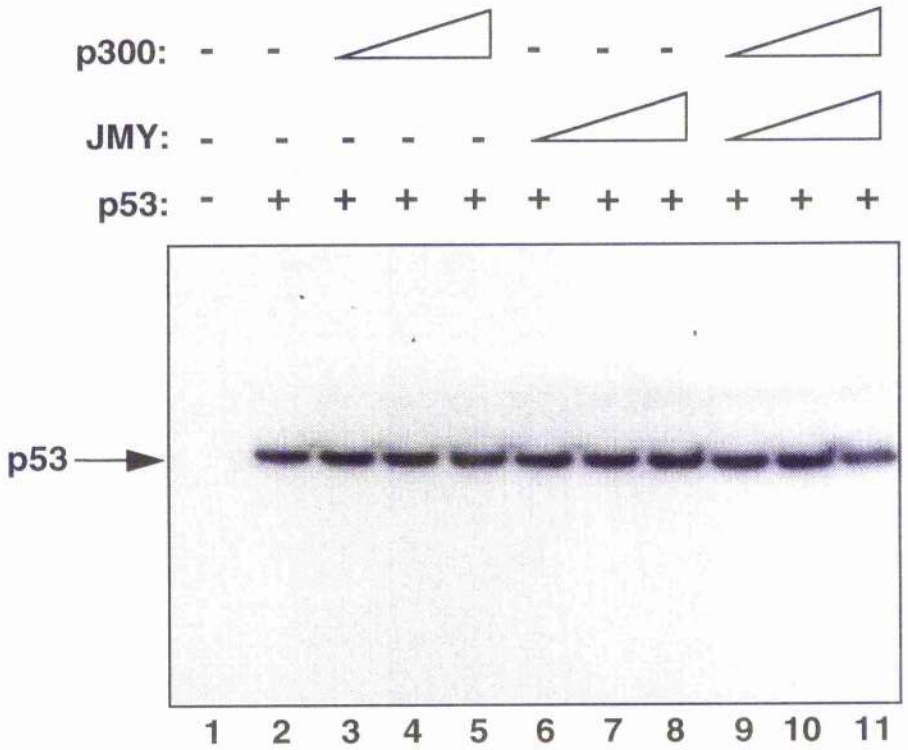

p300: - - - - - -
JMY: - - - - -
p53: - + + + + + + + + + +
p53
1 2 3 4 5 6 7 8 9 10 11

Figure 6-5

The protein expression level of p53 by p300 or JMY.

To assess the level of p53, expression vectors for wild-type p53 (0.3μg), JMY (1, 3 or 6μg) or p300 (1, 6 or 12μg) were transfected either alone or together as indicated into SAOS-2 cells. Extracts from control or transfected cells were prepared and immunoblotted for p53 as described. The p53 polypeptide is indicated.

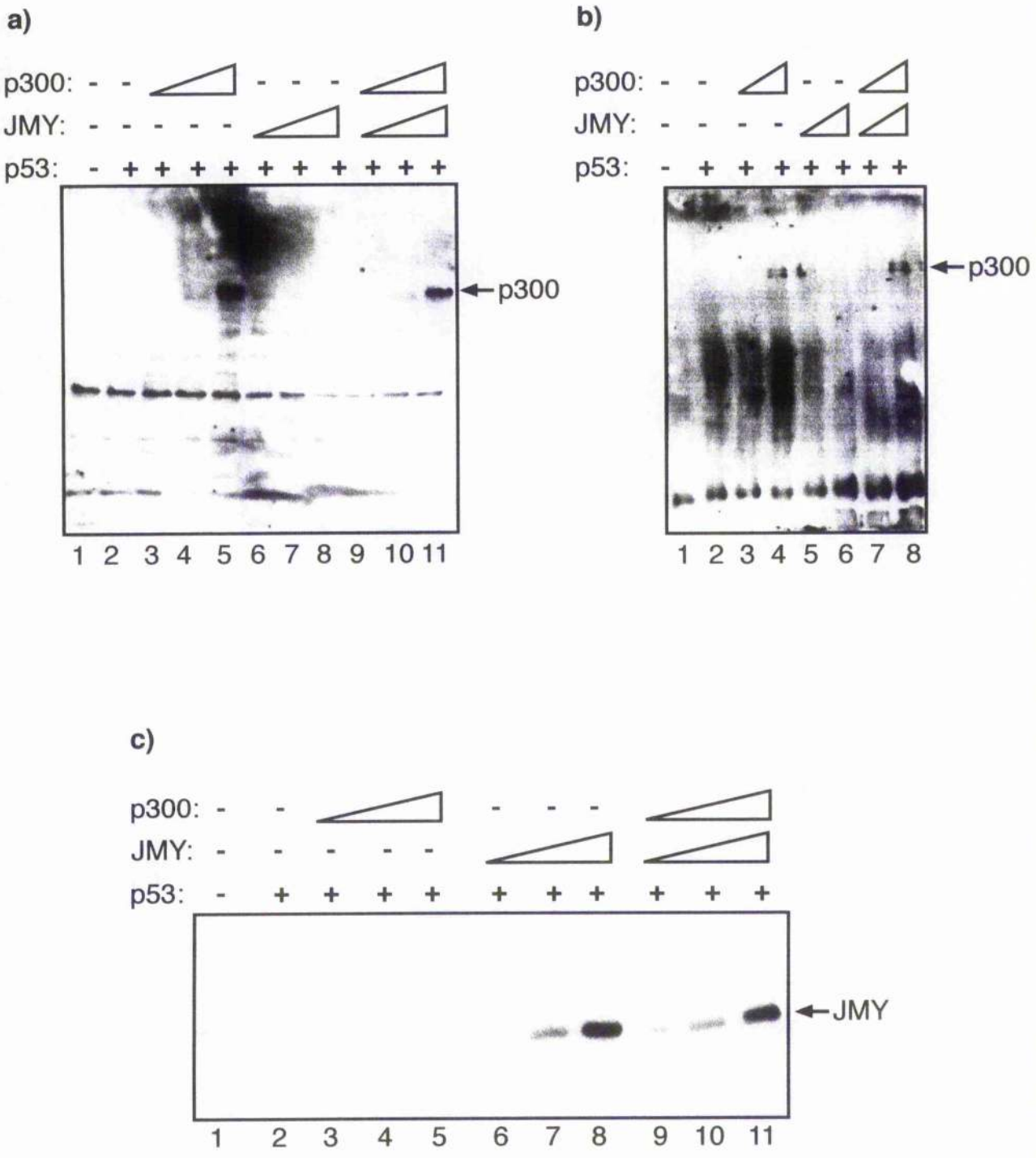

a)
p300: - - ▲ - - - ▲
JMY: - - - - - ▲ ▲
p53: - + + + + + + + + + +
p300
1 2 3 4 5 6 7 8 9 10 11
b)
p300: - - ▲ - - ▲
JMY: - - - - ▲ ▲
p53: - + + + + + + +
p300
1 2 3 4 5 6 7 8
c)
p300: - - ▲ - - - ▲
JMY: - - - - - ▲ ▲
p53: - + + + + + + + + + +
JMY
1 2 3 4 5 6 7 8 9 10 11

Figure 6-6

Levels of p300 and JMY

To assess the levels of p300 and JMY, expression vectors encoding p53 (0.3μg), JMY (1, 3 and 6μg in a), or 1 and 3μg in b)) or Gal4-p300 (1, 6 and 12μg in a), or 1 and 6μg in b)) were transfected into SAOS-2 cells as described. Extracts were prepared and in a) immunoblotted with an anti-Gal4 monoclonal antibody. In b), extracts were firstly immunoprecipitated with the anti-Gal4 monoclonal antibody and thereafter immunoblotted with anti-p300. The vector encoding Gal4-p300 contains the complete p300 protein. In c), expression vectors encoding p53 (0.3μg), JMY (1, 3 and 6μg) or p300 (1, 6 and 12μg) were transfected into SAOS-2 cells as described. Extracts were prepared and immunoblotted with an anti-JMY peptide antiserum.

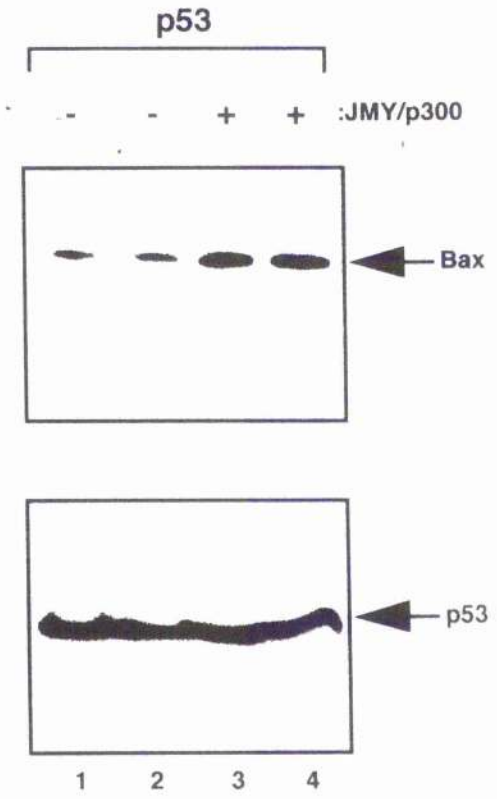

p53
- - + + :JMY/p300
Bax
p53
1 2 3 4

Figure 6-7

Induction of Bax protein by p300/JMY.

To assess the induction of endogenous Bax protein, expression vectors for wild-type p53 (10μg) with or without JMY (40μg) and p300 (40μg) were transfected as indicated into SAOS-2 cells. Extracts from transfected cells were prepared and immunoblotted for Bax and p53 as described. The Bax (upper panel) and p53 (lower panel) polypeptides are indicated.

stress can, in some circumstances, cause apoptosis (Crook *et al.,* 1994; Miyashita and Reed, 1995; Sabbatini *et al.,* 1995; Ko and Prives, 1996). For this analysis I used SAOS-2 cells which become sensitive to apoptosis upon the introduction of wild-type p53 (Chen *et al.,* 1996; Lee *et al.,* 1998). Although apoptosis was evident with p53 alone, co-expression of JMY with p53 significantly enhanced the level of cell death which was not apparent in the absence of p53, indicating that the process is dependent upon p53 (Figures 6-9a and 6-9b and Table 6-1). In contrast, however, in the same conditions p300 failed to affect the apoptotic activity of p53, although the level of apoptosis was significantly greater when JMY and p300 were co-expressed (Figure 6-9b and Table 6-1). Thus, in a similar fashion to its effects on transcription, JMY can augment p53-dependent apoptosis, and the induction of p53-dependent apoptosis caused by JMY can be enhanced by p300.

6-3. Conclusion.

p300/CBP transcriptional co-activators have been demonstrated to interact with a variety of sequence-specific transcription factors (Shikama *et al.*, 1997), and in many cases act to nucleate the assembly of a multi-component co-activator complex that co-ordinates and integrates diverse signals with gene expression (Korzus *et al.,* 1998; Kurokawa *et al.*, 1998). From the data presented here, I conclude that JMY, a newly identified 110kDa polypeptides, forms a physical complex with p300 *in vivo*. By analysing the effects of JMY and p300 on p53, I have obtained data to support the conclusion that p300 and JMY together can effect the transcriptional activation of known target genes, such as *bax*. Moreover, the data also showed that JMY and p300 functionally co-operate promoting p53-dependent apoptosis, a result consistent with a contribution by the p53 *trans* activation domain to apoptosis (Sabbatini *et al.*, 1995; Friedlander *et al.*, 1996; Ko and Prives, 1996)

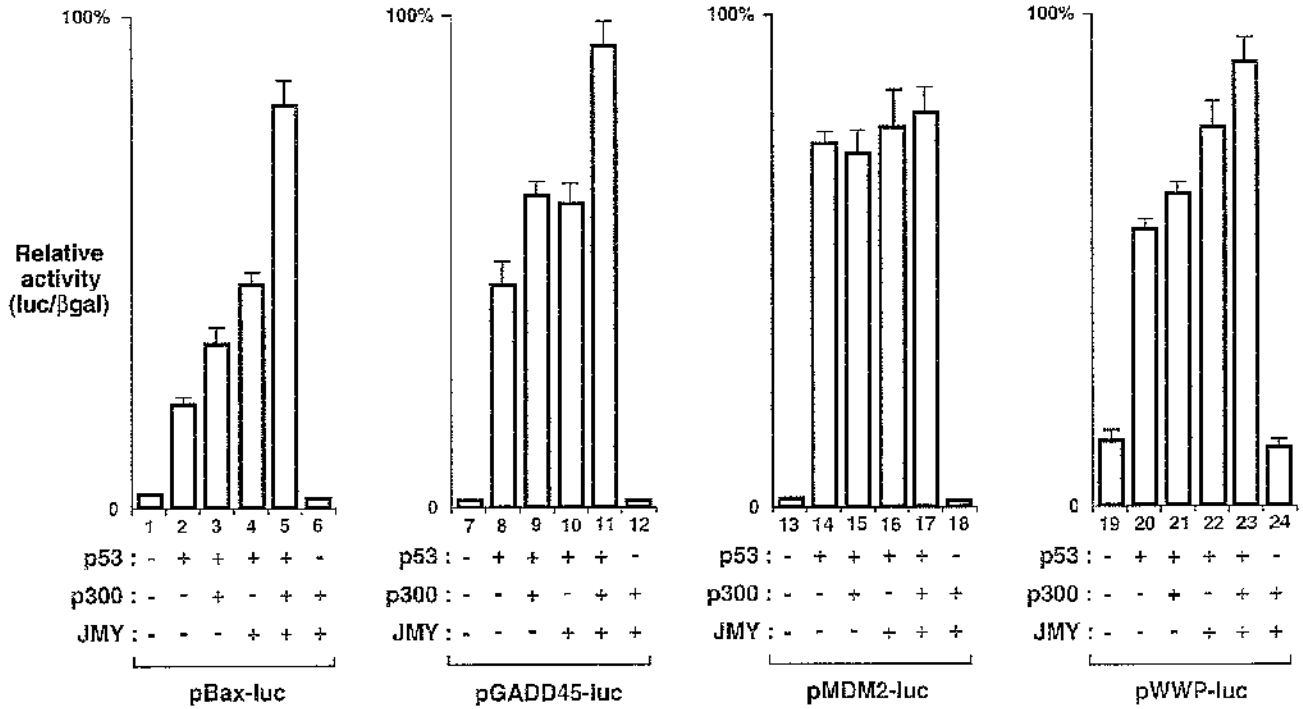
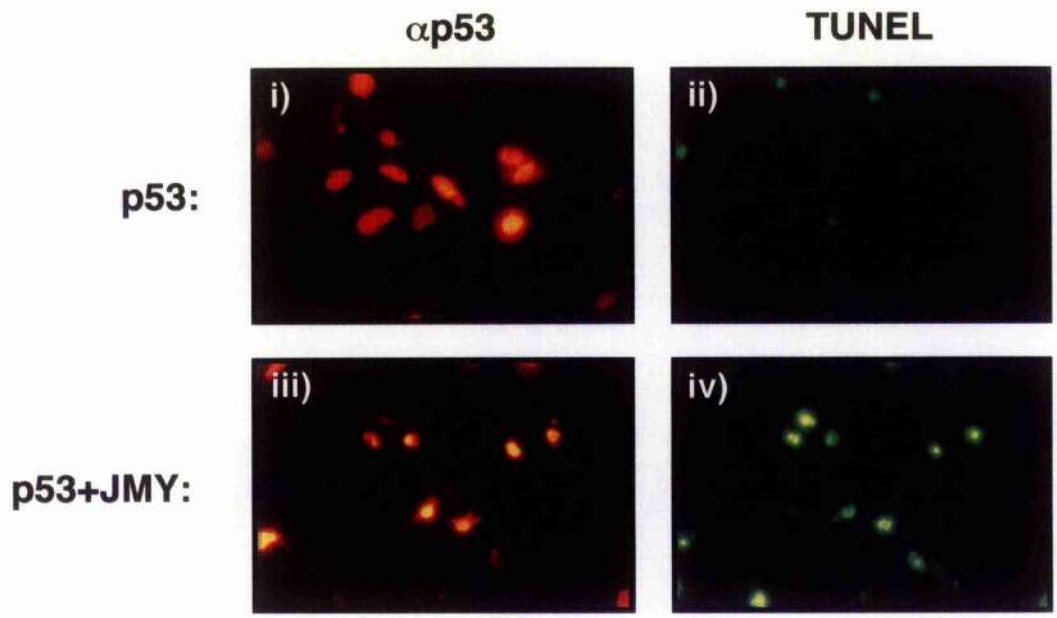

Relative activity (luc/βgal)
100%
0
1 2 3 4 5 6
p53 : - + + + + -
p300 : - - + - + +
JMY : - - - + + +
pBax-luc
7 8 9 10 11 12
pGADD45-luc
13 14 15 16 17 18
pMDM2-luc
19 20 21 22 23 24
pWWP-luc

Figure 6-8

JMY has selective effects on p53-target genes.

The p53 reporters pBax-luc, pGADD45-luc, pMDM2-luc or pWWP-luc (1.0μg) together with expression vectors for wild-type p53 (0.05μg), p300 (2.5μg) or JMY (5μg) were transfected into SAOS-2 cells as indicated. The values shown represent the average of duplicate readings and represent the relative level of luciferase to the β-galactosidase activity from the internal control.


αp53
TUNEL
p53:
i)
ii)
p53+JMY:
iii)
iv)

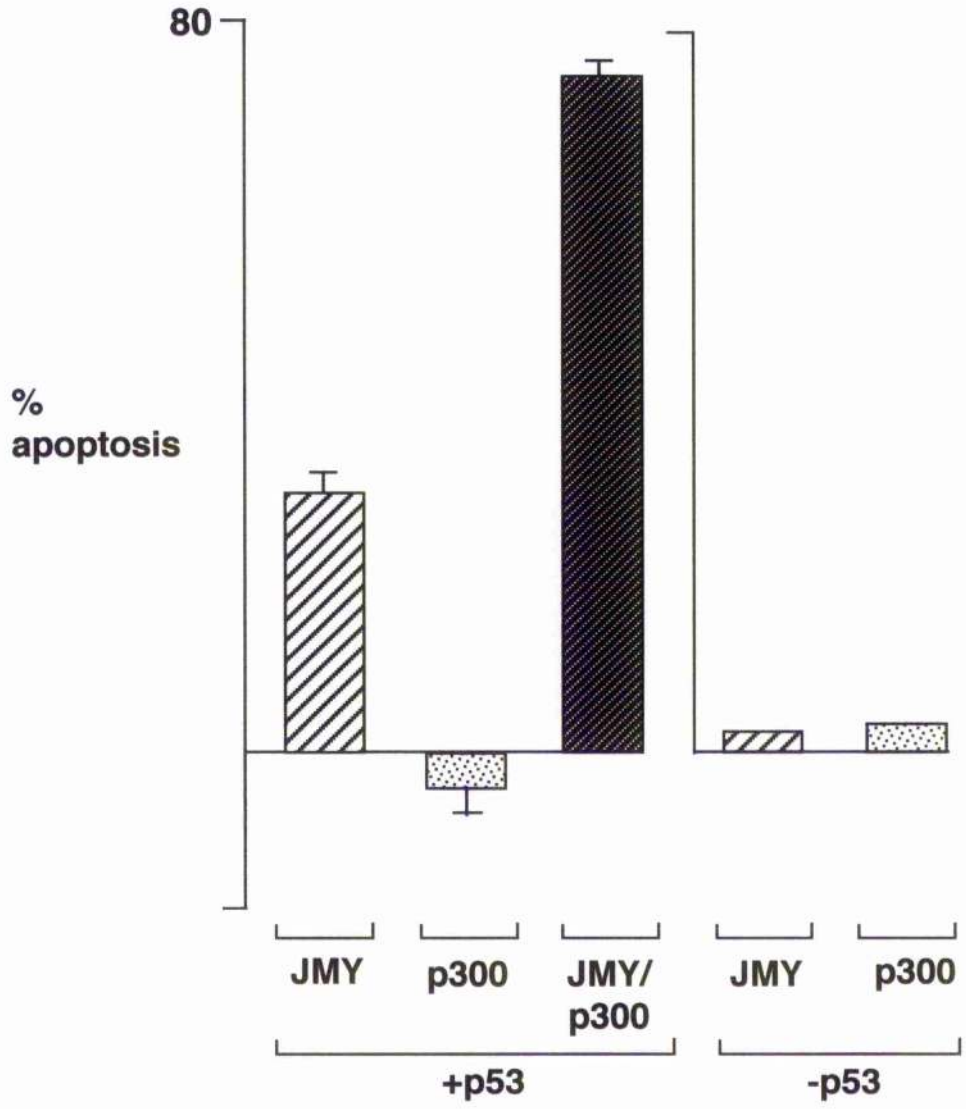

80
% apoptosis
JMY
p300
JMY/ p300
JMY
p300
+p53
-p53

Figure 6-9

JMY augments p53-dependent apoptosis.

a) Expression vectors for p53 either alone (i and ii) or together with JMY (iii and iv) were introduced into SAOS-2 cells as described. Cells were fixed and treated with the anti-p53 monoclonal antibody 421 (i and iii) or assayed for the level of apoptosis by TUNEL (ii and iv); i and ii, and iii and iv, show respectively the same field of cells assayed for p53 and by TUNEL.

b) Quantitative comparison of the effect on apoptosis in SAOS-2 cells caused by JMY, p300 or both in the presence or absence of p53. On the left side, the percentage of p53-positive (determined by monoclonal antibody 421) cells that were TUNEL-positive was derived, and compared to values obtained in the presence of JMY, p300, or both together. In the data, both the absolute percentage level of apoptosis together with the percentage stimulation in apoptosis relative to p53 alone is presented. The level of apoptosing cells represent, the average of three independent readings. The TUNEL-positive population compared to the number of DAPI-positive cells in the absence of p53 was used to assess the level of apoptosis in the presence of JMY and p300, and the values shown were obtained from two separate assays.

Table 6-1 Effect of JMY in apoptosis.

p53	Co-activator	Apoptosis (%)			
		Exp 1 • (TUNEL/DAPI)	Exp 2 (TUNEL/αp53)	Exp 3 (TUNEL/αp53)	Exp 4 (TUNEL/αp53)
+	-	12.4	32.2	17.7	52.5
+	JMY	18.6 (50.0)*	40.5 (25.8)*	23.1 (30.5)*	70.7 (34.7)*
+	p300	13.0 (4.8)*	31.9 (-0.9)*	16.9 (-4.5)*	-
+	JMY/p300	25.3 (104.1)*	53.5 (66.2)*	30.2 (70.6)*	-
-	JMY	2.1	-	2.2 •	-
-	p300	2.4	-	2.9 •	-
-	-	2.1	-	2.4 •	-

• TUNEL/DAPI

* numbers in parenthesis indicate % stimulation in apoptosis relative to wild-type p53

(The values indicated represent the average of readings made on three separate coverslips from the same treatment in which about 10 different fields for each coverslip were assessed, totalling at least 1,000 cells)

and thus with other studies implicating a role for p300/CBP in apoptosis (Muraoka *et al.*, 1996; Shikama *et al.*, 1997; Giles *et al.*, 1998). Given the fact that JMY significantly activated p53-responsive genes and induced p53-dependent apoptosis, it is therefore possible that JMY may be a direct target for p53 transcription. Thus, I have pursued the issue of whether JMY can interact directly with p53. However, I have not been successful in showing a direct interaction between JMY and p53 using mammalian two-hybrid assays and co-immunoprecipitations. Therefore, the data support the notion that JMY through its interaction with p300 is an important effector molecule in directing the cellular response to p53.

Chapter 7.
Discussion

7-1. E2F family members exhibit distinct cellular distributions and functional properties.

It is known that individual E2F activities display distinct effects on cell proliferation and survival, coincident with their differential abilities to activate a large array of endogenous genes that encode proteins important for DNA replication and cell cycle progression (Nevins, 1992; La Thangue, 1994; Muller, 1995). In particular, E2F-1, -2, and -3 can efficiently activite DNA synthesis in quiescent fibroblasts, E2F-4 does so only poorly and E2F-5 has little or no activity in S phase induction (Lukas *et al.*, 1996; DeGregori *et al.*, 1997). The distinct roles for E2F family members, separating E2F-1, -2 and -3 from E2F-4 and -5, are reflected in various properties of the E2F proteins including their different cellular distributions (de la Luna *et al.*, 1996; Allen *et al.*, 1997; DeGregori *et al.*, 1997).

By studying their intracellular distribution, it was possible to divide the E2F family into two distinct categories according to their ability to accumulate in nuclei. The first group, examplified by E2F-1, underwent efficient nuclear accumulation whereas the second, which includes E2F-4 and -5, failed to do so and remained, for the most part, in the cytoplasm. E2F-4 and -5 are likely to rely on other proteins to be localized in nucleus, whereas E2F-1, -2 and -3 possess an intrinsic NLS (Krek *et al.*, 1994; Magae *et al.*, 1996; Allen *et al.*, 1997). In addition, the presence of DP-1 in nuclei, which lacks an intrinsic NLS, is likely therefore to be dependent upon an interaction with the appropriate E2F heterodimeric partner which subsequently causes the efficient nuclear accumulation of the heterodimers (this thesis and de la Luna *et al.*, 1996). Interestingly, Allen *et al.* (1997) reported that upon forming a heterodimer with either DP-3α or -3δ (de la Luna *et al.*, 1996), E2F-4 or -5 could

undergo nuclear accumulation, indicating that in context of the E2F heterodimer, the NLS can be also provided by the DP partner. Furthermore, p107 and p130 could stimulate nuclear localization of E2F-4 and -5, either alone or in combination with DP-1 (Magae *et al.*, 1996; Allen *et al.*, 1997). Although the reason for this effect is not known, it is possible that p107 and p130 stabilize interactions of E2F-4 and -5 with endogenous DP proteins, or facilitate the retention of E2F-4 and -5 within the nucleus (Magae *et al.*, 1996; Allen *et al.*, 1997). Overall, the combined conclusion from these data defines two distinct mechanisms which regulate the levels of nuclear E2F, one in which the NLS is provided by the DP partner and the other where the NLS is supplied by the physical association of a pocket protein.

7-2. An ability of E2F-1 to induce apoptosis.

It is apparent that the primary function of E2F-1 is to promote cell cycle progression from G1 to S phase through the transcriptional activation of genes required for DNA synthesis (Nevins, 1992; La Thangue, 1994; Lam and La Thangue, 1994). Nevertheless, *E2F-1*$^{-/-}$ mice display a defect in thymocyte apoptosis (Field *et al.*, 1996). In addition, these mice also develop a broad spectrum of tumours including reproductive tract sarcoma, lung adenocarcinoma, and lymphomas (Yamasaki *et al.*, 1996), suggesting E2F-1 endowed with tumour suppressor activity. These results indicate that the induction of apoptosis may be one of the mechanisms through which E2F-1 functions as a tumour suppressor.

Previous studies suggest that E2F-1 and p53 co-operate to mediate apoptosis (Qin *et al.*, 1994; Wu and Levine, 1994; Kowalik *et al.*, 1995; Shan *et al.*, 1996; Lee *et al.*, 1998). However, E2F-1 over-expression in SAOS-2 (p53$^{-/-}$) cells can induce apoptosis and therefore this apoptotic activity is not dependent upon p53 (this study and Hsieh *et al.*, 1997; Phillips *et al.*, 1997; DeGregori *et al.*, 1997; Lee *et al.*, 1998). At present, I do not have candidates for the mediators of this E2F-1-induced p53-

independent form of apoptosis. However, the expression of several cellular genes has been shown to be repressed through E2F-binding sites (Muller, 1995; Zwicker and Muller, 1997). For example, among these are the B-myb, cdc25C and cdc2, genes which are also involved in E2F-mediated *trans* activation during the certain stages of the cell cycle (Zwicker and Muller, 1997). Therefore, the importance of DNA-binding to the E2F-1-induced apoptosis described in this thesis suggests that this apoptotic activity may reflect the alleviation of E2F mediated transcriptional repression, rather than the activation of E2F-responsive genes.

I have described that the over-expression of p300 as a co-activator for E2F-1/DP-1 heterodimer dramatically enhanced the E2F-1-induced apoptosis, in contrast to pRb suppressed the E2F-1-induced apoptosis. These results suggest that E2F-1 may directly activaties the expression of some components of the cell death machinery or the genes important for cell survival. Alternatively, inappropriate S phase entry may indirectly trigger cell death, or perhaps increased proliferation may lead to the exhaustion of exogenous factors required for cell survival (Figure 7-1). Similarly, it has been shown that the E2F-1-induced apoptosis is observed following withdrawal of survival factors, and increasing levels of E2F overrides the ability of survival factors to suppress cell death (Hiebert *et al.*, 1995). There is no evidence that E2F-1 regulates the expression of genes involved in apoptosis. However, it has recently been shown that E2F over-expression in *Drosophila* imaginal discs leads to ectopic proliferation, apoptosis and the induction of *reaper*, a known cell death regulator in the fly (Asano *et al.*, 1996).

Interestingly, like p53, E2F-1 has been reported to bind MDM2 (Martin *et al.*, 1995). Thus, E2F-1 could induce cell death by titrating MDM2 away from p53 and stimulating *trans* activation of p53 target genes involved in apoptosis. Although most of apoptosis studies were carried out in $p53^{-/-}$ human cell line, this hypothesis still could be supported by the other observation that p73, a human p53-related protein, can also trigger apoptosis, and its amino-terminal *trans* activation domain

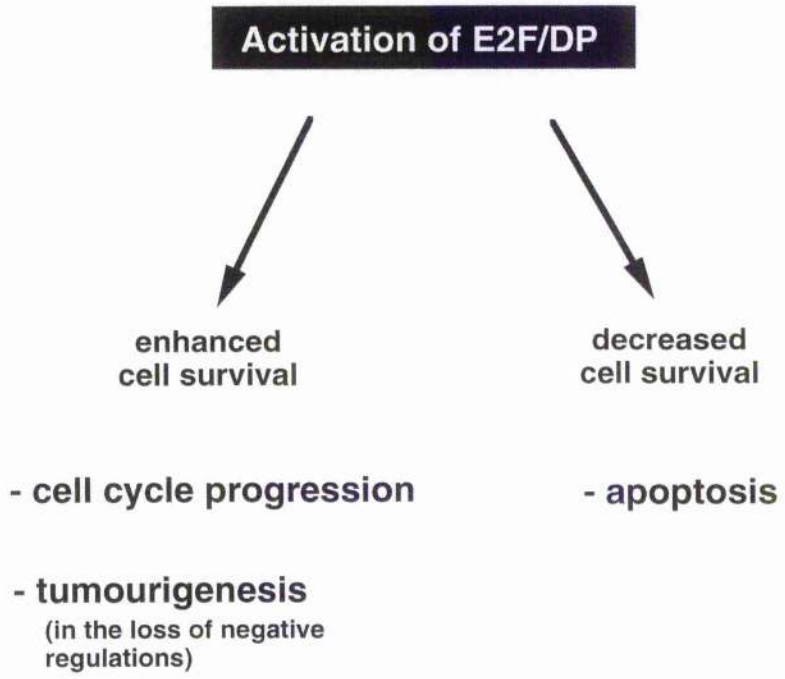


Figure 7-1

The dual functions of E2F/DP on cell cycle control.

Activation of E2F/DP elicits either a proliferative response (left) or an apoptotic response (right). (See text for details)

includes the potential MDM2 binding site (Kaghad *et al.*, 1997; Jost *et al.*, 1997). Therefore, E2F-1 may also compete with MDM2 for p73 to stabilize p73 protein, and thus E2F-1 and p73 co-operate to mediate apoptosis.

Studies of E2F-1 expression have shown recently that E2F-1 can undergo ubiquitination and that the carboxy-terminal region of E2F-1 confer protein instability, and deletion of this region or binding of pRb to this region stabilizes the protein (Hateboer *et al.*, 1996; Hoffmann *et al.*, 1996). Results reported in this study indicated that both E2F-1(1-413) and E2F-1(1-380) induced apoptosis much more strongly than that of the wild-type E2F-1. Therefore, increased stability of E2F-1 caused by the carboxy-terminal truncation of E2F-1 may be responsible for the increased cell death. Deregulated expression of mutant forms of E2F-1 which are defective for pRb binding significantly increased cell death, as shown by the earlier presence and increased absolute number of dead cells observed compared with cells expressing wild-type E2F-1 under identical conditions (in this thesis). These observations are consistent with those of others which indicate that apoptosis induced by E2F-1 can be partially blocked by pRb (Shan *et al.*, 1996; Hsieh *et al.*, 1997).

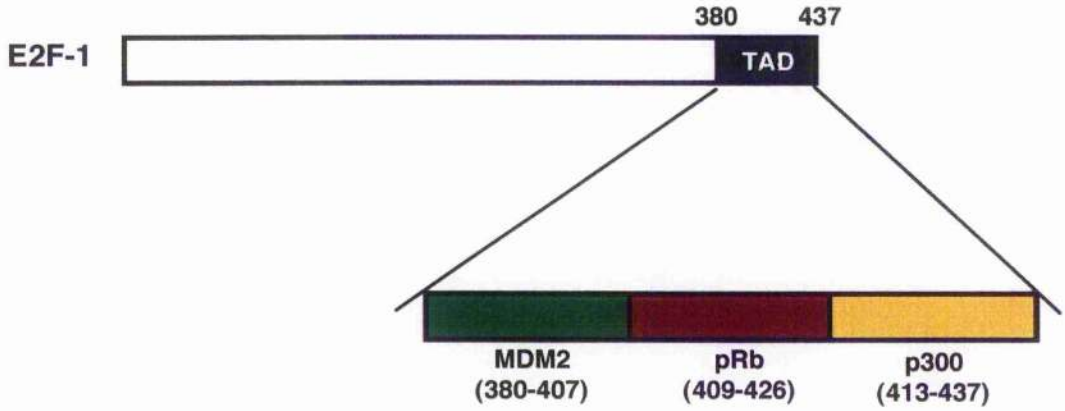
7-3. pRb and p300 have different effects on E2F-1-mediated apoptosis.

Interestingly, recent studies of $pRb^{-/-}$ mice in $p53^{-/-}$ or $p53^{+/+}$ backgrounds have clearly demonstrated p53-independent apoptosis mediated by loss of pRb (Macleod *et al.*, 1996), implying that this may occur from the E2F-1 deregulation mechanism. As this thesis shows that the *trans* activation and the apoptotic functions of E2F-1 are separable, the inhibition of E2F-1-induced apoptosis by pRb cannot be mediated by the suppression of the E2F-1 *trans* activation function. However, it has recently been shown that, when pRb and E2F-1 complex together, the pRb-E2F-1 complex can act as an active repressor to inhibit transcription of

reporters containing E2F-binding sites (Weintraub *et al.*, 1995; Luo *et al.*, 1998). Therefore, the repression function of the pRb-E2F-1 complex may be the mechanism through which pRb inhibits E2F-1-induced apoptosis. This hyphothesis suggests an active role for pRb in E2F-1-induced apoptosis, as well as the necessity for physical interaction between pRb and E2F-1. Nevertheless, it should be pointed out that most of these results were produced in SAOS-2 cells, which fail to express wild-type pRb. In these circumstances, repression of apoptotic target genes may also be carried out by E2F complexes containing other pocket proteins such as p107 and p130.

Even though it was clear that the apoptotic function of E2F-1 requires DNA binding but not *trans* activation, the co-expression of E2F-1 and its transcriptional co-activator, p300, dramatically enhanced the E2F-1 apoptotic activity, consistant with the idea that p300 enhances the transcriptional activity of E2F-1 (this thesis and Lee *et al.*, 1998). Moreover, the interaction of p300 with E2F-1 is mediated through binding to the *trans* activation domain (this thesis and Lee *et al.*, 1998). These results imply that E2F-1-mediated apoptosis can be also augmented by its transcriptional activation pathway. Similarly, it is known that the transcriptional activity of E2F-1 is stimulated through heterodimerization with its partner DP-1, and that this heterodimerization dramatically increased E2F-1-induced apoptosis.

Interestingly, the interacting sites for proteins such as MDM2, p300, and pRb have been mapped to within the last 60 amino acids of the E2F-1 *trans* activation domain (Helin *et al.*, 1992; Hagemeier *et al.*, 1993; Martin *et al.*, 1995; Lee *et al.*, 1998) (Figure 7-2). It is not known whether these three proteins can bind to the *trans* activation domain of E2F-1 simultaneously. However, it is possible that E2F-1 as a common cellular target involved in three distinct pathways of growth control mediated through the binding of the MDM2, pRb or p300 (Chellappan *et al.*, 1991; Helin *et al.*, 1993; Flemington *et al.*, 1993; Martin *et al.*, 1995; Shan *et al.*, 1996; Trouche *et al.*, 1996; Hsieh *et al.*, 1997; Kowalik *et al.*, 1998; Lee *et al.*, 1998; Luo *et al.*, 1998).



Transcription :	stimulate	repress	stimulate
Apoptosis :	inhibit	inhibit	enhance
Cell cycle :	progress(?)	arrest	progress(?)

Figure 7-2

The E2F-1 *trans* activation domain contains three distinct protein-binding sites.

An enlarged bars indicate the regions of E2F-1 required to bind MDM2 (Martin *et al.*, 1995), pRb (Helin *et al.*, 1992; Hagemeier *et al.*, 1993) and p300 (Trouche *et al.*, 1996; Lee *et al.*, 1998). The binding of MDM2, pRb or p300 to the E2F-1 *trans* activation domain (TAD) provides three distinct pathways through which E2F-1 could influence growth control.

Therefore, it is also possible that the competition between these proteins for E2F-1 may be involved in the regulation of E2F-1-induced apoptosis. The functional distinctions between pRb, MDM2 and p300 were exemplified in studies with knockout mice. *pRb*$^{+/-}$ mice develop tumours of the pituitary and thyroid, and *pRb*$^{-/-}$ embryos die between 13 and 15 days of gestation, exhibiting defective erythropoiesis as well as excessive proliferation and cell death in the liver, lens and nervous system (Jacks *et al.*, 1992; Clarke *et al.*, 1992; Lee *et al.*, 1994). *mdm2*$^{-/-}$ mice result in an early embryonic lethality, however, this lethality can be rescued in the absence of p53 (Jones *et al.*, 1995; Montes de Oca Luna *et al.*, 1995). These results suggest that the primary role of MDM2 during the development is to negatively regulate p53, with p53 and MDM2 acting in concert to regulate the cell cycle during early development. However, Lundgren *et al.* (1997) reported that over-production of MDM2 during pregnancy and lactation results in defective development of the mammary gland in both the *p53*$^{+/+}$ and *p53*$^{-/-}$ backgrounds, demonstrating a role for MDM2 that is independent of p53 function. *p300*$^{-/-}$ mice die around mid-gestation despite the presence of normal quantities of highly homologous CBP (Yao *et al.*, 1998). Interestingly, fibroblasts derived from *p300*$^{-/-}$ embryos display specific transcriptional defects and poor proliferation, implying that p300 is essential for cell proliferation and development. Moreover, *p300*$^{+/-}$ / *CBP*$^{+/-}$ mice invariably die in utero (Yao *et al.*, 1998), demonstrating an absolute requirement for a certain combined level of these two closely related proteins for normal animal development.

7-4. The p53 and pRb/E2F pathways of growth control are functionally integrated.

Previous studies with p53 have shown that protein levels can be of critical importance in determining whether a cell undergoes cell cycle arrest or apoptosis

(Chen *et al.*, 1996; Polyak *et al.*, 1996; Macleod *et al.*, 1995). Therefore, it would be interesting to know whether the ability of E2F-1 to promote cell cycle progression or apoptosis also depends on its protein level.

The DNA tumour viruses SV40, adenovirus, and HPV all encode a series of oncogene products that bind to the pRb protein and liberate an active E2F transcription factor (Nevins, 1992; Lam and La Thangue, 1994; Vousden, 1995). As a result, these viral oncogene products signal the cell to enter the replication phase of the cell cycle (Weinberg, 1995; Whyte, 1995; Vousden, 1995; La Thangue, 1994). In addition, each of these viruses encodes a protein which has been shown to bind to the p53 protein (Moran, 1993; Vousden, 1996) and inactivate its function as a transcription factor (Ko and Prives, 1996; Levine, 1997). It was thought that this function may inhibit the tumour-suppressor properties of p53 and facilitate viral transformation. Since activated E2F-1 and p53 co-operate to mediate apoptosis, it could well be that the important function of binding to the p53 protein by viral oncogene products is to block p53-mediated apoptosis as shown with the adenovirus E1A gene product (Debbas and White, 1993; Somasundaram and El-Deiry, 1997).

Several previous studies have shown that E2F-1 over-expression can induce DNA synthesis in otherwise quiescent cells (Kowalik *et al.*, 1995; Almasan *et al.*, 1995). In contrast, the induction of p53 halts the cell cycle which then is believed to allow the cell to repair its DNA or to await more favourable growth conditions (El-Deiry *et al.*, 1993; Dulic *et al.*, 1994; Harper *et al.*, 1993; Waldman *et al.*, 1995; Chen *et al.*, 1996). In addition, a considerable body of research evidence also suggests that the pathways regulated by E2F and p53 are integrated in a way that allows p53 to sense aberrant control of E2F activity. A particularly clear example of this phenomenon relates to the induction of p53-dependent apoptosis in conditions where E2F-1 is expressed at high levels in tissue culture cells (Qin *et al.*, 1994; Wu and Levine, 1994). Other studies, including those where the expression of viral oncoproteins which inactivate pRb occurs in defined cells, such as lens fibre cells

(Pan and Greip, 1994), and the targeted disruption of Rb where the apoptosis is dependent upon p53 (Morgenbesser *et al.*, 1994), support this general idea.

The E2F-1 and p53 proteins are key regulators of cell cycle progression. Therefore, the balance between the activities of E2F-1 and p53 is likely to be crucial to ensure the progression of the cell cycle. In normal cells, the transcriptional activity of p53 and E2F-1 are believed to be regulated mainly at the protein level. Thus, the functional cross-talk between these proteins is very likely to be of great importance in the overall control of the cell cycle.

7-5. p53 physically and functionally associates with DP-1.

In this thesis, I have described the association between DP-1 and p53 *in vitro* and *in vivo*. The region in p53 required for the interaction with DP-1 exists within the amino-terminal 143 amino acid residues. The first 73 residues, which contains the MDM2 binding domain (Momand *et al.*, 1992; Lin *et al.*, 1994; Chen *et al.*, 1994), are not sufficient for the interaction. Although previous studies have suggested that MDM2 can interact with E2F-1 (Martin *et al.*, 1995), our results imply that this interaction is unlikely to be responsible for the association of DP-1 with p53. Interestingly, the region between residues 73 and 143 which is necessary for p53 to bind DP-1 contains residues frequently altered in human tumour cells carrying mutant p53 alleles, such as Lys120, Lys132 and Cys135 mutations (Malkin, 1993; Greenblatt *et al.*, 1994; Ko and Prives, 1996). Based on the results presented here, a potential biological rationale for these mutations could be envisaged whereby they prevent p53 from interacting with DP-1 and thus relieve the negative regulation imposed by p53 on the formation of functional E2F heterodimers and hence cell cycle progression.

Although p53 can associated with DP-1, it was not possible to detect p53 in physiological E2F DNA-binding complexes (Sørensen *et al.*, unpublished

observation). This is consistent with the interaction of p53 with p55U, but not p55L, since p55U is a rare component of physiological E2F DNA-binding activity. The results derived from the *in vitro* assay in which p53 specifically binds to DP-1 support these observations, since they also indicate that p53 binds to an immunochemically distinct form of DP-1. Overall, these data suggest that p53 targets a sub-population of DP-1 and, further, that this form of DP-1 is an infrequent component of E2F DNA-binding complexes. Since it is known that DP-1 undergoes regulated phosphorylation (Bandara *et al.*, 1994; Krek *et al.*, 1994), it is possible that p53 targets a particular post-translationally modified form of DP-1, although further studies are required to clarify this possibility.

Co-expression of p53 specifically inactivated transcription driven by the E2F-1/DP-1 heterodimer. A potential model to explain these results would be that p53 holds DP-1 in a state which prevents it interacting with an E2F family member to form E2F-1/DP-1 heterodimer. Indeed, the region in DP-1 required to interact with p53 is necessary to form a E2F-1/DP-1 heterodimer (Sørensen *et al.*, 1996), and thus binding of p53 to DP-1 could be mutually exclusive with the interaction of DP-1 with E2F family members. Evidence for such a possibility was obtained by demonstrating that p53 and E2F-1 can compete for DP-1 binding and, consequently, that p53 reduces the level of E2F-1/DP-1 DNA-binding activity (Sørensen *et al.*, 1996). These data are compatible with a model in which p53 targets an immunochemically distinct form of DP-1, regulating the formation of E2F-1/DP-1 heterodimers and hence the level of E2F DNA-binding activity (Figure 7-3).

Although p53 is believed to possess the properties of a transcription factor and to *trans* activate of target genes such as *gadd45* and *Waf1/Cip1* (El-Deiry *et al.*, 1993; Kastan *et al.*, 1992; Ko and Prives, 1996) thought to be important in p53-mediated growth arrest, the interaction of p53 with DP-1 provides another potential pathway through which p53 may influence cell cycle progression (Figure 7-3). Thus, since many of the genes regulated by E2F encode proteins required for

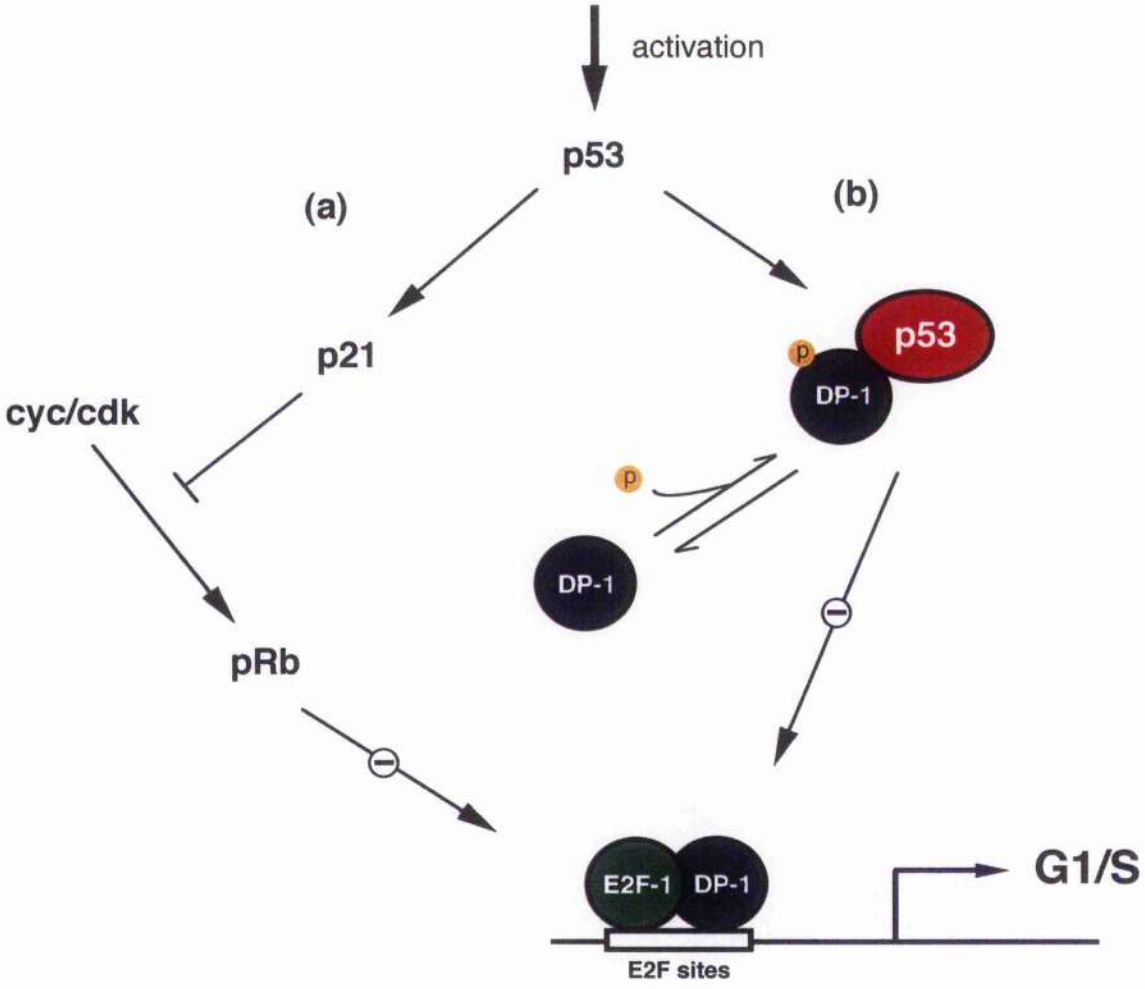

activation
p53
(a)
(b)
p21
cyc/cdk
p53
p
DP-1
p
DP-1
pRb
E2F-1
DP-1
G1/S
E2F sites

Figure 7-3

Repression of E2F/DP transcriptional activity by p53 may occur via two pathways.

p53 regulates the E2F/DP transcriptional activity by pRb-dependent (a) and -independent (b) pathways. (a) Upregulation of $p21^{Waf1/Cip1}$ expression by p53 can inhibit cdk activities. As result of this inhibition, hypophosphorylated pRb accumulates, and a G1 arrest is induced by the consequent repression of E2F responsive genes. (b) p53, which interacts with a distinct form of DP-1 (p55U), competes with E2F-1 for DP-1 leading to a reduction in the level of E2F-1/DP-1 heterodimer transcriptional activity.

cell cycle progression, their transcriptional down-regulation through the interaction of p53 with DP-1 may impede cell cycle progression.

Moreover, the interaction of DP-1 and p53 may help explain the mechanism through which DP-1 exerts high levels of proto-oncogenic activity, a property which is shared by other members of the DP family, and one manifest in the absence of a co-transfected E2F family member (Jooss *et al.*, 1995). It is possible the increased level of DP-1 sequesters p53, titrating out its activity, and thus overriding the growth-regulating effects of p53. In these conditions, DP-1 may act in an analogous fashion to certain viral oncoproteins, such as the adenovirus E1B and papilloma virus E6 proteins, since their ability to inactivate p53 also correlates with oncogenic activity (Moran, 1993; Vousden, 1996).

7-6. Functional cross-talk between p53 and E2F-1 through co-activator p300.

In this thesis, I have also demonstrated a mechanism which can explain the functional interplay between E2F and p53 and which may, in part, influence the physiological outcome of aberrant levels of E2F. A mechanism was suggested when I observed that co-expression of E2F-1 caused a concomitant reduction in p53-dependent transcription, an effect which was overridden by co-expression of pRb. This, combined with the evidence that the *trans* activation domains of E2F-1 and p53 were involved, raised the possibility that competition for a rate limiting transcription target may be responsible for the down-regulation of p53. The p300 protein, which possesses the properties of a transcriptional co-activator and is involved in regulating the activity of a variety of sequence specific transcription factors (Shikama *et al* ., 1997), overcame the down-regulation of p53 and, moreover, enhanced the activity of both E2F-1 and p53-dependent transcription, an effect dependent on a physical association between the *trans* activation domain of each protein and p300. Thus, the data presented here strongly suggest that the reduced

level of p53 transcriptional activity in the presence of E2F-1 is exerted through each *trans* activation domain competing for the p300 co-activator. That co-expression of pRb neutralized the effect of E2F-1 on p53 makes good sense in the light of the regulation of the E2F-1 *trans* activation domain by pRb (Flemington *et al.*, 1993; Helin *et al.*, 1993; Kaelin *et al.*, 1992).

The data clearly indicated that p300 acts co-operatively with p53 to stimulate transcription in a p53-dependent manner. The dominant-negative mutant of p300 ($p300^{1572\text{-}1903}$) containing the region required for the interaction with p53 prevents the activation of a p53-regulated promoter, implying that p300 is a crucial component of p53-directed transcription. In addition, p300 binds to the E2F-1 *trans* activation domain and stimulates the transcriptional activity of E2F-1. The identification of p300 as a component of p53- and E2F-1-mediated transcription, together with the notion that the concentration of p300 is rate limiting in cells (Kamei *et al.*, 1996; Avantaggiati *et al.*, 1997), implies that the functional cross-talk between p53 and E2F-1 through p300 is an important regulatory pathway in cell cycle control. In keeping with my models, recent studies have suggested that a 25% drop in combined p300/CBP levels through the loss of one *p300* or *CBP* allele is enough to interfere seriously with embryonic development, while a 50% drop results invariably in embryonic death (Yao *et al.*, 1998).

Cell cycle arrest mediated by p53 is thought to rely on the ability of p53 to directly activate the transcription of target genes, such as $p21^{Waf1/Cip1}$, which functions as an inhibitor of cyclin-dependent kinases (Chen *et al.*, 1996; El-Deiry *et al.*, 1993; Harper *et al.*, 1993; Macleod *et al.*, 1995) and thus likely occurs through the inactivation of G1 CDK activity and the maintenance of pRb and related proteins in a hypophosphorylated state. In this study, the results define a further level of control in the regulation of $p21^{Waf1/Cip1}$, exerted through a positive autoregulatory feedback mechanism whereby $p21^{Waf1/Cip1}$ enhances the transcriptional activity of the p53/p300 co-activator complex. Although the precise mechanism of action remains to be determined, this effect of $p21^{Waf1/Cip1}$ suggests that the activity of the

p53/p300 co-activator complex is influenced in a negative fashion by CDK phosphorylation. The recent demonstration that p300 associates with cyclin E/CDK2 (Perkins *et al.*, 1997) is consistent with this idea.

A recurring theme in the control of the cell cycle is that of positive autoregulation, which is believed to provide a mechanism for enabling the rapid accummulation of regulatory proteins involved in mediating cell cycle transitions. Numerous examples are known, such as in *Saccharomyces cerevisiae*, where a positive autoregulatory feedback control on transcription by the G1 cyclins favours the rapid induction of CDK activity and thus progression into S phase (Johnston, 1992). Similarly, the mammalian *E2F-1* gene is autoregulated by E2F activity (Johnson *et al.*, 1994) and the product of the *cyclinE* gene, which is regulated by E2F, may positively regulate transcription of its own gene by antagonising the repressive effects of pRb and related proteins (Botz *et al.*, 1996). The autoregulation of p53/p300 activity by $p21^{Waf1/Cip1}$ provides another interesting example of such a mechanism which likely assists the rapid accummulation of $p21^{Waf1/Cip1}$, thus augmenting cell cycle arrest (Figure 7-4). Since it is widely believed that p53 responds to environmental stress, a rapid cell cycle arrest clearly is a desirable response.

7-7. A central role for p300 in cell cycle control and apoptosis.

That p300 functions as a co-activator involved with E2F-1- and p53-dependent transcription and can influence the physiological consequence of p53 induction suggests that p300 plays a central role in co-ordinating early cell cycle events. The results presented in this study demonstrated that p300 could enhance the ability of p53 to cause G1 arrest and, moreover, failed to induce p53-dependent apoptosis. These observations are consistent with a role for p300 as a p53 co-activator in the transcription of $p21^{Waf1/Cip1}$, and in turn suggest that p53 induction

under conditions of limiting levels of p300 may be a signal which causes cells to enter apoptosis. It is known that transcriptionally inactive p53 proteins can induce apoptosis (Caelles *et al.*, 1994; Chen *et al.*, 1996; Haupt *et al.*, 1995b) whereas transcriptional activity correlates with cell cycle arrest (Crook *et al.*, 1994). Further evidence suggests that the level of p53 may dictate the cellular response, with low levels of p53 causing cell cycle arrest and high levels apoptosis (Chen *et al.*, 1996; Polyak *et al.*, 1996; Macleod *et al.*, 1995). Given the data presented in this study, it is possible that at high levels of p53, p300 levels become limiting which, suggested by the data presented here, provides an apoptotic signal.

Of course, some studies have suggested that transcription-dependent mechanisms are involved in p53-dependent apoptosis, and by no means is this study incompatible with this view. For example, the *bax* gene has been implicated as a direct target for p53 (Miyashita and Reed, 1995). However, although *bax*$^{-/-}$ thymocytes fail to respond to certain apoptotic stimuli, they are not deficient in p53-dependent DNA damage induced apoptosis (Knudson *et al.*, 1995). It is likely therefore that p53 can influence apoptosis through different mechanisms. The model proposed in this study suggesting that apoptosis occurs in conditions of p53 induction with limiting levels of p300 may account for one such mechanism. However, other recent studies have suggested that p300 plays a positive role in regulating p53-dependent apoptosis (Avantaggiati *et al.*, 1997; Lill *et al.*, 1997; Somasundaram *et al.*, 1997). Nevertheless, it is possible that the activation and expression of p21$^{Waf1/Cip1}$ and Bax may have not occurred at the same time. Interestingly, my recent unpublished observations are consistent with this speculation, as I found that the accumulation of p21$^{Waf1/Cip1}$ by actinomycin D in SAOS-2 and U2OS cells only occurred at low concentration (1nM) of treatment, whereas cell death was brought about at high concentrations (10nM to 1μM) (data not shown). This is consistent with other similar studies where it was noted that the low levels of p53 was dominant over the growth arrest pathway (Macleod *et al.*, 1995; Chen *et al.*, 1996; Polyak *et al.*, 1996). Therefore, it is possible that the

experimental conditions in this thesis in which cells underwent $p21^{Waf1/Cip1}$-mediated cell cycle arrest may reflect insufficient levels of p53 to activate apoptotic genes such as *bax*. Thus, even though further experiments are required to clarify these issues, it is clear that the dependence of p53 activity on p53 concentration may be a contributory factor.

Although apoptosis is enhanced when p53 and E2F-1 are expressed together, E2F-1 alone was capable of inducing apoptosis in $p53^{-/-}$ tumour cells, suggesting that p53-dependent and -independent mechanisms contribute towards E2F-1 induced apoptosis (this thesis and Hsieh *et al.*, 1997; Phillips *at al.*, 1997). In $p53^{-/-}$ cells, the co-expression of E2F-1 and p300 enhanced apoptosis, in contrast to the effect of p300 in cells expressing p53 and E2F-1 when there was a significant decrease in the proportion of apoptotic cells, suggesting that limiting levels of p300 favour p53-independent apoptosis. Moreover, it is interesting that the partial rescue of apoptosis by p300 is compatible with earlier reports indicating that p53-mediated apoptosis can be overcome by pRb (Haupt *et al.*, 1995a) since, as explained, the presence of p300 will directly favour the maintenance of hypophosphorylated pRb. Interestingly, it is consistent with my models that Thomas and White (1998) have recently reported that rate limiting p300 is required specifically for *trans* activation of the *mdm2* gene by p53 and for regulating p53-mediated apoptosis. In addition, cells expressing E1A were unable to up-regulate MDM2, causing stabilization of high level of p53 that resulted in p53-dependent apoptosis. Furthermore, expressing of anti-apoptotic E1B 19K and Bcl-2 could bypass E1A inhibition of p300 function and restored *mdm2 trans* activation, thereby inhibited p53-dependent apoptosis (Thomas and White, 1998).

The results presented in this study, together with the documented role of p53-dependent transcription in cell cycle arrest, are consistent with a model in which the productive interaction between p53 and p300 and consequent activation of target genes like $p21^{Waf1/Cip1}$ is important in controlling cell cycle arrest. However, it should be noted that a $p21^{Waf1/Cip1}$ deficiency does not completely abbrogate G1

arrest due, for example, to irradiation (Brugarolas *et al.*, 1995; Deng *et al.*, 1993) whereas in *p53*$^{-/-}$ mice G1 arrest is completely absent (Donehower *et al.*, 1992). Thus, p53 likely has other targets in addition to p21$^{Waf1/Cip1}$ which confer cell cycle arrest, or other factors may involve in the regulation of *Waf1/Cip1* gene. Recently, a new p53-related protein, namely p73, has been identified that has the potential to activate p53 target genes (Kaghad *et al.*, 1997; Jost *et al.*, 1997). Interestingly, over-expression of p73 in SAOS-2 and U2OS cells showed elevated levels of p21$^{Waf1/Cip1}$ and GADD45 proteins (Lee and La Thangue, unpublished observation). Moreover, I assessed whether p73 also could utilise p300 as a transcriptional co-activator. The results from mammalian two-hybrid and immunoprecipitation assays have revealed that the amino-terminal *trans* activation domain of p73 directly interacts with the CH3 region of p300 (Lee and La Thangue, unpublished observation), suggesting that p300 is a *bonafide* component of p53 family-mediated transcription in a network of cell cycle arrest and apoptosis.

When the induction of p53 causes cell cycle arrest, for example, in response to genotoxic stress, the data suggests that p53 activates transcription in concert with p300. Further evidence presented here showed that the over-expression of p21$^{Waf1/Cip1}$ enhances the transcriptional activity of the p53/p300 co-activator complex. At the same time, the inhibition of CDK activity by the induction of p21$^{Waf1/Cip1}$ will retain pRb and related proteins in a hypophosphorylated state and thus, by virtue of excluding the E2F-1 *trans* activation domain, enhance the levels of p300 available for p53 (Figure 7-4b).

Previous studies suggest that the cellular level of p53 protein is crucial for determining whether cells arrest or apoptose (Macleod *et al.*, 1995; Chen *et al.*, 1996; Polyak *et al.*, 1996). The apoptotic conditions were also caused by high levels of E2F-1 in conditions which are inappropriate for cell cycle progression or increased level of p53 (Figure 7-4c). However, I would like to emphasise that the data do not rule out the possibility that there are p53 target genes which need to be activated, perhaps through other co-activators, in order to achieve apoptosis.

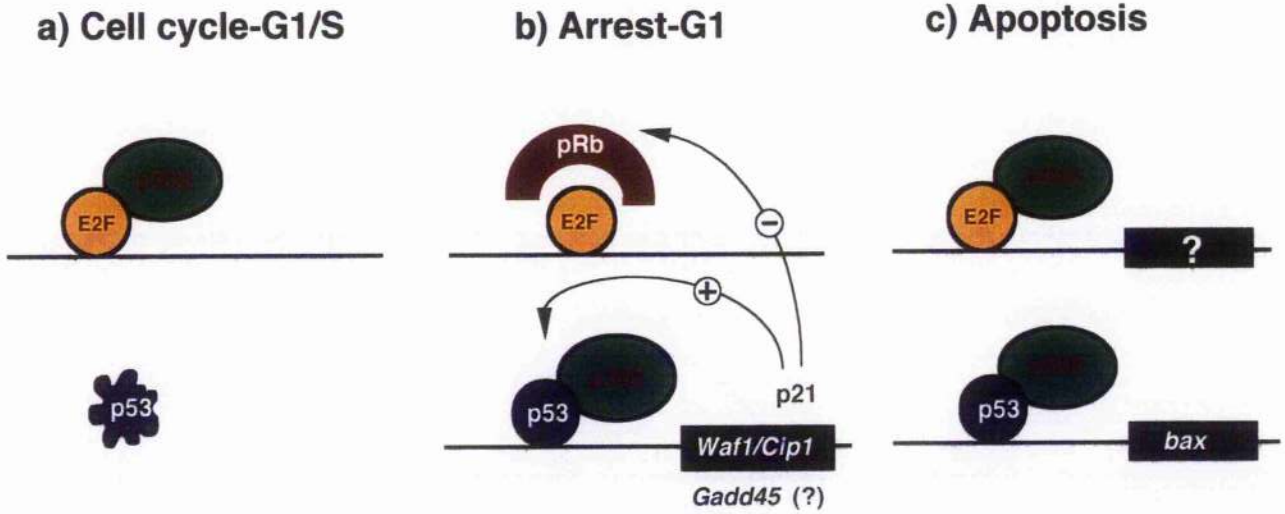

a) Cell cycle-G1/S
b) Arrest-G1
c) Apoptosis
E2F
p53
pRb
E2F
p53
p21
Waf1/Cip1
Gadd45 (?)
E2F
?
p53
bax

Figure 7-4

Model for cell cycle control by p300.

It is envisaged that during normal cell cycle progression (a) p53 remains in a latent state and that p300 functions as a co-activator for transcription factors such as E2F-1. Upon activation and cell cycle arrest by p53 (b), p300 functions as a co-activator for p53-dependent transcription, such as in the activation of the *Waf1/Cip1* gene. The activity of the p53/p300 co-activator complex is enhanced by $p21^{Waf1/Cip1}$ through a positive autoregulatory feedback loop whilst the activity of E2F declines due to the presence of hypophosphorylated pRb. For (c), in conditions which are inappropriate for cell cycle progression aberrantly high levels of E2F compete for p300, resulting in the increased apoptosis. It is envisaged that the high levels of p53, together with E2F, provides a signal for apoptosis.

With respect to this idea, it has been shown that p53 utilizes other co-activator molecules in transcriptional activation (Lu and Levine, 1995; Thut *et al.*, 1995) and thus it is possible that the regulation of p53 activity by p300 relates to a specific group of target genes.

It is noteworthy that the ability of E2F-1 to influence the physiological outcome of p53 activation may bear on the phenotype of the *E2F-1*$^{-/-}$ mice which suffer thymic hyperplasia due to reduced levels of apoptosis during thymocyte ontogeny, together with an increased incidence of tumours in older animals (Field *et al.*, 1996; Yamasaki *et al.*, 1996). Based on the studies presented here, one scenario which may contribute to this phenotype in the absence of E2F-1 would be that a signal (provided by E2F-1) is lost which drives p53-dependent apoptosis, subverting the normal apoptotic process in thymocytes and thus giving rise to hyperplasia. Similarly, the loss of an apoptotic signal will encourage tumour progression, in a similar fashion to the phenotype observed in the *p53*$^{-/-}$ mice (Donehower *et al.*, 1992).

7-8. E1A deregulates p53 and E2F-1 activity through its co-activator p300.

p300 was defined by studies on the mechanisms through which the oncogenic products of DNA tumour viruses subvert normal cellular growth control (Arany *et al.*, 1995). p300 interacts with a domain within the adenovirus E1A protein which is essential for the protein to exert a range of biological effects on cells such as the induction of DNA synthesis and apoptosis (Moran, 1993). Furthermore, the transcriptional activation by p300/CBP could be abolished directly and specifically by binding to E1A (Arany *et al.*, 1995; Yang *et al.*, 1996; Crook *et al.*, 1996). E1A mutants which lack the p300 binding site lose the properties of E1A-driven cell cycle progression and cellular transformation (Moran, 1993).

Taken together, therefore, these results indicate that p300/CBP are key regulators of cell cycle progression and cellular differentiation.

An important and highly interesting feature of p300/CBP is that germline inactivation of one allele or somatic translocation of the gene has been implicated as the cause of many diseases and cancers (Sakai *et al.*, 1992; Eckner *et al.*, 1994; Giles *et al.*, 1995; Petrij *et al.*, 1995; Muraoka *et al.*, 1996; Ida *et al.*, 1997). Thus, it is argued that the level of p300/CBP is physiologically crucial and rate limiting in cells, in consistent with recent studies that the decreased p300 or CBP protein level in $p300^{+/-}$ or $CBP^{+/-}$ mice significantly reduced viability and seriously interefered embryonic development (Yao *et al.*, 1998). Whereas it plays a positive role in cellular differentiation, p53-mediated DNA repair/apoptosis, and E2F-1-mediated apoptosis, it is a negative regulator of cellular growth.

E1A is known to inhibit p53 transcriptional activation function, and binds to the CH3 domain of p300/CBP (Chiou and White, 1997; Somasundaram and El-Deiry, 1997). In this study, it was shown that wild-type E1A specifically inhibited p53-mediated activation, possibly through the interaction of p300/CBP, since the E1A mutant (E1AΔ2-36) failed to repress p53-dependent transcription. Again, the result indicates that E1A inhibition of p53-mediated transcriptional activation correlated with its ability to bind p300/CBP. Indeed, the data make good sense in the understanding of tumourigenesis. The stimulation and activation of p53 by cellular stress causes cell cycle arrest or apoptosis to allow DNA repair or to prevent tumourigenesis. But, the DNA viruses such as adenovirus deregulate p53 activity through its co-activator p300 so as to allow quiescent cells to undergo DNA replication, thereby providing an environment permissive for viral replication.

At the same time, it is known that oncoproteins encoded by these DNA tumour viruses contain a region which mediates binding to the pRb family of proteins to sequester this region from the E2F family, resulting in deregulated cell cycle progression (Weinberg, 1992; Lam and La Thangue, 1994; Vousden, 1995; Wang, 1997). Thus, inappropriate cell cycle entry and deregulated E2F/DP activity

trigger apoptosis (Qin *et al.*, 1994; Wu and Levine, 1994; Shan *et al.*, 1996; DeGregori *et al.*, 1997; Hsieh *et al.*, 1997; Phillips *et al.*, 1997).

In this thesis, it was also demonstrated that E2F-1/DP-1 utilizes p300 as a co-activator through binding to its transcriptional activation domain, resulting in the significant increase of E2F-1-mediated apoptosis. However, the fact that E1A represses E2F-1-mediated transcriptional activation by binding p300 (Trouche and Kouzarides, 1996) may leads to the loss of E2F-1-induced apoptosis, and in turn implies that these cellular conditions may facilitate viral transformation. Moreover, in some environmental circumstances, it is possible that the competition between p300 and pRb for the E2F-1 *trans* activation domain may result in distinct physiological outcomes.

7-9. A novel transcriptional co-activator, JMY, that interacts with p300.

p300/CBP likely plays a series of key regulatory roles in animal cells as described previously, given that binding of p300 by E1A leads to perturbation of the cell cycle, blockade of multiple differentiation processes, failure to suppress transformation, and loss of elements of transcriptional control (Shikama *et al.*, 1997). The biological activity of p300 family members may depend, at least in part, upon their ability to function as co-activators with transcriptional factors such as CREB, c-fos, c-Jun, MyoD, and E2F-1/DP-1 (Arias *et al.*, 1994; Chrivia *et al.*, 1993; Eckner *et al.*, 1996; Torchia *et al.*, 1997; Perkin *et al.*, 1997; Shikama *et al.*, 1997; Lee *et al.*, 1998). Especially, I and others have demonstrated that p300/CBP also acts as a co-activator for p53 and potentiates its transcriptional activity *in vivo* (this study and Lill *et al.*, 1997; Gu *et al.*, 1997; Avantaggiati *et al.*, 1997; Lee *et al.*, 1998). Given this critical and highly specific interaction with this co-activator, it is possible that p300/CBP nucleates different factors so they may functionally interact either negatively or positively.

p300/CBP have been shown to have histone acetyltransferase activity, which can modify chromatin structure and enhance gene expression (Bannister and Kouzarides, 1996; Ogryzko *et al.*, 1996). Recently, p300 also has been shown to acetylate p53 itself (Gu and Roeder, 1997). The acetylation of p53 increases the binding of p53 to specific consensus sequences *in vivo*, this activity may be required for facilitating p53 tetramer interactions with the DNA template, thereby promoting p53-inducible genes transcriptional activation. However, acetylation of p53 by p300 may not be the only regulatory mechanism to control *trans* activation of p53-inducible genes. Interestingly, it has been known that p/CAF, P/CIP, SRC and ACTR interact with p300/CBP and regulate the p300/CBP activity (Kamei *et al.*, 1996; Yang *et al.*, 1996; Chen *et al.*, 1997; Li *et al.*, 1997; Torchia *et al.*, 1997). Therefore, it is possible that different levels of regulation through other factors may play a role in transcriptional activation of p53-inducible genes.

Recently, Shikama *et al.* (1998) have focussed on the identification of additional molecules that functionally interact with p300 that may influence its effects on target transcription factors. In this thesis, it was shown that a novel co-activator, JMY, physically and functionally interacts with p300 in transcriptional activation by p53.

7-10. JMY enhances p53-dependent transcription through co-activator p300.

To investigate the functional properties of JMY, it was considered that JMY together with p300 may modulate the activity of p53. An analysis of the role of p300 and JMY in regulating the *bax* promoter, a gene that responds to p53 (Miyashita and Reed 1995), suggested that p300 and JMY co-operate in activating the transcription of the *bax* gene. In contrast, the effect of JMY on other p53 target genes was less striking and, in the case of *mdm2*, increased levels of JMY failed to significantly affect promoter activity. Thus, although this study has focused on a

limited spectrum of p53-responsive genes, nevertheless the data do support the idea that the co-activator effects of JMY are more pronounced on certain p53 target genes, exemplified by the effect of JMY on *bax*. Overall, therefore, the results suggest a model in which JMY plays a preferential role in mediating the transcriptional activation of a selective group of target genes. However, it should be considered that the activation and accumulation of MDM2 by p53-dependent transcription through its co-activators may lead to negative-regulatory feedback competition between MDM2 and p300 for binding to the p53 *trans* activation domain. Thus, although neither exogenous JMY nor p300 could augment the activity of the *mdm2* promoter, it is highly possible that both p300 and JMY are key integrators of all transcriptional responses of p53.

Recent studies demonstrate that E1A specifically inhibits *mdm2 trans* activation and not other p53-inducible genes such as *bax* and *Waf1/Cip1* (Thomas and White, 1998). Therefore, these results suggest that p300 binding to p53 alone may not regulate the specificity of p53 *trans* activation for *bax* and *Waf1/Cip1*. As numerous protein interactions with p300 occur, JMY may determine the specificity of *trans* activation, accounting for differential regulation of p53-inducible genes by p300.

7-11. JMY augments p53-dependent apoptosis.

Most previous studies support a correlation between transcriptional activation by p53 and the induction of cell cycle arrest, but the dependence of the apoptotic function of p53 on transcriptional activity is more complex. Some studies have found that apoptosis can occur when the p53 *trans* activation domain is compromised whereas other reports support a role for transcriptional activation (Caelles *et al.*, 1994; Crook *et al.*, 1994; Chen *et al.*, 1996; Haupt *et al.*, 1995). The correlation between transcriptional activation of the *bax* promoter and induction of

apoptosis suggests that the *bax*-encoded protein may play a role in p53-mediated apoptosis. This suggestion is consistent with earlier reports (Miyashita and Reed, 1995; Zhan *et al.*, 1994) and with the documented apoptotic effect of Bax over-expression (Oltavi *et al.*, 1993). For example, the *bax* gene is transcriptionally activated by p53 and p53-dependent apoptosis, in some conditions, is compromised by experimental strategies that subvert and inactivate p300/CBP (Lill *et al.* 1997; Avantaggiati *et al.* 1997). It has been reported that expression of Bax can efficiently induce apoptosis in SAOS-2 cells (Ludwig *et al.*, 1996). In this thesis, it was shown that JMY can augment the level of p53-dependent apoptosis in SAOS-2 cells. In sharp contrast, when the effect of exogenous p300 was measured in similar assay conditions, little effect was apparent on apoptosis, suggesting that increasing the level of p300 is not sufficient to promote apoptosis. Rather, the results are consistent with the idea that p300 and JMY act together in the p53 response.

With regard to apoptosis, the need for *trans* activation by p53 appears to depend on cell type. More likely, cell type specific apoptosis may be related with the presence of sufficient amounts of downstream effector molecules of p53-mediated apoptosis, or any additional factors required to activate or modify the death gene products such as Bax. Although the role of p53 in the apoptotic mechanism remains to be elucidated, the data presented in this thesis suggests that both p300 and JMY are potential mediators for p53-induced apoptosis.

Interestingly, I have found that the physiological consequences of p300 and JMY on p53 transcriptional activity can be distinguished from each other. Thus, combined with other results on the requirement for p300 in regulating p53 activity (Lill *et al.*, 1997; Gu *et al.*, 1997; Avantaggiati *et al.*, 1997; Lee *et al.*, 1998), I suggest a general model in which p300 and JMY function together in p53-dependent transcription and that p300 is necessary, but not sufficient, to augment apoptosis. Further, since JMY could augment apoptosis without additional p300, in turn the data suggest that in the conditions of the apoptosis assay JMY, rather than p300, is limiting.

The mechanism of action through which JMY and p300 co-operate in the transcriptional activation of p53 target genes, and the basis of the different effects on promoters, have yet to be elucidated. Nevertheless, the results do point towards a level of control that is likely to be important in the regulation of p53 activity and, specifically, indicate that there are additional co-activator molecules, like JMY, that through an interaction with p300 have a significant impact on the transcriptional activity of target genes and thus physiological outcome.

I do not have any evidence to suggest that JMY is involved in the formation of basal transcriptional complex. However, it is possible that JMY may integrate p300/CBP proteins with components of the basal transcription apparatus, and thereby effect transcriptional activation perhaps through directly modulating basal transcription factor activity.

7-12. Overall conclusion.

In conclusion, I have defined a central role for the p300 co-activator in co-ordinating the interplay between the pathways of control mediated by E2F-1/DP-1 and p53. The data strongly support the idea that p300 functions as a negative regulator of cell cycle progression and that the competition between E2F-1/DP-1 and p53 for p300 is instrumental in influencing whether cell cycle progression, G1 arrest or apoptosis occurs (Figure 7-5). The recent identification of tumour suppressor-like mutations in *p300* genes in human tumour cells provides support for such an idea (Muraoka *et al.*, 1996; Sakai *et al.*, 1992; Giles *et al.*, 1995; Ida *et al.*, 1997).

Finally, a question of central importance relates to the mechanistic and physiological role of p300/CBP in regulating the p53 response. In this thesis, it was demonstrated that JMY, a novel p300 target protein, physically and functionally co-operates with p300 in transcriptional activation by p53 (Figure 7-5). Thus, the data support the notion that JMY is an important effector molecule in directing the

cellular response to p53 and suggest that JMY behaves as a significant determinant in influencing the biological outcome of transcriptional activation by p53.

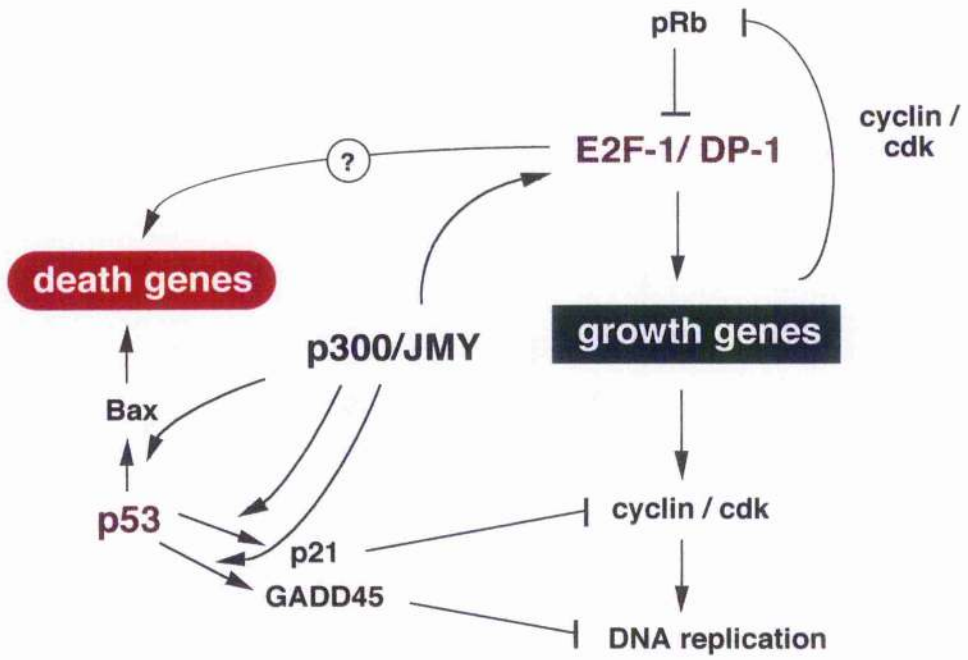

pRb
cyclin /
cdk
E2F-1/ DP-1
?
death genes
growth genes
p300/JMY
Bax
p53
cyclin / cdk
p21
GADD45
DNA replication

Figure 7-5

Regulation of cell cycle and apoptosis by p53 and E2F-1/DP-1 through co-activators p300 and JMY.

E2F/DP may be directly inducing death genes in the absence of pRb and the presence of co-activators. Alternatively, the transcriptional activation of E2F/DP through co-activators may be an indirect trigger for cell death by over-proliferation and exhaustion of survival factors. Activation of p53 transcription through co-activators p300 and JMY can directly induce genes involving in cell cycle arrest and apoptosis. (See text for further details)

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