THE REGULATION OF E2F

A

Thesis

Presented by

Morwenna J. Burden

for

the degree of

DOCTOR OF PHILOSOPHY

at the

UNIVERSITY OF GLASGOW

Division of Biochemistry and Molecular Biology

February 2000

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

Abstract

The cellular transcription factor E2F plays a critical role in co-ordinating the transcription of target genes necessary for cell cycle progression. E2F interacts with important regulators of the cell cycle, such as the Rb tumour suppresser protein, related proteins p107 and p130 and cyclins and cyclin-dependent kinases. The cellular E2F activity is a heterodimer consisting of a DP family member, of which three members have been characterised and an E2F family member, of which six family members have been isolated.

Murine DP-3 differs from the other DP proteins due to striking complexity at the RNA level. RNA analysis has shown that extensive processing gives rise to four different DP-3 proteins, α, β, γ□ and δ. These variants arise via two open reading frame changes, including the insertion of the E region, a 16 residue sequence within the DNA binding domain of DP-3 α and δ, which forms part of a nuclear localisation signal. Additionally, the insertion of single glutamine codon has been noted in the DP-3γ isoform. Extensive splicing within the 5'UTR of DP-3 results in translation initiation at two different methionine codons. Since DP-1 is the major component of the E2F DNA binding activity in mammalian cells and the role of DP-3 remains unclear, the aim of this study was to investigate the expression of DP-3 at the RNA and protein level, in an attempt to understand the role of DP-3 in the E2F heterodimer.

In an effort to understand the post-transcriptional control of DP-3, the murine DP-3 gene was isolated. Analysis of the exon/intron arrangement of the DP-3 gene and comparison of the DP-3 genomic and cDNA sequences provided insight into the post-transcriptional regulation of DP-3, in particular at sites such as the E region. Additionally, comparison with the murine DP-1 gene revealed striking conservation in genomic organisation, suggesting that they are ancestrally related.

Analysis of DP-3 RNA via Northern blotting was performed to study the expression pattern of the different DP-3 RNAs. A range of different mouse tissues and tissue culture cell lines were tested for the presence of DP-3 RNA and DP-3 RNA abundance was analysed during cellular processes such as differentiation.

An investigation into the effects of the different DP-3 5'UTRs on translational regulation of DP-3 protein expression was performed using chimeric DP-3 5'UTR-luciferase reporter constructs. The translational potential of each 5'UTR was analysed by transient transfection in a range of mammalian cells and was found to be different for each 5'UTR. The translational ability of each 5'UTR was also analysed *in vitro*. The effect of the tumour suppresser p53 on the translational ability of the DP-3 5'UTRs was also assessed. p53 is known to influence the translation of both its own RNA and that of cdk4. Analysis suggested that p53 might influence the translation of specific DP-3 isoforms.

To aid the detection of DP-3 protein, anti-peptide polyclonal antibodies were made. These were used to study DP-3 expression by western blotting in a range of different mammalian cells and by immunostaining.

These results imply that the expression of DP-3 is highly regulated at the post-transcriptional level. Although a definite role for DP-3 in E2F mediated processes has yet to be assigned, these results provide insight into the control of DP-3 expression, which may ultimately be linked to the role of DP-3 in cells.

Contents

		Page
Abstract		2-3
Chapter 1:	**Introduction**	17-69
1.1	Transcription	17
1.1.1	RNA polymerase II basal transcription	17
1.1.2	Transcription from TATA-less promoters	18
1.1.3	TFIID	18
1.1.4	*Trans*-acting factors for RNA polymerase II	18
1.1.5	Co-activators and the modulation of chromatin structure	19
1.2	Translational control of gene expression	20
1.2.1	The mechanism of eukaryotic protein synthesis	20
1.2.1.1	The initiation phase	20
1.2.1.1a	Dissociation of 80S ribosome	21
1.2.1.1b	Met-tRNA$_i$ binding to the 40S subunit	21
1.2.1.1c	mRNA binding and recognition of the initiator codon	21
1.2.1.1d	Association of the 60S ribosomal subunit	22
1.2.1.2	Translational elongation	22
1.2.1.3	Termination of translation	22
1.2.1.4	Reinitiation of translation following termination	22
1.2.2	The regulation of translation	23
1.2.2a	Translational regulation by structural features of the UTRs of mRNA	23
1.2.2b	Translational control by upstream AUG codons	24
1.2.2c	Regulation of translation by mRNA/protein interaction	25
1.3	Cell cycle control	26
1.3.1	Cyclin-dependent kinases	26
1.3.2	Cdk inhibitor proteins	27
1.4	The p53 tumour suppresser protein	28
1.4.1	p53 is a transcription factor	29
1.4.2	p53-dependent apoptosis	31
1.4.3	Regulation of p53 activity	31
1.4.5	p53 can modulate translation	33

1.5	The Retinoblastoma gene	34
1.5.1	The Rb protein	34
1.5.2	pRb and the control of G1/S phase progression	35
1.5.3	Phosphorylation of pocket proteins	36
1.6	The E2F transcription factor	37
1.6.1	The E2F family	37
1.6.2	The DP family	39
1.6.3	Murine DP-3	39
1.6.4	Heterodimerisation and DNA binding of E2F and DP family members	40
1.6.5	Binding of pRb family members regulates E2F activity	41
1.6.6	Transcriptional regulation by E2F	42
1.6.6a	E2F target genes	42
1.6.6b	*Trans*activation of E2F target genes by E2F complexes	43
1.6.7	The regulation of E2F activity	44
1.6.7a	Regulation of E2F by phosphorylation	44
1.6.7b	Regulation of E2F by subcellular location	44
1.6.7c	Regulation of E2F activity by protein degradation	46
1.6.8	E2F and proliferation	46
1.6.9	Overexpression of E2F-1 leads to apoptosis	47
1.7	Aims	48
Chapter 2:	**Materials and Methods**	70-91
2.1	Mammalian cell culture	70
2.1.1	Differentiation of F9 embryonal teratocarcinoma cells (F9EC)	70
2.1.2	Transfection of mammalian cells	70
2.1.3	Reporter assays	72
2.1.3.a	Luciferase reporter assays	72
2.1.3.b	β-Galactosidase reporter assay	72
2.2	Bacterial culture	72
2.3	Manipulation of nucleic acids	73
2.3.1	Determination of nucleic acid concentration	73

2.3.2	DNA preparation	73
2.3.2a	Preparation of murine brain genomic DNA	73
2.3.2b	Purification of phage DNA	74
2.3.3	RNA extraction	74
2.3.3a	Extraction of nuclear and cytoplasmic RNAs	74
2.3.3b	Isolation of polyadenylated mRNA	75
2.3.4	Genomic library screening	75
2.3.5	Restriction endonuclease digestion	76
2.3.6	Polymerase chain reactions (PCR)	76
2.3.7	Phosphatase treatment of vector DNA	76
2.3.8	Ligation reactions	77
2.3.9	Transformation of competent bacteria	77
2.3.10	DNA Sequencing	77
2.3.11	Construction of genomic plasmid clones	77
2.3.12	Construction of DP-3 type IV 5'UTR-luciferase expression vector	78
2.3.13	Vectors used	78
2.4	Agarose Gel Electrophoresis	79
2.4.1	Electrophoresis of DNA samples	79
2.4.2	Electrophoresis of RNA samples	79
2.4.3	Gel purification of DNA fragments	80
2.5	Southern and Northern blots	80
2.6	Dot Blotting	80
2.7	Probe preparation	80
2.7.1	Random priming	80
2.7.2	5' end labelling of oligonucleotides	81
2.7.3	DP-3 E region probe	81
2.7.4	Probe Purification	81
2.8	Hybridisation of Southern, Northern and dot blots	81
2.8.1	Southern, Northern and dot blot hybridisation washes	82
2.9	Autoradiography	82
2.10	Stripping Northern and Southern blots	82
2.11	Protein analysis	83

2.11.1	Preparation of cellular protein extracts	83
2.11.2	*In vitro* transcription/translation	83
2.11.3	SDS PAGE	83
2.11.4	Western blotting	84
2.12	Antibody production	84
2.12.1	Coupling of peptides to BSA	84
2.12.2	Rabbit immunisations	85
2.12.3	Peptides used in this study	85
2.12.4	Antibody Reagents	85
2.12.5	Immunofluoresence	85
2.13	Buffers and solutions	89

Chapter 3:	**Analysis of the DP-3 Gene**	92-113
3.1	Introduction	92
3.2	Results	93
3.2.1	The DP-3 gene is a single copy gene	93
3.2.2	The isolation and restriction enzyme mapping of the DP-3 gene	94
3.2.3	The DP-3 genomic structure	94
3.2.4	The E region of DP-3α and δ is a discrete 48 bp alternatively spliced exon	95
3.2.5	DP-3 Q+ arises via the use of an alternative 3' splice acceptor site	95
3.2.6	hDP-2 protein isoforms arise via alternative splicing of equivalent exons	95
3.2.7	Murine DP-1 and DP-3 have identical exon structure	96
3.3	Discussion	97
3.3.1	Alternative splicing of the E region influences the cellular location of DP-3	97
3.3.2	The insertion of the glutamine residue (Q) may represent a site of post translational modification	97
3.3.3	Murine and human DP-3 cDNAs suggest similar genomic organisations	98

3.3.4	DP-1 and DP-3 have a common evolutionary origin	99
Chapter 4:	**Analysis of DP-3 RNA Expression**	114-131
4.1	Introduction	114
4.2	Results	115
4.2.1	DP-3 specific transcripts are expressed in a range of mouse tissues	115
4.2.2	DP-3 transcripts are expressed in different cell lines	116
4.2.3	DP-3 transcripts are polyadenylated, cytoplasmic mRNAs	116
4.2.4	DP-3 RNA is expressed throughout differentiation of F9EC cells	117
4.2.5	DP-3 transcripts contain the E region	117
4.3	Discussion	118
4.3.1	DP-3 expression differs in different mouse tissues	118
4.3.2	DP-3 transcripts are expressed in a range of tissue culture cell lines	119
4.3.3	DP-3 transcripts are cytoplasmic, poly A+ messenger RNAs	120
4.3.4	Multiple DP-3 transcripts are observed by northern analysis	120
4.3.5	RNA analysis suggests extensive processing of DP-3 RNA	121
Chapter 5:	**The regulation of translation by the 5'untranslated regions**	132-156
5.1	Introduction	132
5.2	Results	132
5.2.1	Analysis of DP-3 translation by the different 5'UTRs	132
5.2.2	The DP-3 5'UTRs are equally translated *in vitro*	133
5.2.3	The luciferase activity reflects the quantity of luciferase protein	134
5.2.4	DP-3 5'UTRs have translational activities	134

5.2.5	Translation of DP-3 type I and IV 5'UTRs was compromised in U2OS cells	135
5.3	Discussion	136
5.3.1	DP-3 5' untranslated regions influence protein expression by a post-transcriptional mechanism	136
5.3.2	DP-3 expression is cell type dependent and mediated by the 5'UTRs	137
5.3.3	M1 of DP-3 type II 5'UTR may be used preferentially *in vivo*	137
5.3.4	All DP-3 5'UTRs are translated equally *in vitro*	138
5.3.5	DP-3 translation could be regulated via different mechanisms	138
5.3.6	A novel post-transcriptional mechanism may influence subunit composition of the E2F heterodimer	140
Chapter 6:	**The effect of p53 on DP-3 translation**	157-167
6.1	Introduction	157
6.2	Results	158
6.2.1	DP-3 5'UTRs are regulated in SAOS-2 cells	158
6.2.2	p53 represses translation of DP-3 type IV 5'UTR transcripts	158
6.3	Discussion	159
6.3.1	The translation of DP-3 type IV 5'UTR is enhanced in SAOS-2 cells	159
6.3.2	p53 may influence DP-3 translation via direct RNA/protein interaction	160
6.3.3	p53 dependent transcription activity may influence translation	160
6.3.4	p53 may target the expression of specific DP-3 protein isoforms	161
6.3.5	DP-3 isforms may function during DNA damage or repair	162

Chapter 7:	**Analysis of DP-3 protein**	168-181
7.1	Introduction	168
7.2	Results	169
7.2.1	Peptide design	169
7.2.2	Analysis of α7.6 antisera	169
7.2.2.1	α7.6 antisera detects E+ DP-3 isoforms and not E- isoforms	169
7.2.2.2	α7.6 antisera specifically recognises DP-3 E+ isoforms	170
7.2.2.3	α7.6 antisera specifically detects DP-3 E+168 isoforms by immunostaining	170
7.2.3	α7.7 antisera specifically detected DP-3 E+ isoforms by western blotting	170
7.2.4	Truncated DP-3 polypeptides are detected with α7.6 and α7.7 antisera	171
7.3	Discussion	171
7.3.1	DP-3 anti-peptide antisera detect exogenously expressed DP-3 proteins, but not endogenous DP-3	171
7.3.2	DP-3 protein is expressed at low levels in cells	172
7.3.3	DP-3 protein levels may be influenced by post-transcriptional regulation	172
7.3.4	DP-3 protein expression may be regulated by degradation	172
7.3.5	Regulation of DP-3 protein may impact on E2F activity	173
Chapter 8:	**General discussion**	182-201
8.1	Implications of DP-3 alternative splicing	182
8.2	DP-3 RNA expression, regulation and functions	185
8.3	DP-3 translation maybe regulated by RNA/protein interaction	187
8.4	The potential regulation of DP-3 translation by reinitiation	188
8.5	The role of DP-3 during activation of p53	190
8.6	A second DP family member, why?	192
8.7	Future Work	194

8.7.1 Translational regulation of DP-3 expression 194
8.7.2 The generation of DP-3 null mice 194
Acknowledgements 202
References 203-237

List of Figures

1.1	The assembly of RNA polymerase II transcription initiation complex on a TAT containing promoter	49-50
1.2	*Trans*activation by DNA-binding transcription factors	51-52
1.3	The mechanism of translation initiation	53-54
1.4	The mammalian cell cycle	55-56
1.5	The E2F family	57-58
1.6	The DP family	59-60
1.7	DP-3 protein isoforms	61-62
1.8	The organisation of the 5' untranslated regions of DP-3 RNA	63-64
1.9	The regulation of E2F heterodimers by stable binding to members of the pRb family	65-66
1.10	The regulation of E2F by subcellular localisation	67-68
Table 1.1	E2F target genes	69
Table 2.1	Summary of genomic plasmid clones and sequencing	87
Table 2.2	Oligonucleotides used	88
3.1	Analysis of the DP-3 gene	100-101
3.2	A SacI restriction map of DP-3 phage clones	102-103
3.3	The murine DP-3 genomic structure	104-105
3.4	The E region of DP-3 is a single, alternatively spliced exon	106-107
3.5	The Q insertion of DP-3γ arises from use of an alternative 3' acceptor site	108-109
3.6	Comparison of hDP-2 cDNA sequences and DP-3 genomic structure	110-111
3.7	Comparison of murine DP-1 and DP-3 genomic organisation	112-113
4.1	Northern blot analysis of DP-3 and DP-1 transcripts in mouse tissues	122-123
4.2	Northern blot analysis of DP-3 in a range of cell lines	124-125

4.3	Analysis of DP-3 transcripts	126-127
4.4	Analysis of DP-3 transcripts during F9EC differentiation	128-129
4.5	Analysis of E+ DP-3 transcripts	130-131
5.1	Translational analysis of DP-3 5'UTRs	141-142
5.2	The DP-3 5'UTRs are translated equally *in vitro*	143-144
5.3	The luciferase activity reflects luciferase protein	145-146
5.4	The expression of DP-3 5'UTRs in C33A cells	147-148
5.5	The expression of DP-3 5'UTRs in COS-7 cells	149-150
5.6	The expression of DP-3 5'UTRs in NIH 3T3 cells	151-152
5.7	The expression of DP-3 5'UTRs in F9EC cells	153-154
5.8	The expression of DP-3 5'UTRs in U2OS cells	155-156
6.1	The expression of DP-3 5'UTRs in SAOS-2 cells	164-165
6.2	The effect of co-expression of p53 on DP-3 5'UTR activity in SAOS-2 cells	166-167
7.1	DP-3 peptides	174-175
7.2	Analysis of α7.6 antisera	176-177
7.3	Immunostaining analysis with α7.6 antisera	178-179
7.4	Analysis of α7.7 antisera	180-181
8.1	The insertion of a glutamine residue disrupts a potential DP-3 phosphorylation site	196-197
8.2	The composition of E2F heterodimers may influence E2F activity	198-199
8.3	Summary of the potential influence of p53 on DP-3	200-201

Abbreviations:

A	adenine
AML	acute myelogenous leukaemia
AP	alkaline phosphatase
APS	ammonium persulphate
ARF	alternative reading frame
ATP	adenosine 5'-triphosphate
AUG	translation start signal (methionine codon)
BSA	bovine serum albumin
C	cytosine
CAK	cdk-activating kinase
cdc	cell division cycle
CDE	cell cycle dependent element
cdk	cyclin dependent kinase
cdkI	cyclin dependent kinase inhibitor
cDNA	complementary deoxyribonucleic acid
CHR	cell cycle homology region
cm	centimetre
cpm	counts per minute
CTP	cytosine 5'-triphosphate
DCB	DP conserved box
DEPC	diethyl pyrocarbonate
DHFR	dihydofolate reductase
DIP	DP interacting protein
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
E.coli	*Eschericha coli*
E2F	E2 factor
EC	embryonal carcinoma
EDTA	ethylenediaminetetraacetic acid
eIF	eukaryotic initiation factor
FCS	foetal calf serum
G	guanine

GAPDH	gylceraldehyde-3-phosphate dehydrogenase
GMC	ganglion mother cell
GTP	guanosine 5'-triphosphate
HAT	histone acetyl-transferase
H_2O	distilled water
HCl	hydrochloric acid
HEPES	N-[2-Hydroxethyl]piperazine-N'-[2-ethanesulfonic acid])
HPV	human papilloma virus
HRP	horseradish peroxidase
Inr	Initiator
IBMX	3-isobutyl-1-methyl-xanthine
IRE	iron response element
IRES	internal ribosome entry site
IRF	iron regulatory factor
kD	kilodalton
l	litre
LAP	liver activating protein
LIP	liver inhibitory protein
M	molar
MBP	myelin basic protein
MEFS	mouse embryo fibroblasts
mg	milligram
ml	millilitre
mM	millimolar
mRNA	messenger RNA
ng	nanogram
nm	nanometres
NP-40	Nonidet P40
NTP	nucleotide triphosphates
OD	optical density
ONPG	o-nitrophenyl-β-D-galactopyranoside
ORF	open reading frame

PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	parietal endoderm
pol I/II/III	RNA polymerase I/II/III
pRb	retinoblastoma gene product
Q	glutamine residue
RNA	ribonucleic acid
RNAse	ribonuclease
rRNA	ribosomal RNA
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
snRNA	small nuclear RNA
SV40	simian virus 40
T	thymine
TAF	TBP-associated factor
TBP	TATA binding protein
TdT	terminal deoxynucleotidyl transferase
TF	transcription factor
Tm	melting temperature
Tris	tris(hydroxymethyl)methylamine
Tween20	polyoxyethylene sorbitan monolaurate
U	units/uracil
uAUG	upstream translational intitation site
uORF	upstream open reading frame
UTR	untranslated region
uv	ultra violet
v/v	volume/volume
w/v	weight/volume
wt	wild-type
µg	microgram
µl	microlitre
µM	micromolar

Chapter 1

Introduction

The regulation of gene expression

The phenotype of a cell is dependent on the nature of the genes expressed. Gene expression is controlled at a variety of levels, including transcription, messenger RNA processing, translation and protein modification and finally at the level of protein turnover.

1.1 Transcription

Transcription in eukaryotes is mediated by three RNA polymerases (pol I, II and III), which transcribe three classes of genes. RNA pol I transcribes ribosomal RNAs (rRNA) whereas pol III transcribes transfer RNA and small nuclear RNA (snRNA). RNA pol II is responsible for the transcription of protein coding genes and will be discussed in the most detail.

1.1.1 RNA polymerase II basal transcription.

Transcription by RNA pol II is influenced by three types of transcription factor; basal factors, DNA binding *trans*-acting factors and coactivators. Initially basal transcription factors were identified termed, TFIIA, TFIIB, TFIID, TFIIE, TFIIH and TFIIF, which were necessary for basal transcription from TATA containing promoters (Zawel and Reinberg, 1993). Basal transcription factors have been shown to assemble on the TATA box in a sequential manner to initiate transcription (Figure 1.1). Thus, TFIID initially binds directly to the TATA box, and under certain conditions this is stimulated by TFIIA (Buratowski *et al.*, 1989, Reinberg, 1996). However, in certain cases TFIIA is dispensable for basal transcription. Once TFIID has bound the TATA box, TFIIB binds and recruits RNA pol II to the complex, which is followed by the association TFIIE and TFIIH (summarised in Figure 1.1; Buratowski *et al.*, 1989). Additional factors for basal transcription have also been identified for example; TFIIF may stimulate binding of pol II to TFIIB (Tschochner *et al.*, 1992; Ha *et al.*, 1993). Additionally, TFIIJ can bind to the final complex to form a fully primed pre-initiation complex, although ATP is required for transcription to begin (Reinberg, 1996).

1.1.2 Transcription from TATA-less promoters

Not all polymerase II transcribed genes contain a TATA element, although TFIID is still required for transcriptional initiation (Pugh and Tijan, 1991). Several models have been proposed to explain how transcription is initiated from these promoters. A novel sequence originally identified in the terminal deoxynucleotidyltransferase (TdT) promoter, termed an initiator element (Inr), was shown to be necessary to direct transcription (Smale and Baltimore, 1989). Subsequently many Inr elements have been identified which fall into different families, but these elements are also found in TATA containing promoters (Zawel and Reinberg, 1993). It has been suggested that Pol II directs the assembly of a pre-initiation complex though interacting weakly with Inr elements.

1.1.3 TFIID

The TFIID basal transcription factor is composed of several subunits; the TATA binding protein TBP and several TBP-associated factors (TAFs, Figure 1.1). TBP plays a role in transcription for all RNA polymerases and may also regulate transcription from TATA-less promoters.

TBP alone can only mediate basal levels of transcription, although when associated with TAFs, activated transcription can be induced by *trans*-acting transcription factors (Hoey *et al.*, 1993). Originally TAFs were thought to act as "bridges" between *trans*-acting factors and the basal machinery (Goodrich and Tjian, 1994). Further analysis has revealed that several TAFs are in fact structurally related to histones H2B, H3 and H4 (Burley and Roeder, 1996) suggesting a possible role for TAFs in modulating chromatin. Furthermore, $TAF_{II}250$ has been shown to possess histone acetyl-transferase (HAT) activity (Mizzen *et al.*, 1996). Histone acetylation is believed to be an important step in the generation of transcriptionally active chromatin and the regulation of chromatin structure is an important mechanism for regulating transcription (Grunstein, 1997).

1.1.4 *Trans*-acting factors for RNA polymerase II

Transcription factors that activate transcription consist of a heterogeneous group of proteins that can be cell type specific or ubiquitously expressed. They are usually modular in structure and can be separated into at least two domains; a DNA

binding domain and a transcriptional activation domain (Mitchell and Tjian, 1989). The DNA binding domain confers sequence specific DNA binding properties and usually contains basic residues that contact the DNA (Mitchell and Tjian, 1989).

Many transcription factors bind DNA as dimers through conserved interaction motifs. Often two different proteins bind to form heterodimeric transcription factors, for example AP-1, which is composed of the c-fos and c-jun proteins that dimerise via a leucine zipper motif (Neuberg *et al.*, 1989). Likewise, the E box binding proteins E12 and E47 dimerise via helix-loop-helix structure (Murre *et al.*, 1989).

The *trans*activation domains of transcription factors can vary in structure. For example, Sp1 contains a glutamine rich region, whereas the *trans*activation domain of CTF is proline rich (Mitchell and Tjian, 1989). In general, *trans*-acting factors induce transcription by binding to DNA sequences upstream of the transcriptional start site and contacting the basal transcription machinery (Figure 1.2). Thus, the Sp1 *trans*activation domain can bind to $TAF_{II}110$ part of the TFIID complex, and regulate transcription (Hoey *et al.*, 1993). However, under some circumstances intermediate or coactivator proteins are required (Section 1.1.5; Figure 1.2).

1.1.5 Coactivators and the modulation of chromatin structure

A number of nuclear hormone receptors interact with the coactivator CBP (CREB-binding protein) and activate transcription. Other transcription factors such as p53 and MyoD, have been shown to use CBP or the related protein, p300 as coactivators of transcription (Eckner *et al.*, 1996; Lill *et al.*, 1996; Lee *et al.*, 1997). Coactivators may therefore function by aiding contact between the basal machinery and *trans*-acting factors. Additionally, some coactivators have been shown to exhibit HAT activity (Hampsey, 1997; Hassig and Schreiber, 1997; Pazin and Kadonaga, 1997). Therefore, like $TAF_{II}250$ coactivators may have dual functions, promoting protein/protein interactions and also in regulating chromatin.

1.2 Translational control of gene expression

The control of gene expression at the level of translation permits the direct and rapid expression of proteins in response to various cellular signals. When compared to the regulation of gene expression by transcription alone, translational regulation allows an immediate alteration in the level of a particular protein, whereas transcription, splicing, nuclear export and then translation can result in a delay in protein expression. Additionally, genes such as p53 are under both transcriptional and translation regulation, enabling the fine control of protein level. Some genes that are very large can take a considerable time to be transcribed, for example dystrophin mRNA is more than 2000 nucleotides long, therefore the protein needs to be expressed rapidly, this could be achieved via a translational mechanism, rather than by transcriptional control. A variety of mechanisms exist for translational control, including the regulation of translation by RNA/protein interaction and the regulation via structural or sequence elements in the untranslated regions (UTR) of an mRNA. Additionally, the regulation of activity of general protein factors of the translation machinery can also influence the level of translation in a cell.

1.2.1 The mechanism of eukaryotic protein synthesis

Translation in eukaryotes can be divided into three phases: initiation, elongation and termination. Most translational control mechanisms occur at the level of translation initiation, although some examples of regulation at the level of elongation have been observed.

1.2.1.1 The initiation phase

Translational initiation involves the selection of an mRNA and the formation of a ribosome initiation complex, in which the anti-codon of the initiator Met-tRNA interacts with the initiator codon of the mRNA. It is this interaction that establishes the reading frame of the mRNA. The interaction of the Met-tRNA and mRNA to ribosomes is assisted by numerous initiation factors (eIFs) and requires energy (Merrick and Hershey, 1996; Figure 1.3).

The translation efficiency of different mRNAs is different and is often dictated by the rate of initiation. Structural elements such as the m^7G cap, the context of the initiator codon, the length of the 5'untranslated region (5'UTR) of the

mRNA, secondary structure within the 5'UTR and the presence of multiple upstream AUGs (uAUGs) or open reading frames (uORFs) in the 5'UTR can all influence the initiation step of translation (Pain, 1996).

The initiation of translation can be divided into four major stages; dissociation of 80S ribosomes into subunits, recognition of Met-tRNA$_i$ to the 40S ribosomal subunit, binding of mRNA and recognition of the initiator codon and finally, association of the 60S ribosomal subunit (Figure 1.3).

1.2.1.1.a Dissociation of 80S ribosomes

Initiation of translation commences with the dissociation of the 80S ribosomes. The mechanism of ribosome dissociation is poorly understood, however three eIFs are involved. eIF1A and eIF3 bind the 40S subunit and prevent association with the 60S subunit. eIF6 binds to the 60S subunit and inhibits association with the 40S subunit (Merrick and Hershey, 1996; Figure 1.3).

1.2.1.1.b Met-tRNA$_i$ binding to the 40S subunit

Before binding to the 40S subunit, the initiator Met-tRNA$_i$ forms a complex with eIF2 and GTP. This ternary complex then binds to the 40S ribosomal subunit to form a 43S preinitiation complex (Pain, 1996).

1.2.1.1.c mRNA binding and recognition of the initiator codon

Binding of the mRNA to ribosomes can occur via two methods. Firstly, the binding of the preinitiation complex to the 5' end of the mRNA followed by 'scanning' along the RNA until the initiator codon is reached, or internal ribosome entry, where the preinitation complex binds at the initiation codon (Sachs *et al.*, 1997). Generally the 'scanning' mechanism of initiator codon recognition is thought to occur, this involves the recognition of the m^{7}G cap by eIF4E, which forms the cap binding complex with eIF4A and eIF4G (eIF4F) (Merrick and Hershey, 1996; Sachs *et al.*, 1997). eIF4A has RNA helicase activity and can disrupt mRNA secondary structure to allow the binding of the 40S preinitiation complex (Pain, 1996; Sachs *et al.*, 1997).

The scanning model suggests that the most 5' AUG will be the initiator codon, however if the context of the AUG does not match the consensus

ACCAUGG, leaky scanning occurs (Kozak, 1991) and may result in an AUG being passed over and initiation taking place at another AUG.

1.2.1.1.d Association of the 60S ribosomal subunit

After formation of the preinitiation complex and interaction of the AUG codon with Met_i-tRNA, eIF5 recognises the complex and promotes the hydrolysis of the GTP bound to eIF2 (Pain, 1996). Once GTP hydrolysis has occurred eIF2 is released bound to GDP, which is then recycled (Figure 1.3). After the initiation factors have dissociated from the complex, the 60S ribosomal subunit combines with the 40S subunit to form the 80S initiation complex, which can then enter the elongation stage of translation (Pain, 1996).

1.2.1.2 Translational elongation

Translation elongation involves the addition of amino acids to the carboxy-terminal end of the emerging peptide. Elongation involves the binding of the aminoacyl-tRNA complex containing eEF1A and GTP (ternary complex), on the ribosomal surface. When codons are matching, GTP hydrolysis and guanine nucleotide exchange occurs on eEF1A and a peptide bond is formed and the stripped tRNA leaves the ribosome. The mRNA and the peptidyl-tRNA then move along on the surface of the ribosome, such that the next codon is exposed and ready for aminoacyl-tRNA binding and addition of the next amino acid (Merrick and Hershey, 1996).

1.2.1.3 Termination of translation

When a termination codon is reached on the ribosomal surface, no ternary complex can bind; in which case a release factor binds and promotes hydrolysis of the peptidyl-tRNA (Merrick and Hershey, 1996).

1.2.1.5 Reinitiation of translation following termination

After termination of translation the 80S ribosome is released and then dissociates into the 40S and 60S subunits and can then take part in further protein synthesis. Reinitiation by the terminating ribosome is not thought to occur for most eukaryotic mRNAs (Merrick and Hershey, 1996). However the reinitiation after

translational termination of short upstream ORFs can occur for example, the yeast GCN4 mRNA (Geballe and Morris, 1994; Section 1.5.2b).

1.2.2 The regulation of translation

The control of translation represents an important regulatory control point in gene expression. There are many mechanisms by which a cell can regulate translation and many involve sequence elements within the UTRs of transcripts and the influence these have on translation initiation. The sequence context of the AUG initiator codon, the presence of secondary structure, upstream AUG codons, internal ribosome entry sites and sequence elements recognised by RNA binding proteins in the UTRs of an mRNA may all influence initiation of translation. Furthermore, translation may also be regulated via phosphorylation of translation factors, in response to changes in growth conditions.

1.2.2.a Translational regulation by structural features of the UTRs of mRNA.

Analysis of the sequence surrounding the AUG codon of many mRNAs from higher eukaryotes was found to conform to a consensus sequence, GCC$^{A}/_{G}$CCAUGG (Kozak, 1991). Although the full consensus sequence is rarely found, two highly conserved positions are observed and are critical for function, the purine at position –3 (usually an adenine) and the G at position +4 (the A of AUG is +1) (Kozak, 1991). Conformation to the consensus sequence results in efficient translation initiation. A few vertebrate mRNAs contain non-consensus nucleotides in the critical positions, such as genes encoding growth factors. Therefore, poor initiator context may act to restrain the expression of potentially harmful proteins.

The formation of stable stem-loop secondary structures within the 5'UTR of mRNA can influence translation and is thought to prevent binding of initiation factors, resulting in inefficient translation (Kozak, 1991; Pain, 1996). Analysis of mRNAs containing strong secondary structure revealed that, like those with poor initiator sequences, many mRNAs that have secondary structure code for proteins involved in cell growth (Kozak, 1991). The relief of translation repression is important to switch on protein expression when required. This may be achieved by activation of translation initiation factors. Indeed, several signalling pathways have been shown to phosphorylate elements of the translation machinery in response to

growth signals, an example being the control of translation by insulin. Insulin induces the phosphorylation of the eIF4E-binding proteins, the 4E-BPs. Once phosphorylated 4E-BPs cannot inhibit eIF4E binding to the 5' end of mRNAs (Section 1.2.1.1c) and translation proceeds (Proud and Denton, 1997).

1.2.2b Translational control by upstream AUG codons

The presence of uAUGs and uORFs can influence the translation of the major ORF. The presence of uORFs often has a suppressive influence on translation; however, translation can also be upregulated by uORFs (Geballe and Morris, 1994). An example of an mRNA with suppressive uORFs is the 5'UTR of the *Saccharomyces cerevisae* GCN4 mRNA, which is regulated by reinitiation and by phosphorylation of eIF2. GCN4 is a transcription factor required for amino acid biosynthesis. The GCN4 mRNA is constitutively expressed, however GCN4 protein is only produced during conditions of nutrient deprivation. The 5'UTR of GCN4 mRNA contains four short uORFs. When amino acids are plentiful, the level of active eIF2 is high, since the kinase that phosphorylates eIF2 is activated only when the levels of amino acids are low. Translation of the GCN4 mRNA begins at ORF1 and when complete, rapid reinitiation of translation occurs at ORF4. The termination site of ORF4 does not allow reinitiation and translation of this mRNA ceases and GCN4 is not produced. During amino acid deprivation eIF2 becomes phosphorylated slowing the rate of translation initiation. The rate of reinitiation is also slower and it takes longer for the reinitiation complex to form and scanning continues past ORF4 and initiation takes place at the GCN4 ORF (Kozak, 1992; Geballe and Morris, 1994).

The influence of uAUG codons can be overcome via the presence of internal ribosome entry sites (IRES). IRES elements were first defined by construction of artificial dicistronic mRNAs containing a poliovirus sequence which allowed translation of the second cistron, even when cap-mediated translation of the first cistron was blocked (Pelletier and Sonenberg, 1988; Mountford and Smith, 1995). The IRES is thought to enable ribosomes to associate with the mRNA at the IRES site and thereby avoid uAUG sites that could inhibit translation. This mechanism of translation has been observed in piconavirus RNAs and also occurs in some mammalian mRNAs such as the immunoglobulin heavy chain binding protein (BiP)

(Maccjak and Sarnow, 1991). It has been observed that those genes regulated by internal ribosome entry are often involved in cellular regulation, like many others regulated by structural features of the 5'UTR (Kozak, 1991; Pain, 1996). The 5'UTRs of mammalian mRNAs with IRES do not however resemble those of the picornaviruses and a different mechanism may exist in mammals (Pain, 1996). Additionally, eukaryotic IRES sequences may be common since analysis of functionally defined IRES elements does not reveal any sequence homology and conserved features and hence at present IRES can only be identified by functional analysis (Mountford and Smith, 1995).

1.2.2c Regulation of translation by mRNA/protein interaction

Sequence elements within the UTRs of mRNAs may be binding sites for protein factors that can influence translation. The binding of proteins to the 5'UTR of an mRNA may prevent initiation or scanning by the translation machinery. Alternatively, the binding of proteins to an mRNA may promote translation. In mammalian cells the synthesis of ferritin is regulated by the binding of a repressor protein, the iron regulatory factor (IRF) to the iron response element (IRE) in the 5'UTR of the ferritin mRNA. The IRE has a stable secondary structure and a stem-loop structure forms, situated at the 5' end of the ferritin 5'UTR. The binding of the IRF to the IRE under conditions of low iron results in repression of ferritin translation. It is thought that the presence of the IRF interferes with translational initiation and several models have been proposed (Melefors and Hentze, 1993). An increase in cellular iron levels causes in a change in the affinity or availability of the IRF for the IRE and translation is de-repressed (Melefors and Hentze, 1993). RNA-binding proteins that promote translation have been characterised, however it is thought that these promote translation via an indirect mechanism rather than directly activating translation (Kozak, 1992).

1.3 Cell Cycle Control

1.3.1 Cyclin-dependent kinases

The cell cycle in eukaryotic cells is divided into four distinct phases (Figure 1.4). DNA synthesis (S-phase) and mitosis (M-phase) are separated by two gap phases termed G1 and G2. The G2 phase occurs before mitosis and allows assessment of the DNA replication process whereas G1 occurs after mitosis and is a major control point for entry into the cell cycle. Cell cycle progression is co-ordinated by a family of related cyclin-dependent kinases (cdks) that phosphorylate substrates necessary for cellular proliferation. The cdk consists of a catalytic kinase subunit, a cyclin subunit and a regulatory subunit (cyclin-dependent kinase inhibitor cdkI). The importance of these kinases is reflected in their conservation through a variety of organisms ranging from yeast to humans. To date eight cdk subunits have been identified in mammalian cells termed cdk1-8. Furthermore thirteen cyclin subunits have been isolated (A-H) and seven cdk inhibitors have been identified (Pines, 1995).

The cyclins are related gene products containing a cdk binding domain and some contain a PEST sequence, necessary for ubiquitin-mediated degradation (Pines, 1995). As their name suggests cyclins undergo cyclical synthesis and destruction during cell cycle progression. Thus cyclin B is induced at the G2/M boundary whereas cyclin E is induced in G1 suggesting that each may be required for a different phase of the cell cycle. Indeed this appears to be the case since overexpression of cyclin E causes a reduction in length of G1 and entry into S-phase (Ohtsubo and Roberts, 1993; Ohtsubo *et al.*, 1995). In contrast cyclin B in association with cdc2 is necessary for cells to enter mitosis. Not all cyclins are cell cycle regulated, for example the D-type cyclins are only induced upon growth factor stimulation (Matsushime *et al.*, 1991), although cyclin D1 will co-operate with cyclin E to promote G1 progression and S-phase entry (Resnitzky *et al.*, 1994). Other cyclins appear to be associated with specific cellular processes, such as cyclin H, which in combination with cdk7, is component of TFIIH and is also the cdk activating kinase (Fesquet *et al.*, 1993; Poon *et al.*, 1993; Roy *et al.*, 1994; Serizawa *et al.*, 1995). For some cyclins such as cyclin C, a clear function remains to be assigned. Cyclin C was originally identified as a potential G1 cyclin; however, it does not appear to play a role in cell cycle progression (Lew *et al.*, 1991).

The cdks are also related gene products containing a cyclin binding domain, a catalytic domain and an ATP-binding motif. Cdks are activated when bound to a cyclin partner. Interestingly, the binding of cyclin to cdk results in a conformational change to allow ATP binding (Jeffery *et al.*, 1995). While cyclin binding has substantial influences on and is required for cdk activation, full activation also requires phosphorylation on cdk threonine-160 (Fisher and Morgan, 1994). Phosphorylation of Thr-160 is catalysed by another cyclin/cdk complex termed CAK (cdk activating kinase), which consists of cdk7 together with cyclin H (Fesquet *et al.*, 1993; Poon *et al.*, 1993).

Unlike the cyclin component the cdks are generally more stable and are not subject to cell cycle mediated destruction. Therefore the timing of cdk activation is thought to be dictated by the availability of cyclins. As might be expected, specific cyclins associate with particular cdks to promote cell cycle progression. For example, cyclin E associates exclusively with cdk2 and therefore the cyclin E induced G1 acceleration is thought to be mediated by cdk2 (Sherr, 1993). In contrast, the D-type cyclins associate with cdk 2, 4 and 6, whereas cyclin B binds cdc2/cdk1 (Xiong *et al.*, 1992; Sherr, 1993; Bates *et al.*, 1994). Since cyclin E/cdk2 kinase activity is required for G1 to S transition specific substrates must exist. One such substrate is the retinoblastoma protein (pRb). The pRb protein can be phosphorylated by cyclinE/cdk2 and the D-type cyclins (Ewen *et al.*, 1993), causing inactivation of growth suppressing functions and allows cell cycle progression from G1 to S-phase (Figure 1.4; Section 1.5). Similarly cyclin A/cdk2 is necessary for DNA synthesis (Girard *et al.*, 1991; Pagano *et al.*, 1992). The replication factor RF-A is a substrate for cyclin A/cdk2 and is phosphorylated in S-phase (Dutta and Stillman, 1992). Phosphorylation of RF-A enhances helicase activity, which may explain the basis for cyclin A/cdk2 mediated S phase progression (Dutta and Stillman, 1992).

1.3.2 Cdk inhibitor proteins

The identification of a family of cdk inhibitor polypeptides has proposed new mechanisms of cell cycle control. Two families of cdk inhibitors (cdkI) have been identified based on sequence similarity and mode of action, the p16 and the p21 family (Sherr and Roberts, 1999).

The p16 family consists of p16 (INK4a), p15 (INK4b), p18 (INK4c) and p19 (INK4d/ARF) (Serrano *et al.*, 1993; Guan *et al.*, 1994; Hannon and Beach, 1994; Kamb *et al.*, 1994; Chan *et al.*, 1995; Hirai *et al.*, 1995). The p16 family can bind to either monomeric cdk4/6 or directly to the cyclin D/cdk4/6 complex to inhibit kinase activity. The importance of p16 in growth control is underscored by the results obtained from transgenic knockout mice. Homozygous deletion of p16 in mice results in increased susceptibility to tumours and mouse embryo fibroblasts (MEFs) derived from these mice grow faster with an increased S-phase population (Serrano *et al.*, 1996).

Unlike the p16 family, the p21 family are general inhibitors of cdks. The p21 family includes p21 (kip/waf1/cip1), p27 and p57 (Polyak *et al.*, 1994; Harper *et al.*, 1995; Lees *et al.*, 1995; Matuoka *et al.*, 1995). The p21 family bind to cyclin/cdk complexes and inhibit kinase activity partly by occupying the ATP binding site (DeBondt *et al.*, 1993; Jeffery *et al.*, 1995; Russo *et al.*, 1996). Interestingly, p21 expression is induced by p53 and contributes to p53 mediated growth arrest. Transgenic mice lacking p21 and p27 have also been generated. Surprisingly p21 null mice do not show increased malignancies, however the p53-dependent G1 arrest is impaired (Brugarolas *et al.*, 1995; Deng *et al.*, 1995). In contrast, mice lacking p27 are larger than normal mice, with bigger organs (Fero *et al.*, 1996; Kiokawa *et al.*, 1996; Nakayama *et al.*, 1996).

In conclusion, multiple related cdkI exist which function though regulating the activity of cdks. In general these inhibitors do not function during cell cycle progression but play a unique role in exit and entry into the cell cycle

1.4 The p53 tumour suppresser protein

The human tumour suppresser protein p53 is an important regulator of cellular proliferation. For example, the loss of p53 through mutation or inactivation by viral oncoproteins is observed in over half of all human cancers. Furthermore, the inheritance of a mutant *p53* allele results in Li-Fraumenti syndrome and predisposition to various cancers (Malkin, 1993). The disruption of both *p53* alleles in mice does not result in developmental defects but in a high predisposition to diverse tumours (Donehower *et al.*, 1992).

Functional analysis of p53 has revealed a central role in the cellular response to DNA damage. The accumulation of p53 following DNA damage and other genotoxic stress, results in either arrest of the cell cycle at G1 or in cell death by apoptosis (Kastan *et al.*, 1992; Donehower and Bradley, 1993; Lu and Lane, 1993; Macleod *et al.*, 1995; Ko and Prives, 1996). Once detection of DNA damage and signalling to p53 have occurred, p53 functions to arrest cells at either the G1/S phase or G2/M phase checkpoints of the cell cycle, allowing DNA repair to take place before DNA synthesis or division. If DNA repair is unsuccessful, p53 initiates apoptosis, thereby preventing the transmission of the genetic damage to the next and successive generations. However p53 is not required for all forms of apoptosis (Clarke *et al.*, 1993; Macleod *et al.*, 1996).

1.4.1 p53 is a transcription factor

The p53 protein is a sequence specific transcription factor that can bind and activate promoters containing p53-binding sites. The DNA binding domain of p53 has been mapped to the central region of the p53 protein. Since more than 90% of missense mutations in p53 are found in this region, the DNA binding and transcriptional functions of p53 are considered central to its biological functions (Ko and Prives, 1996). Mutation of residues such as R248 and R273 result in poor contacts with DNA and result in loss of transcriptional activity of p53 (Ko and Prives, 1996). Other mutations disrupt the structure of the DNA binding domain (Hupp *et al.*, 1992; Levine, 1997).

The N-terminus of p53 contains the transcriptional activation domain required for activation of p53 target genes. Numerous p53 target genes have been identified and are observed to be induced on activation of p53, for example after DNA damage. p53 responsive genes have functions linked to the known functions of p53, for instance cell cycle arrest. The p21 gene has been identified as a p53 target gene (El-Diery *et al.*, 1993; Harper *et al.*, 1993). p21 is a general cyclin-dependant kinase inhibitor (Pines, 1995; Section 1.3.2) and upregulation of p21 results in the inhibition of cyclin-dependant kinase phosphorylation of pRb. Consequently, hypophosphorylated pRb accumulates and the repression of E2F responsive genes results in cell cycle arrest at the G1/S phase boundary (DeGregori *et al.*, 1995; Lukas *et al.*, 1995). p21 also inhibits other cyclin-dependant kinase complexes and

can prevent the phoshorylation of other members of the pocket protein family, p107 and p130, thereby inhibiting the activity of E2F complexes (La Thangue, 1994). p21 also interacts with PCNA (proliferating cell nuclear antigen) (Waga *et al.*, 1994; Gulbis *et al.*, 1996), this interaction blocks PCNA function in DNA replication and therefore, p21 can act via two mechanisms to inhibit S phase entry and DNA replication.

Other p53 responsive genes include the *mdm2* proto-oncogene (Momand *et al.*, 1992; Oliner *et al.*, 1992). The MDM2 protein interacts directly with p53, inhibiting transcriptional activity by masking the transactvation domain (Momand *et al.*, 1992; Oliner *et al.*, 1992). Furthermore, MDM2 regulates the stability of p53 and targets p53 protein for proteosome mediated degradation (Haupt *et al.*, 1997; Kubbutat *et al.*, 1997) and suggesting an autoregulatory feedback loop.

The *GADD45* gene is another p53 transcriptional target that is induced in response to DNA damage (Kastan *et al.*, 1992; Lu and Lane, 1993). GADD45 protein interacts with PCNA and inhibits the entry of cells into S phase (Smith *et al.*, 1994) and may contribute to p53 mediated cell cycle arrest.

The induction of apoptosis by p53 is thought to involve the activation of other transcriptional targets such as the *bax* gene. The Bax protein binds to the cell survival factor Bcl-2 and promotes apoptosis (Miyashita and Reed, 1995).

Other p53 responsive genes include the insulin-like growth factor binding protein 3 (*IGF-BP-3*) gene, the product of which inhibits mitotic signalling by insulin-like growth factor (Buckbinder *et al.*, 1995) and cyclin G, a cyclin of unknown function (Okamoto and Beach, 1994).

p53 can also repress transcription of several genes which do not have p53 binding sites in their promoters, including *c-fos, c-jun, Rb*, and *Bcl-2* and it is thought that the functions of p53 as a repressor may be important in it's role as a tumour suppresser (Ko and Prives, 1996). The N-terminal *trans*activation of domain of p53 interacts with basal transcription factors such as TBP (Horikoshi *et al.*, 1995), and TBP-associated factors including, TAF40, TAF60 and TAF31 (Lu and Levine, 1995; Thut *et al.*, 1995). It is thought that these interactions may be involved in p53-mediated repression of transcription. Also, p53 has been shown to interact with components of TFIIH, and through this interaction transcriptional inhibition by p53 in the absence of a p53 DNA binding site is overcome (Leveillard *et al.*, 1996).

1.4.2. p53-dependant apoptosis

p53 mediates apoptosis in many cell types and is an important mechanism for suppression of proliferation and tumour formation (Ko and Prives, 1996). The transcriptional functions of p53 are important for induction of cell cycle arrest and also for mediating apoptosis. However, some groups have shown that mutant p53 proteins which cannot activate transcription can induce apoptosis, whilst others suggest that transcription defective p53 mutants cannot induce apoptosis (Haupt *et al.*, 1995; Sabbatini *et al.*, 1995; Ko and Prives, 1996). Such data suggests that p53 may induce apoptosis via different transcription dependent and independent mechanisms and perhaps is influenced by cell or tissue specific differences. However, the expression of proteins that block p53-dependent apoptosis such as Bcl-2 or adenovirus E1B 19KD protein, have been observed to cause a decrease in p53 mediated transcriptional repression but have no effect on transcriptional activation (Shen and Shenk, 1994; Sabbatini *et al.*, 1995). In addition, the p53 protein interacts with a range of cellular and viral proteins (Ko and Prives, 1996; Levine, 1997), and it is possible that these interactions may be involved in the induction of apoptosis.

How cells decide whether to arrest or apoptose in response to p53 activation appears to be dependent on several factors including cell type, growth factors, the presence of viral proteins, pRb function and overexpression of E2F (Ko and Prives, 1996). Loss of pRb function results in failure to arrest in G1 and subsequent over expression of E2F-1 leads to apoptosis, which is enhanced in the presence of p53 (Shan and Lee, 1994; Wu and Levine, 1994). The cellular status with respect to both pRb and E2F-1 may influence the balance between apoptosis and cell cycle arrest.

1.4.3 Regulation of p53 activity

The activation and accumulation of p53 protein in response to stimuli such as DNA damage, hypoxia, nutrient deprivation and other stresses are vital for induction of cell cycle arrest and apoptotic pathways. Wild-type p53 protein levels are low in cells due to a rapid turnover rate, and the activation of p53 results in an increase in protein levels, via post-transcriptional mechanisms (Kastan *et al.*, 1991). The stability and transcriptional activity of p53 is regulated by interaction with MDM2, which binds to the N-terminal region of p53 and prevents transcriptional activation

by masking the transcriptional activation domain (Oliner *et al.*, 1993). MDM2 targets p53 for proteosome-mediated degradation and indeed MDM2 can function as an E3 ubiquitin ligase (Haupt *et al.*, 1997; Honda *et al.*, 1997; Kubbutat *et al.*, 1997). Furthermore, MDM2 must bind to the transcriptional coactivator p300 to mediate p53 degradation (Grossman *et al.*, 1998) and shuttle from the nucleus to the cytoplasm to be degraded (Roth *et al.*, 1998). Since MDM2 is a transcriptional target of p53, the activation of p53 also induces MDM2 expression, which subsequently inhibits p53 function, thereby forming an autoregulatory feedback loop (Wu *et al.*, 1993). Therefore, the disruption of the MDM2-p53 interaction is likely to be important in activating p53.

Recent work has revealed that p53 is induced on expression of $p19^{ARF}$ (alternative reading frame, ARF), an alternative product of the INK4a (p16) locus (Kamijo *et al.*, 1998; Section 1.3.2). ARF binds to MDM2 and forms a trimeric complex with p53 *in vivo*; however, it is not clear how this leads to stabilisation of p53 (Kamijo *et al.*, 1998; Pomerantz *et al.*, 1998; Stott *et al.*, 1998; Zhang *et al.*, 1998). It is interesting to note that in cells that have a homozygous deletion of ARF, p53 is induced in response to DNA damage (Kamijo *et al.*, 1997), suggesting that other mechanisms exist to regulate p53 in conditions of DNA damage. ARF is thought to be induced by viral and cellular oncogenes and may represent another signalling pathway through which p53 controls proliferation.

The p53 protein contains many phosphorylation sites. DNA damage can induce the phosphorylation of p53 on Ser-15 (Shieh *et al.*, 1997; Siliciano *et al.*, 1997). DNA-PK has been shown to phoshorylate Ser-15 and Ser-37 *in vitro* (Lees-Miller *et al.*, 1992) and in cells that lack DNA-PK, p53 cannot bind DNA, suggesting that DNA-PK may contribute to activation of p53 transcription (Woo *et al.*, 1998). p53 is also phosphorylated on Ser-15 by ATM, a kinase that is defective in patients with ataxia telangiectasia, a disorder resulting in extreme sensitivity to ionising radiation (Banin *et al.*, 1998; Canman *et al.*, 1998). In addition, the ATM related kinase ATR can phosphorylate Ser-15 and Ser-37 *in vitro* and a catalytically inactive form of ATR can inhibit Ser-15 phosphorylation *in vivo* (Tibbetts *et al.*, 1999). Such data suggests that the phosphorylation of p53 at Ser-15 is important in the activation of p53 transcriptional activity. Phosphorylation of p53 at Ser-15 weakens the interaction between MDM2 and p53 and the ability of MDM2 to

repress p53 transcriptional activation (Shieh *et al.*, 1997). Therefore, multiple DNA damage induced protein kinases target the p53-MDM2 interaction to activate p53 transcriptional activity.

Other sites in the N-terminus of p53 are also phosphorylated in response to DNA damage (Siliciano *et al.*, 1997; Sakaguchi *et al*, 1998). The C-terminus of p53 is also modified in response to some forms of DNA damage, where both phosphorylation and acetylation have been reported to occur in response to DNA damage (Sakaguchi *et al.*, 1998), and also de-phosphorylation of a site has been observed after exposure to ionising radiation (Waterman *et al.*, 1998). The C-terminus of p53 can negatively regulate DNA binding and it is possible that these modifications de-repress p53 DNA binding. It is therefore likely that multiple kinases, phosphatases and acetylases modify p53 and influence function in response to various cellular stresses.

1.4.5 p53 can modulate translation

Analysis of p53 protein levels in human acute myelogenous leukemia (AML) cells revealed differences in the levels of p53 protein and p53 mRNA, and further analysis suggested that the 3'UTR of human p53 transcripts may repress p53 translation (Fu *et al.*, 1996). In addition, the induction of p53 protein in response to γ-radiation has been shown to be dependent on the 3'UTR of human p53 mRNA in AML cells (Fu and Benchimol, 1997). Murine p53 negatively regulates p53 expression through the 5'UTR of p53 transcripts and physically interacts with the 5'UTR of p53 mRNA *in vitro* (Mosner *et al.*, 1995). Murine p53 has also been shown to be important in the repression of cyclin dependant kinase 4 (CDK4) translation in TGF-β1-induced G1 cell cycle arrest, an effect that is mediated by the 5'UTR of the CDK4 transcript (Ewen *et al.*, 1995). Hence p53 may accumulate via mechanisms other than protein stabilisation, such as an increase in translation. Indeed, p53 may influence not only p53 protein levels, but also other targets by both transcriptional and translational mechanisms.

1.5. The Retinoblastoma Gene

Retinoblastoma is a rare childhood cancer that arises from inactivating mutations on both alleles of the *retinoblastoma* (Rb) gene. Additionally, the *Rb* gene is often found to be mutated in cancers such as osteosarcomas, small-cell lung carcinomas, cervical and breast carcinomas and some types of leukaemia, suggesting that Rb has tumour suppressing function in many different cell types (Herwig and Strauss, 1997). The Rb gene product (pRb) acts as a tumour suppresser protein; an important function of pRb is to restrain uncontrolled growth by inhibiting progression through G1 phase of the cell cycle. pRb is a member of a family of related proteins; p107 and p130 have similar growth suppressive functions to pRb, but not considered to be tumour suppresser genes, as they are not mutated in human tumours (Cobrinik *et al.*, 1993; Hannon *et al.*, 1993; Li *et al.*, 1993; Zhu *et al.*, 1993).

1.5.1 The Rb protein

Human pRb is a 105 KD nuclear phosphoprotein that interacts with many cellular proteins including, E2F and the D-type cyclins, and in addition is targeted and inactivated by several viral oncoproteins such as adenovirus E1a, human papilloma virus type 16 (HPV) E7 protein and simian virus 40 (SV40) T antigen. The regions of E1a, E7 and T antigen required to bind pRb are also necessary for immortalisation and transformation (Whyte *et al.*, 1988; Dyson *et al.*, 1989). All of these proteins interact directly with pRb through a conserved region, named the pocket (Herwig and Strauss, 1997). The pocket region of pRb is frequently found mutated in human tumours and these mutants are unable to interact with E1A, T antigen, E7, E2F and the D-type cyclins. A comparison of the primary sequence of pRb and relatives, p107 and p130 revealed good sequence conservation in the pocket region and for this reason these proteins are often referred to as the pocket protein family. It is notable that no tumour derived mutants of p107 and p130 have been characterised that impair the functions of these two pRb related proteins, although the pocket region is still required to bind E2F (Cobrinik *et al.*, 1993; Hannon *et al.*, 1993; Li *et al.*, 1993; Zhu *et al.*, 1993).

1.5.2. pRb and the control of G1/S phase progression

In tumour cells lacking pRb, overexpression of wild-type pRb causes cell cycle arrest (Huang *et al.*, 1988; Bookstein *et al.*, 1989). pRb regulates the progression of cells from G1 to S phase of the cell cycle. The activity of pRb is influenced by phosphorylation state, where hypophosphorylated pRb can repress and control entry into S phase. Hyperphosphorylated pRb is unable to inhibit cell cycle progression and cells enter S phase and progress through the cell cycle (Mittnacht, 1998). Likewise, p107 and p130 are also phosphorylated in a similar manner and like pRb, it is likely that phosphorylation influences function (Dyson, 1998).

The pocket proteins regulate the cell cycle through interaction with the E2F family of transcription factors (Bandara and La Thangue, 1991; Zamanian and La Thangue, 1992, 1993). The pocket proteins bind E2F via the pocket region and repress E2F transcriptional activity. E2F target genes include those required for S phase progression and DNA replication and repression of these genes prevents cells from entry into S phase (Section 1.6; Table 1.1). The oncogenic activity of E1A, E7 and T antigen is considered to arise from the sequestration of pocket proteins, releasing active E2F and allowing uncontrolled cell cycle progression. Likewise, tumour derived mutant pRb cannot bind and inhibit E2F, resulting in uncontrolled proliferation (Bandara *et al.*, 1991).

The pocket proteins associate with specific members of the E2F family of proteins (Section 1.6.4). In addition, pocket protein-E2F complexes are observed at different stages of the cell cycle. For example, and consistent with the role of pRb in regulating S phase entry, pRb complexes are observed in G1 phase. In contrast, p130-E2F complexes are observed in quiescent cells (Dyson, 1998).

Homozygous disruption of the *Rb* gene in mice results in an embryonic lethal phenotype, with defects observed in the liver, central nervous system, lens and erythropoiesis (Clarke *et al.*, 1992; Jacks *et al.*, 1992; Lee *et al.*, 1992) which suggests that pRb may function in differentiation in some cell types. The disruption of either p107 or p130 in mice results in no abnormalities (Cobrinik *et al.*, 1996; Lee *et al.*, 1996), although inactivation of both p107 and p130 results in neonatal lethality and defective bone development (Cobrinik *et al.*, 1996). The observation that disruption of pRb and p107/p130 result in different phenotypes suggests the pocket proteins may have different E2F target genes. This correlates with analysis of

E2F targets in mouse embryo fibroblasts (MEFs) derived from knockout mice (Hurford *et al.*, 1997). The analysis of MEFs derived from pRb knockout mice revealed deregulation of a subset of E2F target genes, whereas either p107 or p130 knockouts did not reveal changes in E2F targets, although deregulation of E2F targets was observed in the MEFs from mice deficient for both p107 and p130. Interestingly, the target genes affected by the loss of pRb and by the loss of 107/p130 were different (Hurford *et al.*, 1997).

1.5.3. Phosphorylation of pocket proteins

The activity of pocket proteins is linked to the cell cycle via phosphorylation by cdks, which are activated by interaction with cyclins (Section 1.3). The G1 phase of the cell cycle is regulated by the D-type cyclins, which activate cdk4 and cdk6, and by cyclin E, which activates cdk2 in late G1. The D-type cyclins can interact with all pocket protein family members via the pocket region (Dowdy *et al.*, 1993; Ewen *et al.*, 1993). The cyclin D-dependent kinases can phosphorylate pRb, which results in dissociation of E2F/pRb complexes and loss of G1 arrest functions of pRb (Ewen *et al.*, 1993). Inhibition of cyclin D-dependent kinases by the expression of cdk inhibitors such as p16 results in hypophosphorylated pRb and G1 arrest (Lukas *et al.*, 1995b). However, cells that do not express pRb do not arrest when cyclin D-dependent kinase function is inhibited, suggesting that the phosphorylation of pRb by cyclin D-dependent kinases is important for cell cycle progression (Lukas *et al.*, 1994, 1995a). Similarly, the expression of cyclin E can induce the phosphorylation of pRb, and cyclin E can induce S phase in cells which lack functional pRb and therefore may phosphorylate other targets required for S phase entry (Ohtsubo *et al.*, 1995; Hofmann and Livingstone, 1996).

The phosphorylation of p107 and p130 is also cell cycle regulated (Beijerbergen *et al.*, 1995; Mayol *et al.*, 1995), and the growth suppressing functions of p107 and p130 are overcome by cyclin D-dependent phosphorylation (Beijersbergen *et al.*, 1995; Zhu *et al.*, 1993).

1.6 The E2F transcription factor

E2F was originally identified as a cellular transcription factor able to bind to a sequence in the adenovirus E2 promoter (Kovesdi *et al.*, 1987; La Thangue and Rigby, 1987). E2F DNA binding activity was found to be high in undifferentiated F9EC cells declining as cells differentiated to parietal endoderm-like cells, suggesting a role in embryogenesis. Furthermore, E2F was also implicated in adenovirus replication as a DNA binding activity, induced during infection.

The importance of E2F in the control of cellular proliferation was highlighted when it was identified as a target for pRb (Bandara and La Thangue, 1991; Chellapan *et al.*, 1991). E2F forms complexes with pRb that can be disrupted by the adenovirus E1a protein (Bandara and La Thangue, 1991). The regions of E1a required for binding pRb coincide with that necessary for cell transformation suggesting that sequestration of pRb from E2F plays a role in E1a-mediated oncogenesis. In support of this hypothesis it has been shown that pRb can repress E2F-dependent transcription, which in turn can be alleviated by co-expression of E1a (Zamanian and La Thangue, 1992). The p107 and p130 proteins, like pRb, also form complexes with E2F and can repress E2F transcription (Zamanian and La Thangue, 1993; Vairo *et al.*, 1994)

The cellular E2F DNA binding activity is a heterodimer composed of an E2F family member and a DP family member. To date, six E2F genes and two DP genes have been isolated in mammals. E2F and DP family members have also been cloned in other species including *Drosophila melanogaster*, *Xenopus laevis* and chicken (Dynlacht *et al.*, 1994; Girling *et al.*, 1994; Ohtani and Nevins 1994; Philpot and Friend, 1994; Hao *et al.*, 1995; Pasteau *et al.*, 1995).

1.6.1 The E2F family

The first E2F family member isolated was E2F-1 (Helin *et al.*, 1992; Kaelin *et al.*, 1992; Shan *et al.*, 1992). Two further clones with significant homology to E2F-1 were also isolated, E2F-2 and E2F-3 (Ivey-Hoyle *et al.*, 1993; Lees *et al.*, 1993). E2F-1, 2 and 3 all have a highly conserved DNA binding and dimerisation domains, a C-terminal transactivation domain and an N-terminal domain, which contains a nuclear localisation signal and cyclin A binding domain (Figure 1.5). In addition, E2F-1, 2 and 3 all bind preferentially to pRb, through a C-terminal domain.

Additional E2F family members, E2F-4 and E2F-5, have also been characterised (Beijersbergen *et al* 1994; Ginsberg *et al.*, 1994; Buck *et al.*, 1995; Hijmans *et al.*, 1995; Sardet et *al.*, 1995). Although all the E2F family members share a similar domain structure, sequence comparison shows greater similarity between E2F-4 and 5 than with the other E2F family members and suggests they represent a subfamily of E2Fs. E2F-4 and 5 have a truncated N-terminus and therefore lack the cyclin A binding domain present in E2F-1, 2 and 3 (Figure 1.5). E2F-4 and 5 are regulated by interaction with p107 and p130 via a C-terminal domain (Figure 1.5), and E2F-4 can also associate with pRb under certain conditions (Beijersbergen *et al* 1994; Ginsberg *et al.*, 1994; Vairo *et al.*, 1994; Moberg *et al.*, 1995). E2F-5 preferentially interacts with p130 (Hijmans *et al.*, 1995). In comparison to all the other E2Fs, the sixth E2F family member isolated E2F-6, lacks the N-terminus of E2F-1, 2 and 3 and consists of the DNA binding and dimerisation domains. Perhaps more importantly, E2F-6 lacks the C-terminal pocket protein binding and transactivation domains. Therefore, E2F-6 does not interact with any members of the pocket protein family and is thought to act as a repressor of E2F dependent transcription (Morkel *et al.*, 1997; Cartwright *et al.*, 1998; Trimarchi *et al.*, 1998).

Presently, only a few differences have been observed between the individual E2F family members. Differences have been observed in the RNA expression patterns of mammalian E2Fs in a range of tissues and cell lines. For example in human tissues E2F-2 RNA is highly expressed in placenta, lung and kidney, and E2F-3 RNA levels are higher in liver and muscle and absent in the brain (Lees *et al.*, 1993). E2F-1 is expressed in most tissues during mouse embryo development, and in some tissues is often co-localised with DP-1 (Tevosian *et al.*, 1996). Analysis of E2F-2 and 4 expression by *in situ* hybridisation during murine development suggests expression is highest in proliferating cells and in contrast, E2F-5 is expressed in differentiated cells such as the choroid plexus (Dagnino *et al.*, 1997; Lindeman *et al.*, 1998). Homozygous deletions of E2F-1 and E2F-5 in mice have resulted in different tissue specific effects and suggest that each E2F may have particular functions in tissues or cell types (Field *et al.*, 1996; Yamasaki *et al.*, 1996; Lindeman *et al.*, 1998).

1.6.2 The DP Family

DP-1, the first DP family member to be isolated was identified as an E2F site-specific DNA binding polypeptide in murine F9EC cells (Girling *et al.*, 1993) and subsequently human DP-1 was also isolated (Helin *et al.*, 1993). Analysis of E2F DNA binding complexes has revealed that DP-1 is the most frequent component of the E2F DNA binding activity (Girling *et al.*, 1993; Bandara *et al.*, 1994). The isolation of related DP proteins, DP-2 in *X. laevis* and a second murine DP, named DP-3, suggest that not all E2F complexes will contain DP-1. All the DP family members isolated have a high level of conservation across the DNA binding domain and in the DEF box (region of DP and E2F similarity), a domain required for dimerisation with E2F family members (Girling *et al.*, 1993, 1994; Figure 1.6). The DEF box contains the region of greatest similarity with the E2F family. Additionally, all DP proteins show good conservation in the DP-conserved boxes 1 and 2 (DCB 1 and 2) and in an acidic C-terminal domain, the negatively charged box (NCB; Figure 1.6).

Analysis of the expression patterns of the DP family members revealed that human DP-1 and DP-2 are expressed in many different cell lines and tissues (Wu *et al.*, 1995; Zhang and Chellapan, 1995). In addition, *in situ* hybridisation analysis of DP-1 during mouse embryogenesis revealed DP-1 to be expressed in most tissues, at a consistently higher level than E2F-1 (Tevosian *et al.*, 1996). Analysis of *X. laevis* DP expression suggested that DP genes might be tissue restricted (Girling *et al.*, 1994).

1.6.3 Murine DP-3

DP-3 differs from other DPs due to a striking complexity at the RNA level. RNA analysis has suggested that extensive processing of DP-3 RNA gives rise to four potentially different DP-3 proteins; α, β, γ, δ, which can all dimerise with E2F-1 and activate E2F-dependent transcription (Ormondroyd *et al.*, 1995). These variants arise via two changes in the ORF; namely the inclusion of a 16 residue sequence (named the E region) within the DNA binding domain in the DP-3α and δ isoforms, and a single a glutamine amino acid (Q) insertion in the DP-3 γ isoform between the DNA binding domain and the E2F dimerisation domain (Ormondroyd

et al., 1995; Figure 1.7). Analysis of the E region revealed that it functions as part of a nuclear localisation signal (de la Luna *et al.*, 1996; Section 1.6.7b).

Extensive splicing in the 5'untranslated region (UTR) of DP-3 RNAs gives rise to several different 5'UTRs and results in the initiation of translation at two different translational start sites, named M1 and M2 (Ormondroyd *et al.*, 1995) (Figure 1.8). Initiation at M1 results in translation of the DP-3α isoform and an extended N-terminus, containing sequence homology to the N-terminus of DP-1 (Ormondroyd *et al.*, 1995). All other DP-3 isoforms result from initiation at M2. To date, four different DP-3 5'UTRs have been characterised and RNase protection data suggests that the expression is tissue restricted (Ormondroyd *et al.*, 1995; Figure 1.8). In addition, particular ORFs are found associated with specific 5'UTRs, suggesting another level of complexity (Ormondroyd *et al.*, 1995). Human clones with a high degree of homology to DP-3 have been found, named DP-2 and suggest that many of the variations observed for murine DP-3 are conserved in human DP-2 (Wu *et al.*, 1995; Zhang and Chellapan, 1995, 1996; Rogers *et al.*, 1996).

1.6.4 Heterodimerisation and DNA binding of E2F and DP family members

Individual E2Fs or DPs can bind to DNA weakly (Helin *et al.*, 1992; Kaelin *et al.*, 1992; Shan *et al.*, 1992; Girling *et al.*, 1993). However, heterodimerisation of DP and E2F family member results in synergistic DNA binding and *trans*activation of E2F-dependent transcription (Bandara *et al.*, 1993; Helin *et al.*, 1993). Heterodimerisation requires the DEF box of DP proteins and, in addition, residues outside this region contribute to dimerisation (Bandara *et al.*, 1993; Helin *et al.*, 1993). The region of E2F-1 necessary for heterodimerisation with DP-1 has also been defined (Helin *et al.*, 1993). The relevance of E2F homodimerisation *in vivo* remains to be elucidated.

The crystallisation and subsequent structural analysis of E2F-4/DP-2 heterodimer-DNA complex has been achieved and revealed that the E2F DNA binding domain is related to the winged-helix DNA binding motif, a motif that has been noted in several eukaryotic transcription factors. The structure also revealed that E2F-4/DP-2 heterodimers bind the major groove of DNA and that the residues that contact the DNA are well conserved in all E2F and DP family members (Zheng *et al.*, 1999). Furthermore, the DEF box and N-terminal sequences of DP-2 are

required for heterodimerisation with E2F-4 and an extensive protein-protein interface is formed. The structures formed between E2F-4 and DP-2 are asymmetrical and it has been suggested that this contributes to DNA binding as a heterodimer rather than as homodimers (Zheng *et al.*, 1999).

1.6.5 Binding of pRb family members regulates E2F activity

E2F heterodimers form stable complexes with members of the pRb family of proteins (pocket proteins) (Bagchi *et al.*, 1991; Bandara and La Thangue, 1991; Cao *et al.*, 1992; Cobrinik *et al.*, 1993; Figure 1.9). This is via direct interaction between the pocket protein and an E2F family member, however this interaction can be influenced by the contacts between the C-terminus of DP-1 and the pocket protein (Bandara *et al.*, 1994). The pocket protein binding domain of E2F family members is highly conserved and has been mapped to the C-terminus, overlapping with the *trans*activation domain (Figure 1.5), suggesting that binding of a pRb family member to E2F masks the *trans*activation domain and prevents activation of transcription by E2F.

E2F/pocket protein complexes are observed at different stages of the cell cycle. pRb containing E2F complexes are present during G1 phase of the cell cycle, when pRb is hypophosphorylated (Buchkovich *et al.*, 1989; Chellapan *et al.*, 1991; Mittnacht, 1998) and decrease as cells enter S phase, correlating with increasing phosphorylation of pRb. E2F/p107 complexes are observed during G0/G1 and increase in abundance as cells enter S phase. On the other hand, p130 complexes are detected principally in quiescent or differentiated cells (G0 cells), some complexes being observed in G1 phase (Dyson, 1998).

The phosphorylation state of pocket proteins is thought to regulate the formation of E2F complexes. The phosphorylation of pocket proteins disrupts E2F/pocket protein complexes, and the affinity of pocket proteins for E2F decreases as pocket proteins become hyperphosphorylated, releasing free E2F and resulting in an increase in E2F activity. The phosphorylation state of pocket proteins fluctuates as cells cycle and is regulated by the action of cdks. pRb, p107 and p130 all have multiple cdk phosphorylation sites, and the regulation of pRb phosphorylation has been studied in the most detail. The phosphorylation of pRb occurs in stages, firstly in early G1 phase, via action of cyclin D–dependant kinases, followed by the cyclin

E-dependant kinases at the G1/S phase transition (Mittnacht, 1998). The conversion of pRb from a hypophosphorylated form to a hyperphosphorylated form at the G1/S phase boundary correlates with an increase in E2F activity. p107 and p130 are also phosphorylated in a cell cycle-dependent fashion (Beijersbergen *et al.*, 1995; Mayol *et al.*, 1995) suggesting that E2F complexes containing p107 and p130 may also be regulated by pocket protein phosphorylation. Indeed, p107 complexes containing cyclin A/cdk2 and cyclin E/cdk2 have been noted (Faha *et al.*, 1992; Schwartz *et al.*, 1993).

1.6.6 Transcriptional regulation by E2F

1.6.6.a E2F target genes

Analysis of cellular promoters identified E2F sites in genes involved in cell cycle regulation. These genes include those required for DNA replication such as *DNA polymerase α, dihydrofolate reductase* (*DHFR*), *thymidine kinase* and *ORC1* and genes involved in cell cycle control including, *cyclin A*, *cyclin E*, *p107*, *E2F-1* and 2 and *B-myb* (Herwig and Strauss, 1997; Helin, 1998; Table 1.1). The E2F sites in target genes can mediate either activation or repression of transcription; since mutation of E2F sites in target genes can result in either an increase or decrease in promoter activity when compared to the wild-type promoter. For example, activation of the *DHFR* promoter is lost when an E2F site is mutated (Means *et al.*, 1992), although mutation of an E2F site in the *B-myb* promoter results in loss of repression during G0 (Lam and Watson, 1993). This suggests that E2F can target some genes for silencing during specific stages of the cell cycle whilst at the same time activating others. The E2F sites in these genes are nearly identical but act in opposing ways, suggesting that other sequence elements or factors involved in the complex cell cycle regulation of E2F target genes. For example, the *CDC25C* promoter has multiple sequence elements required for the observed cell cycle regulation of expression. A cell cycle-dependent element (CDE) and a cell cycle homology region (CHR) element are both present in the *CDC25C* promoter and are required for cell cycle-dependant expression (Zwicker and Müller, 1997). Mutation of these promoter elements abolishes cell cycle-dependent expression of this gene and suggests that proteins may bind to these elements. Indeed, *in vivo* footprinting analysis of the *CDC25C* promoter during G0/G1 phase of the cell cycle revealed the

CDE to be occupied by an unknown repressor protein (named CDF; Lucibello *et al.*, 1995a). In addition, footprinting analysis of other E2F target genes, such as *CDC2* and *cyclin A* revealed G0/G1 specific occupation of both the CDE and the CHR. Mutation of the CDE and the CHR sites results in a reduction in the repression of these promoters in G0 (Zwicker *et al*, 1995). Furthermore, it has been suggested that the CDF may compete with E2F for promoter binding (Lucibello *et al.*, 1995b; Liu *et al.*, 1998).

1.6.6.b *Trans*activation of E2F target genes by E2F complexes

At present little information is available as to how individual E2F complexes mediate activation or repression of particular E2F target genes. However, different combinations of E2F and DP family members have been shown to bind specifically to particular sequences using the CASTing method of selection of random oligonucleotides (Tao *et al.*, 1997). This suggests that subtle differences in E2F sites may influence which E2F heterodimers preferentially bind. The recruitment of pocket proteins to the DNA by E2F prevents activation of the target gene and results in the formation of a repressive complex on the DNA, therefore pocket proteins not only block E2F transcription by masking the *trans*activation domain but also actively repress transcription (Hamel *et al.*, 1992).

E2F may co-operate with other transcription factors to activate transcription. For example, it has been noted that E2F target promoters often have binding sites for the Sp1 transcription factor and that E2F-1, 2 and 3, but not E2F-4 or 5, can physically interact with Sp1. It is notable that mutation of these Sp1 sites can decrease the cell cycle related activity of E2F target genes (Karlseder *et al.*, 1996; Lin *et al.*, 1996). In addition, the CBP transcriptional co-activator stimulates E2F activity and directly interacts with the activation domain of E2F-1 (Trouche *et al.*, 1996). Furthermore, *in vitro* binding of E2F-1 with TBP, TFIIB and TFIIH has been shown (Hagemeier *et al.*, 1993; Pearson and Greenblatt, 1997), suggesting that interaction between E2F-1/DP heterodimers and the basal transcription machinery may enhance activation of transcription. It is likely therefore that a combination of promoter elements, different E2F/pocket protein complexes and interactions with other transcription factors act in concert to confer the opposing regulation of E2F target genes.

1.6.7 The regulation of E2F activity

1.6.7.a Regulation of E2F by phosphorylation

The DNA binding and *trans*activation functions of E2F heterodimers are compromised when DP and E2F partners are phosphorylated. Phosphorylation of E2F-1 and DP-1 has been shown to occur via interaction with cyclin A/cdk2, which forms a stable complex with an N-terminal domain of E2F-1 (Dynlacht *et al.*, 1994; Krek *et al.*, 1994; Xu *et al.*, 1994). It is thought that both DP-1 and E2F-1 are phosphorylated through the formation of this cdk complex. Mutation of either the cyclin A binding domain of E2F-1 or the phosphorylation sites of DP-1 causes the formation of stable E2F complexes and an S phase arrest in transfected cells (Krek *et al.*, 1995). The phosphorylation of E2F-4, which does not have the cyclin A binding domain, occurs through association with p107/cyclin A/cdk2 or p107/ cyclin E/cdk2 complexes (Zhu *et al.*, 1995).

The binding of pRb and E2F-1 may also be regulated by phosphorylation of E2F-1. E2F-1 is phosphorylated on Ser-332 and Ser-337 *in vitro* and mutation of these residues prevents the formation of E2F-1/pRb complexes (Fagan *et al.*, 1994). In addition, phosphorylation of E2F-1 on Ser-375 by cyclin A/cdk2 enhances association with pRb (Peeper *et al.*, 1995). It is possible therefore that phosphorylation of E2F-1 allows the disruption of E2F-1/pRb complexes without pRb phoshorylation.

1.6.7.b Regulation of E2F by subcellular location

The subcellular location of individual DP and E2F family members differs when overexpressed in transfected cells. E2F-1, 2 and 3 are all nuclear proteins, whereas E2F-4 and 5 are cytoplasmic (de la Luna *et al.*, 1996; Magae *et al.*, 1996; Allen *et al.*, 1997; Müller *et al.*, 1997; Verona *et al.*, 1997). Correspondingly, the subcellular location of the DP proteins differs when overexpressed; DP-1 is a cytoplasmic protein, although the subcellular location of DP-3 differs for the individual isoforms. DP-3α and DP-3δ are located in the nucleus and, in contrast, DP-3β and DP-3γ have a cytoplasmic location (de la Luna *et al.*, 1996). DP-3α and δ differ from DP-3β and γ due to the inclusion of the E region, a 16 residue sequence in the DNA binding domain of the DP-3α and δ isoforms (Ormondroyd *et al.*,

1995). Subsequent analysis of this region revealed that together with additional C-terminal residues (named NLS II; Figure 1.7), the E region functions as a nuclear localisation signal (NLS; de la Luna *et al.*, 1996). DP-1 does not contain a domain resembling the E region, although the cytoplasmic location of this protein is not due to the presence of a cytoplasmic retention signal, since fusion of the NLS of the Bel 1 protein resulted in nuclear accumulation (Ormondroyd *et al.*, 1995; de la Luna *et al.*, 1996).

An NLS has also been identified for E2F-1, since an N-terminal deletion mutant of E2F-1, lacking amino acids 85-91, within the cyclin A binding domain, results in cytoplasmic staining when overexpressed in U2OS cells (Muller *et al.*, 1997). Likewise, an NLS is located in the same N-terminal region of E2F-2 as for E2F-1 and it has been postulated that an NLS exists in same N-terminal region of E2F-3 due to high amino acid conservation (Verona *et al.*, 1997). E2F-4 and 5 lack the N-terminal cyclin-A binding domain and do not contain an NLS, and like DP-1, both E2F-4 and 5 can locate in the nucleus via the addition of an NLS (Müller *et al.*, 1997; Verona *et al.*, 1997).

The expression of a nuclear E2F (E2F-1, 2 or 3) with a cytoplasmic DP protein (DP-1, DP-3β, or DP-3γ) results in the nuclear localisation of both proteins (Figure 1.10). Likewise, the expression of E2F-4 or 5 with DP-3α or δ, results in nuclear accumulation of both the E2F and the DP. However, the co-expression of E2F-4 with DP-1 results in cytoplasmic location of both proteins (de la Luna *et al.*, 1996; Magae *et al.*, 1996; Allen *et al.*, 1997; Müller *et al.*, 1997; Verona *et al.*, 1997). Hence, an E2F or DP protein that contains an NLS can recruit a partner that does not contain an NLS to the nucleus (Figure 1.10).

It has been demonstrated that dimerisation is required for recruitment of DP-1 to the nucleus by E2F-1 (Magae *et al.*, 1996). DP-1 is a frequent component of the E2F heterodimer in many cell types and is often found complexed with E2F-4 or 5 (Bandara *et al.*, 1993, 1994; Girling *et al.*, 1993; Wu *et al.*, 1995). Although these complexes do not enter the nucleus via an intrinsic NLS sequence on either protein, the subcellular location of endogenous E2F-4 and 5 does vary during the cell cycle (Allen *et al.*, 1997; Müller *et al.*, 1997; Verona *et al.*, 1997), suggesting that mechanisms must exist for nuclear entry of DP-1/E2F-4 or 5 complexes. The co-expression of DP-1 with E2F-4 or 5 with p107 or p130 results in nuclear

accumulation of both DP-1 and E2F-4 or 5 and requires the pocket protein-binding domain (Allen *et al.*, 1997; Magae *et al.*, 1996; Verona *et al.*, 1997). Therefore, two possible mechanisms regulate the nuclear entry of E2F-4 and 5, the formation of a heterodimer with DP-3α or δ (NLS containing DP-3 isoforms) and formation of complexes with p107 or p130. The mechanism of nuclear entry can influence the cell cycle; nuclear accumulation of E2F-5 mediated by pocket proteins results in cell cycle arrest and in contrast nuclear entry via dimerisation with DP-3α or δ results in cell cycle progression (Allen *et al.*, 1997). Analysis of E2F-dependent transcription supports this data; expression of E2F complexes that contain an NLS in cells results in activation of transcription and p107-mediated nuclear localisation of E2F-4 complexes results in repression of E2F transcription (Müller *et al.*, 1997; Verona *et al.*, 1997).

1.6.7.c Regulation of E2F activity by protein degradation

The ubiquitin-mediated degradation of E2F represents another level of control of E2F activity. When overexpressed E2F-1 and 4 are degraded by the ubiquitin-proteosome pathway (Hateboer *et al.*, 1996; Hofmann *et al.*, 1996). The stability of E2F-1 and 4 is increased when associated with members of the pocket protein family and loss of the transcription activation domain and pocket protein binding domain of E2F-1 and 4 also results in increased stability.

1.6.8 E2F and proliferation

It is thought that the growth suppressive properties of the pocket protein family members involves repression of E2F-dependent transcription and that deregulation of E2F activity promotes S phase entry and stimulates proliferation. The overexpression of E2F family members supports this model; the forced expression of E2F-1 promotes S phase entry, both alone and when DP-1 is co expressed (Johnson *et al.*, 1993; Shan and Lee, 1994; Wu and Levine, 1994). Similar results have been observed for the other E2F family members, although E2F-4 and 5 require co-expression of DP-1 (Lukas *et al.*, 1996). In addition, the expression of E2F-1 can overcome G1 arrest imposed by expression of the CDK inhibitors p16, p21 and p27 (DeGregori *et al.*, 1995), and can also be overcome by the expression of E2F-2 and 3 alone, but E2F-4 and 5 require co-expression of DP-1 (Lukas *et al.*,

1996). The ability of E2F-1 to promote S phase entry depends on the dimerisation, DNA binding and *trans*activation domains and therefore it is assumed that E2F-1 is involved in activating genes essential for S phase entry (Johnson *et al.*, 1993).

The ability of E2F to drive cells into S phase would suggest that E2F might have oncogenic activity. Indeed DP-1 can cooperate with activated *ras* to transform rat embryo fibroblasts (Jooss *et al.*, 1995) and E2F-1 overexpression in rat embryo fibroblasts can induce neoplastic transformation (Singh *et al.*, 1994). In addition, cooperation of E2F-4/DP-1 with activated *ras* can transform rat embryo fibroblasts (Beijersbergen *et al.*, 1994). A mutant E2F-4 that cannot interact with pocket proteins can transform NIH 3T3 fibroblasts and cause tumour formation in nude mice (Ginsberg *et al.*, 1994). It is therefore possible that E2F family members may function as oncogenes when deregulation is coupled with other mutations such as *ras* activation, in tumour cells.

1.6.9 Overexpression of E2F-1 leads to apoptosis

The overexpression of E2F-1 causes S phase entry and proliferation, although in many cell types overexpression of E2F-1 leads to apoptosis. In fibroblasts in which E2F-1 is inducible, E2F-1 expression results in apoptosis when cells are grown in low serum (Shan and Lee, 1994). The induction of apoptosis by E2F-1 is p53-dependent as co-expression of wild-type p53 but not mutant p53 with E2F-1 stimulates apoptosis (Wu and Levine, 1994). The induction of apoptosis by E2F-1 is unique, as over-expression of the other E2Fs does not result in apoptosis (DeGregori *et al.*, 1997). In addition, both E2F-1 and DP-1 physically interact with p53 and this interaction decreases E2F-dependant transcription (O'Connor *et al.*, 1995; Sørensen *et al.*, 1996); E2F-1 also interacts with MDM2 (Martin *et al.*, 1995). However, it is unclear how these interactions influence induction of apoptosis by E2F-1.

E2F-1 can also induce apoptosis in cells that lack p53 (Hsieh *et al.*, 1997; Phillips *et al.*, 1997), suggesting that a p53-independent mechanism exists. This apoptosis requires DNA binding but not *trans*activation, and the expression of pRb can inhibit E2F-1 induced apoptosis in the absence of p53 (Hsieh *et al.*, 1997). In addition, p53-independent apoptosis by E2F-1 does not require S phase entry (Phillips *et al.*, 1997). Since the DNA binding but not *trans*activation functions of

E2F-1 are required for p53-independent apoptosis, E2F-1 may act to repress transcription of target genes.

The analysis of mice with homozygous deletion of E2F-1 revealed defects in apoptosis in thymocytes (Field *et al.*, 1996) and in older mice, a high incidence of sporadic tumours was observed (Yamasaki *et al.*, 1996). Such data strongly supports a role for E2F-1 as a regulator of apoptosis and in suppression of proliferation. It has also been noted that in pRb-deficient embryos high levels of apoptosis are observed (Jacks *et al.*, 1992; Lee *et al.*, 1996). Analysis of embryos that lack both p53 and pRb revealed apoptosis in the lens and central nervous system is p53-dependent whereas, apoptosis in the peripheral nervous system was p53-independent (Morgenbesser *et al.*, 1994; Macleod *et al.*, 1996).

1.7 Aims

The aims of this project were to study the expression of murine DP-3 isoforms at both the RNA and protein level. The identification of cellular circumstances under which DP-3 is expressed would enable further investigation into the functions of DP-3 under physiological conditions and the elucidation of the role of DP-3 in the E2F heterodimer and in growth regulation.

Figure 1.1

The assembly of RNA polymerase II transcription initiation complex on a TATA containing promoter

A schematic representation of the assembly of basal transcription factors on a TATA containing promoter.

a) Assembly of the TFIID complex on the TATA box. TFIID is composed of the TATA-binding protein (TBP, red) and TBP-associated factors (TAFs, orange).

b) TFIID targets other basal transcription factors to the transcriptional start site; TFIIB (green) binds and recruits RNA polymerase II (yellow), this interaction is thought to be stimulated by TFIIF (white).

c) TFIIE (lilac) and TFIIH (blue) then associate with the complex. In addition, TFIIJ (pink) may bind to the complex to form a fully primed initiation complex and transcription can proceed.

Figure 1.1

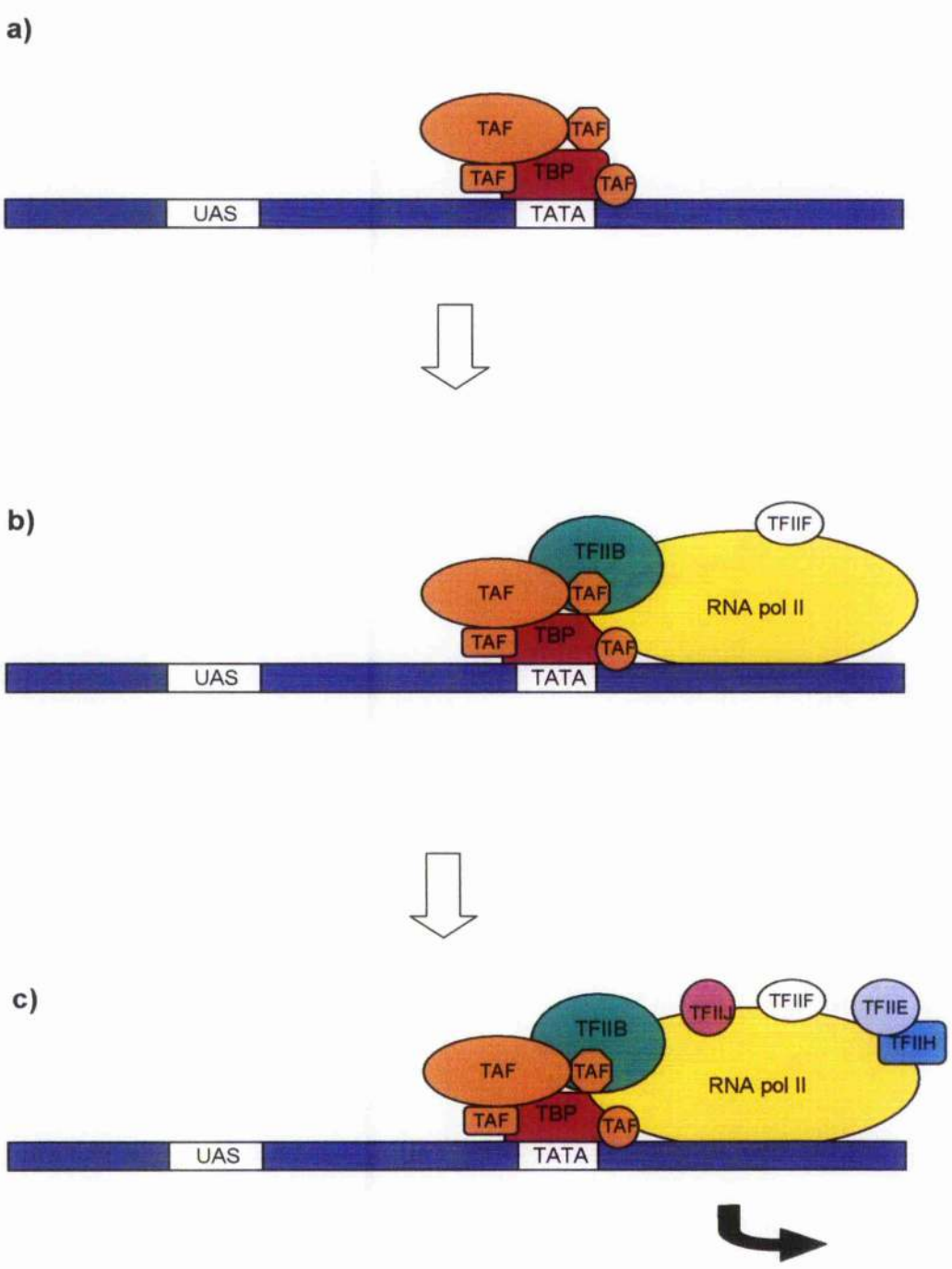

a)
TAF
TAF
TAF
TBP
TAF
UAS
TATA
b)
TFIIB
TFIIF
TAF
TAF
RNA pol II
TAF
TBP
TAF
UAS
TATA
c)
TFIIJ
TFIIF
TFIIE
TFIIB
TFIIH
TAF
TAF
RNA pol II
TAF
TBP
TAF
UAS
TATA

Figure 1.2

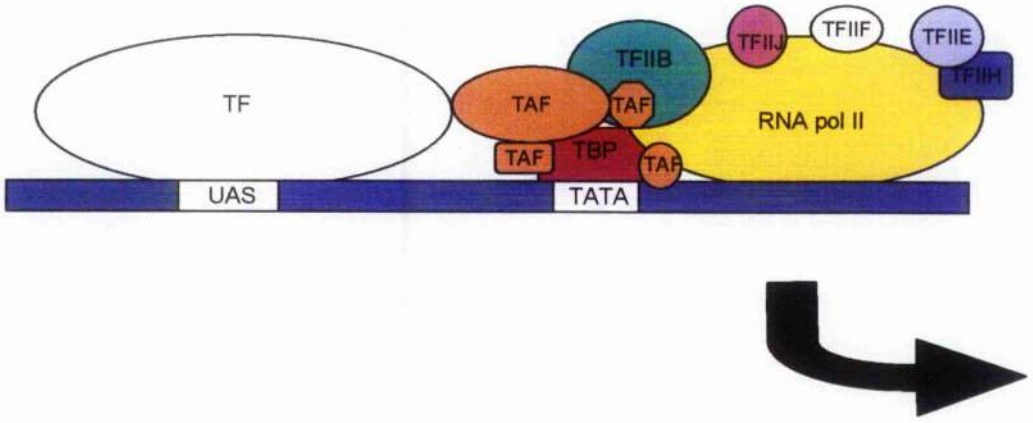
***Trans*activation by DNA-binding transcription factors**

A schematic representation of the activation of transcription by transcription factors. a) Direct *trans*activation. Transcription factor (TF, white) binds to the upstream activating sequence (UAS) and stimulates transcription by contacting the basal transcription machinery. The transcription factor contacts the TAFs (orange), components of TFIID (See Figure 1.1).

b) Transcription via co-activators. The transcription factor (white) stimulates transcription via the intermediate co-activator (blue). The co-activator forms a bridge between the TF and the basal transcription machinery.

Figure 1.2

a) CO-ACTIVATOR NOT REQUIRED



b) CO-ACTIVATOR REQUIRED

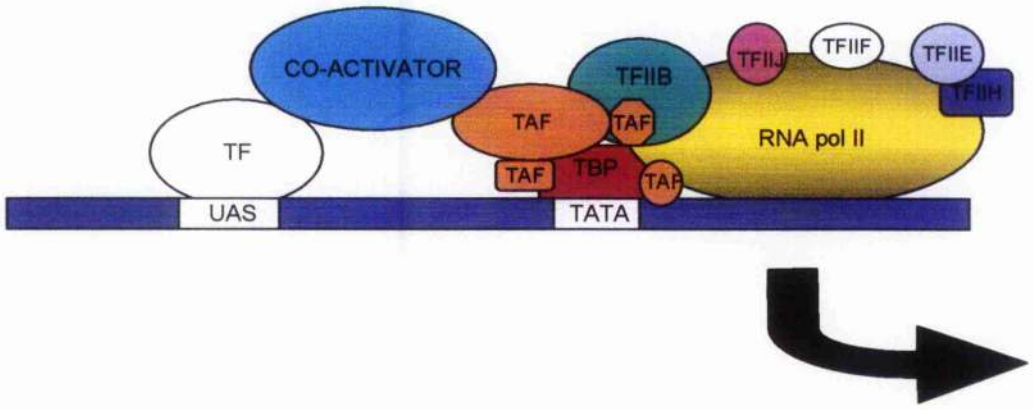


Figure 1.3

The mechanism of translation initiation

Diagrammatic representation of the initiation phase of eukaryotic translation. (Adapted from Pain, 1996)

a) The 80S ribosome dissociates, the 40S subunit binds both eIF1A and eIF3 (43S) and the 60S subunit binds to eIF6. Met-tRNA$_i$ binds to eIF2-GTP forming a ternary complex, which subsequently binds to the 40S ribosomal subunit. Thus, the 43S preinitiation complex is formed.

b) The 43S preinitation complex binds to the mRNA, usually via the m^{7}G cap structure, and scans along the RNA until the initiator codon is recognised. This process involves several initiation factors, required for the formation of the cap-binding complex and also to inhibit any mRNA secondary structure.

c) eIF5 recognises the preinitiation complex and promotes the hydrolysis of the GTP bound to eIF2. eIF2-GDP is then released and recycled. Initiation factors dissociate from the 40S subunit and the 60S subunit joins, forming the 80S initiation complex and elongation proceeds.

Figure 1.3

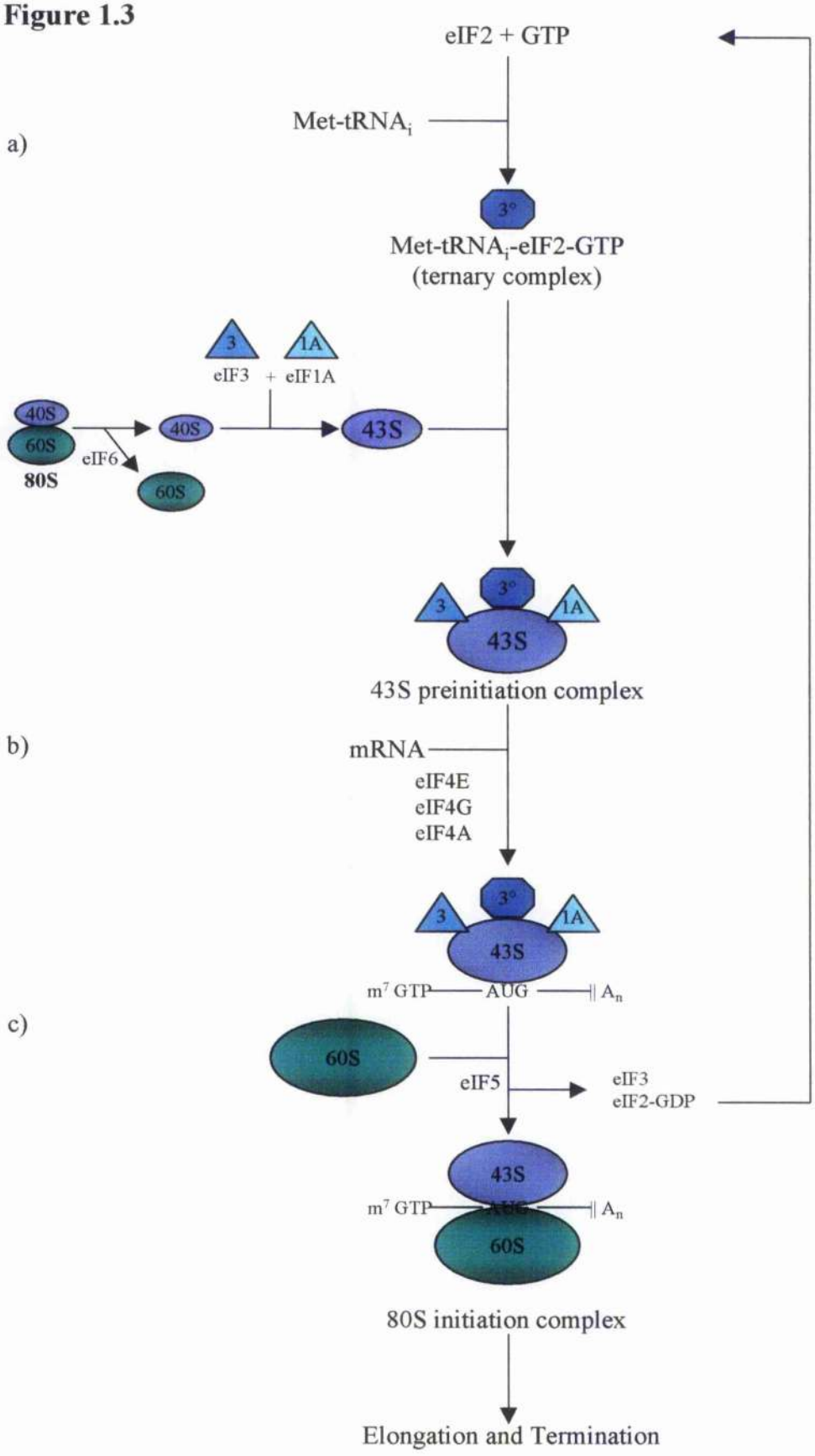

eIF2 + GTP
Met-tRNA$_i$
a)
3°
Met-tRNA$_i$-eIF2-GTP
(ternary complex)
3
1A
eIF3 + eIF1A
40S
60S
80S
eIF6
40S
60S
43S
3°
3
1A
43S
43S preinitiation complex
b)
mRNA
eIF4E
eIF4G
eIF4A
3°
3
1A
43S
m^7 GTP
AUG
A$_n$
c)
60S
eIF5
eIF3
eIF2-GDP
43S
m^7 GTP
A$_n$
60S
80S initiation complex
Elongation and Termination

Figure 1.4

The mammalian cell cycle

A schematic representation of the mammalian cell cycle.

The mammalian cell cycle is separated into distinct phases. G1 (Gap 1), S (S phase, DNA synthesis phase), G2 (Gap 2) and M (mitosis). G0 represents a non-dividing or differentiated state. Cell cycle progression is coordinated by the cyclin-dependent kinases (cdk) and their cyclin partners, which phosphorylate important substrates such as the retinoblastoma gene product (pRb, orange), in a cell cycle dependent manner.

Figure 1.4

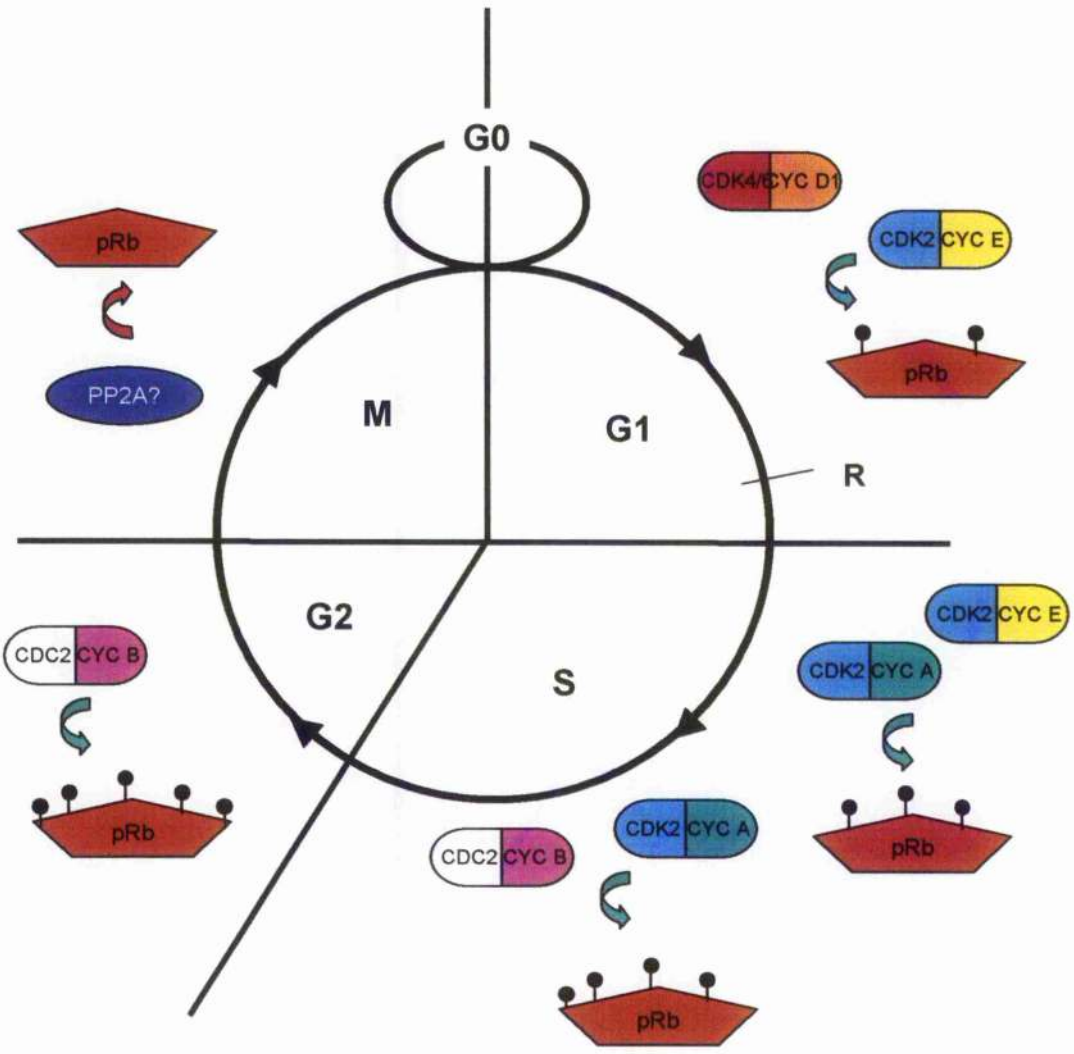


Figure 1.5

The E2F family

A diagram summarising the members of the E2F family of proteins.
The conserved domains of the E2F family are indicated, including the pocket protein binding domain (pp binding, red), the region of similarity with the DP family (orange). The heptad (blue) and marked (green) boxes are also shown; these are regions of sequence similarity, however at present their functions are unknown. The DNA binding domain is also indicated (Adapted from Lam and La Thangue, 1994; Dyson, 1998).

Figure 1.5

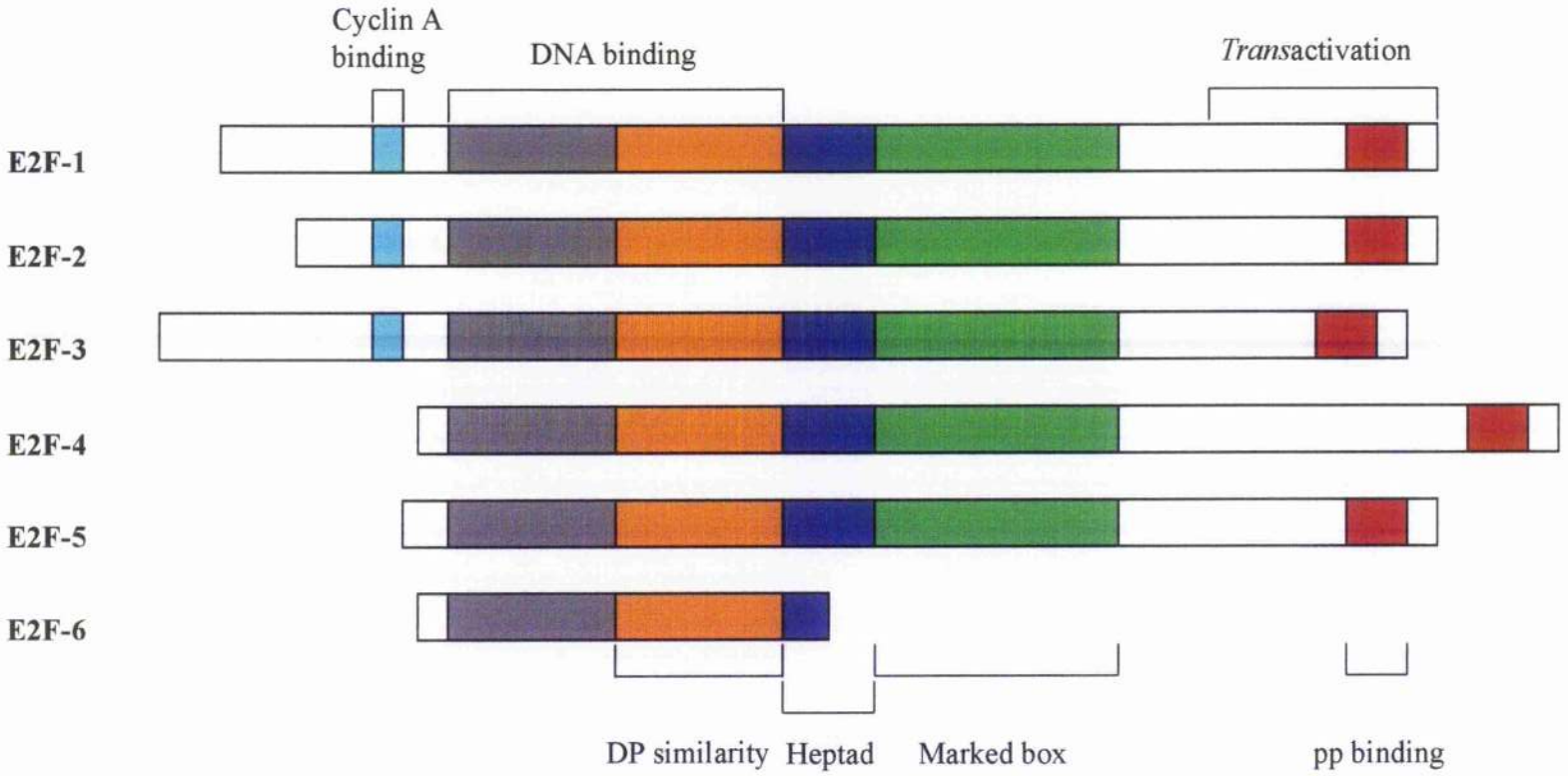

Cyclin A binding
DNA binding
*Trans*activation
E2F-1
E2F-2
E2F-3
E2F-4
E2F-5
E2F-6
DP similarity
Heptad
Marked box
pp binding

Figure 1.6

The DP family

A schematic summary of the DP family members.

The conserved domains DCB1 (lilac), DCB2 (purple, DP conserved boxes 1 and 2) and NCB (pink, negatively-charged box) are indicated. The DEF box (blue, DP-E2F homology region) contains the region of greatest similarity to E2F family members. The positions of the DNA binding and dimerisation domains are indicated (Adapted from Lam and La Thangue, 1994).

Figure 1.6

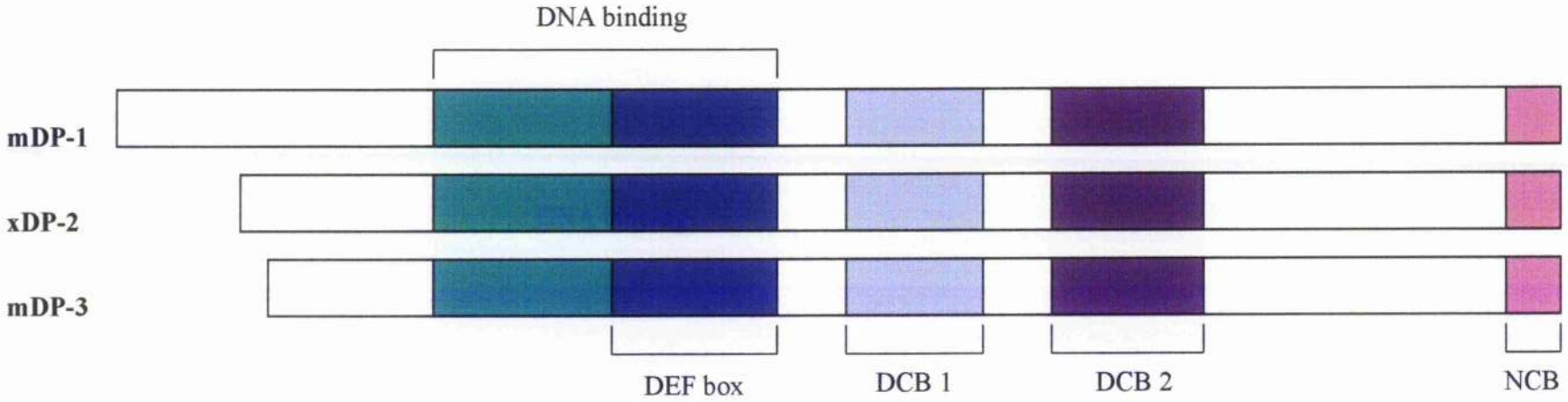


Figure 1.7

DP-3 protein isoforms

A schematic representation of the DP-3 protein isoforms, indicating the site of the E region (blue) and the glutamine (Q) insertion. The sequence required in addition to the E region, for nuclear localisation is also indicated (NLS II, yellow), this region is common to all DP proteins.

Figure 1.7

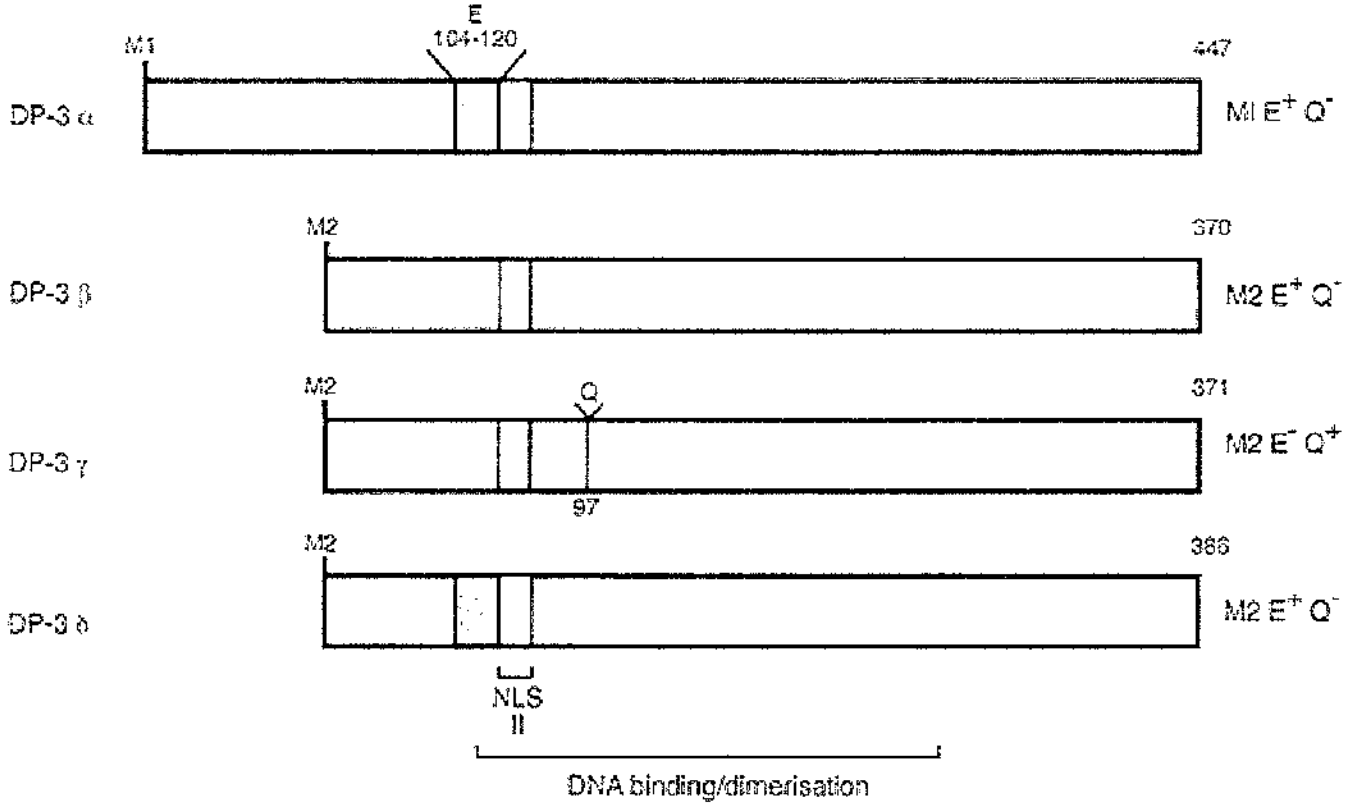

E
104-120
M1
447
DP-3 α
M1 E+ Q-
M2
370
DP-3 β
M2 E+ Q-
M2
Q
371
DP-3 γ
97
M2 E- Q+
M2
366
DP-3 δ
M2 E+ Q-
NLS
II
DNA binding/dimerisation

Figure 1.8

The organisation of the 5' untranslated regions of DP-3 RNA.

A diagrammatic representation of the 5"UTRs of DP-3 RNA

a) The 5'UTR arise from rearrangement of the five segments numbered 1-5. These sequences contain two different initiation signals (M1 and M2).

b) The 5'UTRs of DP-3. Arrows represent possible sites of translational initiation and X corresponds to the sites of translational termination. ⊗ indicates sites of translational stop signals.

Figure 1.8

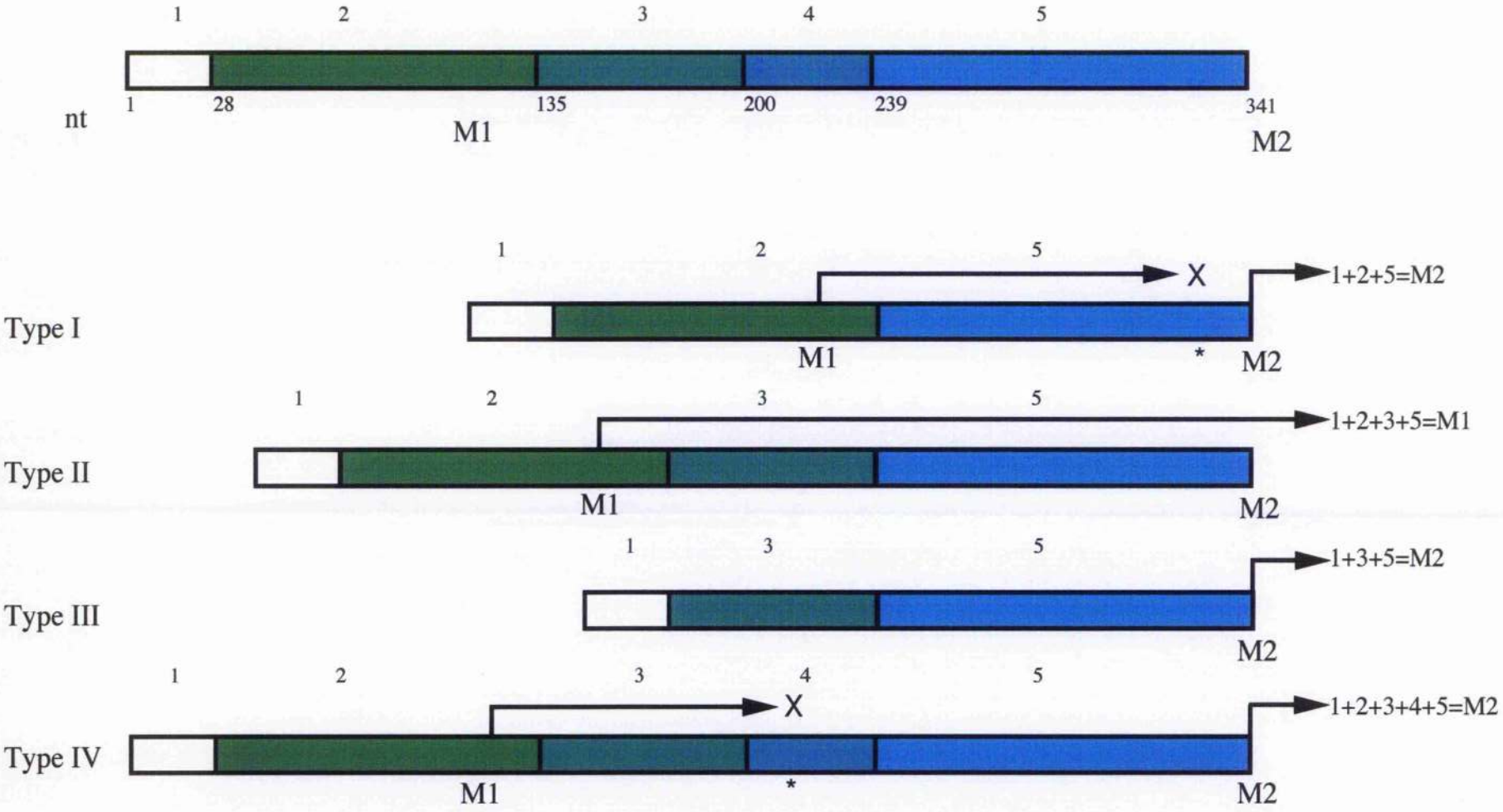

1
2
3
4
5
nt
1
28
135
200
239
341
M1
M2
Type I
X
1+2+5=M2
*
Type II
1+2+3+5=M1
Type III
1+3+5=M2
Type IV
1+2+3+4+5=M2

Figure 1.9

The regulation of E2F heterodimers by stable binding to members of the pRb family

A diagrammatic representation of the regulation of E2F by interaction with members of the pRb family (pocket proteins).

a) DP (blue) and E2F (red) heterodimers bind to promoters containing E2F binding sites and activate transcription.

b) Members of the pocket protein family, exemplified by pRb, bind to E2F heterodimers and the transcription of genes containing E2F sites ceases. It is thought that the binding of pocket proteins to E2F result both in the formation of inhibited complexes, through masking of the E2F *trans*activation domain. Additionally, the recruitment of pocket proteins to the DNA may also result in the formation of a repressive complex.

Figure 1.9

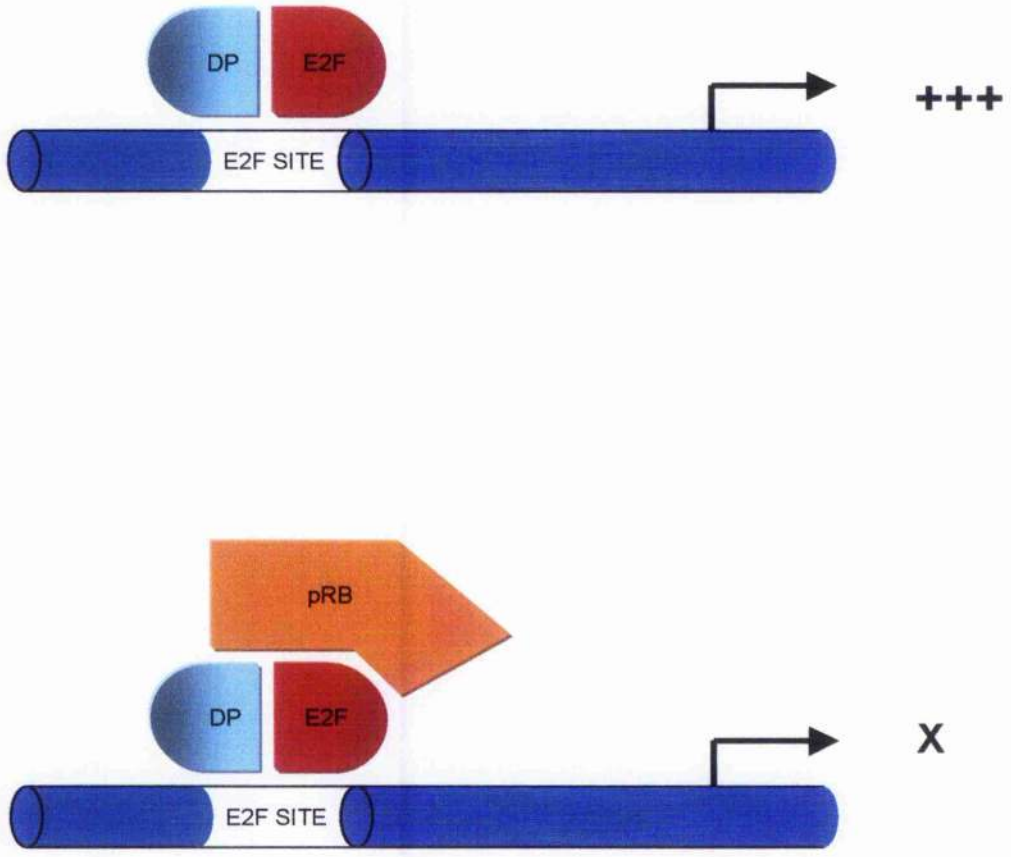


Figure 1.10

The regulation of E2F by subcellular localisation

A schematic summary of the regulation of E2F by subcellular location and by heterodimer composition.

a) The formation of E2F heterodimers between E2F4/5, which do not contain an NLS and DP-3 E+ isoforms (α and δ) which have an NLS, results in nuclear localisation of both the E2F and the DP partners.

b) E2F heterodimers composed of DP-3 isoforms that do not contain the E region and E2F4/5, which also do not have an NLS would not b able to accumulate in the nucleus. However, association with members of the pocket protein family results in the localisation of the E2F heterodimer to the nucleus. The formation of E2F pocket protein complexes localised to the nucleus results repression of E2F target genes.

c) The formation of heterodimers composed of E2F1, 2 or 3, which contain an NLS and DP proteins which lack an NLS (E minus DPs; DP-1, DP-3 β and δ), results in the accumulation of both partners in the nucleus. Such a complex would then able to activate E2F target genes.

Figure 1.10

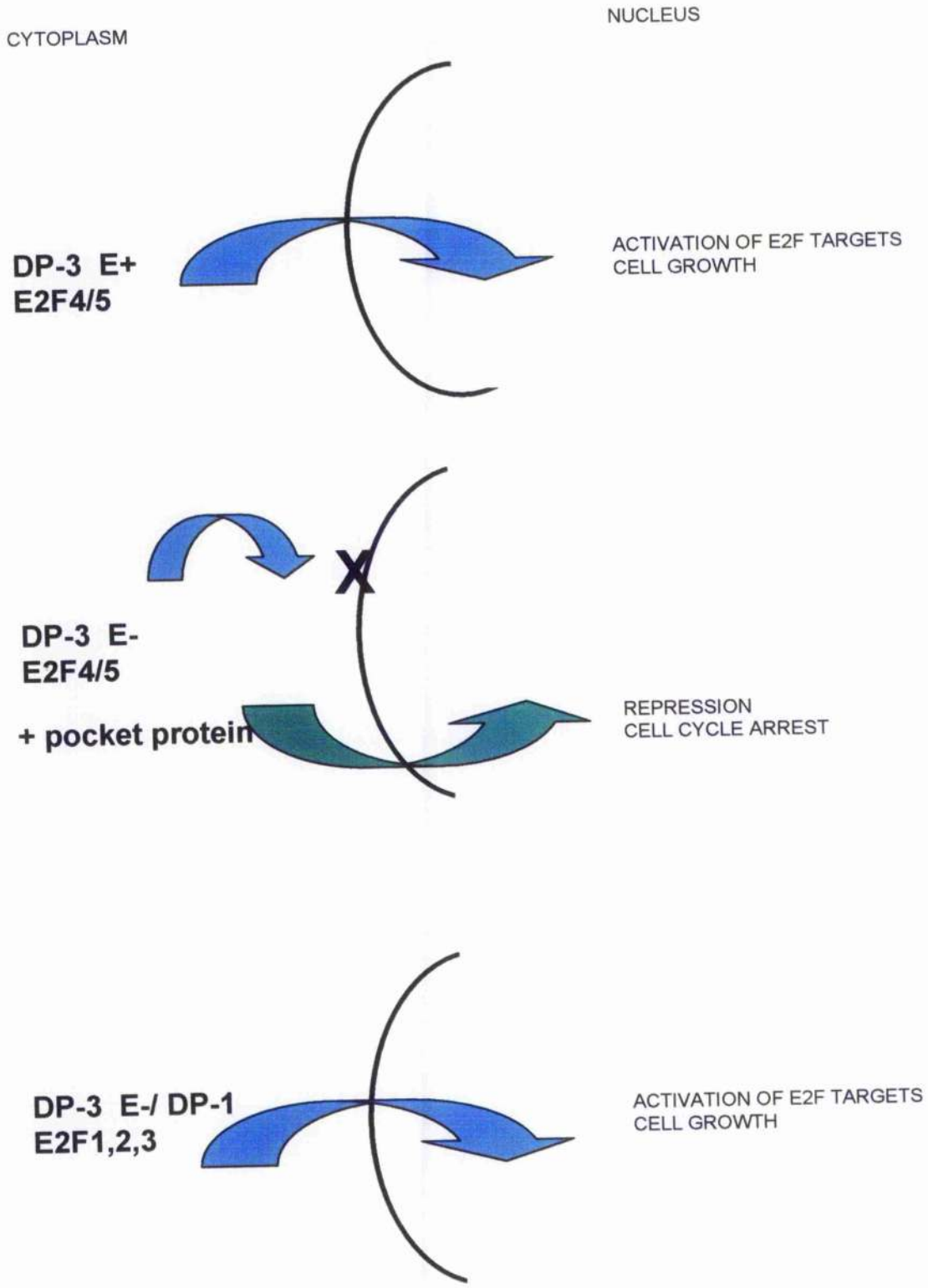

CYTOPLASM
NUCLEUS
DP-3 E+
E2F4/5
ACTIVATION OF E2F TARGETS
CELL GROWTH
DP-3 E-
E2F4/5
+ pocket protein
REPRESSION
CELL CYCLE ARREST
DP-3 E-/ DP-1
E2F1,2,3
ACTIVATION OF E2F TARGETS
CELL GROWTH

Table 1.1 E2F Target Genes

Genes involved in DNA replication and S phase
DNA polymerase α
Thymidine kinase
Thymidylate synthase
Dihydrofolate reductase (DHFR)
ORC1
CDC6
Histone H2A
PCNA
Proto-oncogenes and transcription factors
E2F-1
E2F-2
pRb
p107
c-myc
N-myc
B-myb
jun
Cyclins and CDKs
Cyclin-A
Cyclin-E
Cyclin D
cdc2

Compiled from Helin, 1998; Herwig and Strauss, 1997.

Chapter 2.

Materials and Methods.

2.1 Mammalian cell culture

All tissue culture cell lines were cultured in Dulbecco's modified Eagle's Medium (DMEM) (Gibco BRL), supplemented with 10% fetal calf serum (Gibco BRL) and 100U/ml penicillin, 10mg/ml streptomycin (Gibco BRL), at 37°C in a 5% CO_2/ H_2O saturated atmosphere. Cells were passaged by washing with sterile phosphate buffered saline (PBS) and treatment with trypsin/EDTA (Gibco BRL). Detached cells were resuspended in DMEM with FCS to arrest the trypsin and cells diluted and re-plated in tissue culture treated flasks. Alternatively, cells were pelleted by centrifugation at 900rpm at room temperature for 5 minutes, resuspended in fresh medium, counted in a haemocytometer and plated in tissue culture dishes. For immunostaining analysis (Section 2.12.4) sterile glass coverslips were introduced during the passage of cells and removed for staining before passage of cells.

2.1.1 Differentiation of F9 embryonic teratocarcinoma cells (F9EC).

F9EC cells were stimulated to differentiate to F9 parietal endoderm like cells (F9PE) by culture in DMEM with 10% fetal calf serum, supplemented with 1mM N^6,2'-O-dibutyladenosine 3':5' cyclic monophosphate (cAMP), 0.05μM trans retinoic acid and 0.1mM 3- isobutyl-1 methyl-xanthine (IBMX). F9EC cells typically were seeded at $0.5x10^6$ cells per 10cm dish and harvested at 3, 5 and 7 days after addition of cAMP, retinoic acid and IBMX.

2.1.2 Transfection of mammalian cells

Transient transfection of U2OS, C33A, F9EC and SAOS-2 cells with expression vectors was performed using the calcium phosphate co-precipitation method (Maniatis *et al.*, 1989). The transfection of NIH 3T3 was performed using lipofectamine (Gibco BRL) and COS-7 cells were transfected using lipofectin (Gibco BRL).

Cells for transfection by the calcium phosphate method were plated at $1x10^6$ cells per 100mm tissue culture dish or $5x10^5$ cells per 60mm tissue culture dish the

day before transfection and left to attach overnight. Four hours prior to transfection, fresh medium was added. The DNA/calcium phosphate precipitates were made by mixing 7.5μg DP-3 5'UTR-luciferase expression vector, 0.5μg pCMVβ-gal expression vector and high molecular weight DNA (Bochringer Mannheim) to a final concentration of 10μg. To this, 25μl of 2.5M $CaCl_2$ was added and the volume made up to 250μl with dH_2O. The DNA solution was then added dropwise, with mixing to 250μl of sterile 2xHBS. The precipitates were allowed to form at room temperature for 15-30 minutes and then added dropwise to the cells and left overnight. The following day the medium was removed and the cells washed twice with either sterile PBS or DMEM (no FCS) and fresh DMEM supplemented with FCS was added. The cells were harvested at 48 hour post-transfection by scraping in 1ml PBS, 750μl of cell suspension was pelleted and used for RNA extraction, the remaining 250μl of cell suspension was pelleted and resuspended in 1x reporter lysis buffer (Promega Corporation) and used for reporter assays (Section 2.1.3) and protein analysis.

The transfection of SAOS-2 cells with p53 was performed using 12μg of DP-3 5'UTR-luciferase vector, 1μg pCMVβ-gal expression vector and variable amounts of p53 expression vector or the corresponding backbone vector. High molecular weight DNA was added to give a final concentration of 20μg DNA, 50μl 2.5M $CaCl_2$ was then added and the volume made up to 500μl. The DNA solution was then added to 500μl 2xHBS as before. The preciptitate was then split between two 60mm tissue culture dishes. Cells were harvested by scraping in 1x reporter lysis buffer and reporter assays performed.

NIH 3T3 cells were plated at $1x10^6$ cells per 100mm tissue culture dish and left to attach overnight. Lipofection was performed by mixing 20μl lipofectamine and 400μl of DMEM (no FCS) and incubation for 30 minutes at room temperature. The DNA, usually 10μg of DP-3 5'UTR-luciferase expression vector and 1μg pCMVβ-gal expression vector was mixed with 400μl DMEM (no FCS) and the added to the lipofectamine mix and incubated at room temperature for 10 minutes. The cells were then washed twice with DMEM to remove FCS. 6ml of DMEM was then added to the DNA and lipofectamine mixes and added to the cells (final volume medium, approximately 6.8ml). The following day the cells were washed and

DMEM supplemented with FCS replaced. The lipofection of COS-7 cells was performed in the same manner except the cells were plated onto 60mm dishes, 14µl of lipofectin was added to 200µl of DMEM and the DNA was mixed with 200µl DMEM. After the final incubation, 3ml of DMEM was added and this was then added to the cells. Cells were harvested the same manner as the other cell lines.

2.1.3 Reporter assays

Transfected cell lysates were pelleted briefly to remove cell debris and the supernatant used for luciferase and β-galactosidase reporter assays.

2.1.3.1 Luciferase reporter assay

Half the cell lysate was assayed for luciferase enzymatic activity using the luciferase substrate solution (Promega Corporation) in an automated luminometer. The luciferase activity for each sample was then divided by the β-galactosidase activity of the same sample to give the relative luciferase enzyme activity.

2.1.3.2 β-galactosidase reporter assay

The cell lysate was made up to 150µl with dH_2O and mixed with 150µl 2x β-gal assay buffer and incubated at 37°C until the yellow colour had fully developed. 0.5ml of 1M $NaCO_3$ was then added to stop the reaction and the optical density (OD) immediately measured at 420nm. The OD obtained was then used to normalise the luciferase enzyme activity.

2.2 Bacterial culture

Bacterial liquid cultures were prepared by inoculation of an appropriate volume of LB media containing ampicillin (50-100µg/ml) as required and incubation at 37°C, overnight, with shaking. Bacterial colonies were maintained on LB agar plates with ampicillin where necessary, stored at 4°C. For long term storage, an aliquot of an overnight liquid culture supplemented with 15% glycerol, was stored at -80°C.

2.3 Manipulation of nucleic acids

2.3.1 Determination of nucleic acid concentration

The concentration of nucleic acid solutions was determined by absorption at 260nm. Concentration was calculated using the formulae:

An OD of 1_{260nm} = 50μg/ml for double stranded DNA solutions

An OD of 1_{260nm} = 10μg/ml for DNA oligonucleotide solutions

An OD of 1_{260nm} = 40μg/ml for RNA solutions.

2.3.2 DNA Preparation

Mini preparations of plasmid DNA were performed using SV Mini prep kits (Promega Corporation), using an appropriate volume of overnight culture of *E. coli* cells, according to the manufacturer's instructions. Alternatively, plasmid DNA was isolated from 1.5ml of overnight culture of *E. coli* cells by alkaline lysis, organic extraction and ethanol precipitation via standard protocols (Sambrook *et al.*, 1989). Plasmid DNA was resuspended in 50μl of TE or sterile distilled water (dH_20).

Large scale preparations of plasmid DNA were performed using Qiagen maxi DNA preparation kits, from an appropriate volume of overnight culture of *E. coli* cells, according to the manufacturer's instructions.

2.3.2a Preparation of murine brain genomic DNA.

One freshly dissected mouse brain was homogenized in PBS with a small Dounce homogeniser. The homogenate was pelleted and gently resuspended in 0.5ml PBS and 10ml proteinase K buffer was added. 358μl proteinase K (10mg/ml) was then added and gently mixed and incubated overnight at 37°C. An equal volume of phenol (pH8.0) was added and gently mixed for 1 hour and then centrifuged at 3000rpm at room temperature for 10 minutes. The upper aqueous phase was then re-extracted with an equal volume of phenol, mixed by hand for 5 minutes and centrifuged as before. The upper aqueous phase was re-extracted with an equal volume of chloroform as before. The DNA was precipitated by the addition of an equal volume of isopropanol and mixed gently by hand for 5 minutes. 0.25 volumes of 5M $NaClO_4$ were added and mixed gently, the DNA forms a ball from which the supernatant was removed by decanting. The DNA was then washed with 70%

ethanol by gentle mixing and the supernatant removed by decanting. The genomic DNA was then briefly air dried and dissolved in 1ml sterile TE at 37°C for an hour.

2.3.2b Purification of phage DNA

A single plaque was picked using a sterile pasteur pipette and cultured in 50ml LB media supplemented with 10mM $MgSO_4$, overnight at 37°C, with shaking. Once bacterial lysis was clearly observed 0.5ml chloroform was added and the culture incubated at 37°C, with shaking for 5 minutes. The culture was then centifuged at 4000rpm at room temperature for 20 minutes and the supernatant retained. Bacterial RNA and DNA was digested by the addition of DNase I and RNase to 10µl/ml and incubated at room temperature for 30 minutes, or at 4°C overnight. Phage particles were pelleted at 16 000rpm, 4°C for 2 hours (Sorvall SS34). The supernatant was completely removed and the pellet resuspended in 0.5ml 0.1M Tris (pH 7.9), 0.3 M NaCl and transferred to an eppendorf tube. The phage coat was digested by the addition of proteinase K to 1µl/ml and incubated at 37°C for 30 minutes, the proteinase K was inactivated by the addition of EDTA to a final concentration of 10mM. The DNA was then extracted three times with phenol/chloroform/iso-amyl-alcohol (ratio 25:24:1, $^{v}/_{v}$), pre-equilibrated with 1M NaCl, 20mM Tris/HCl (pH 8.0), 1mM EDTA. DNA was then precipitated with 2 volumes of ethanol at –20°C for at least 1 hour, and pelleted at 13000rpm at room temperature. The purified phage DNA briefly air dried and resuspended in 50µl T.E and stored at 4°C.

2.3.3 RNA Extraction

Total cellular RNA was extracted from tissue culture cells using Tri Reagent (Molecular Research Center), following the manufacturer's instructions.
All purified RNAs were solubilised in Formazol (Molecular Research Center) and stored at –80°C.

2.3.3a Extraction of nuclear and cytoplasmic RNAs.

NIH 3T3 cells were harvested PBS and pelleted at 3000rpm, 4°C. Cell pellets were resuspended in 0.5ml fractionation buffer per $6x10^6$ cells, incubated on

ice for 5 minutes and centrifuged at 3000rpm for 2 minutes, 4°C. The supernatant was retained (cytosolic fraction) and stored on ice. The pellet (nuclear fraction) was washed with 0.5 ml fractionation buffer and incubated on ice for 5 minutes, centrifuged at 3000rpm at 4°C for 2 minutes, the supernatant was retained and added to the cytosolic fraction and stored on ice.

The cytosolic RNAs were purified using Tri Reagent LS (Molecular Research Center), following the manufacturer's instructions. Nuclear RNAs were isolated from the pellets using Tri Reagent, following the manufacturer's instructions. RNA was solubilised in Formazol (Molecular Research Centre) and stored at –80°C.

2.3.3b Isolation of polyadenylated mRNAs

Polyadenylated mRNA was purified from total RNA using an Oligotex mRNA mini kit (Qiagen) following manufacturer's instructions and stored at -80°C.

2.3.4 Genomic library screening

A λGEM12 genomic library prepared from embryonic stem cell line SV129D3 was kindly supplied by J.M. Garnier and P. Chambon. The phage titre was determined by serial dilution, plating and counting of phage plaques. The bacterial strain LE392 (Promega Corporation) was used as the phage host strain and was cultured overnight in LB, the cells were then harvested by centrifugation at 3000 rpm and resuspended in 10mM $MgSO_4$. The phage library was diluted in λ diluent and gently mixed with plating bacteria and incubated at 37°C for 20 minutes. A solution of 0.7%agarose in LB was autoclaved and stored at 50°C, prior to use, $MgSO_4$ was added to a final concentration of 10mM. 50ml of top agarose was then added to the phage, mixed and then plated on LB agar plates, which has been dried and warmed to 45°C. Once the top agarose had set, the plates were inverted and incubated at 37°C until plaques had formed, after which plates were stored at 4°C.

Phage lifts were made in duplicate, using Hybond-N membrane (Amersham International) and each membrane denatured in denaturing solution for 1 minute. The membranes were then placed in neutralising solution for 5 minutes and rinsed in 2xSSC, air dried and UV crosslinked using a UV crosslinker (Amersham

International), set to autocrosslink mode and stored dry at room temperature. The filters were then hybridised using a DP-3 cDNA probe, washed (Section 2.8) and visualised by autoradiography (Section 2.9). Positive plaques were picked using a sterile pasteur pipette and phage eluted in λ diluent. The phage were re-plated until pure and phage DNA then purified (Section 2.3.2b).

2.3.5 Restriction endonuclease digestion

Single enzyme digests were performed in 1x enzyme reaction buffer supplied with the restriction endonuclease, at the recommended temperature (usually 37°C). Double digests were performed in 1x reaction buffer suitable for both enzymes. Where no buffer was compatible with both enzymes, DNA was digested with one enzyme and then phenol/chloroform extracted and ethanol precipitated and digested with the other enzyme. The quantity of restriction endonuclease added to a digest varied depending on the source of DNA, however generally 1unit of enyzme was added per μg of DNA in a 20μl reaction, at the appropriate temperature. When digesting genomic DNA an excess of enzyme was usually added to ensure adequate DNA restriction.

2.3.6 Polymerase chain reactions (PCR)

PCR reacts were all performed using the same conditions in a programmable thermal controller. A typical reaction contained 10ng plasmid template, 100ng of each primer, 10mM of mixed dNTP and 1xPCR reaction buffer (supplied with enzyme). To this, 2 units of Taq polymerase (Promega Corporation) was added and overlaid with paraffin oil. Typically, samples were initially heated to 94°C for 2 minutes and then for another 30 seconds, then cooled to the annealing temperature for 30 seconds, and then incubated at 72°C for 1 min and then back to 94°C for 30 seconds. This cycle was then repeated 30 times. The annealing temperatures were calculated using the formula for the T_m of an oligonucleotide, section 2.8.

2.3.7 Phosphatase treatment of vector DNA

Vector DNA digested with a single enyzme was de-phosphorylated prior to ligation to prevent re-ligation of the vector. Calf intestinal alkaline phosphatase was

added to digested DNA, approximately 0.1units /pmol of DNA ends, was used per reaction. Reactions were performed in 1x alkaline phosphatase buffer, supplied with the enzyme (Boehringer Mannheim) at 37°C for 15 minutes. The vector DNA was purified by phenol/chloroform extraction and ethanol precipitation. The vector DNA was then checked on a gel and the concentration estimated.

2.3.8 Ligation reactions

Ligation reactions were performed in 10µl volume containing 1xT4 DNA ligase buffer supplied with the enyzme and 10 units of T4 DNA ligase (Promega Corporation). Generally 50ng of vector DNA was used with a 3x molar excess of insert added. Reactions were performed either at 16°C overnight or at room temperature for 2 hours.

2.3.9 Transformation of competent bacteria

Transformations were performed using purchased competent cells, generally *E.coli* strains XL-1Blue (Stratagene) and DH5α (Gibco BRL) were used. 5µl of ligation reaction was transformed by mixing with 50µl competent cells and incubating on ice for 20 minutes. The bacteria were then heat shocked at 42°C for 20 seconds and returned to ice for a further 2 minutes. 500- 1000µl warmed LB was then added and the bacteria incubated at 37°C for 30 minutes with shaking. 200µl was plated on LB agar containing 50-100µg/ml ampicillin and incubated at 37°C.

2.3.10 DNA Sequencing

Plasmid DNA was sequenced using a Sequenase version 2.0 DNA sequencing kit (USB) following manufacturer's instructions. Alternatively, plasmid DNA was sequenced using an automated DNA sequencer. PCR reactions were performed as recommended (Applied Biosystems) and were analysed on ABI sequencers by the Glasgow University sequencing service.

2.3.11 Construction of genomic plasmid clones

Each genomic phage DNA was digested for four hours with SacI. The restriction digests were then phonol/chloroform extracted, precipitated and ligated

into SacI digested and phosphatase treated pBluescript KS II (Stratagene). The clones were named by phage number (pλ), restriction enzyme used and mini-preparation number, for example pλ1-SacI-3. The inserts were the mapped by Southern blotting using oligonucleotides from the DP-3 ORF (Table 2.2) and sequenced (Table 2.1). The E exon clone pBKS λ3–17, was constructed by digestion of phage 3 with NotI and XhoI, gel purification of the 5Kb fragment and ligation into pBluescript KS II (Stratagene) cut with NotI and XhoI.

2.3.12 Construction of DP-3 type IV 5'UTR-luciferase expression vector

DP-3 type IV 5'UTR-luciferase expression vector was constructed using PCR. The first round of PCR used a sense oligonucleotide primer from the 5' terminus of a type IV clone, with a HindIII restriction site (7.10H, Table 2.2) and an antisense oligonucleotide primer from exon 5 spanning the M2 ATG and fused to the 5' end of the luciferase ORF (Luc 3) with a type IV vector as template. The second round of PCR used a sense oligonucleotide primer from the 5'end of the luciferase ORF fused to the M2 region of DP-3 (Luc 1) and an antisense oligonucleotide primer from the EcoRI site of luciferase (Luc 2), using pGL2Control (Promega Corporation) as a template. The products of each PCR were purified by agarose gel electrophoresis and both used as templates in the third PCR reaction, using 7.10H and Luc 2 as primers. The product of this PCR was then chloroform extracted, precipitated and digested with HindIII and EcoRI. The restricted DNA was gel purified and cloned into the HindIII and EcoRI sites of pGL2Control (Promega Corporation) and sequenced. The type IV-luciferase was digested with HindIII and BamHI, gel purified and subcloned into the HindIII and BamHI sites of pBluecript KS II (Stratagene) for use in *in vitro* translation experiments.

Luc 1 $^{5'}$GGACCCCAAATGATTGAAGACGCCAAAAAC$^{3'}$
Luc 2 $^{5'}$GTAGATCCAGAGGAATTC$^{3'}$
Luc 3 $^{5'}$GTCTTCAATCATTTGGGGTCC$^{3'}$

2.3.13 Vectors used

DP-3 5'UTR-luciferase constructs containing type I, type II and type III were prepared as described in section 2.3.12 and kindly provided by S. de la Luna. The

control vector was pGL2Control (Promega Corporation) and the mock vector used was pSG5.

The p53 expression vector contains BamHI fragment of p53 in pCMV-neo BAM vector, kindly supplied by D. Lane (Baker *et al.*, 1990).

The DP-3 type I, type II and type III 5'UTR and control vectors in pBluescript KS II (Stratagene) were kindly supplied by S. de la Luna.

2.4 Agarose Gel Electrophoresis

2.4.1 Electrophoresis of DNA samples

Agarose gels were prepared for the analysis of DNA samples. Typically 1% gels were prepared for the analysis of plasmid DNAs, 0.8% gels were used to electrophorese genomic and phage DNA samples. Agarose gels were prepared by dissolving an appropriate amount of agarose in 1xTBE by warming. Once cooled ethidium bromide was added to a final concentration of 0.2μg/ml. Gels were run in 1xTBE and run at an appropriate voltage at room temperature. DNA samples were prepared by addition of DNA sample buffer to 1x. Electrophoresed DNA was visualised using an UV transilluminator with a digital imaging system.

2.4.2 Electrophoresis of RNA samples

Denaturing 1% agarose gels were prepared for the analysis of RNA samples. These were prepared by melting an appropriate amount of agarose in DEPC-treated water. Once cooled 10x MOPS and formaldehyde were added to final concentrations of 1x and 2.2M respectively. RNA was prepared by adding 10μl deionised formamide, 2μl 10xMOPS, 3.5μl 37% formaldehyde and 1μl ethidium bromide (2μg/μl). Prior to loading RNA was denatured at 65°C for 15 minutes and placed on ice. 2μl formaldehyde gel loading buffer was added and samples loaded immediately. Denaturing gels were run in 1xMOPS/DEPC-H_2O at 4°C, usually overnight at a low voltage. Electrophoresed RNA was visualised using an UV transilluminator. RNA gels for Northern blotting were then rinsed briefly in DEPC-H_2O to remove excess formaldehyde and used for Northern blotting.

2.4.3 Gel purification of DNA fragments

Fragments generated from restriction digests were purified by electrophoresis in low melting point agarose gels prepared in 1xTAE as detailed (Section 2.4.1). The required fragments were excised using a scalpel and the DNA extracted by melting the agarose at 65°C for 5 minutes. EDTA and NaCl were added to 10mM and 100mM respectively and the DNA was extracted with phenol (pH 8) and preciptated with 2 volumes of ethanol at -20°C. The DNA was then pelleted, washed with 70% ethanol and resuspended in TE. An aliquot of purified DNA was checked on an agarose gel to ensure extraction was successful and DNA concentration estimated.

2.5 Southern and Northern blots

Southern and Northern blots were prepared from agarose gels containing DNA and RNA samples. All blots were performed using Hybond-N (Amersham International) according to the manufacturer's protocol. Blotting was usually performed overnight. Blots were then UV crosslinked using a UV crosslinker (Amersham International), set to autocrosslink mode.

2.6 Dot Blotting

RNA samples were prepared for dot blotting in 1x dot blotting solution and denatured at 65°C for 15 minutes and then placed on ice. Samples were blotted under vacuum onto Hybond N (Amersham International), soaked in 2x dot blotting solution, using Biorad dot blotting apparatus. Blotting samples were washed twice in 200µl 2x dot blotting solution under vacuum. The blotting apparatus was then dismantled and the blot left to dry. The RNA was fixed by UV-crosslinking as before.

2.7 Probe preparation

2.7.1 Random priming

All DNA probes were prepared by random priming using 50-100ng gel purified template DNA, (α-^{32}P)dNTP (usually dCTP, Amersham International) and

the Megaprime kit (Amersham International) according to the manufacturer's instructions.

2.7.2 5' end labelling of oligonucleotides

Oligonucleotides were end labelled using (γ-^{32}P)ATP (Amersham International). Each labelling reaction consisted of 10pmol of oligonucleotide, 10pmol of (γ-^{32}P) ATP, 10U of T4 polynucleotide kinase (Promega Corporation) and 1xT4 polynucleotide kinase buffer (supplied with the enzyme) in a final reaction volume of 10µl. Reactions were incubated at 37°C for 30 minutes.

2.7.3 DP-3 E region probe

An E region probe was made using a primer to the 5' end of the E region sequence (MB-E^{+}) and template DNA, pBKSDP-3α digested with BsrI. BsrI cuts DP-3 cDNA in several places including the 3' end of the E region. 3µg pBKSDP-3α digested with BsrI (New England Biolabs) and 20pmol MB-E^{+} primer were mixed and denatured by boiling for 5 minutes. The primer and template were annealed by heating to 65°C for 2 minutes and leaving to cool gradually to 37°C. 3µl OLB (minus dCTP and dGTP), 12 µg BSA, 3µl (α-^{32}P) dCTP (3000Ci/mmol, Amersham International), 3µl (α-^{32}P) dGTP (3000Ci/mmol, Amersham International), 10U Klenow fragment was then added. The reaction was incubated at 37°C for 1 hour.

MB-E^{+} $^{5'}$TCGTTTACTTTCTGAGA$^{3'}$

2.7.4 Probe Purification

Probes were purified using Quick Spin columns (Boehringer Mannheim), DNA probes were purified through Sephadex G50 columns and oligonucleotide probes through G25 columns. On occasion, oligonucleotide probes were purified by phenol/chloroform extraction and precipitation.

2.8 Hybridisation of Southern, Northern and dot blots

Southern, Northern and dot blots were rinsed briefly in sterile H_2O and hybridised in an appropriate volume of Quickllyb (Stratagene) in hybridisation bottles (Hybaid), in a hybridisation oven with shaking platform (Hybaid), at an

appropriate temperature. Typically DNA probes synthesised by random priming were hybridised at 65°C. End-labelled oligonucleotide probes were hybridised at temperatures determined by the melting temperature (Tm) of the oligonucleotide. The Tm was calculated by the formula:

$$Tm = [(G+C)x4] + [(A+T)x2] - 5°C$$

Blots were prehybridised for at least 1 hour and probes added. 1µl each probe was counted to determine the number of counts per minute (cpm) per µl of probe, $5x10^5$ cpm of probe was added per ml of hybridisation solution. Probes were hybridised overnight.

2.8.1 Southern, Northern and dot blot hybridisation washes

Blots hybridised with random primed labelled DNA probes were rinsed and then washed twice in 2xSSC/0.1%SDS for 10 minutes at 60°C, in roller bottles. The blot was then removed from the bottles and placed in a sandwich box. A high stringency wash was then performed in 0.1xSSC/0.1%SDS for 30 minutes at 60°C, on a shaking platform. Likewise, oligonucleotide probes were rinsed and washed twice in roller bottles in 6xSSC/0.1%SDS at 37°C. Higher stringency washes were performed at a temperature 5°C lower then the calculated Tm or the hybridisation temperature chosen, using 6xSSC/0.1%SDS for 30 minutes on a shaking platform.

2.9 Autoradiography

Washed blots were sealed in Saran wrap and exposed medical X-ray film (Fuji), in autoradiography cassettes with intensifying screens. Autoradiography was carried out at -80°C until satisfactory images were obtained.

2.10 Stripping Northern and Southern blots

Random primed generated DNA probes were removed from blots by washing in 50% formamide/10mM NaH_2PO_4 (pH6.5), at 65°C for 30 minutes. Oligonucleotide probes were stripped by washing in 0.1xSSC/0.1%SDS at 65°C for 30 minutes. All blots were re-exposed to ensure all the probe had been removed.

2.11 Protein analysis

2.11.1 Preparation of cellular protein extracts

Whole cell extracts of transfected tissue culture cells were prepared by harvesting in PBS and pelleting at 3000rpm, 4°C. Pellets were resuspended in 1x SDS gel loading buffer and stored at -20°C.

2.11.2 *In vitro* transcription/translation

Protein was translated *in vitro* from plasmid DNA templates using the TNT T3 coupled reticulocyte system (Promega Corporation), following manufacturer's instructions. 1µg of plasmid DNA template was used in 50µl reaction mixture with ^{35}S- methionine (10mCi/ml) and incubated at 30°C for 60-120 minutes. *in vitro* translated proteins were analysed by SDS PAGE (Section 2.11.3), vacuum dried and visualised by autoradiography (Section 2.9)

2.11.3 SDS PAGE

Protein samples were analysed by SDS PAGE (Laemmli, 1970). 10% denaturing polyacrylamide resolving gels with a low acrylamide/low pH stacking gel were prepared and electrophoresed in 1x SDS running buffer. Protein samples were prepared in 1x SDS gel loading buffer and boiled prior to loading. Samples were electrophorised through the stacking gel at 80 volts, after which the voltage was increased to 110-130 volts, until the dye front had reached the bottom of the gel. Gels were immediately either vacuum dried or used for western blotting.

Resolving gel:

10% acrylamide/0.27% bis-acrylamide

0.75M Tris-HCl (pH 8.8)

0.1% SDS

Stacking gel:

5.1% acrylamide/0.186% bis-acrylamide

0.1M Tris-HCl (pH 6.8)

0.1%SDS

Gels were polymerised by the addition of ammonium persulphate (APS) and TEMED (Sigma)

2.11.4 Western blotting

Western blots were prepared from SDS polyacrylamide gels as described (Sambrook *et al.*, 1989). Polypeptides were transferred onto 0.45μm pore nitrocellulose membrane (Biorad) at 400mA at 4°C, for 60-75 minutes, using a mini protein trans-blotter (Biorad). Transferred proteins were visualised by Ponceau-S (Sigma) staining of the membrane and destained by washing in PBS. The membrane was then blocked in 10% milk powder/PBS at room temperature for at least 30 minutes, with shaking and incubated overnight in primary antibody, prepared in 5% milk powder/PBS. Where peptide competition was required, specific or non specific peptides were added to 25mg/ml final concentration. After incubation the membrane was washed three times for 15 minutes in PBS/0.25%Tween-20 (Sigma) and incubated in suitable secondary antibody, conjugated either to alkaline phosphatase (AP) (Promega Corporation) or horseradish peroxidase (HRP). Secondary antibody was incubated at room temperature for at least 30 minutes after which the membrane was washed three times for 15 minutes in PBS/0.25% Tween-20 and then developed for either AP or HRP activity.

Alkaline phosphatase activity was detected by incubating in NBT/BCIP solution prepared from an NBT/BCIP tablet (Sigma) dissolved in 10ml H_2O.

Horseradish peroxidase activity was detected using the SuperSignal system (Pierce and Warriner) following the manufacturer's instructions and visualised by autoradiography (Section 2.9).

2.12 Antibody production

2.12.1 Coupling of peptides to BSA.

1ml peptide 7.6 or 7.7 (5mg/ml) was mixed with 1ml bovine serum albumin (BSA) (5mg/ml in PBS), 2mls 0.2% glutaraldehyde/ PBS was added slowly whilst mixing at room temperature. The peptide was then mixed for 2 hours at room temperature. Glycine (pH 7.2, in PBS) was added to a final concentration of 200mM and mixed at room temperature for 1 hour. The peptide was then dialysed exhaustively in PBS at 4°C, and aliquoted and stored at –80°C.

2.12.2 Rabbit immunisations

Antigen samples for immunisation were prepared by vortexing equal volumes of coupled peptide and Freunds complete adjuvant (Sigma) until thoroughly mixed. 1ml of antigen was prepared per rabbit. Rabbits were immunised and then boosted with antigen prepared in Freunds incomplete adjuvant (Sigma), four times at three week intervals. Test bleeds were taken and tested by western blotting. All the rabbits immunised elicited a response to DP-3 peptides and antibodies were therefore harvested by terminal bleed-out by cardiac puncture under general anaesthesia. Blood samples were left at room temperature to coagulate and then the coagulate was pelleted. The serum was removed, aliquoted and stored at -20°C.

2.12.3 Peptides used in this study

The following peptides were used in this study:

7.6	S D R K R A E F I D S D F S E
7.7	E A A Q W V P S D R K R A
C	D Y S T R R P L S P S N Q L Q E K H V

2.12.4 Antibody Reagents

Rabbit polyclonal anti-DP-3 antibody α7.2 has been described (Ormondroyd *et al.*, 1995; de la Luna *et al.*, 1996). The rabbit polyclonal antibodies α7.6 ad α7.7 were made as described in Chapter 7. The rabbit polyclonal anti-luciferase antibody was purchased from the Promega Corporation and the anti-β-galactosidase monoclonal antibody purchased from Boehringer Mannheim.

2.12.5 Immunofluorescence

Coverslips from transfected U2OS cells were removed from culture dishes and washed in PBS. All washing of coverslips was performed by dipping each individual coverslip into a universal tube containing the wash solution, 10 times per wash. Cells were then fixed by treatment with 4% paraformaldehyde/PBS for 15 minutes at room temperature and washed twice in PBS, coverslips were then transferred to a damp sponge, cell side facing upwards. The coverslips were then treated with 1%TritonX-100/PBS for 10 minutes at room temperature and washed twice with PBS. Blocking was performed by the addition of 100μl 5%FCS/PBS and

incubation for 15 minutes at room temperature, followed by two washes in PBS. The primary antibody, either anti-7.2, (de la Luna *et al.*, 1996) or anti-7.6 (Chapter 7) was diluted 1:200 in 1%FCS/PBS, containing either peptide 7.6 or C at a 25mg/ml final concentration and cells treated for 30 minutes at room temperature, followed by four washes in PBS. Secondary antibody (anti-rabbit-FITC, Europath Ltd.) was diluted 1:200 in 1%FCS/PBS and cells treated for 15 minutes at room temperature. Coverslips were then washed four times in PBS, the final wash solution containing DAPI stain. Coverslips were then mounted in Citifluor (Agar Scientific) on glass slides and analysed by fluorescence microscopy.

Table 2.1

Summary of genomic plasmid clones and sequencing

Oligonucleotide	Exon	Phage	Genomic plasmids
7.12A	6	3	pλ3 SacI-1
7.16S	6	3	pλ3 SacI-1
7.18S	6	3	pλ3 SacI-1
E1-S	7	3	pBK+ λ3-17
E2-A	7	3	pBK+ λ3-17
7.17A	8	3/5	pBK+ λ3-17
7.17S	8	3/5	pλ5 SacI-7, pλ5 SacI-11
7.13A	8	5	pλ5 SacI-2
7.13S	9	5	pλ5 SacI-11, pλ5 SacI-2
7.28A	9	5	pλ5 SacI-2
7.27S	10	5	pλ5 SacI-2, pλ5 SacI-3
R7-A	10	5	pλ5 SacI-3
7.31S	11	5	pλ5 SacI-7
7.4S	12	1	pλ1 SacI-3
7.30S	12	1	pλ1 SacI-3
7.26S	13	1	pλ1 SacI-8
7.26A	13	1/6	pλ6 SacI-3
7.5A	14	1/6	pλ1 SacI-8, pλ6 SacI-3
7.9S	14	6	pλ1 SacI-3, pλ6 SacI-3
7.9A	14	1	pλ1 SacI-8

Table 2.2

Oligonucleotides used

Oligonucleotide	Sequence (5'-3')	Position (DP-3 type I E-Q+)
7.10H	GCTGAAGAGAGAG	1-13
7.12A	CCAATCAGAACACTTCCTG	268-286
7.16S	CACCCGCAATGGTCACT	304-320
7.18S	GCTGGCTGGGTTCCCAG	342-358
E1-S	GATAGAAAACGAGCTAGAG	E Region
E2-A	TTCTGAGAAATCAGAGTCTA	E Region
7.17S/A	GGGAAAGGCTTGAGACAT	387-404
7.7S	CTGGCAGCTGATTCGCAG	507-524
7.13A	CATCATAAACTCTTCGTCTA	545-564
7.13S	CGAAGAGTTTATGATGC	549-565
7.28A	CTGAGCAGAATTGGTAGG	633-650
7.27S	TCGAGAAGCAGAGGCGG	670-686
R7S/A	GAGCCCAGCTACAAGAACTTC	709-729
7.31S	CCTGGTACAGAGAAATCGAC	755-774
7.4S	GGCAAATGCTCTCTGGA	984-1000
7.30A	CAGGGATCTTGCGATTTTC	1007-1025
7.26S/A	GAACTCTACCCAATCAGT	1100-1117
7.5A	TGCTAAGGCCACTTCAG	1188-1205
7.9A	TCCTGTCTTTATTCTGGG	1337-1354
7.9S	CAGAATAAAGACAGGAG	1339-1355

2.13 Buffers and solutions

1xL-Broth media (LB, per litre)

10g bacto-tryptone

5g bacto-yeast extract

10g NaCl

Phosphate Buffered Saline (PBS)

136mM NaCl

2.7mM KCl

4mM Na_2HPO_4

1.8mM KH_2PO_4

pH adjusted to 7.2

50 x TAE

2M Tris base

1M glacial acetic acid

50mM EDTA pH 8.0

10 x TBE

1M Tris base

1M boric acid

20mM EDTA pH 8.0

5 x SDS-PAGE Running Buffer

125mM Tris base

1.25mM glycine

17mM SDS

pH adjusted to 8.3

10x Blotting Buffer

250mM Tris base

2M glycine

17mM SDS

pH adjusted to 8.3

6 x Agarose Gel Sample Buffer

0.25% (w/v)bromophenol blue

0.25% (w/v)xylene cyanol FF

50% (v/v)glycerol

1mM EDTA pH 8.0

2 x SDS Gel Loading Buffer

4% (w/v)SDS

125mM Tris-HCl pH 6.8

0.002% (w/v)bromophenol blue

20% glycerol

10 x MOPS Running Buffer

0.2M MOPS buffer

50mM sodium acetate

10mM EDTA

(in DEPC-treated H_2O)

pH adjusted to 7.0

6 x Formaldehyde-Gel Sample Buffer

0.25% (w/v)bromophenol blue

0.25% (w/v)xylene cyanol FF

50% (v/v)glycerol

1mM EDTA pH 8.0

(in DEPC-treated H_2O)

DEPC-treated H_2O

0.1% diethyl pyrocarbonate
in H_2O, treated overnight and
autoclaved

20 x SSC

3M NaCl
300mM sodium citrate
pH adjusted to 7.0

TE

10mM Tris-HCl pH 7.5
1mM EDTA

λ Diluent

100mM Tris pH 7.5
10mM $MgSO_4$

Denaturing Solution

1.5M NaCl
0.5M NaOH

Neutralising solution

1.5M NaCl
0.5M Tris-HCl pH 7.2
1mM EDTA pH 8.0

RNA Cell Fractionation Buffer

150mM NaCl
10mM Tris pH 8.5
5mM $MgCl_2$
0.5% IGPAL (Sigma)
in DEPC-treated H_2O

2x Dot Blot Solution

15% formaldehyde
10 x SSC
in DEPC-treated H_2O

2x β-galctosidase Assay Buffer

200mM sodium phosphate buffer pH 7.3
2mM $MgCl_2$
100mM β-mercaptocthanol
1.33mg/ml ONPG (o-nitrophenyl-β–D-
galactopyranoside)

2x HBS

280mM NaCl
50mM Hepes
1.5mM NaH_2PO_4
pH adjusted to 7.12
Filter sterilised, stored -20°C

Proteinase K Buffer

50mM Tris pH 8.0

100mM EDTA pH 8.0

100mM NaCl

1% SDS

OLB

Solution A

625µl 2M Tris-HCl pH 8.0

125µl 1M $MgCl_2$

250µl H_2O

18µl β-mercaptoethanol

5µl each dATP, dTTP, dGTP

Solution B

2M HEPES pH 6.6

Solution C

Random hexamers in 3mM Tris-HCl, 0.2mM EDTA, pH 7.0

Solutions A, B and C were combined in the ratio 2:5:3

Stored -20°C.

Chapter 3
Analysis of the DP-3 Gene

3.1 Introduction

The isolation and analysis of genes at the genomic level can provide insight into the regulation of expression at several levels. The isolation and analysis of the 5' regulatory region of a gene permits study of the transcriptional regulation. In addition, post-transcriptional regulation can be investigated; the exon structure of a gene can contribute to the understanding of splicing and other RNA processing events and the isolation of these regulatory regions allows study and manipulation *in vitro*. Finally, genomic sequence can be used to generate transgenic and gene knockout animals for the study of genes *in vivo*.

The genomic sequence for several E2F family members has been determined. Analysis of the mouse E2F-1 gene revealed multiple promoter elements required for trancriptional regulation at the G1/S phase boundary (Li *et al.*, 1994; Hsiao *et al.*, 1994; Neuman *et al.*, 1994). Regulation of the E2F-1 gene is in part mediated via E2F binding sites suggesting that this gene may be subject to autoregulation. Likewise the isolation of the human E2F-2 gene has shown a similar pattern of regulation (Sears *et al.*, 1997). In contrast, the murine DP-1 gene does not appear to be cell cycle regulated and contains a TATA-less promoter with multiple transcription initiation sites (Gopalkrishnan *et al.*, 1996), and is regulated by p53 (Gopalkrishnan *et al.*, 1998).

Extensive RNA analysis has suggested that DP-3 transcripts are highly processed and several different RNA species have been characterised. Sequence analysis suggests at least four different protein isoforms may exist (Figure 1.7) (Ormondroyd *et al.*, 1995). The DP-3 RNA variants arise from the inclusion or exclusion of two open reading frame changes. The insertion of a short 48 bp sequence within the DNA binding domain has been observed in some RNAs; this sequence, named the E region, has been shown to form part of a nuclear localisation signal in E+DP-3 proteins (de la Luna *et al.*, 1996). Furthermore, the presence or absence of a single glutamine residue (Q) has been noted between the DNA binding and E2F dimerisation domains of some DP-3 protein isoforms, (Ormondroyd *et al.*, 1995; Zhang and Chellapan 1995). In addition, four different 5' untranslated regions

have been identified, which result from rearrangements of five different exons (Figure 1.8). This regulation in the 5' region of RNA has the potential to result in translation intiation at one of two different initiating methionines and as a consequence one of the predicted protein isoforms, DP-3α, has an N-terminal extension which, interestingly, shows some homology to the N terminus of DP-1 (Girling *et al.*, 1993; Ormondroyd *et al.*, 1995). Two alternative 3'untranslated regions have also been characterised, which are likely to result in translational termination at the same translation termination signal.

The diverse and complex range of DP-3 RNAs could arise via several regulated events. The various different DP-3 RNAs may result from extensive post-transcriptional processing. It is also possible that different DP-3 RNAs result from the use of alternative transcriptional start sites and that there are several different promoter regions within the 5' end of the DP-3 gene. Since the processes of transcription and mRNA splicing take place simultaneously and are structurally linked (Xing *et al.*, 1993), the regulation described for DP-3 RNA may be influenced by a combination of these events.

The aim of this study was to isolate and characterise the murine DP-3 gene in order to provide information that may be useful in understanding its regulation.

3.2 Results

3.2.1 The DP-3 gene is a single copy gene

Prior to screening a genomic library to isolate the DP-3 gene, Southern blot analysis of mouse genomic DNA was performed with DP-3 probes to establish how many copies of the DP-3 gene existed in the genome. Mouse brain genomic DNA was isolated, blotted and thereafter probed for DP-3. This analysis revealed a simple pattern of genomic fragments (Figure 3.1), suggesting that DP-3 was a single copy gene. The blots were reprobed with different probes. The DP-3 FL probe spans the complete open reading frame (ORF) of DP-3α and produced a seven of bands of similar intensity. Digestion with KpnI yielded four bands of 14.1Kb, 9.8Kb, 7.6Kb and 5.2Kb, whereas HindIII digestion revealed three bands of 9.8Kb, 7.1Kb and 2.6Kb. These data suggest the ORF of DP-3 spans at least 36.7Kb of DNA. Analysis with a probe from the 5'untranslated region revealed a 14Kb HindIII fragment and a KpnI fragment of 4.1Kb. The 5' end of the DP-3 gene might therefore span more

than 14Kb. The DP-3 3'end probe revealed that the 9.4Kb KpnI and the 2.6Kb HindIII fragments observed with the full length probe contain the 3'end of DP-3 ORF. These results are consistent with DP-3 being a single copy gene. Hence, the DP-3 gene appears to be a large gene and is likely to be single copy, since a more complicated pattern would have been expected for a multi-copy gene.

3.2.2 The isolation and restriction enzyme mapping of the DP-3 gene

A λGEM12 genomic library, constructed from the mouse embryonic stem cell line SV129D3, was plated and 10^6 plaques were screened with DP-3FL probe (Figure 3.1b). The DNA from seven phage was isolated and comparison of the restriction digestion with SacI suggested that two of the phage were identical; in total six different phage were isolated. Southern blotting of phage DNA and subsequent hybridisation with DP-3 cDNA oligonucleotides confirmed that the phage contained DP-3 genomic sequence and that they overlapped. From these data a SacI map was constructed (Figure 3.2) which contained the ORF and the 3'UTR of the DP-3 gene. Blotting analysis of restriction digests with oligonucleotide probes derived from both 5'UTR and ORF sequence indicated that the first exon after the 5' UTR (exon 6) was the most 5' exon isolated of the phage. The SacI map of the phage DNAs (Figure 3.2) therefore implied that an intron of at least 8.9Kb existed 5' of exon 6 and suggested that the 5'UTR and promoter regions of DP-3 had not been isolated.

3.2.3 The DP-3 genomic structure

The phage clones were shotgun cloned into pBluescript (Stratagene) as SacI fragments and then sequenced to determine the positions of the exon/intron junctions (Figure 3.3). Sequence analysis revealed the splice junction of the most 5'exon isolated was just after the second initiating methionine codon (M2) of DP-3 (Ormondroyd *et al.*, 1995) (Figure 1.8). Hence, these data confirm that the second exon of the DP-3 ORF was the most 5' exon isolated. Since five exons are predicted from analysis of the 5' untranslated region (Ormondroyd *et al.*, 1995), this exon was designated as exon 6 (Figure 3.3a). All the splice junctions sequenced (Figure 3.3b) conformed to the 'GT' 5'splice donor and 'AG' 3' splice acceptor consensus intron sequences.

3.2.4 The E region of DP-3α and δ is a discrete 48 bp alternatively spliced exon

The isolation of the DP-3 gene enabled a study of the relationship between mRNA and genomic sequence. Previous studies of the DP-3 cDNAs had predicted four different protein isoforms, named α, β, γ and δ (Ormondroyd *et al.*, 1995) (Figure 1.7). DP-3α and δ have an extra 16 residues inserted close to the DNA binding domain of the E region (Ormondroyd *et al.*, 1995). Subsequently, the E region has been shown to be part of a NLS and DP-3α and δ accumulate in the nucleus. However, E minus DPs such as DP-3β, γ and DP-1 are cytoplasmic (de la Luna *et al.*, 1996; Magae *et al.*, 1996). Sequencing and PCR analysis of the DP-3 gene revealed the E region to be a discrete 48 bp exon (exon 7, Figure 3.3a) surrounded by a 5' intron of 2.6Kb and a 3' intron of 3Kb (Figure 3.4a). Conventional splice donor and acceptor sites exist at the exon boundaries (Figure 3.4b).

3.2.5 DP-3 Q+ arises via the use of an alternative 3' splice acceptor site.

The DP-3γ isoform differs from the other DP-3 isoforms due to the insertion of a single glutamine (Q) residue (Ormondroyd *et al.*, 1995). The Q insertion occurs between the DNA binding and dimerisation domains, in a region not conserved between DP family members. It is likely that the insertion in DP-3γ arises through alternative selection of two adjacent 3'splice acceptor sequences at the 3' end of the intron between exons 8 and 9 (Figure 3.3). The selection of the first AG, reading 5'-3', results in a Q+DP-3, whereas selection of the second results in a Q-DP-3 (Figure 3.5).

3.2.6 hDP-2 protein isoforms arise via alternative splicing of equivalent exons

The human homologue of DP-3, human DP-2, has been reported (Wu *et al.*, 1995; Zhang and Chellapan, 1995; Rogers *et al.*, 1996). The cDNAs so far published include the additional E region and are therefore homologous to DP-3α and δ. Furthermore, the inclusion of the Q residue has also been reported (Zhang and Chellapan, 1995). The human dbest database (Altschul *et al.*, 1997) contains data generated from a sequencing project; cDNA is generated randomly from human mRNA and sequenced. The sequence data is entered into the database directly, with

little or no analysis, the sequence data therefore may contain unidentified sequencing errors. Searches of the human dbest database with a DP-3 cDNA sequence have identified sequences that suggest other potential human DP-2 isoforms may exist (Figure 3.6). These human DP-2 isoforms are different to those published and since all the sequences arise from combinations of the DP-3 exons described in Figure 3.3, it is likely that these are previously unidentified splice variants. When the translation products of these splice variants were predicted, it was interesting to note that some of the RNAs give rise to short N terminal polypeptides and, in addition, translational initiation at downstream methionines may occur. An example is sequence N23047 (Figure 3.6), where translation could initiate at methionine 1 in exon 2. However, the absence of exon 6 results in a frameshift resulting in a termination signal in exon 7, in which case translation could be re-initiated the next methionine (M4) in exon 8. Furthermore, a unique exon not found in mouse DP-3 transcripts was observed in one transcript (Figure 3.6, AA039977) between exons 5 and 6, that may represent a human specific sequence. An examination of the sequence suggests that it is not an intron that was not successfully spliced out, since the sequence does not contain splice donor and acceptor signals. This sequence, contains a termination signal, therefore, the insertion of this exon may result in early translational termination, resulting in a short N terminal polypeptide.

3.2.7 Murine DP-1 and DP-3 have identical exon structure

Comparison of DP-3α exon structure and the DP-1 exon structure (Gopalkrishnan *et al.*, 1996), revealed striking similarity, since all the exon/intron junctions are observed in exactly the same positions (Figure 3.7). The exon arrangement diverges at the 3' end of the gene, which is the region of least similarity between DP-1 and DP-3 at the amino acid level (Girling *et al.*, 1993; Ormondroyd *et al.*, 1995). However the highly conserved domains, such as the DEF box, DCB1, DCB2 and the N-terminus (Lam and La Thangue 1994) are all situated on exons of exactly the same size in both proteins. The 5' regions of DP-3 were not present in the genomic phage isolated and exons 2, 3 and 5 were predicted from studies of splicing arrangements of the 5' untranslated regions (Chapter 1; Ormondroyd *et al.*, 1995).

The strong conservation in exon structure between these two genes suggests a common evolutionary origin, perhaps one gene arising via duplication and subsequent divergence of the first gene. However, an assessment of DP gene evolution requires extensive analysis of a large number of family members, which so far are available from DP proteins cloned in humans (Helin *et al.*, 1993; Wu *et al.*, 1995; Zhang and Chellapan, 1995; Rogers *et al.*, 1996) and mice (Girling *et al.*, 1993; Ormondroyd *et al.*, 1995). DP homologues have also been characterised in *Xenopus laevis* (Girling *et al.*, 1994) and in *Drosophila melanogaster* (Dynlacht *et al.*, 1994; Hao *et al.*, 1995). More family members and more species are needed for comprehensive phylogenetic analysis of the sequences.

3.3 Discussion

3.3.1 Alternative splicing of the E region influences cellular the location of DP-3

Analysis of the genomic organisation of the E region revealed an alternatively spliced 48 bp exon (exon 7, Figure 3.3). The inclusion of the protein sequence resulting from this exon generates an NLS in DP-3α and DP-3δ (de la Luna *et al.*, 1996). In addition, DP-3α and δ can recruit cytoplasmic E2F-4 and 5 to the nucleus and likewise, E2F-1, 2 and 3 can recruit E minus DPs such as DP-1 and DP-3β and γ to the nucleus (de la Luna *et al.*, 1996; Magae *et al.*, 1996; Allen *et al.*, 1997). It has been shown that the mechanism of nuclear localisation of the E2F heterodimer can have different functional consequences on the cell cycle (Allen *et al.*, 1997) and therefore, the functional consequence of the alternative splicing of the E region may be very important in the growth regulating functions of E2F.

3.3.2 The insertion of the glutamine residue (Q) may represent a site of post translational modification

DP-3γ contains an extra Q residue. The most likely reason for this is because of an alternative 3'splice acceptor sequence at the 3' end of the intron between exons 8 and 9 (Figure 3.3). The selection of either of two splice acceptor sites might result from random slippage by the splicing machinery (Padgett *et al.*, 1986). Alternatively, the selection process may be regulated and in this respect, only a subset of DP-3 RNA variants would select the first splice acceptor site (Ormondroyd *et al.*, 1995). Furthermore, when DP family members were compared, it was noticed

that the Q residue resides in an unconserved region between two conserved helical domains, which may form a loop that represents a potential site of post-translational modification. Indeed, the insertion of this Q residue results in the destruction of a theoretical casein kinase II phosphorylation site and the creation of a putative DNA-PK phosphorylation site. It is possible that the glutamine insertion may therefore represent a site of importance, for example in post-translational modifications by DNA-PK.

The insertion or exclusion of a single glutamine has been observed in other genes such as the mouse obese gene (Zhang *et al.*, 1994) where a Q residue is located in a highly conserved region of the protein. However, the significance of the insertion is unknown. The insertion of Q residues in two different positions in the human *brca 1* gene has also been observed (Miki *et al.*, 1994), where the insertion occurs at a splice junction and may therefore represent a similar situation to that in DP-3. Likewise, the functional relevance of the Q insertion in BRCA 1 protein has not been determined.

3.3.3 Murine and human DP-3 cDNAs suggest similar genomic organisations

The analysis of available human DP-2 sequences predicted several different protein isoforms, some of which are homologous to the murine DP-3 protein isoforms (Figure 3.6). In addition, some cDNAs suggested that truncated hDP-2 proteins exist (Figure 3.6). The comparison of the hDP-2 cDNAs and the DP-3 gene exons revealed all the hDP-2 sequence variants arise from rearrangements of exons equivalent to those characterised for the murine DP-3 gene (Figures 3.3 and 3.6). Therefore, it is likely that the exon structure of the hDP-2 gene will be similar to the mouse DP-3 gene.

The expression level of both hDP-2 sequence variants and the resultant truncated protein products might be low in cells. However, it is possible that these short polypeptides may represent as yet uncharacterised functional DP-3/DP-2 proteins and it is conceivable that they could influence E2F activity. Truncated polypeptides that lack important regulatory domains could form repressive complexes with E2F family members, preventing activation of E2F dependant transcription. Indeed, an E2F family member, E2F-6, that lacks a transactivation domain, has been proposed to function as a transcriptional repressor (Morkel *et al.*,

1997; Cartwright *et al.*, 1998; Trimarchi *et al.*, 1998). It is relevant to consider that truncated DP-3/DP-2 proteins may function in a similar manner.

During the analysis of hDP-2 sequences, a cDNA containing an extra exon sequence, not yet observed for either DP-3 or hDP-2, was identified (Figure 3.6). This short sequence may represent an artefact that arose during the cDNA production or a real exon. It is feasible that this exon also exists in the murine DP-3 gene but has not yet been isolated, as hDP-2 sequence variants arise from selection of exons equivalent to those characterised for the murine DP-3 gene. Furthermore, comparisions of DP-3 and hDP-2 cDNAs has revealed a striking level of similarity. Consequently, it would not be unexpected for future work to reveal that this exon exists in the murine DP-3 gene.

3.3.4 DP-1 and DP-3 have a common evolutionary origin

An interesting and important question is why did a second DP gene evolve? At present the role for DP-3 in cells is ill defined. In contrast, DP-1 has been well characterised and its regulation exhaustively studied. DP-1 is the major DP partner in the E2F heterodimer, and to date the role DP-3 in E2F regulation has not been clarified. Further analysis of DP-3 function is required before questions such as these can begin to be answered.

Figure 3.1

Analysis of the DP-3 gene

a) A Southern blot was prepared with 10μg brain genomic DNA digested with KpnI and with HindIII was sequentially hybridised with different DP-3 specific probes as indicated. The blot was completely stripped between hybridisations.

b) The DP-3 probes used for Southern hybridisations in (a). The DP-3 full length (FL) probe was purified from pBKSDP-3α digested with EcoRI and XhoI. The DP-3 5'UTR probe was purified from pBKS-B5 (type II 5'UTR), digested with EcoRI. The DP-3 3'end probe was purified from digestion of a pBKSDP-3γ with EcoRV and XhoI. The nucleotide numbers are given for a DP-3γ cDNA.

Figure 3.1

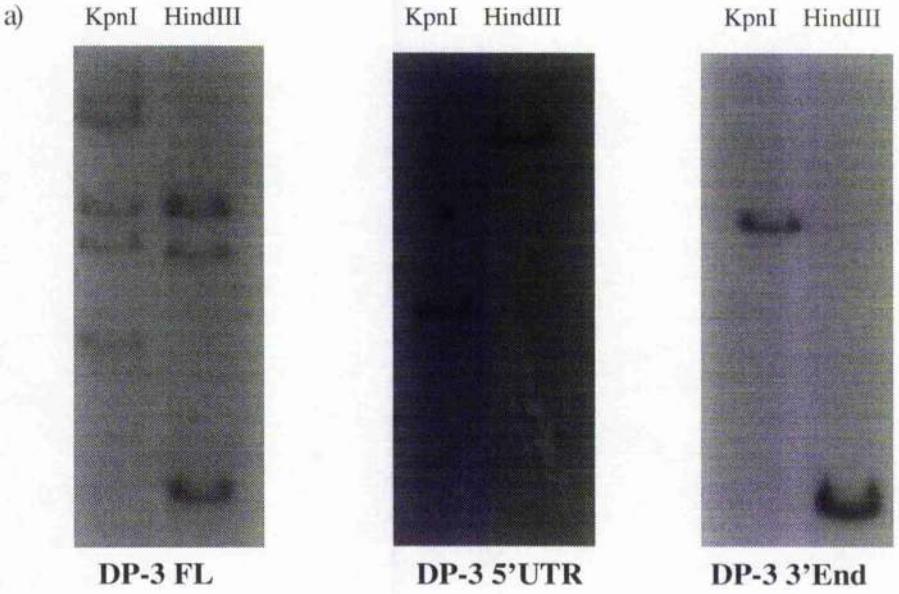

a)
KpnI HindIII
KpnI HindIII
KpnI HindIII
DP-3 FL
DP-3 5'UTR
DP-3 3'End
b)
Probes
DP-3 cDNA Type IV E-Q+
1
M1
M2
1493
DP-3 FL
118
1493
DP-3 5'UTR
1
340
DP-3 3'End
1163
1493

Figure 3.2

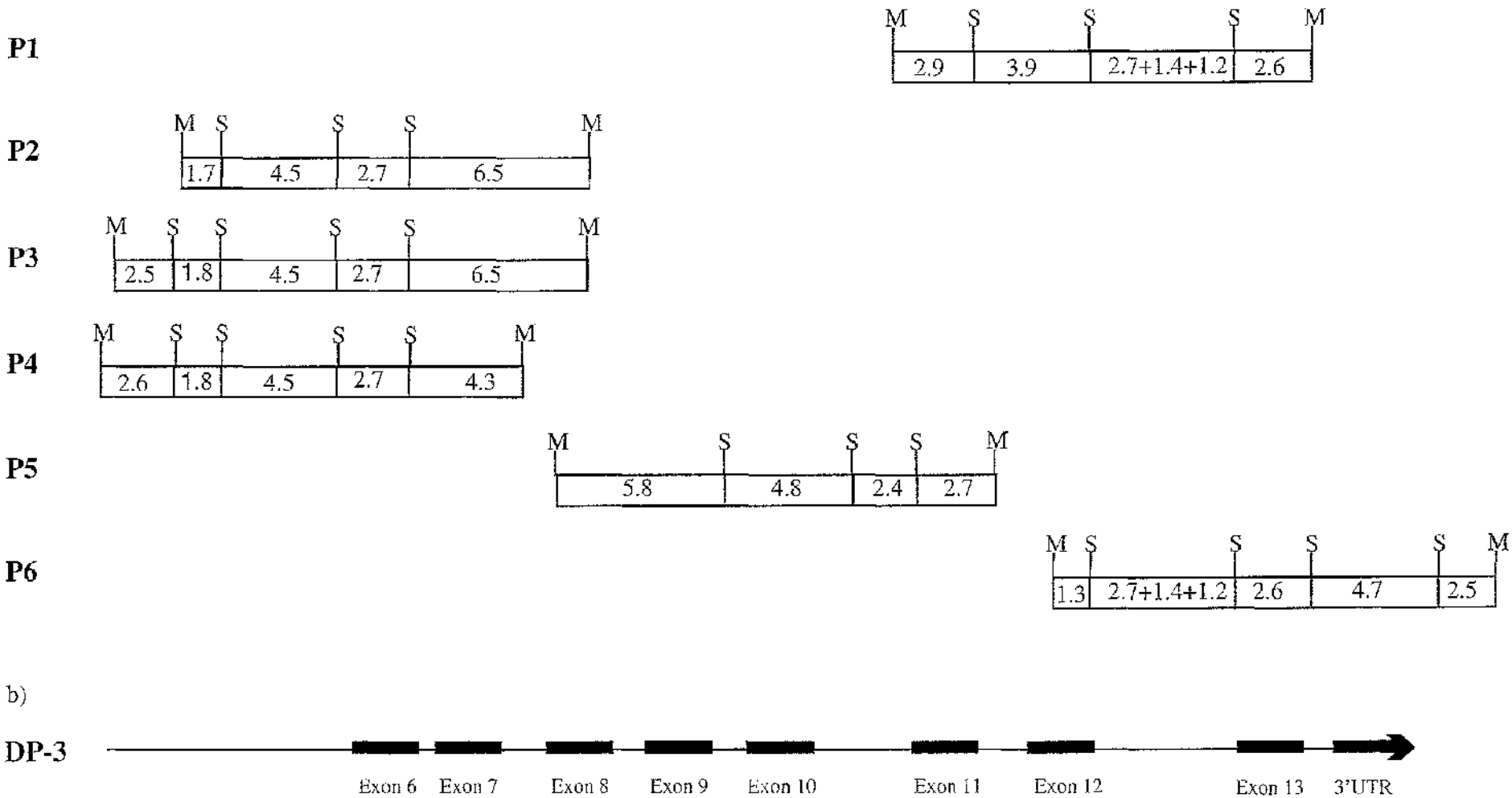
A SacI restriction map of DP-3 phage clones

a) Genomic digests of the DP-3 phage clones derived from data obtained from restriction analysis of the DP-3 and Southern blot analysis with oligonucleotide probes (Chapter 2, Table 2.2). S denotes SacI restriction sites and M denotes an MboI restriction site (Chapter 2).

b) The positions of the DP-3 exons on the phage clones are represented by black bars. The thin lines represent intron sequences (not to scale).

Figure 3. 2

a)


P1
M S S S M
2.9 3.9 2.7+1.4+1.2 2.6
P2
M S S S M
1.7 4.5 2.7 6.5
P3
M S S S S M
2.5 1.8 4.5 2.7 6.5
P4
M S S S S M
2.6 1.8 4.5 2.7 4.3
P5
M S S S M
5.8 4.8 2.4 2.7
P6
M S S S S M
1.3 2.7+1.4+1.2 2.6 4.7 2.5

b)

DP-3
Exon 6
Exon 7
Exon 8
Exon 9
Exon 10
Exon 11
Exon 12
Exon 13
3'UTR

Figure 3.3

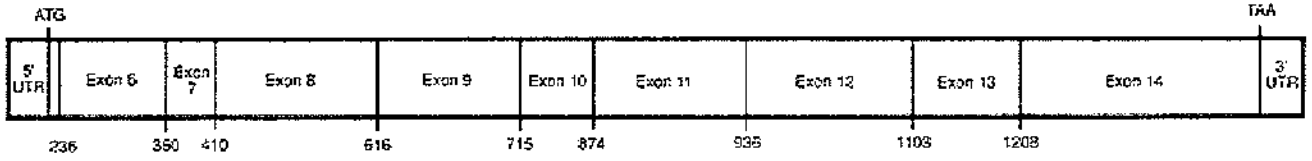
The murine DP-3 genomic structure

a) The DP-3 gene exon arrangement. Exons are represented as open rectangles. The numbers denote the nucleotide position of the first base of the exons and correspond to the DP-3δ cDNA (Ormondroyd *et al.*, 1995).

b) The DP-3 genomic sequences at each exon/intron junction. The intron sequences are boxed; the nucleotide positions are given of the first and last nucleotides of the exon. The splice donor and acceptor sequences are coloured red. The black boxes denote the position of the alternative 3'splice acceptor sites at the 3' end of the intron between exons 8 and 9.

Figure 3.3

a)


ATG
TAA
5' UTR
Exon 6
Exon 7
Exon 8
Exon 9
Exon 10
Exon 11
Exon 12
Exon 13
Exon 14
3' UTR
236
350
410
616
715
874
935
1103
1208

b)

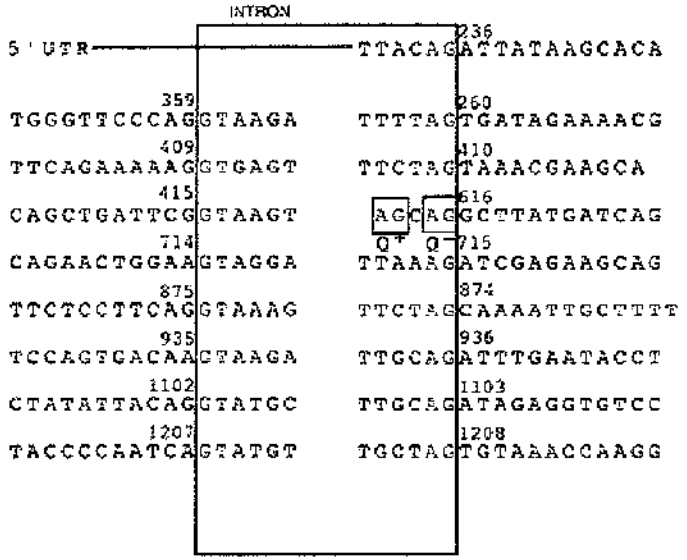

INTRON
5' UTR
236
TTACAGATTATAAGCACA
359
TGGGTTCCCAGGTAAGA
260
TTTTAGTGATAGAAAACG
409
TTCAGAAAAAGGTGAGT
410
TTCTAGTAAACGAAGCA
415
CAGCTGATTCGGTAAGT
616
AGCAGGCTTATGATCAG
Q+
Q-
714
CAGAACTGGAAGTAGGA
715
TTAAAGATCGAGAAGCAG
875
TTCTCCTTCAGGTAAAG
874
TTCTAGCAAAATTGCTTTT
935
TCCAGTGACAAGTAAGA
936
TTGCAGATTTGAATACCT
1102
CTATATTACAGGTATGC
1103
TTGCAGATAGAGGTGTCC
1207
TACCCCAATCAGTATGT
1208
TGCTAGTGTAAACCAAGG

Figure 3.4

The E region of DP-3 is a single, alternatively spliced exon

a) Diagram summarising the genomic DP-3 E region. Exons are labelled and represented by boxes and intron sequence by a solid line.

b) Sequence of the splice junctions of the DP-3 genomic region together with the resulting protein sequences. The top sequence is E minus, the bottom E plus. Exon boundaries are indicated by ▪.

Figure 3.4

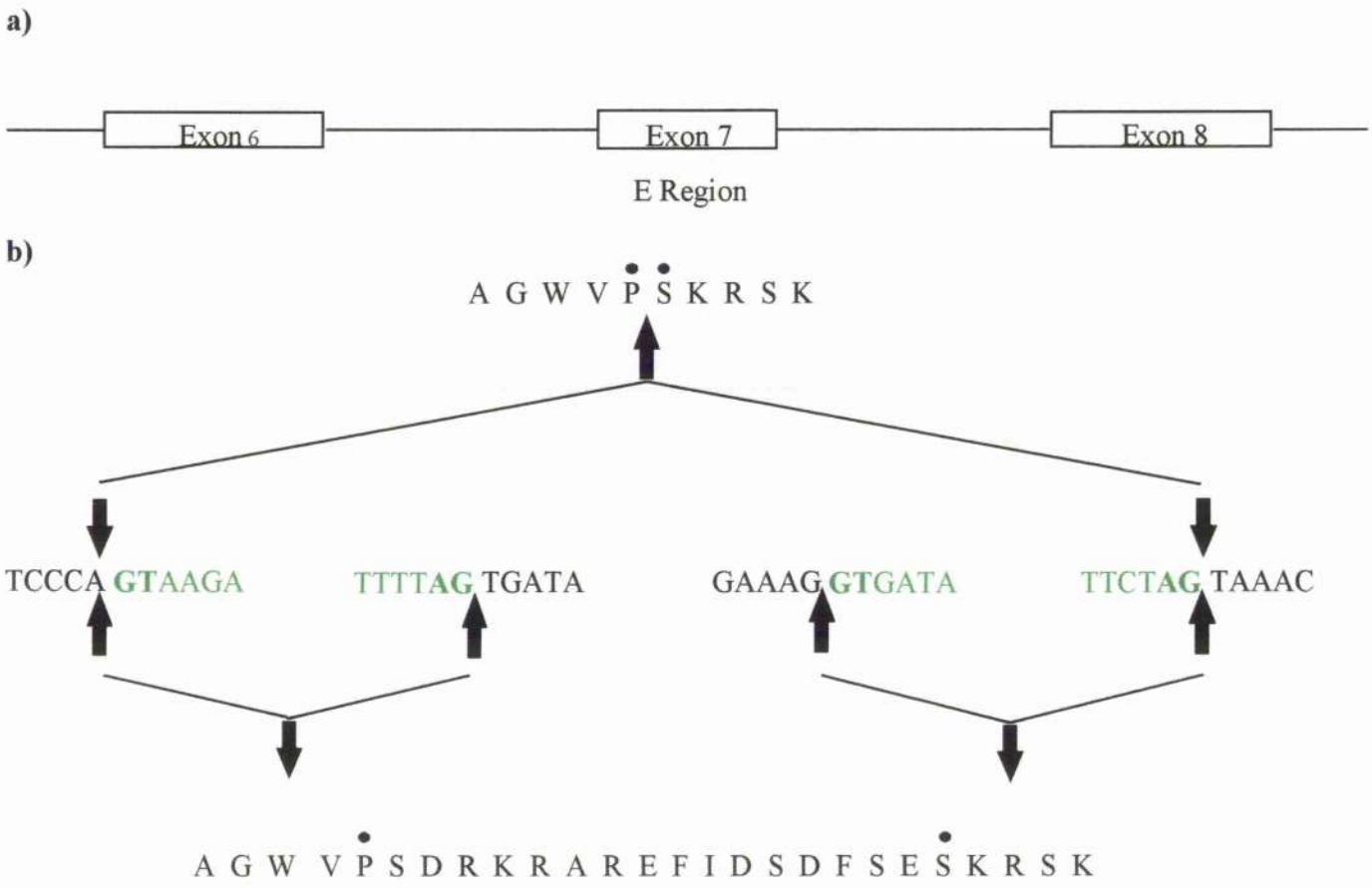

a)
Exon 6
Exon 7
Exon 8
E Region
b)
A G W V P S K R S K
TCCCA GTAAGA
TTTTAG TGATA
GAAAG GTGATA
TTCTAG TAAAC
A G W V P S D R K R A R E F I D S D F S E S K R S K

Figure 3.5

The Q insertion of DP-3γ arises from use of an alternative 3'acceptor site

A diagram summarising how Q+ and Q- DP-3 mRNAs arise. The sequence of the 3' end of the intron between exons 8 and 9 is given, together with the resulting amino acid sequences, Q- at the top and Q+ at the bottom.

Figure 3.5

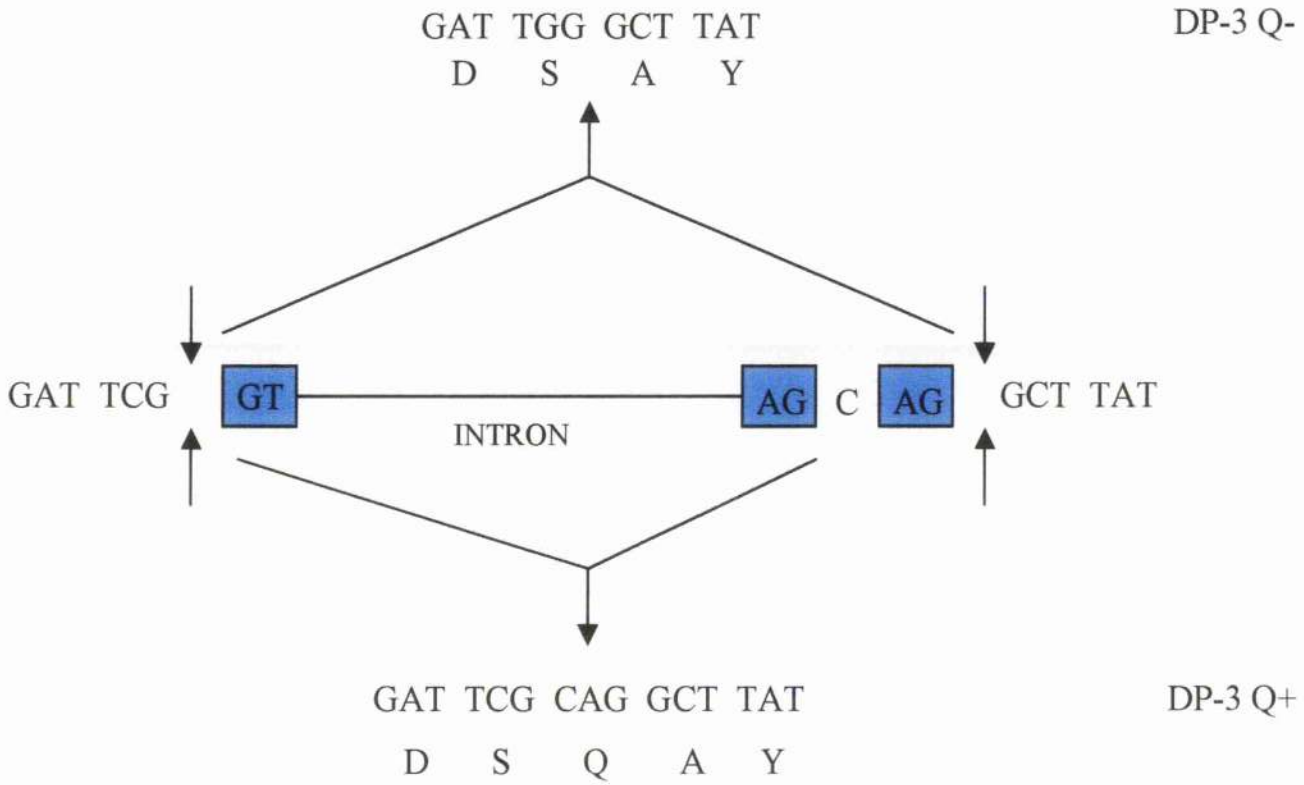

GAT TGG GCT TAT
D S A Y
DP-3 Q-
GAT TCG
GT
AG
C
AG
GCT TAT
INTRON
GAT TCG CAG GCT TAT
D S Q A Y
DP-3 Q+

Figure 3.6

Comparison of hDP-2 cDNA sequences and DP-3 genomic structure

Diagrammatic representation of cloned hDP-2 sequences compared to mDP-3 genomic structure. The shaded box indicates the E region. Numbered boxes indicate the arrangement of DP-3 exons, corresponding to the hDP-2 sequences.

Figure 3.6

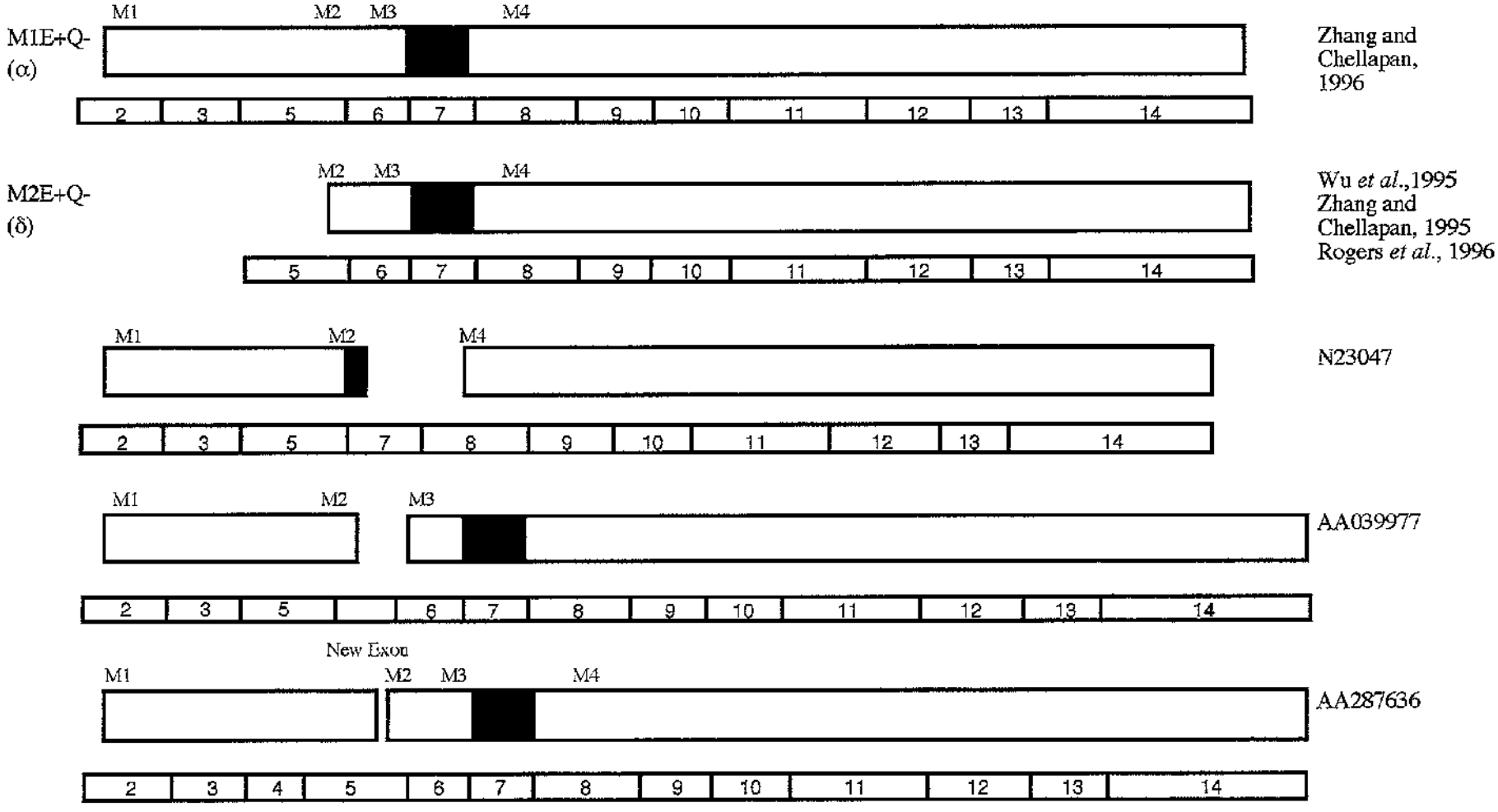
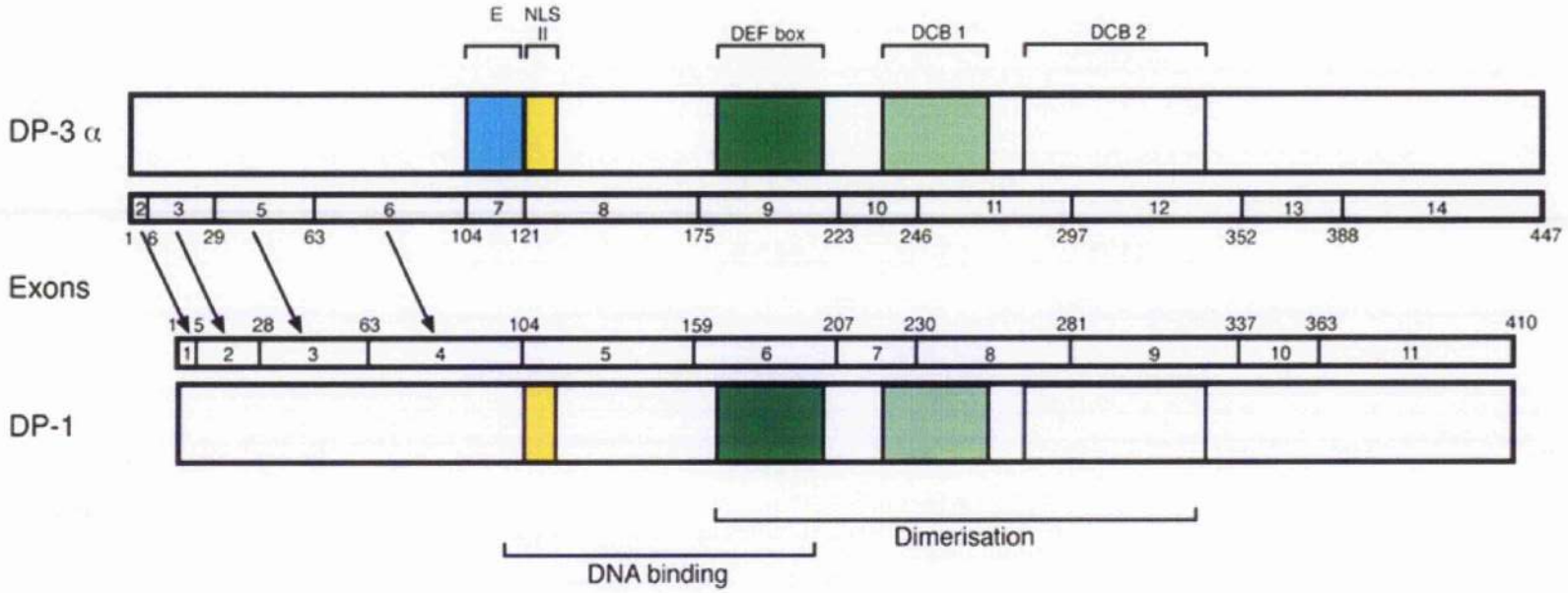

M1E+Q-
(α)
M1
M2
M3
M4
2
3
5
6
7
8
9
10
11
12
13
14
Zhang and Chellapan, 1996
M2E+Q-
(δ)
M2
M3
M4
5
6
7
8
9
10
11
12
13
14
Wu et al.,1995
Zhang and Chellapan, 1995
Rogers et al., 1996
M1
M2
M4
2
3
5
7
8
9
10
11
12
13
14
N23047
M1
M2
M3
2
3
5
6
7
8
9
10
11
12
13
14
AA039977
New Exon
M1
M2
M3
M4
2
3
4
5
6
7
8
9
10
11
12
13
14
AA287636

Figure 3.7

Comparison of murine DP-1 and DP-3 genomic organisation

Alignment of DP-3α exons and protein sequence, compared with analogous alignment of DP-1. Amino acid positions of the exon boundaries are indicated. Arrows indicate homologous exons of the 5'end. Shaded areas denote domains conserved in DP family members and are labelled.

Figure 3.7


E
NLS
II
DEF box
DCB 1
DCB 2
DP-3 α
2
3
5
6
7
8
9
10
11
12
13
14
1
6
29
63
104
121
175
223
246
297
352
388
447
Exons
1
5
28
63
104
159
207
230
281
337
363
410
1
2
3
4
5
6
7
8
9
10
11
DP-1
Dimerisation
DNA binding

Chapter 4

Analysis of DP-3 RNA Expression

4.1 Introduction

Analysis of cellular mRNA provides an indication of the regulation of the gene of interest. Analysis of mRNA levels can reflect the transcriptional activity of a gene and provide an indication of the potential expression level of the protein. The analysis of cellular RNA by Northern blotting aids determination of the size and abundance of RNA transcripts. Although Northern blotting can detect changes in transcript abundance it does not indicate the mechanism of RNA accumulation. RNA can be regulated transcriptionally and by RNA processing and stability, and RNA detected by Northern blotting gives a representation of the steady state levels of RNA. Therefore, Northern blotting represents a useful technique for study of RNA transcripts. The information obtained can contribute to the understanding of the regulation of a particular gene and additionally, used in subsequent protein studies.

Northern blotting analysis of RNA from cells at different stages of the cell cycle indicated that DP-1 transcript levels remained unchanged during the cell cycle, although E2F-1, 4 and 5 transcripts were induced at the G1/S boundary (Johnson *et al.*, 1994; Li *et al.*, 1994; Sardet *et al.*, 1995). Analysis of other E2F family members has revealed some differences in RNA expression patterns. For example, in human tissues E2F-2 RNA is highly expressed in lung and kidney, whereas E2F-3 RNA is high in muscle and liver but absent in brain (Lees *et al.*, 1993). Analysis of DP-1 and E2F-1 by *in situ* hybridisation during mouse embryo development indicated that E2F-1 is expressed in many tissues and is often expressed in the same tissues as DP-1 (Tevosian *et al.*, 1996).

DP-3 differs from the other DPs due to a striking complexity at the RNA level. RNA analysis during the cloning of DP-3 cDNAs suggested that extensive processing of DP-3 RNA may give rise to four different DP-3 protein isoforms, $\alpha,\beta,\gamma,\delta$ (Ormondroyd *et al.*, 1995) . These variants arise through two ORF changes and include the insertion of a 48 bp sequence within DNA binding domain. In addition, extensive processing gives rise to several different 5'untranslated regions (5'UTRs). Furthermore, two different 3'UTRs have also been identified (Chapter 1).

In an effort to study the expression of DP-3, an investigation of DP-3 RNA was instigated. The aim of this study was to identify physiological conditions under which DP-3 RNA levels change and therefore conditions under which DP-3 proteins could be analysed.

4.2 Results

4.2.1 DP-3 specific transcripts are expressed in a range of mouse tissues

Analysis of DP-3 RNA via Northern blotting revealed that DP-3 exists as three transcripts of different mobility, approximately 8Kb, 3Kb and 2Kb in size (Figure 4.1a). Similar analysis of DP-3 on a Northern blot of mRNA prepared from mouse tissues showed that DP-3 mRNA varies in abundance between tissues, not only were the overall levels of DP-3 message variable but the abundance of the individual transcripts also fluctuated. Comparison with the β-actin transcript (Figure 4.1c) indicated that the quantity of mRNA analysed from each tissue type was approximately the same, suggesting that DP-3 transcripts may therefore be regulated in a tissue specific manner. For example, liver contained the 3Kb and 2Kb transcript, whereas the 8Kb transcript was poorly expressed (Figure 4.1a, lane 5). In contrast, brain RNA contained more of the 8Kb transcript than 3Kb and 2Kb transcripts (Figure 4.1a, lane 2). It is interesting to note that the mobility of the 3Kb and 2Kb transcripts were altered in the spleen (Figure 4.1a, lane 3), however, the mobility of the β-actin transcript was not changed in spleen RNA, suggesting that it was not due to experimental differences in the RNA samples (Figure 4.1c, lane 3). This may, therefore, represent a particular level of regulation unique to spleen DP-3 RNA.

The DP-1 gene gives rise to a single transcript of 3Kb (Figure 4.1b), which does not cross-hybridise with the DP-3 probe used, arguing that the multiple transcripts described for DP-3 were not influenced by cross-hybridisation with DP-1. The DP-3 transcripts were less abundant than the DP-1 and the β-actin transcripts. Overall, these results indicate that DP-3 transcripts are expressed in a wide range of mouse tissues and the levels of individual transcripts may be regulated in a tissue specific manner.

4.2.2 DP-3 transcripts are expressed in different cell lines

The abundance DP-3 transcripts were also investigated in a range of tissue culture cell lines (Figure 4.2). The F9 embryonal carcinoma (F9EC) and NIH 3T3 mouse cell lines clearly expressed DP-3 (Figure 4.2a, lanes 1 and 2). In the human tumour cell lines C33A, U2OS and HeLa, DP-3 transcripts were of a different mobility to the transcripts detected in mouse cell lines, although three transcripts were still observed (Figure 4.2a, lanes 3, 4 and 5). HeLa cells differ from the other cell lines due to a unique transcript of 4.4Kb (Figure 4.2a, lane 5). Comparison of the control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and DP-3 transcript levels suggested that there was some variation in the levels of DP-3 RNA in the human and mouse cell lines (Figure 4.2a and b). However, DP-3 RNA is expressed in all the cell lines tested.

4.2.3 DP-3 transcripts are polyadenylated, cytoplasmic mRNAs

The Northern blot analysis of DP-3 revealed a complicated pattern of RNA transcripts. In order to characterise the DP-3 transcripts in greater detail, it was determined if they possessed the properties of mRNA, namely being cytoplasmic and polyadenylated. Thus, polyadenylated RNA was selected from total RNA, and nuclear and cytosolic fractions of RNA were isolated, the purified RNA analysed by Northern blotting (Figure 4.3). A DP-3 specific probe revealed that DP-3 transcripts were polyA+, cytoplasmic (Figure 4.3a). A comparison of the poly A- and poly A+ samples (Figure 4.3a, lanes 2 and 3) suggested that the 3Kb transcript might be a set of transcripts of similar size, since a subset of these appeared to either lack polyadenylation, or were polyadenylated less efficiently than the other DP-3 messages. However, since some of the GAPDH mRNA were not completely selected it is possible that the selection of polyadenylated transcripts was not complete and this effect was observed on two occasions (Figure 4.3b). Hybridisation with histone 3 (H3, an unpolyadenylated, cytosolic RNA) and U6 RNA (an unpolyadenylated, nuclear RNA) indicated that the polyadenylated fraction had not been contaminated with unpolyadenylated RNA (Figure 4.3c and d). Likewise, the nuclear and cytosolic fractionation suggests that DP-3 transcripts are cytoplasmic transcripts and little contamination of fractions had occurred. In conclusion, the majority of DP-3 transcripts appear to be polyadenylated and cytoplasmic and

therefore the DP-3 transcripts possess the properties of typical mRNA, derived from protein-encoding genes.

4.2.4 DP-3 RNA is expressed throughout differentiation of F9EC cells

DP-3 transcripts were expressed in all the cell lines and tissues tested. To explore the possibility that DP-3 mRNA levels may be regulated during specific cellular processes, F9 embryonal carcinoma (F9EC) cells were stimulated to differentiate into parietal endoderm-like cells via treatment with retinoic acid and cAMP (Strickland *et al.*, 1980). This system is thought to represent a tissue culture based model used widely for studying differentiation. Analysis of the DP-3 transcripts in F9EC cells and F9 parietal endoderm-like cells (F9PE) at 3, 5 and 7 days after addition of retinoic acid and cAMP, revealed little change in DP-3 transcript abundance (Figure 4.4a). A decrease occurred at day five however, since this was reflected in the GAPDH transcript (Figure 4.4b, lane 3) it was likely to result from experimental variation. Consequently, it is probable that DP-3 transcript abundance does not change significantly in F9EC cells stimulated to differentiate to parietal endoderm-like cells.

4.2.5 DP-3 transcripts contain the E region

The earlier analysis of DP-3 (Figure 4.1) indicated that there was some variation in the expression pattern of DP-3 transcripts in different tissues. Since analysis of DP-3 cDNA suggested several protein isoforms (Ormondroyd *et al.*, 1995), fluctuation in the level of a particular transcript might correlate with a change in DP-3 protein isoform. In an effort to relate transcript to cloned cDNAs, the F9EC blot (Figure 4.4) was stripped and re-probed with a probe specific for the E region, a 48bp sequence observed in a subset of DP-3 cDNAs (Ormondroyd *et al.*, 1995; Chapter 1). A probe for the E region suggested that all the transcripts contained the E region (Figure 4.5a). Since it was known that the DP-3 transcripts possess the properties of mRNA, it was likely that these transcripts represented mature RNA and not unspliced RNA. As suggested earlier (Section 4.2.3), the 3Kb transcript may be composed of a heterogeneous set of transcripts of very similar mobility. Indeed, a comparison of the hybridisation pattern with the E region and the DP-3 probes revealed a slight difference in size (Figure 4.4 and 4.5) and combined with the

polyadenylation analysis of DP-3 transcripts (Figure 4.3) suggests that not all the DP-3 3Kb transcripts contain the E region.

The 8Kb and 2Kb DP-3 transcripts also contain the E region, and the hybridisation pattern observed was similar for both the E region and DP-3 probes (Figure 4.4 and 4.5). Hence, some of the DP-3 transcripts contain the E region and the 3Kb DP-3 transcript may be a heterogeneous mixture of E+ and E- transcripts. Further analysis of the variation between transcripts has not been successful.

4.3 Discussion

4.3.1 DP-3 expression differs in different mouse tissues

The results indicate that the expression of DP-3 transcripts is different in different tissues since fluctuations were observed both in the overall levels and the abundance of the individual transcripts (Figure 4.1). It is likely that, as a result of these differences, the levels of the DP-3 protein isoforms will vary correspondingly, suggesting perhaps that they have specific roles in different tissues. In contrast to DP-3, the expression of DP-1 RNA was not found to be variable, as DP-1 was observed in all mouse tissues tested. Possibly, DP-1 and DP-3 have different roles in specific tissues and it is conceivable that they influence the function of E2F in distinct ways and in turn, perhaps cause subtle differences in E2F activity.

Although at present little is known about the expression and functions of individual E2F family members in different tissues, mice have been generated with homozygous deletions of the E2F-1 and 5 genes (Field *et al.*, 1996; Yamasaki *et al.*, 1996; Lindeman *et al.*, 1998). These mice have exhibited different tissue specific effects and suggested that some tissues may have specific E2F requirements; for example E2F-5 null mice displayed defects in the choroid plexus, implying an important role for E2F-5 in this tissue during development (Lindeman *et al.*, 1998). Analysis of E2F-5 RNA expression by *in situ* hybridisation revealed high levels in the choroid plexus, in addition, E2F-5 RNA levels were also high in other tissues, such as the bowel and skin, however these tissues were normal in E2F-5 null mice (Lindeman *et al.*, 1998). Such data indicates that a high level of RNA in a particular tissue does not necessarily correlate with an essential role in that tissue. Additionally, although a high degree of similarity exists between family members,

gene knockout data suggests that they do not necessarily have degenerate functions. Therefore, DP-1 and DP-3 may have distinct roles in specific tissues.

The human DP-3 homologue has been cloned and named hDP-2 (Wu *et al.*, 1995; Zhang and Chellapan, 1995; Rogers *et al.*, 1996). Work published by several groups reaches similar conclusions. For example, an analysis of human tissues revealed five hDP-2 transcripts, ranging from 9.5Kb to 1.35Kb (Zhang and Chellapan, 1995), some of similar size to DP-3 transcripts presented here. Likewise, the variation in abundance of hDP-2 and DP-3 transcripts was similar in some tissues, for example, both human and murine brain RNA exhibited a similar pattern of DP-3 transcripts (Zhang and Chellapan, 1995). DP-1 expression was detected in all human tissues tested, with some variation in abundance (Wu *et al.*, 1995; Zhang and Chellapan, 1995). Interestingly, the levels of both hDP-1 and hDP-2 transcripts were low in some tissues, such as pancreas and lung (Wu *et al.*, 1995), perhaps reflecting differing roles for hDP-1, hDP-2 and the E2F activity in these tissues. It is interesting to note that analysis of the results suggests that the lower level of expression of one DP cannot be compensated by increased levels of the other DP transcript, implying the DPs may have functionally distinct roles.

Since DP-3 transcripts are expressed differently in tissues, different DP-3 protein isoforms may correspondingly be expressed to different levels. In addition DP-1 is uniformly expressed in all the tissues, whereas DP-3 is not, and it is therefore possible that DP-3 has a unique function and this may have implications for the role of the E2F heterodimer in tissues.

4.3.2 DP-3 transcripts are expressed in a range of tissue culture cell lines

Analysis of DP-3 transcripts in tissue culture cell lines revealed that DP-3 transcripts are expressed in all the lines tested. The mouse cell lines, F9EC and NIH 3T3, both expressed similar levels of DP-3 RNAs. The human cell lines expressed lower levels of the human DP-3 transcripts (hDP-2) and in addition the human transcripts were of different mobility. Comparison with the published analyses of hDP-2 RNA transcripts revealed similarities; hDP-2 is expressed in a range of human cell lines and some variation in abundance was observed between cell lines and particular transcripts. Unlike analysis of hDP-2 in tissues, three hDP-2 transcripts were observed in human cell lines (Wu *et al.*, 1995). The level of hDP-2

RNA in the ML1 cell line was extremely high (Wu *et al.*, 1995), and subsequent studies have shown ML-1 cells to express hDP-2 protein to a detectable level (Rogers *et al.*, 1996). DP-1 was found to be expressed in all the cell lines tested (Wu *et al.*, 1995; Zhang and Chellapan, 1995) whereas hDP-2 expression was variable. As discussed (Section 4.3.1), this may reflect different roles for DP-1 and DP-3 in E2F cellular activity.

4.3.3 DP-3 transcripts are cytoplasmic, poly A+ messenger RNAs

Analysis of DP-3 transcripts revealed that they are cytoplasmic RNAs and the majority of species are polyadenylated. DP-3 transcripts therefore possess the properties expected for mRNAs, with the potential to be translated into protein. Selection of polyadenylated RNAs suggested that a subset of the 3Kb message may not be polyadenylated or has a shorter poly A tail, which may have an influence on DP-3 expression. Since polyadenylation can influence nucleocytoplasmic transport, mRNA stability, decay and translation (Manley and Proudfoot, 1994; Huang and Carmichael, 1996; Wickens *et al.*, 1997), it is possible that regulated polyadenylation may influence DP-3 protein expression.

4.3.4 Multiple DP-3 transcripts are observed by northern analysis

Analysis of hDP-2 in tissues revealed five transcripts of different mobility (Zhang and Chellapan, 1995). In contrast, northern blot analysis with a DP-3 specific probe revealed only three transcripts. However, the analysis of DP-3 transcripts suggested that there might indeed be more than three DP-3 transcripts. The selection of polyadenylated RNAs revealed that a subset of the 3Kb transcript contained either a short polyadenylated tail or lacked polyadenylation (Figure 4.3). In addition, the identification of E+ transcripts (Figure 4.5) suggested that a subset of the 3Kb transcripts were E+. Detection of all transcripts with the E region probe would not be expected, as some DP-3 transcripts should be E- since E- cDNAs have been cloned (Ormondroyd *et al.*, 1995). Consequently, it is possible that there are more than three DP-3 transcripts, which represent different splice forms of DP-3 that have yet to be resolved and are similar to the five transcripts observed in human tissue analysis (Zhang and Chellapan, 1995).

4.3.5 RNA analysis suggests extensive processing of DP-3 RNA

The DP-3 cDNA analysis revealed a complex regulation at the RNA level, as at least four different 5'UTRs and two 3'UTRs, with variations in the open reading frame, have been described. A complicated pattern of RNA would have therefore been predicted by northern analysis.

Overall, the results have indicated that the DP-3 gene gives rise to a variety of transcripts that differ dramatically in size. This information, combined with the fact that DP-3 RNA is subject to alternative splicing both in the coding sequence and the 5'UTR, suggests that DP-3 RNA is subject to complex regulation.

Figure 4.1

Northern blot analysis of DP-3 and DP-1 transcripts in mouse tissues.

a) A northern blot containing 2μg polyA+ RNA per lane prepared from the indicated mouse tissues was probed with the DP-3 3'end probe (Chapter 2). The RNA markers are shown.

b) The northern blot from (a) was stripped and reprobed with the DP-1 3'end probe (Chapter 2).

c) The northern blot from (a) was stripped and reprobed with a β-actin control probe.

Figure 4.1

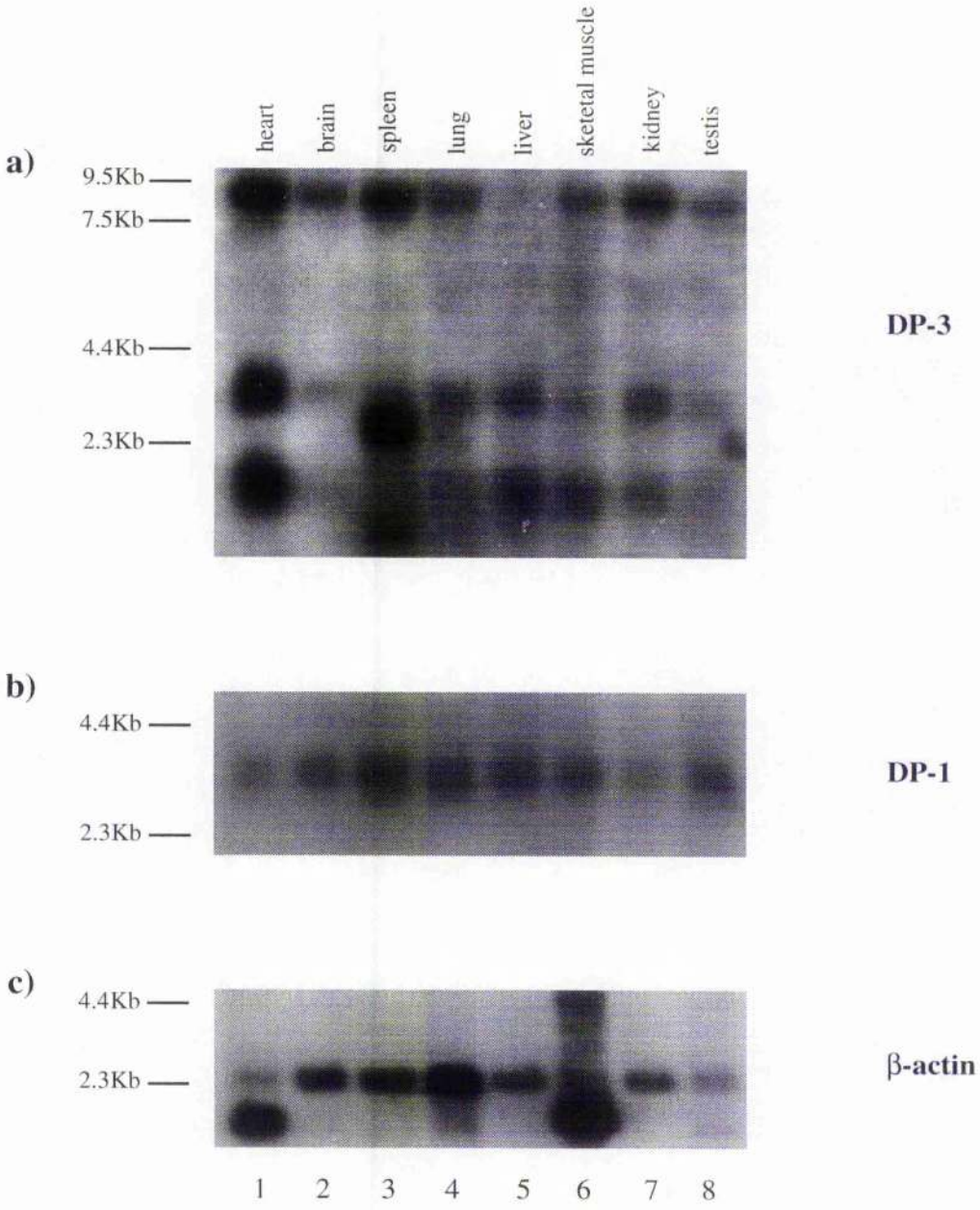

a)
heart
brain
spleen
lung
liver
sketetal muscle
kidney
testis
9.5Kb
7.5Kb
4.4Kb
2.3Kb
DP-3
b)
4.4Kb
2.3Kb
DP-1
c)
4.4Kb
2.3Kb
β-actin
1 2 3 4 5 6 7 8

Figure 4.2

Northern blot analysis of DP-3 in a range of cell lines

a) Analysis of a northern blot prepared from 25μg total cellular RNA from the indicated tissue culture cell lines, probed with the DP-3 3' end probe. The positions of the RNA markers are shown.

b) The northern blot from (a) was stripped and reprobed with a GAPDH control probe.

Figure 4.2

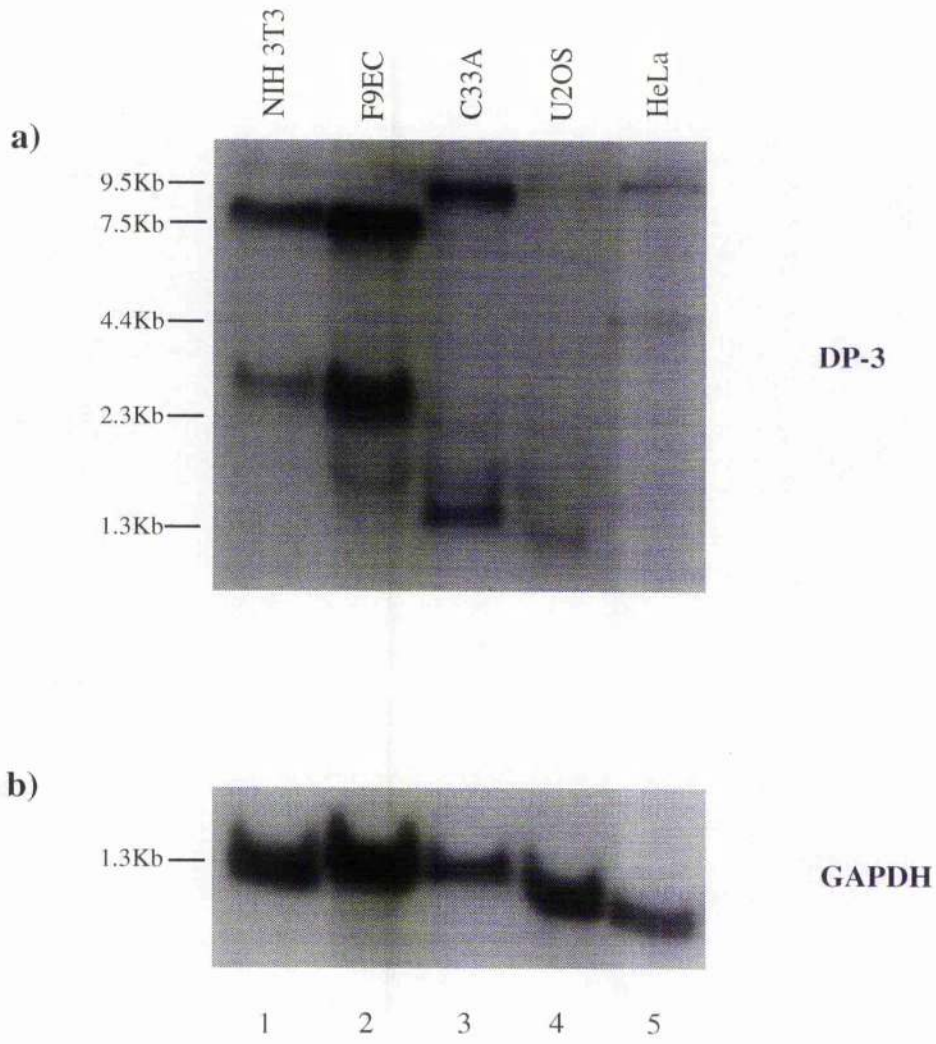

a)
NIH 3T3
F9EC
C33A
U2OS
HeLa
9.5Kb
7.5Kb
4.4Kb
2.3Kb
1.3Kb
DP-3
b)
1.3Kb
GAPDH
1
2
3
4
5

Figure 4.3

Analysis of DP-3 transcripts

a) A northern blot was prepared with 25μg NIH 3T3 cell total RNA and polyadenylated and non-polyadenylated RNA fractions were isolated from NIH 3T3 cell total RNA. Nuclear and cytosolic RNAs were extracted from NIH 3T3 cells. The total amount of polyadenylated and non-polyadenylated RNAs and nuclear and cytosolic RNAs loaded was 25μg. The northern blot was hybridised with the DP-3 3'end probe (Chapter 2). The RNA size markers are indicated.

b) The northern blot from (a) was stripped and reprobed with a GAPDH probe.

c) The northern blot from (a) was stripped and reprobed with a histone 3 probe.

d) The northern blot from (a) was stripped and reprobed with a U6 probe.

Figure 4.3

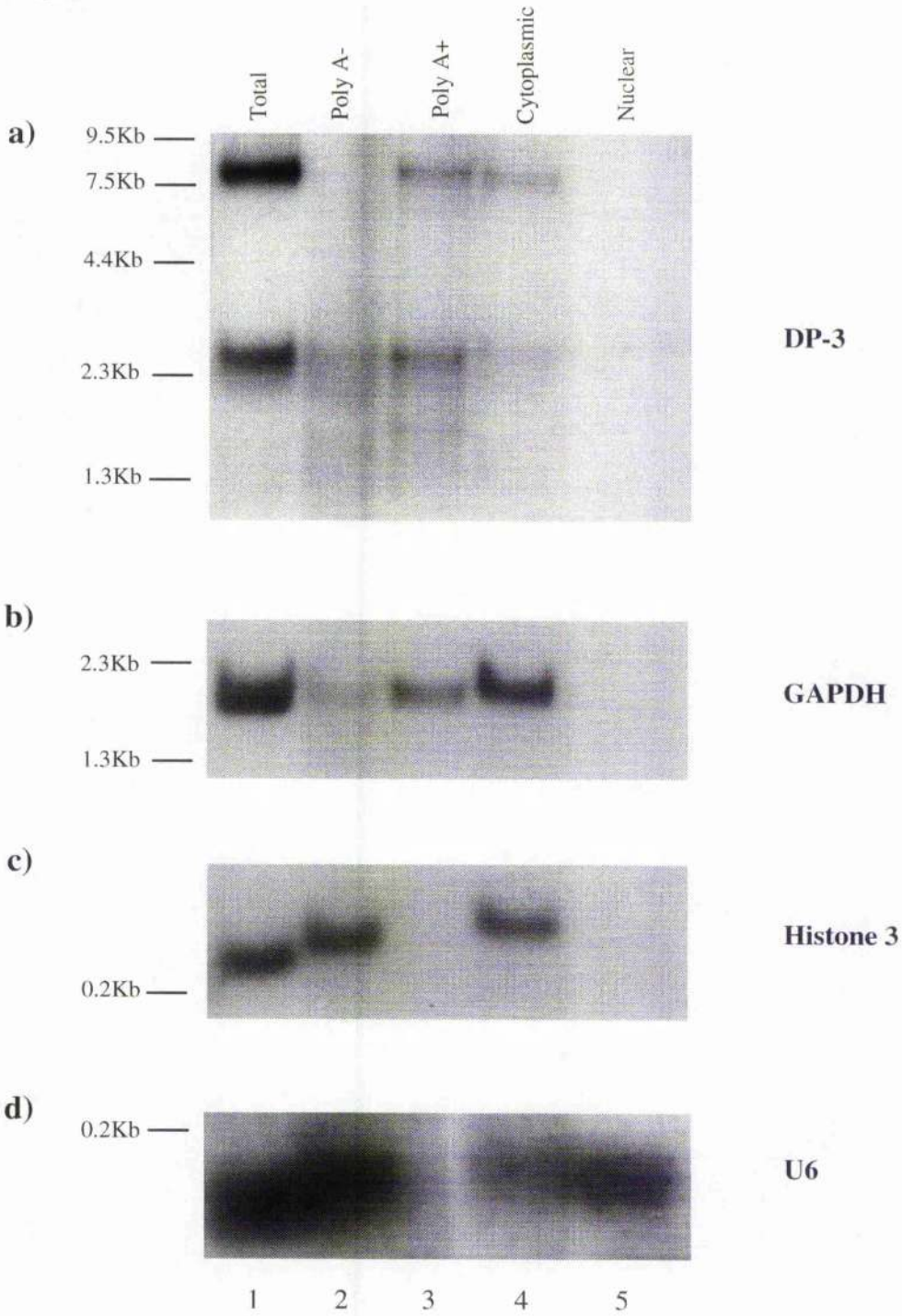

Total
Poly A-
Poly A+
Cytoplasmic
Nuclear
a)
9.5Kb
7.5Kb
4.4Kb
2.3Kb
1.3Kb
DP-3
b)
2.3Kb
1.3Kb
GAPDH
c)
0.2Kb
Histone 3
d)
0.2Kb
U6
1
2
3
4
5

Figure 4.4

Analysis of DP-3 transcripts during F9EC cell differentiation

a) A northern blot was prepared from 25μg of total RNA purified from F9EC cells and F9PE cells at days 3, 5 and 7 of differentiation. The blot was hybridised with the DP-3 3'end probe (Chapter 2). The positions of the RNA size markers are shown.

b) The northern blot from (a) was stripped and reprobed with a GAPDH probe and washed at high stringency.

Figure 4.4

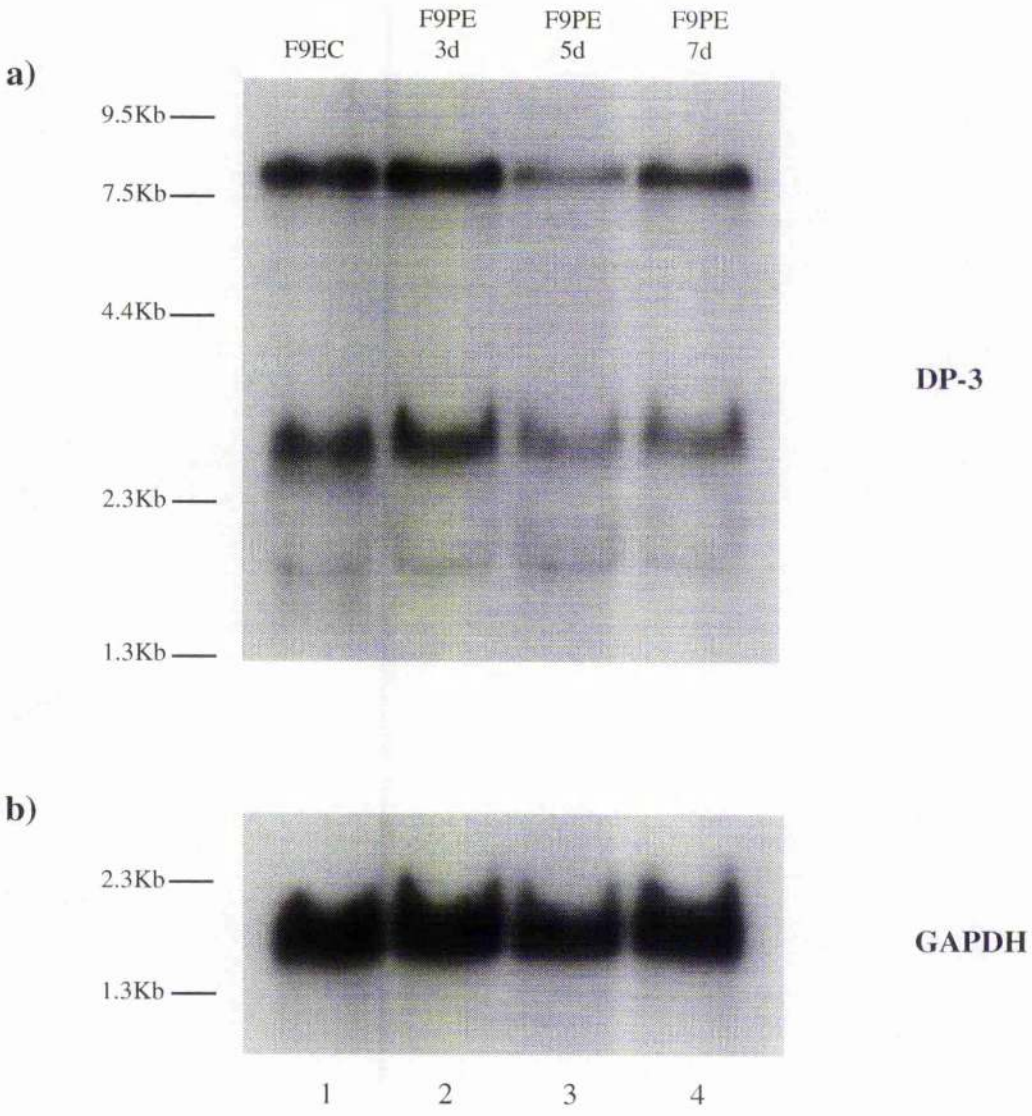

a)
F9EC
F9PE 3d
F9PE 5d
F9PE 7d
9.5Kb
7.5Kb
4.4Kb
2.3Kb
1.3Kb
DP-3
b)
2.3Kb
1.3Kb
GAPDH
1
2
3
4

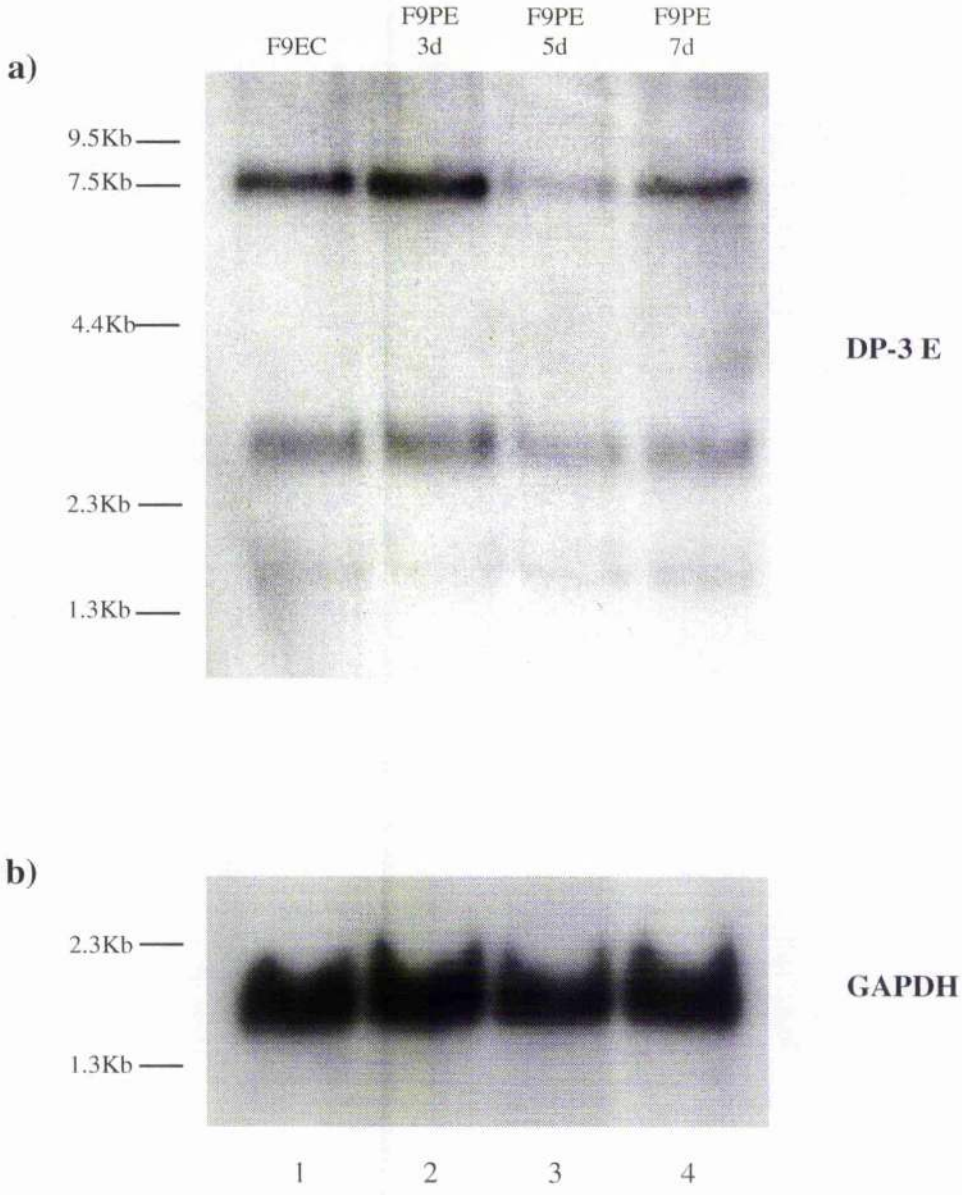
Figure 4.5

Analysis of E+ DP-3 transcripts

a) The northern blot of F9EC and F9PE cells from Figure 4.4 was stripped and hybridised with a probe specific for the E region (Chapter 2). The size markers are shown.

b) The hybridisation of the GAPDH probe, from figure 4.4b.

Figure 4. 5


a)
F9EC
F9PE 3d
F9PE 5d
F9PE 7d
9.5Kb
7.5Kb
4.4Kb
2.3Kb
1.3Kb
DP-3 E
b)
2.3Kb
1.3Kb
GAPDH
1
2
3
4

Chapter 5

The regulation of DP-3 translation by the 5' untranslated regions

5.1 Introduction

The regulation of gene expression in eukaryotic cells occurs at many levels including transcription, mRNA processing, nuclear mRNA export, mRNA stability, translation and post-translational modifications of proteins. Several studies have shown that genes involved in cell cycle control are regulated via a translation mechanism. An example is p27, a cyclin-dependant kinase inhibitor; accumulation of this protein is regulated during the cell cycle at both at the level of translation and protein turnover (Hengst and Reed, 1996). The translation of specific mRNAs may be influenced by structural features of the mRNAs, for example, the translational regulation of cyclin-dependant kinase 4 (CDK4) by TGF-β1 is p53-dependant and is mediated by the 5'UTR of the CDK4 mRNA (Ewen *et al.*, 1995). In addition, murine p53 binds to the 5'UTR of its own mRNA and negatively regulates p53 translation (Mosner *et al.*, 1995).

The isolation and characterisation of DP-3 cDNAs revealed a complex 5'UTR structure. DP-3 5'UTRs are both tissue restricted and associated with particular DP-3 protein isoforms (Ormondroyd *et al.*, 1995). Since DP-3 transcripts are expressed in a range of tissues and cell lines and possess the properties of mRNAs with the potential to be translated (Chapter 4), it is possible that the 5'UTR of a transcript influences the translation of DP-3 isoforms. The aim of this study was to investigate the influence of the different DP-3 5'UTRs on translation of DP-3 transcripts.

5.2 Results

5.2.1 Analysis of DP-3 translation by the different 5'UTRs

A set of mammalian expression vectors were constructed with the luciferase reporter gene fused to the four different 5'UTRs of DP-3, (types I-IV) (Figure 5.1). Since three of the DP-3 protein isoforms are translated from the second methionine (M2, exon/segment 5), the DP-3 5'UTRs were fused at M2, in which case initiation of translation at M2 would result in active luciferase protein (Chapter 2). Initiation of translation at the first methionine (M1) of a type II 5'UTR would result in

luciferase fused to the N-terminus of DP-3α, which might influence the conformation of the luciferase protein and it was conceivable that the enzymatic activity of a fused luciferase protein might be impaired.

The measurement of luciferase activity in cells transfected with the chimeric reporter constructs, combined with analysis of luciferase RNA, was extracted to provide an indication of the level of translation from each luciferase transcript. The backbone vector (pGL2Control), with a short 5'UTR of vector sequence, was included as a control for luciferase expression. A set of vectors under the control of the bacteriophage T3 promoter were also constructed for studying the translation of the DP-35'UTR-luciferase fusions *in vitro* (Chapter 2).

The 5'UTR-luciferase expression vectors were transiently transfected into several cell lines of different origins and the luciferase levels assayed. In each transfection a second reporter vector, pCMVβ-galactosidase (Tassios and La Thangue, 1991) was included to enable determination of transfection efficiency and identify any non-specific effects on gene expression. Furthermore, RNA was extracted from the transfected cells and the levels of luciferase and β-galactosidase mRNA determined by dot blot analysis. Hence, the translational potential of each chimeric transcript was assessed by comparison of the luciferase activity and the level of luciferase transcript.

5.2.2 The DP-3 5'UTRs are equally translated *in vitro*

A set of vectors under the control of the bacteriaophage T3 promoter, for studying the translation of the DP-35'UTR-luciferase transcripts *in vitro* were constructed from the mammalian expression vectors (Chapter 2). The *in vitro* translation of these vectors in a coupled transcription/translation system in a rabbit reticulocyte lysate resulted in an equal level of translation in from all the DP-3 5'UTRs (Figure 5.2). Furthermore, all the *in vitro* translated luciferase proteins were enzymatically active (data not shown). In addition, translation was initiated at both M1 and M2 of a type II 5'UTR and no apparent difference in the abundance of the luciferase protein initiated from either methionine was observed. Hence, the DP-3 5'UTRs are translated equally *in vitro* and are all of similar enzymatic activity.

5.2.3 The luciferase activity reflects the quantity of luciferase protein

It was assumed that the luciferase activity measured in cell extracts accurately reflects the amount of luciferase protein translated. Since the expression vectors had been constructed using PCR (Chapter 2) it was possible that mutations may have been introduced which could affect the enzymatic activity of the reporter gene. To ensure that the observed enzymatic activity is a true reflection of the levels of luciferase and β-galactosidase protein, extracts from transfected NIH 3T3 cells (Figure 5.6) were analysed by western blot (Figure 5.3). The levels of luciferase protein detected by an anti-luciferase antibody (Chapter 2) closely reflected changes in luciferase activity (Figure 5.3; Figure 5.6).

Luciferase protein levels were very high in the type I and IV 5'UTRs transfected cells and the level of luciferase was lower in cells transfected with pGL2Control (Figure 5.6). Luciferase protein was not detected in type III transfected cell extracts, and the level of luciferase activity was somewhat lower than that of the control, suggesting that the level of protein is too low to be detected by the antibody. Interestingly, luciferase protein was detected in type II transfected extracts, despite a very low level of luciferase activity. The luciferase protein appears to be of a slightly slower mobility than the luciferase protein in the in other lanes (Figure 5.3a, lane 4 *). Translation initiation was possible at either M1 or M2 of DP-3 type II 5'UTR RNA, where initiation at M1 would result in expression of a DP-3 N terminus-luciferase fusion protein, which would be larger in size than luciferase. It was possible that the protein detected is a DP-3 N-terminal-luciferase fusion protein. The levels of β-galactosidase protein also closely reflect the β-galactosidase activity (Figure 5.3c) and are similar in each lane suggesting that the efficiency of transfection is comparable.

5.2.4 DP-3 5'UTRs have translational activities

In most cell lines tested, the type I 5'UTR RNA had the highest translational activity, for example, in C33A cells (Figure 5.4) the type I 5'UTR was approximately 70% higher than the activity derived for the control. In the other cell lines tested, COS-7 cells and NIH 3T3 cells (Figures 5.5 and 5.6) the type I 5'UTR-luciferase exhibited greater than 90% higher activity than the pGL2control. In F9EC

cells (Figure 5.7) the type I 5'UTR was over 200% translationally more active than the control.

The type II and III 5'UTRs RNAs were generally expressed at a much lower level than the control. In most cell lines type II 5'UTR translation was reduced, in comparison with the control expression revealed up to 99% reduction in luciferase activity (C33A cells, COS-7 cells, NIH 3T3 cells and F9EC cells, Figures 5.4, 5.5, 5,6 and 5.7). More luciferase was expressed from type III 5'UTR RNA than type II 5'UTR. However, in most experiments expression of type III 5'UTR RNA resulted in between 65% (F9EC cells, Figure 5.7) and 90% decrease in luciferase expression when compared to the control activity (C33A cells, COS-7 cells NIH3T3 cells, Figures 5.4, 5.5 and 5.6).

The translation of the type IV 5'UTR RNA was the most variable of all the 5'UTRs in the cell lines tested. Generally type IV was expressed approximately 30% higher than the control (COS-7 cells and NIH3T3 cells, Figures 5.5 and 5.6), however in F9EC cells (Figure 5.7) the type IV 5'UTR was over 50% higher than the control. In C33A cells (Figure 5.4) a type IV 5'UTR was translated 20% less than the control.

In the cell lines tested, the four DP-3 5'UTRs examined were all translated differently. The type I 5'UTR-luciferase was expressed to a higher level than the control, pGL2control. The type IV 5'UTR was usually translated to a similar level as the control. The type III 5'5'UTR was translated to a much lower level than the control, and the type II 5'UTR was only marginally active. In each cell line the analysis of luciferase and β-galactosidase mRNA revealed little variation, suggesting the differences observed in protein level, were not due to differences in transcription or mRNA stability in each transfection. Hence, the expression of DP-3 directed by the 5'UTRs may be cell type dependent. As observed with the other cell lines the levels of luciferase and β-galactosidase mRNA remained constant in each transfection.

5.2.5 Translation of DP-3 type I and IV 5'UTRs was compromised in U2OS cells

The translation of the different DP-3 5'UTR fused to luciferase was different in U2OS cells, an osteosarcoma cell line in which the control exhibited the highest

level of translation (Figure 5.8). The translational activity of type I and IV 5'UTRs was lower than the control and therefore reduced in these cells (Figure 5.8). Similar activity was noted for type II and type III as was observed in the other cell lines Hence, the expression of DP-3 directed by the 5'UTRs may be cell type dependent. However, analysis of mRNA was not successful in this cell line, therefore it is conceivable that the differences observed are attributable to non-translation mechanisms.

5.3 Discussion

5.3.1 DP-3 5' untranslated regions influence protein expression by a post-transcriptional mechanism

The influence of the 5'untranslated regions of DP-3 on translation has been assessed both *in vivo* and *in vitro*. *In vivo*, in transfected mammalian cells, the expression of luciferase protein is dependent on the DP-3 5'UTR to which it was fused. Comparison of RNA levels suggests that there were few differences between 5'UTR transcript levels; hence the differences observed in enzyme activity are not due to variation in transcription and mRNA stability of the different luciferase transcripts. Therefore, the DP-3 5'UTRs are regulated by a post-transcriptional mechanism, probably at the level of translation.

The DP-3 type I 5'UTR was translated most efficiently in the four cell lines, suggesting that the most abundant DP-3 isoforms in cultured cells may be translated from DP-3 transcripts with a type I 5'UTR. A type IV 5'UTR expression was variable, however, expression was generally lower than type I 5'UTR and at approximately the same level as the control. On occasion type IV 5'UTR was expressed at a very high level, higher than type I and it is possible that the translation of this 5'UTR is dependant on the cell state, for example the stage in the cell cycle. Type IV 5'UTR may therefore be regulated under specific cellular conditions. The type II and III 5'UTRs were translated much less efficiently than either the control or types I and IV and therefore lower levels of DP-3 isoforms expression would be predicted from transcripts that possess types II and III 5'UTRs.

5.3.2 DP-3 expression is cell type dependent and mediated by the 5'UTRs

Transfections in U2OS cells revealed a strikingly different pattern of translation. The translation of types II and III were similar to that observed in other cell lines with respect to the control activity. In contrast, translation of types I and IV was lower than control in this cell type. It is therefore conceivable that specific cell or tissue types may express different levels of DP-3 via a specific translational control mechanism, mediated by the 5' untranslated regions.

It is however possible that the observed expression pattern is not due to differences in translational ability but because of regulation at another level. Gene expression can be regulated at several instances both at the level of transcription and via post-transcriptional mechanisms. Analysis of RNA levels suggested that similar amounts of RNA was expressed in each experiment. Therefore, it was unlikely that differences in RNA stability resulted in the observed differences in protein level. Additionally, differences in polyadenylation could affect translation (Pain, 1996; Sachs *et al.*, 1997) and regulation at the level of RNA nuclear transport could also influence translation. The RNA analysis undertaken in this study would not detect these subtle changes in RNA. However, the pGL2control contains a polyadenylation site and since the vector has not been manipulated in this region it is unlikely that the regulatory sequences in the 3' region have been impaired.

5.3.3 M1 of DP-3 type II 5'UTR may be used preferentially *in vivo*

The different DP-3 5'UTRs can direct the initiation of translation at either M1 or M2. Initiation at M1 results in an extended N-terminus, homologous to the N-terminus of DP-1. Initiation of translation at M1 is possible in type I, II and IV 5'UTRs, however in types I and IV, in frame stop codons terminate translation before M2 and subsequently translation can re-initiate at M2. Translation at M1 from a type II 5'UTR-luciferase transcript will result in a luciferase protein fused to the DP-3 N- terminus, which may affect the protein conformation and influence enzymatic activity. Type II 5'UTR had the lowest translational activity and it is feasible that a luciferase fusion protein would not be enzymatically active. Therefore, it is possible that M1, rather than M2 is used *in vivo*, since initiation at M2 would result in active luciferase protein. Hence M1 might be preferentially used *in vivo*. However, the absence of detectable M2 translated luciferase protein does not

indicate that translation from M2 does not occur *in vivo*. Analysis of protein on western blots from transfected cells revealed the presence of a luciferase polypeptide that appeared slightly larger than the other luciferase proteins, suggesting an M1-luciferase protein, however the identity of this polypeptide must be further confirmed before any firm conclusions can be made. Therefore, further investigation into the translational activity of type II 5'UTR transcripts is required.

5.3.4 All DP-3 5'UTRs are translated equally *in vitro*

Analysis of the 5'UTRs *in vitro* revealed that all the 5'UTRs are translated equally, and that translation is initiated at both M1 and M2 in the type II end, in an equivalent manner. Therefore, the difference in expression observed *in vivo* and *in vitro* suggests that cellular factors not present in rabbit reticulocyte lysates are required for the regulated expression observed *in vivo*.

5.3.5 DP-3 translation could be regulated via different mechanisms

There are multiple mechanisms for the regulated translation of mRNA in eukaryotes (Kozak, 1992; Pain, 1996). Structural features of an mRNA and the *trans*-acting factors that interact with mRNA influence translation. The context of the AUG initiation signal can also influence translation (Kozak, 1989) and other sequence elements such as the presence of upstream AUG codons (uAUG) and ORFs (uORFs) can also affect the translational efficiency of an mRNA. For example, uORFs in the 5'UTR of the yeast GCN4 gene suppress the translation of GCN4 when nutrients are plentiful and only during amino acid starvation is the translation of GCN4 de-repressed (Hinnebusch, 1996).

Secondary structure within the 5'UTR can also influence translational activity (Kozak, 1989) and additionally, RNA/protein interactions can regulate the translation of a few specific mRNAs, for example the synthesis of ferritin in mammalian cells is regulated by the secondary structure of the iron response element (IRE) within the 5'UTR of ferritin mRNA. The iron regulatory factor (IRF) binds to the IRE and represses translation (Melefors and Hentze, 1993).

DP-3 5'UTRs could be regulated by any of these mechanisms. DP-3 5'UTRs contain uORFs and uAUGs that may influence translation. Type I, II and IV transcripts all contain M1 (Figure 5.1) and initiation at M1 from type I and IV

transcripts results in translation of short uORFs that terminate before M2. Reinitiation at M2 may then occur resulting in translation of DP-3 M2 products. The distance between the stop codon of the uORF and M2 is different in type I and type IV and this intercistronic distance may influence reinitiation at M2 and could account for the differences observed in translation from transcripts with these 5'UTRs. Therefore, the splicing arrangement of DP-3 5' exons may determine the probability of reinitiation at M2.

The DP-3 type II 5'UTR contains two in frame initiation signals M1 and M2. A type II 5'UTR is only associated with an E+ Q- ORF, therefore translation at M1 or M2 would result in DP-3α or δ respectively. The differential use of in-frame initiation codons may have implications on DP-3 expression. The mRNA of LAP protein, a liver-enriched transcription factor, contains two uAUG in-frame codons in the 5'UTR of the *lap* mRNA and results in synthesis of either LAP protein or an inhibitor of LAP called LIP (Descombes and Schibler, 1991). A change in the use of AUG codons results in a change in the ratio of LAP to LIP altering the transcriptional properties of the gene. Likewise, it is possible that initiation at M1 and M2 of type II may also be regulated and have a potentially dramatic influence on E2F formation and function.

Additionally, DP-3α and DP-3δ have been shown to interact with a DP interacting protein (DIP), a POZ domain containing protein that influences the cell cycle (de la Luna *et al.*, 1999). It is possible therefore that the interaction between DP-3α and DIP could be influenced by translational initiation, selection of M2 of type II 5'UTR transcripts would result in translation of DP-3δ which only weakly interacts with DIP . A switch between translation initiation at M1 and M2 may occur under specific cellular conditions and that this could influence the interaction of DP-3 with DIP or other cellular factors.

DP-3 expression could be regulated by RNA/protein interactions. DP-3 5'UTRs are translated differently *in vivo*, however *in vitro* translation of each 5'UTR is equal: this suggests that specific cellular factors may be required for the differences in translation observed in transfected mammalian cells. It is possible that repressor proteins bind to type II and III 5'UTR and inhibit translation, and that these proteins are unable to bind to type I or IV. Conversely activators may bind to type I 5'UTR and not to the other 5'UTRs. Therefore the variation in the level of

type IV translation could be via altered affinity of a repressor or activator protein for type IV 5'UTR, perhaps in response to changes in cellular environment

5.3.6 A novel post-transcriptional mechanism may influence subunit composition of the E2F heterodimer

The translational regulation of DP-3 expression via the different 5'UTRs may have an impact upon E2F. Regulating of translation enables cells to rapidly 'turn on' protein expression. DP-3 transcripts are detected in many tissues and cell lines, it is possible that these transcripts are present at all times and then in response to changes in cellular conditions for example in response to growth signals or stress, DP-3 protein is rapidly translated. The timely expression of DP-3 protein may be important for E2F function under such specific cellular conditions. Alternatively the 5'UTRs of DP-3 may exist to maintain precise levels of each isoform. Each untranslated region is associated with different protein isoforms (Ormondroyd *et al.*, 1995) and the 5'UTRs are translated to different levels in different cell lines. It is therefore possible that they are just translated differently to ensure that cells have the correct levels of each isoform. For example type I 5'UTR is found associated with DP-3 γ isoform and a type III 5'UTR with DP-3β isoform. Since a type I 5'UTR is highly expressed in cells and a type III 5'UTR is, on comparison, expressed to a much lower level, cells might require more DP-3γ than DP-3β. Such regulation may act to maintain the correct cellular levels of DP-3 and reflect its requirement as an E2F partner and ultimately, its physiological role.

The translational regulation of DP-3 via its 5'untranslated regions represents a level of regulation unique within the E2F family of transcription factors. Distinct cellular circumstances may dictate the expression of DP-3 isoforms and influence E2F heterodimer formation and activity.

Figure 5.1

Translational analysis of DP-3 5'UTRs

Luciferase reporter constructs. Each of the DP-3 5'UTR sequences was fused to luciferase at methionine 2. Arrows represent possible sites of translation initiation. × represents sites of translational termination. * represents the site of a stop signal.

Figure 5.1

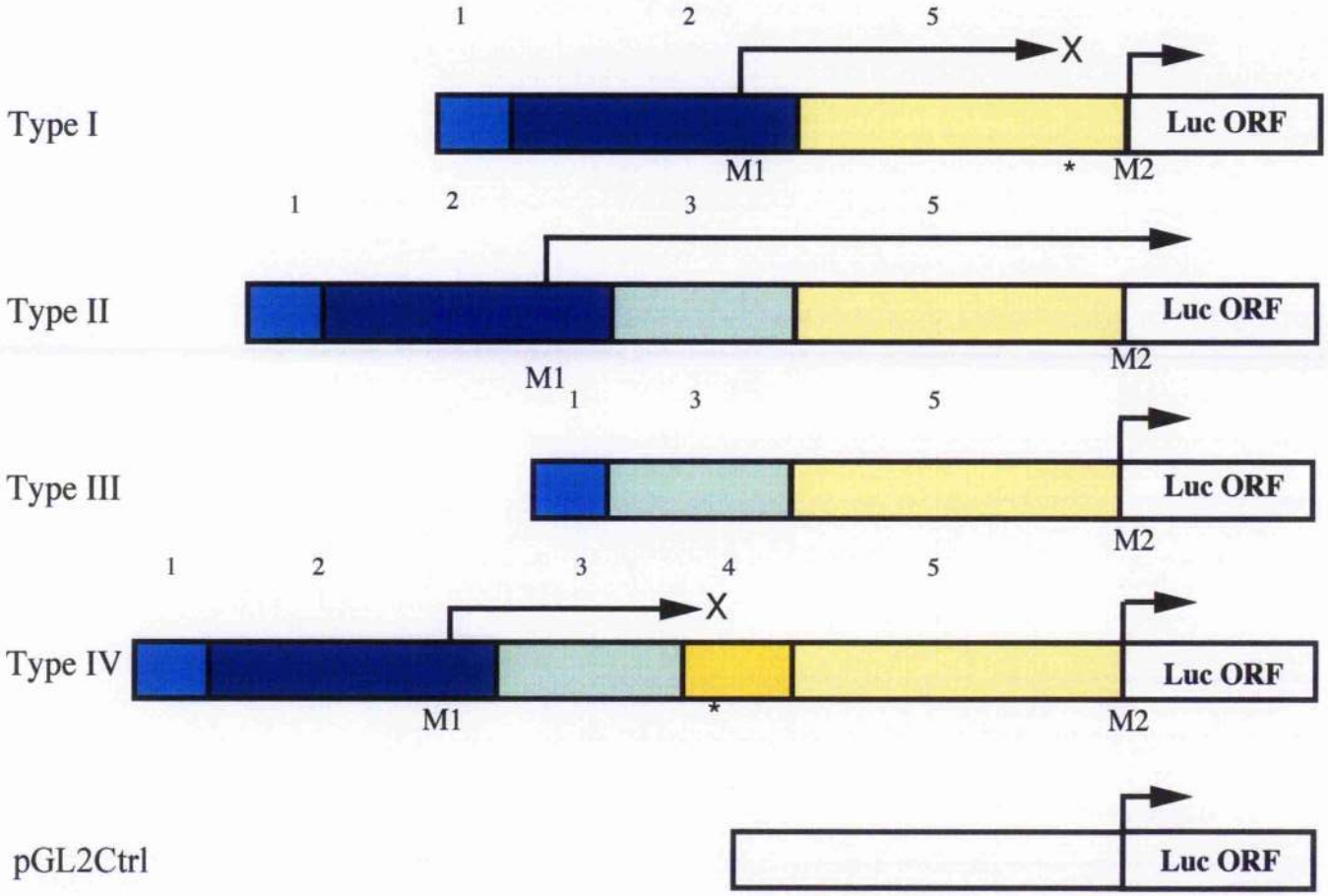

1
2
5
X
Type I
Luc ORF
M1
*
M2
1
2
3
5
Type II
Luc ORF
M2
M1
1
3
5
Type III
Luc ORF
M2
1
2
3
4
5
X
Type IV
Luc ORF
M1
*
M2
pGL2Ctrl
Luc ORF

Figure 5.2

The DP-3 5'UTRs are translated equally *in vitro*

1μg of each plasmid DNA, as indicated, was *in vitro* translated in the presence of ^{35}S-methionine. A tenth of each reaction was analysed by PAGE and autoradiography.

Figure 5.2

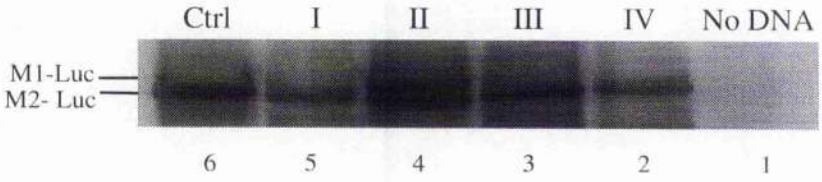


Figure 5.3

The luciferase activity reflects luciferase protein

a) Extracts from transfected NIH 3T3 cells (figure 5.6) were immunoblotted with an α luciferase antibody. The positions of M1 and M2 initiating luciferase proteins are indicated. * denotes M1-luciferase.

b) A shorter exposure of the immunoblot in a). The position of luciferase protein is given.

c) The immunoblot from a) was stripped and β-galactosidase protein detected with α β-galactosidase antisera.

Figure 5.3

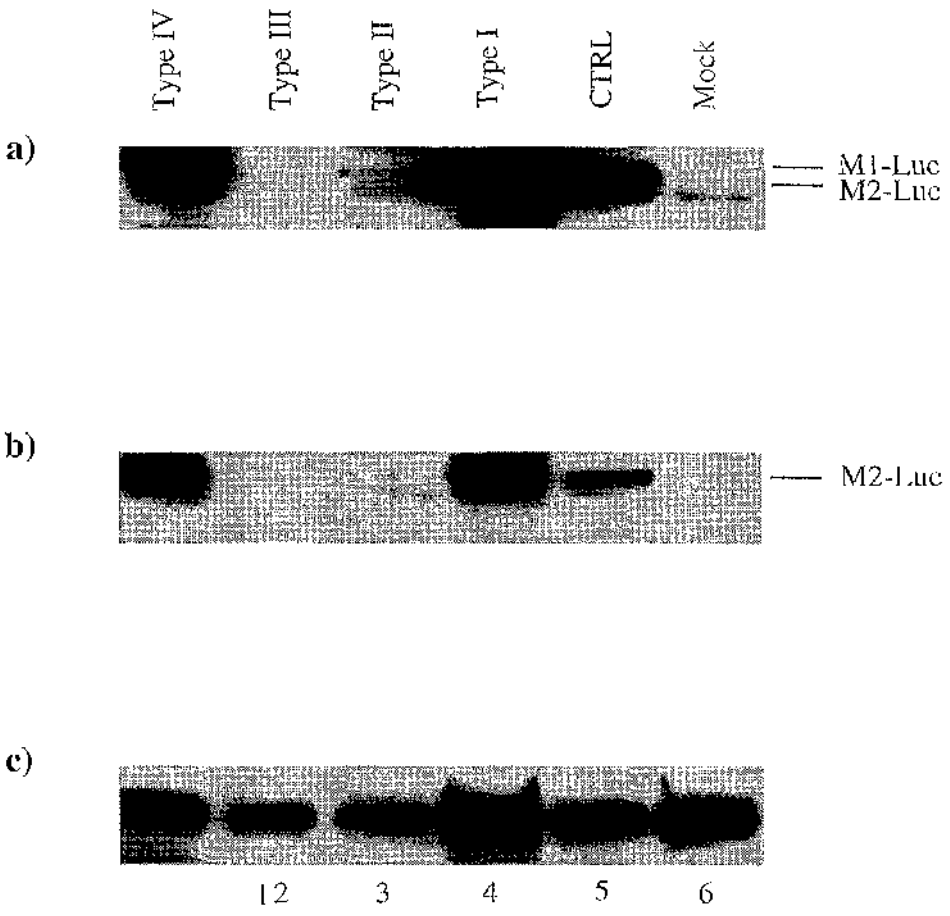

Type IV
Type III
Type II
Type I
CTRL
Mock
a)
M1-Luc
M2-Luc
b)
M2-Luc
c)
1 2
3
4
5
6

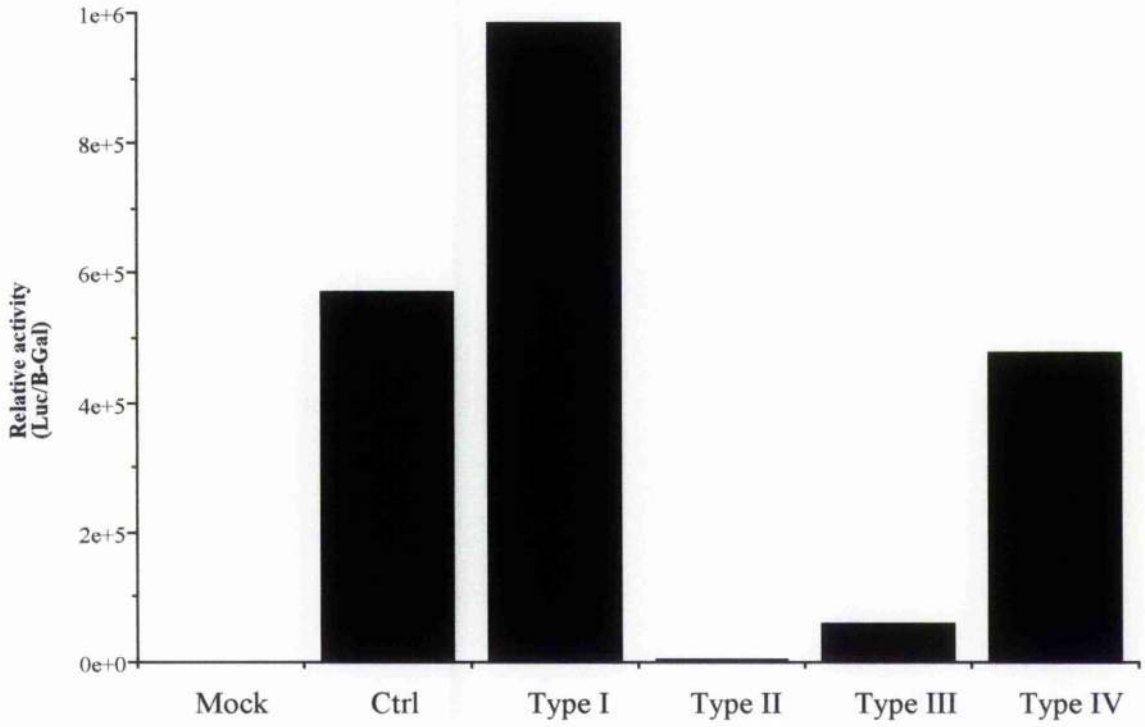
Figure 5.4

The expression of DP-3 5'UTRs in C33A Cells

a) C33A cells were transfected with 7.5μg 5'UTR-luciferase construct as indicated and 2.5μg pCMV-β-galactosidase by the calcium phosphate method. Cells were harvested at 48 hours post transfection, for both RNA and protein. The values plotted are based on the activity of luciferase relative to the activity of the control, β-galactosidase.

b) RNA was extracted from C33A cells from (a) and dot blotted. Dot blots were hybridised with a luciferase and a β-galactosidase probe.

Figure 5.4


1e+6
8e+5
6e+5
4e+5
2e+5
0e+0
Relative activity
(Luc/B-Gal)
Mock
Ctrl
Type I
Type II
Type III
Type IV

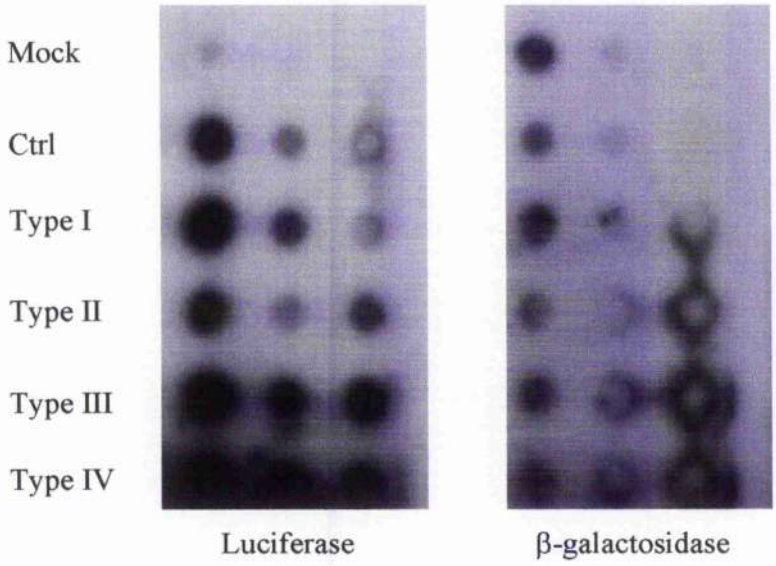

Mock
Ctrl
Type I
Type II
Type III
Type IV
Luciferase
β-galactosidase

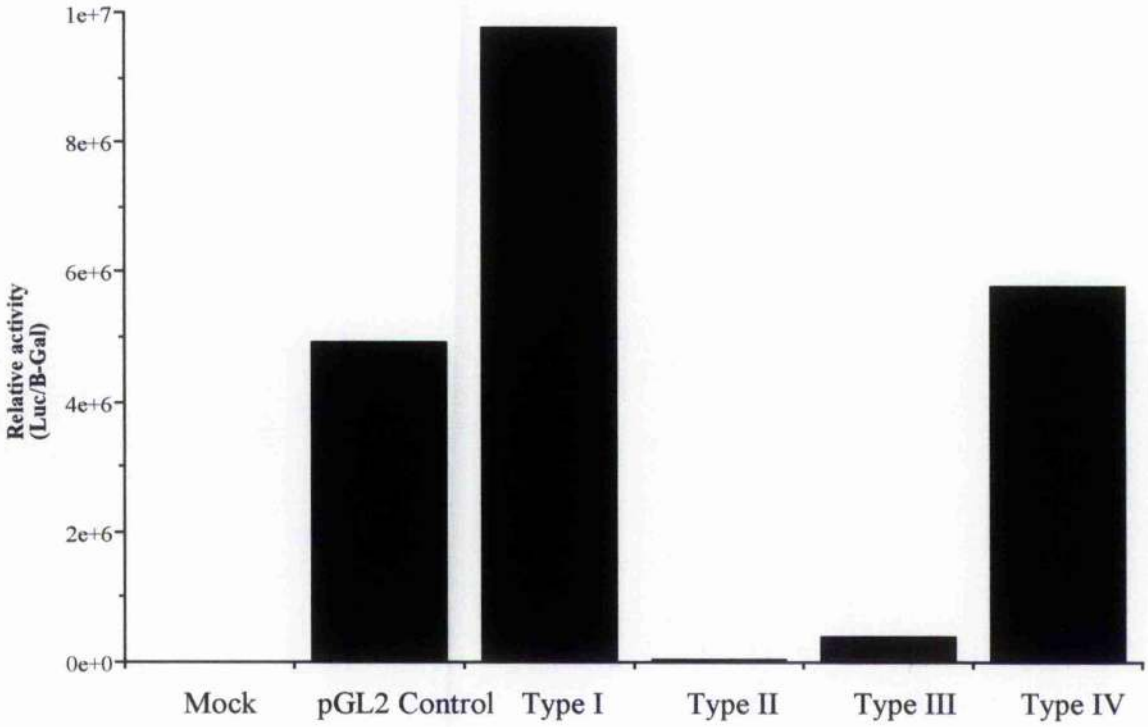
Figure 5.5

The expression of DP-3 5'UTRs in COS 7 Cells

a) COS-7 cells were transfected with 7.5μg 5'UTR-luciferase construct as indicated and 2.5μg pCMV-β-galactosidase. Cells were harvested at 48 hours post transfection, for both RNA and protein. The values plotted are based on the activity of luciferase relative to the activity of the control, β-galactosidase.

b) RNA was extracted from COS-7 cells from (a) and dot blotted. Dot blots were hybridised with luciferase and β-galactosidase probes.

Figure 5.5


1e+7
8e+6
6e+6
4e+6
2e+6
0e+0
Relative activity (Luc/B-Gal)
Mock
pGL2 Control
Type I
Type II
Type III
Type IV

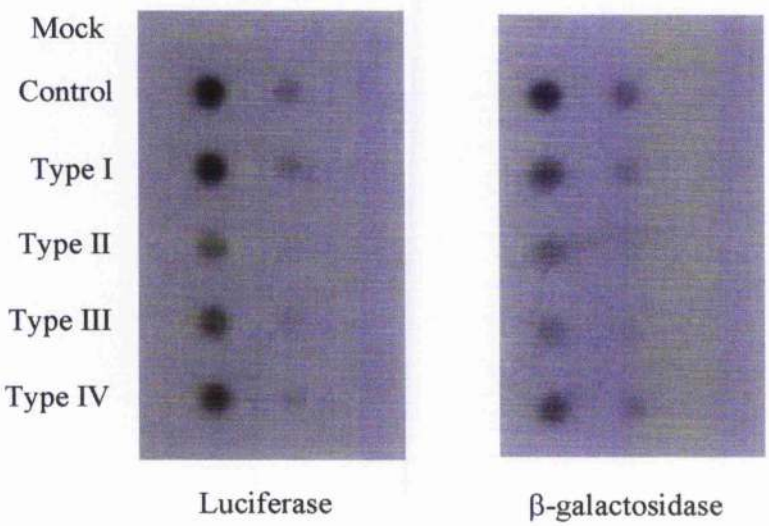

Mock
Control
Type I
Type II
Type III
Type IV
Luciferase
β-galactosidase

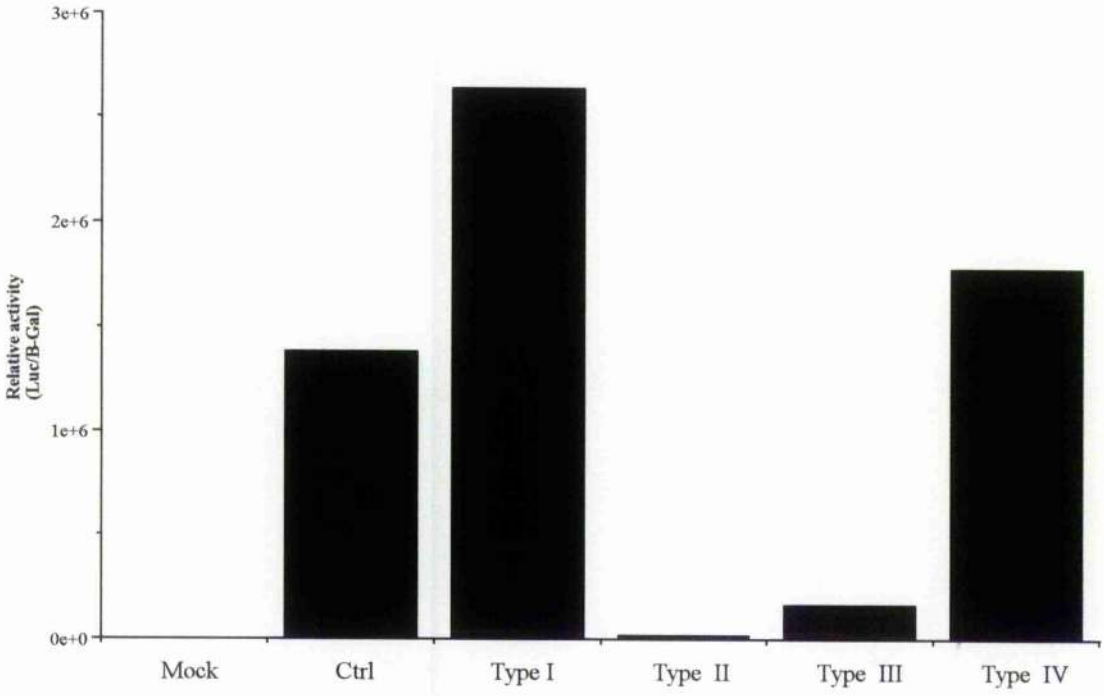
Figure 5.6

The expression of DP-3 5'UTRs in NIH 3T3 Cells

a) NIH 3T3 cells were transfected with 10μg 5'UTR-luciferase construct as indicated and 2μg pCMV-β-galactosidase. Cells were harvested at 48 hours post transfection, for both RNA and protein. The values plotted are based on the activity of luciferase relative to the activity of the control, β-galactosidase.

b) RNA was extracted from NIH 3T3 cells from (a) and dot blotted. Dot blots were hybridised with a luciferase and a β-galactosidase probe.

Figure 5. 6


3e+6
2e+6
1e+6
0e+0
Relative activity
(Luc/B-Gal)
Mock
Ctrl
Type I
Type II
Type III
Type IV

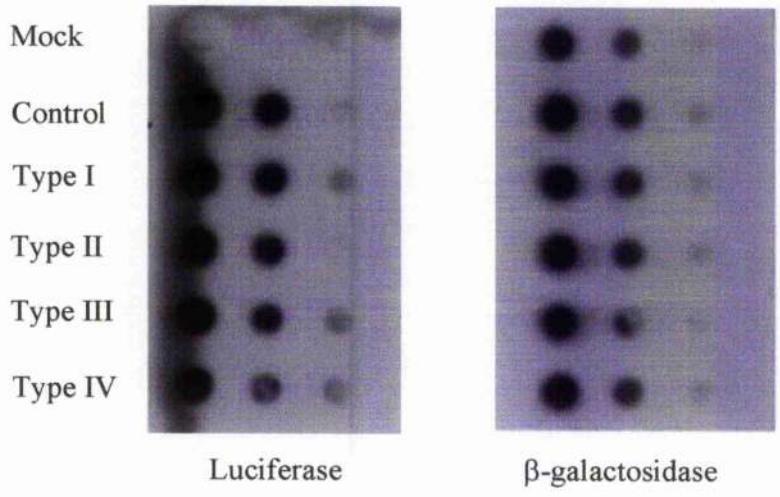

Mock
Control
Type I
Type II
Type III
Type IV
Luciferase
β-galactosidase

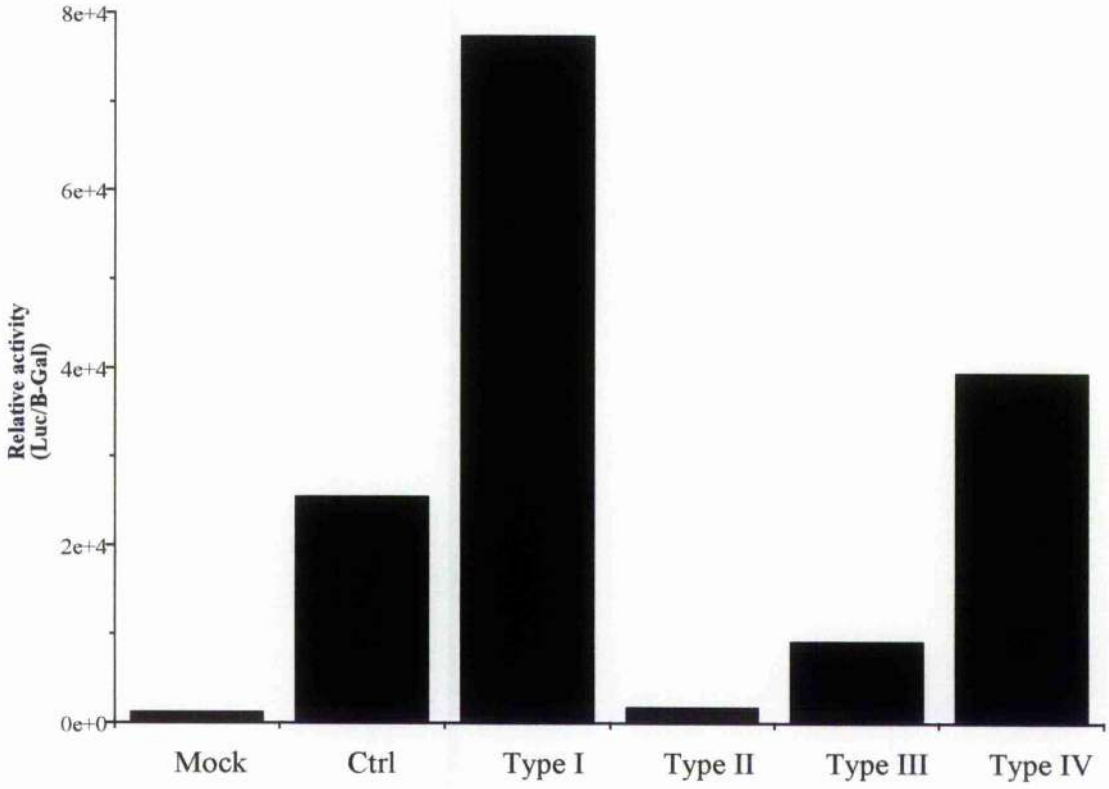
Figure 5.7

The expression of DP-3 5'UTRs in F9EC Cells

a) F9EC cells were transfected with 7.5μg 5'UTR-luciferase construct as indicated and 2.5μg pCMV-β-galactosidase by the calcium phosphate method. Cells were harvested at 48 hours post transfection, for both RNA and protein. The values plotted are based on the activity of luciferase relative to the activity of the control, β-galactosidase.

b) RNA was extracted from F9EC cells from (a) and dot blotted. Dot blots were hybridised with both luciferase and β-galactosidase probes.

Figure 5.7


8e+4
6e+4
4e+4
2e+4
0e+0
Relative activity (Luc/B-Gal)
Mock
Ctrl
Type I
Type II
Type III
Type IV

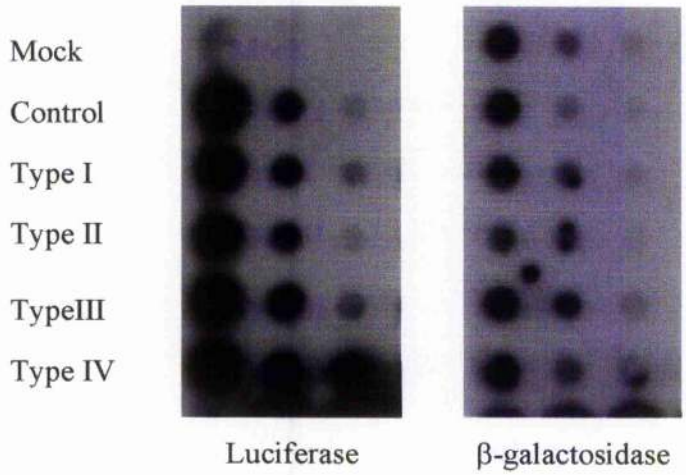
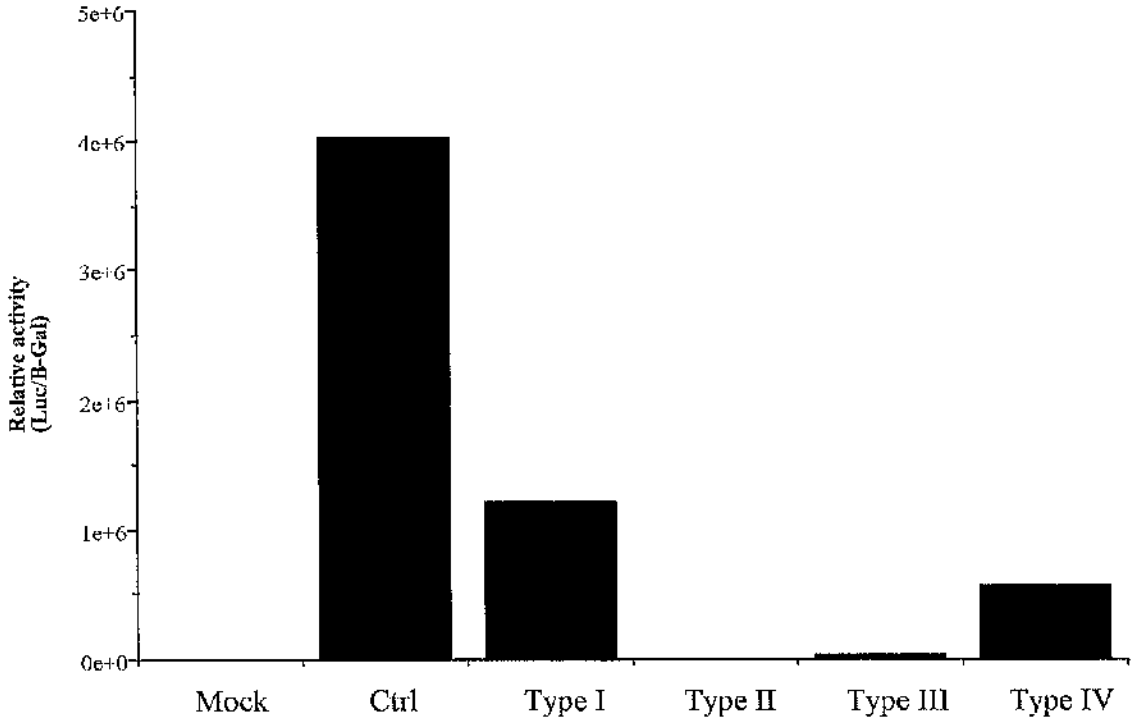

Mock
Control
Type I
Type II
TypeIII
Type IV
Luciferase
β-galactosidase

Figure 5.8

The expression of DP-3 5'UTRs in U2OS Cells

a) U2OS cells were transfected with 7.5μg 5'UTR-luciferase construct as indicated and 2.5μg pCMV-β-galactosidase by the calcium phosphate method. Cells were harvested at 48 hours post transfection, for both RNA and protein. The values plotted are based on the activity of luciferase relative to the activity of the control, β-galactosidase.

Figure 5.8


5e+6
4e+6
3e+6
2e+6
1e+6
0e+0
Relative activity
(Luc/B-Gal)
Mock
Ctrl
Type I
Type II
Type III
Type IV

Chapter 6

The effect of p53 on DP-3 translation

6.1 Introduction

The 5' untranslated region of a transcript can influence translation of eukaryotic mRNAs. The level of translation can be influenced both by the mechanism of initiation site selection and by RNA-protein interactions within the 5' untranslated regions (Jackson and Wickens, 1997). In addition, short upstream open reading frames can also influence translation of the major open reading frame (ORF) (Geballe and Morris, 1994). Therefore the 5'UTR of a transcript can have an impact on the expression of protein at a translation level.

The p53 tumour suppressor protein is an important regulator of the cellular response to DNA damage, the induction of active p53 protein resulting in either cell cycle arrest or apoptosis (Ko and Prives, 1996). The p53 protein is a sequence specific transcription factor, whose responsive genes are involved in apoptosis and cell cycle regulation. In addition, p53 interacts with a number of cellular and viral proteins (Ko and Prives, 1996). Genes regulated by p53 include $p21^{Waf1/Cip1}$, a cyclin-dependant kinase (cdk) inhibitor that induces cell cycle arrest (El-Deiry *et al.*, 1993; Harper *et al.*, 1993). The p21 protein modulates the activity of cdks by preventing phosphorylation of pRb. Hypophosphorylated pRb forms an inhibitory complex with E2F, thereby preventing progression into S phase of the cell cycle and arrest in G1 phase results (Dulic *et al.*, 1994). Another transcriptional target of p53 is the *mdm2* gene (Momand *et al.*, 1992). MDM2 is an oncoprotein that interacts with p53, inhibiting the transactivation functions of p53 and also targeting p53 for proteosome-mediated degradation (Haupt *et al.*, 1997; Kubbutat *et al.*, 1997).

The pRb/E2F and p53 pathways appear to be linked, not only via induction of p21 and pRb induced cell cycle arrest but via p53 regulated apoptosis. High expression of E2F-1 can cause quiescent cells to enter S phase (Johnson *et al.*, 1993) and subsequently apoptose, a process which is enhanced in the presence of p53 (Wu and Levine 1994). Furthermore, components of the E2F/pRb and p53 pathways physically interact, suggesting further integration of these pathways (Martin *et al.*, 1995; O'Connor *et al.*, 1995; Sørensen *et al.*, 1996).

Murine p53 negatively regulates it's own expression through the 5'UTR of p53 transcripts and physically interacts with the 5'UTR of p53 mRNA *in vitro* (Mosner *et al.*, 1995). Murine p53 has also been shown to be important in the repression of cyclin-dependent kinase 4 (CDK4) translation in TGF-β1-induced G1 cell cycle arrest; this effect is mediated by the 5'UTR of the CDK4 transcript (Ewen *et al.*, 1995). In addition, the 3'UTR of human p53 mRNA inhibits the expression of p53 protein (Fu *et al.*, 1996) and participates in activation of p53 translation in response to γ-irradiation (Fu and Benchimol, 1997). It is therefore apparent that p53 may be able to exert its cell cycle regulatory effects both by transcriptional and translational mechanisms.

An investigation into the expression of the DP-3 5'UTR-luciferase vectors in SAOS-2 cells revealed striking differences in expression when compared with the results obtained in other cell lines tested (Chapter 5). SAOS-2 cells lack functional pRb and p53 (Shew *et al.*, 1990). Since the p53 and E2F pathways are integrated and p53 is involved in translational regulation of cell cycle related targets, an investigation into the effect of p53 on DP-3 5'UTR mediated translation was instigated.

6.2 Results

6.2.1 DP-3 5'UTRs are regulated in SAOS-2 cells

The chimeric DP-3 5'UTR luciferase reporter vectors (Chapter 5) were transiently transfected into SAOS-2 cells. The results (Figure 6.1) revealed differences in expression when compared to the other cell lines assayed (Chapter 5). The translation of the type I 5'UTR was observed to be 60% less than pGL2control. The type III 5'UTR was 95% lower than the control, similar to all other cell lines tested. However, a striking difference was observed for the type IV 5'UTR. The translation of this 5'UTR was 60% higher than the control, this elevated level of type IV activity was not observed in any other cell lines (Chapter 5). Such data suggests that the translation of DP-3 type IV 5'UTR is regulated in SAOS-2 cells.

6.2.2 p53 represses translation of DP-3 type IV 5'UTR transcripts

The expression of the chimeric DP-3 5'UTRs-luciferase was different in SAOS-2 cells when compared to the other cell types tested (Chapter 5). The high

level of expression of type IV 5'UTR was unique to this cell line. All the other cell lines tested (Chapter 5) expressed pRb and p53, except C33A cells which lack pRb (Zhu *et al.*, 1993). On comparison with the other cell lines, the absence of pRb did not result in any differences in 5'UTR expression in C33A cells, since the expression of all the DP-3 5'UTRs was similar to that observed in the other cell lines (Chapter 5, Figure 5.4). Hence, it was possible that the absence of p53 in SAOS-2 cells, resulted in the high level of luciferase expression from type IV transcripts. In an effort to further investigate the regulation of DP-3 translation via the DP-3 5'UTRs, p53 was co-expressed with the chimeric DP-3 5'UTR-luciferase vectors in SAOS-2 cells.

Co-expression of p53 with the DP-3 5'UTR-luciferase vectors in SAOS-2 cells resulted in several effects on DP-3 translation (Figure 6.2). Co-transfection of p53 did not effect the expression of type I 5'UTR. However co-transfection of p53 resulted in a 65% reduction in type IV 5'UTR activity. The translation of type IV 5'UTR in the presence of p53 was similar to the level of type I 5'UTR activity. Furthermore, expression of the type IV 5'UTR in SAOS-2 cells co-transfected with p53 was 50% less than the control and resembled the levels observed in other cell lines (Chapter 5). Such data suggests that p53 may repress the translation of DP-3 transcripts with a type IV 5'UTR.

The translation of DP-3 type III 5'UTR transcripts was enhanced in the presence of p53 and resulted in a 60% increase in luciferase expression (Figure 6.2). However the level of type III translation was still 90% lower than the control value and therefore similar to the level observed in other cell lines (Chapter 5).

6.3 Discussion

6.3.1 The translation of DP-3 type IV 5'UTR is enhanced in SAOS-2 cells

The high level of translational of a DP-3 type IV transcript was unique to SAOS-2 cells, since all other cell lines tested revealed lower levels of type IV 5'UTR translation (Chapter 5). Such data suggests that the translation of type IV 5'UTR is regulated in a specific manner in SAOS- 2 cells. The absence of pRb or p53 or both proteins in this cell line may be responsible for the phenotype observed. Transient transfection experiments in C33A cells, which lack functional pRb but express p53, revealed similar regulation to other cell lines tested, suggesting that the

absence of functional pRb may not be solely responsible for the phenotype observed in SAOS-2 cells. Indeed, addition of p53 represses the expression of type IV transcripts to the levels observed in other cell lines (Chapter 5). It is conceivable that the absence of endogenous p53 in SAOS-2 cells prevents the repression of translation of type IV transcripts, and hence high levels of translation were observed. Alternatively, the expression pattern and regulation of translation factors in SAOS-2 cells may dictate the level of translation.

6.3.2 p53 may influence DP-3 translation via direct RNA/protein interaction

There are several possible mechanisms through which p53 could exert its effects on DP-3 translation. p53 may directly regulate DP-3 translation by binding to the 5'UTR of a particular DP-3 transcript. A direct interaction between the DP-3 type IV 5'UTR and p53 could prevent efficient translation. Since direct RNA/protein interactions can influence translation (Jackson and Wickens, 1997) and murine p53 protein has been shown to bind directly to p53 transcripts *in vitro* (Mosner *et al.*, 1995), p53 may specifically bind to type IV 5'UTR RNA and repress translation. In contrast, an interaction between p53 and type III RNA may result in the enhancement of type III translation observed in the presence of p53, however activators of translation are rare in eukaryotic translation (Kozak, 1992).

6.3.3 p53 dependent transcription activity may influence translation

p53 may influence translation indirectly by interaction with cellular proteins or via the transcriptional activation of a range target genes. Once such target is $p21^{Waf1/Cip1}$, increased levels of this cdk inhibitor induces cell cycle arrest (El-Deiry *et al.*, 1993; Harper *et al.*, 1993). It is conceivable that the translation of any of the DP-3 5'UTRs may be regulated during the cell cycle and that p53 mediated cell cycle arrest causes the change in translation of DP-3 5'UTRs observed. Thus, the effect of p53 on DP-35'UTR translation may be an indirect effect of p53 rather than a direct physical interaction between DP-3 RNA and p53 protein.

The *mdm2* proto-oncogene is a target of p53 transcription. MDM2 protein has been implicated in translational regulation; thus, MDM2 interacts with the ribosomal L5 protein-5srRNA nucleoprotein complex, both in the presence and absence of p53 (Marechal *et al.*, 1994). Furthermore, the RING finger domain of

MDM2 binds specifically to RNA and this interaction is independent of the L5-MDM2 interaction (Elenbaas *et al.*, 1996). MDM2 may influence translation by the binding of specific RNAs and influence translation through the L5-5srRNA interaction. If DP-3 5'UTRs were specifically bound and their translation influenced by MDM2, increased p53 expression could result in the modification of DP-3 5'UTR translation observed. However, preliminary results in SAOS-2 cells suggest that over expression of MDM2 protein does not result in the changes observed in DP-3 5'UTR translation, when p53 is co-expressed (data not shown).

6.3.4 p53 may target the expression of specific DP-3 protein isoforms

The expression of p53 in SAOS-2 cells influenced the translation of specific DP-3 5'UTRs in opposing ways. Type I 5'UTR translation did not change in the presence or absence of p53. Type III translation was increased and in contrast, type IV translation was reduced in the presence of p53. Extensive analysis of DP-3 RNA has revealed that particular 5'UTRs are found associated with particular ORFs (Ormondroyd *et al.*, 1995). For example, type III 5'UTR is associated with an M2 E-Q- ORF and results in translation of DP-3β isoform (Ormondroyd *et al.*, 1995). Likewise, a type IV 5'UTR is associated with an M2 E-Q+ ORF and a DP-3γ isoform would result from translation of such a transcript. Based upon the difference in response to p53 in SAOS-2 cells of the DP-3 5'UTR, it is likely that during a p53 response the translation of DP-3β would be enhanced and that DP-3γ translation would be reduced. It is interesting to note that the only difference between these two DP-3 protein isoforms is the inclusion of a single glutamine residue (Q) in the DP-3γ isoform. This glutamine residue may represent a site of post-translational modification; the inclusion of the glutamine residue in DP-3γ disrupts a putative casein kinase II phosphorylation site in DP-3β and creates a putative DNA-PK phosphorylation site (Chapter 3; Figure 8.1). At present, comparisons of the transcriptional activity of DP-3β and DP-3γ with different E2F partners and with various E2F responsive promoters is underway.

6.3.5 DP-3 isforms may function during DNA damage or repair

p53 is induced and activated in response to a range of stimuli, including DNA damage. Several protein kinases phosphorylate p53, enhancing p53 accumulation and functional activation in response to DNA damage. The kinases that phosphorylate p53 include DNA-PK, which has been shown to phosphorylate p53 *in vitro* (Lees-Miller et al., 1992) DNA-PK is also known to be active during DNA repair (Anderson and Carter, 1996). It is possible therefore that p53 decreases the expression of DP-3γ (via type IV 5'UTR) and in addition, DNA-PK phosphorylates DP-3γ, perhaps inhibiting any active DP-3γ in the cell. Such regulation suggests that this DP-3 isoform needs to be switched off during DNA damage and repair.

The enhancement of DP-3β expression in the presence of p53 suggests that this isoform may function in cellular conditions where p53 levels are elevated, such as during DNA damage and repair. Subtle changes in the levels of DP-3 isoforms may serve to activate or repress particular E2F target genes. Many of the E2F target genes are required for DNA replication (Helin, 1998) and therefore, turning off E2F transcription would be expected, thereby allowing DNA repair before replication. This would imply that DP-3β expression might have a negative influence on E2F and reduce E2F-dependent transcription. Perhaps DP-3β dimerises with specific E2F partners and forms inhibitory complexes that cannot bind to DNA or that cannot activate transcription. Indeed, an E2F-4 or 5/DP-3β complex would only accumulate in the nucleus via the action of pocket proteins and is thought repress E2F target genes (Allen *et al.*, 1997), therefore, expression of DP-3β may indeed cause the formation of repressive E2F complexes. Alternatively, DP-3β may activate as yet undiscovered genes required for DNA repair. Additionally, phosphorylation of DP-3γ by DNA-PK during DNA repair may modify the affinity of DP-3γ for particular E2F partners or alter DNA binding ability and like DP-3β, influence E2F transcription. p53 functions as a sensor to cellular stresses, for example, DNA damage, metabolic changes and deregulated cell cycle progression, it is possible that p53 might target E2F activity by altering translation of particular DP-3 isoforms, potentially assisting growth arrest or apoptosis.

At present no definite function has been found for DP-3. Endogenous DP-3 protein is difficult to detect in cells and therefore, DP-3 isoforms may have a short protein half-life. It is possible that DP-3 is only required under specific circumstances, such as DNA damage or repair. The formation of specific E2F complexes during conditions of DNA damage may be mediated by p53 dependant translational control of DP-3 isoforms and that rapid degradation of DP-3 prevents the maintenance of E2F complexes when no longer required.

Figure 6.1

The expression of DP-3 5'UTRs in SAOS-2 Cells

a) SAOS-2 cells were transfected with 7.5μg 5'UTR-luciferase construct as indicated and 2.5μg pCMV-β-galactosidase. Cells were harvested at 48 hours post transfection. The values plotted are based on the activity of luciferase relative to the activity of the control, β-galactosidase.

Figure 6.1

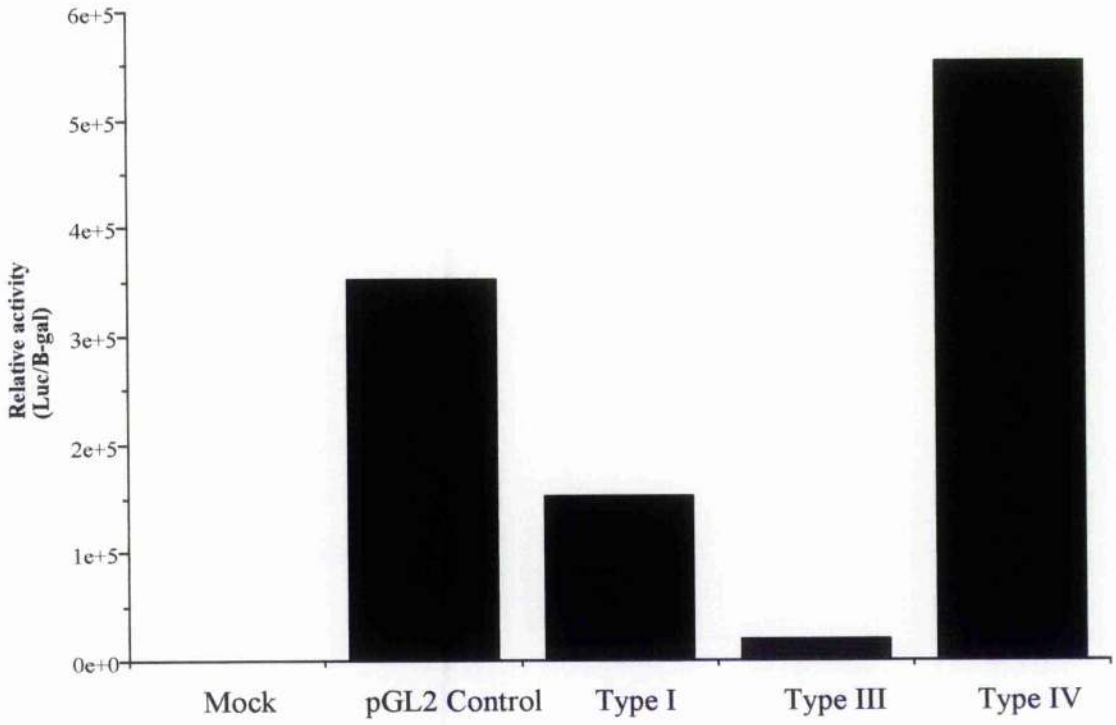
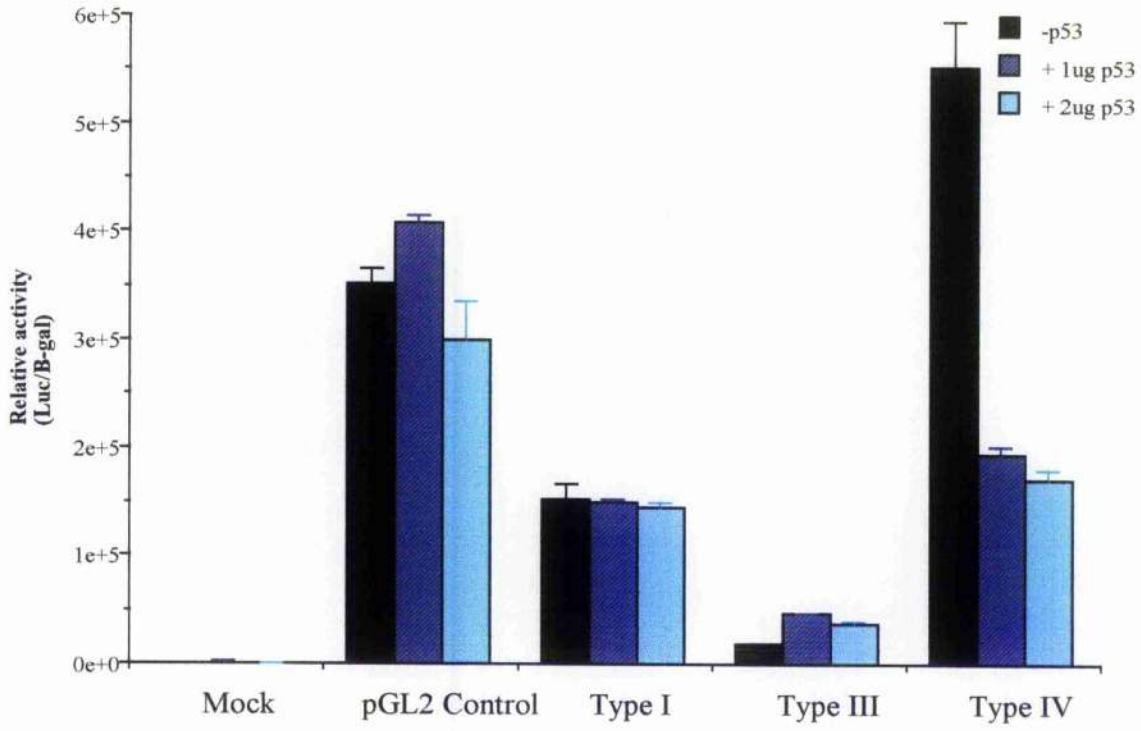

6e+5
5e+5
4e+5
3e+5
2e+5
1e+5
0e+0
Relative activity
(Luc/B-gal)
Mock
pGL2 Control
Type I
Type III
Type IV

Figure 6.2

The effect of co-expression of p53 on DP-3 5'UTRs activity in SAOS-2 cells

a) SAOS-2 cells were transfected with 3μg 5'UTR-luciferase vector as indicated and 0.5μg pCMV-β-galactosidase, in duplicate. Cells were harvested at 48 hours post transfection. The values plotted are based on the average activity of luciferase relative to the activity of the control, β-galactosidase.

Figure 6.2


6e+5
5e+5
4e+5
3e+5
2e+5
1e+5
0e+0
Relative activity
(Luc/B-gal)
-p53
+ 1ug p53
+ 2ug p53
Mock
pGL2 Control
Type I
Type III
Type IV

Chapter 7

Analysis of DP-3 protein

7.1 Introduction

Antisera have been important in the characterisation of E2F. Antisera have been raised to the different E2F components and used to gain insight into the cellular E2F activity. For example, analysis of E2F DNA binding activity during the cell cycle, using E2F-1 and E2F-3 specific antisera has shown that E2F-3/DP-1 heterodimers form the E2F activity as cycling cells enter S phase (Leone *et al.*, 1998). Likewise E2F-4 and p130 antisera have been used to characterise early cell cycle complexes (Vairo *et al.*, 1996), and antisera has been useful in the study of the subcellular location of E2F and DP proteins (de la Luna *et al.*, 1996: Magae *et al.*, 1996: Müller *et al.*, 1997). Antisera specific for DP-3 or a particular DP-3 isoform were therefore likely to be useful for the studying the role of DP-3 in E2F.

DP-3 cDNAs suggested four different protein isoforms may exist (Ormondroyd *et al.*, 1995). From the amino acid sequence, DP-3α was estimated to be approximately 50 KDa and DP-3 β,γ and δ approximately 43 KDa in size. During the early analysis of DP-3 protein, several different rabbit anti-peptide antisera produced (S. de la Luna personal communication and Figure 7.1) Western blot analysis revealed that these polyclonal antisera all recognised bacterially expressed GST-DP-3 fusion proteins. In addition, the antisera detected exogenously expressed DP-3 proteins in transiently transfected mammalian cell extracts, in both immunoblots and immunostaining studies, but did not react with exogenous or endogenous DP-1 in western analysis. However, detection of endogenous DP-3 protein had proved difficult. Whilst competition experiments performed with specific and non-specific peptides in western blots found that all the anti-peptide polyclonal antisera react with polypeptides in whole cell extracts, none of the antisera detected the same polypeptides. Therefore confirmation that any of the polypeptides were indeed endogenous DP-3 protein was not possible.

The detection of DP-3 protein was essential for the characterisation of its role in the E2F heterodimer. Thus, in an attempt to analyse DP-3 protein expression, rabbit polyclonal antisera were raised to different regions of the DP-3 amino acid

sequence. These antisera were characterised by western blotting and by immunostaining of transfected cells.

7.2 Results

7.2.1 Peptide design

Several peptides had been designed and made during the initial cloning of DP-3. In an effort to expand the repertoire immunochemical reagents available to study DP-3 proteins, new antisera were raised. The amino acid sequence of peptide 7.6 and 7.7 was taken from the E region of DP-3α and δ, 7.6 spans the entire 16 residues of the E region (Figure 7.1). The peptide 7.7 sequence begins 8 residues before the E region and overlaps the 7.6 sequence (Figure 7.1c). These peptides were designed on the basis that the resultant antisera would react specifically with E+ DP-3s and permit a distinction between E+ and E- protein isoforms. It was predicted that antisera raised to 7.6 and 7.7 peptide sequences would not recognise DP-1, since the E region is absent (Figure 7.1b and c).

7.2.2 Analysis of α7.6 antisera

7.2.2.1 α7.6 antisera detects E+ DP-3 isoforms and not E- isoforms

Western analysis with extracts from COS-7 cells transiently transfected with DP-3 suggested that the α7.6 antisera could specifically distinguish between E+ and E- forms of DP-3 (Figure 7.2a). Antisera from both rabbits immunised with peptide 7.6 detected polypeptides in COS-7 cell extracts expressing DP-3α and δ (Figure 7.2a, lanes 3 and 7, Figure 7.2b, lanes 11 and 15). However, extracts from cells transfected with DP-3β did not detect polypeptides with antisera from either rabbit (Figure 7.2a, lane 5 and Figure 7.2b, lane 13) showing a similar appearance to the negative control, (mock transfected extracts, Figure 7.2a, lanes 1 and 2, Figure 7.2b, lanes 9 and 10). As expected the positive control α7.2 antiserum specifically detected all DP-3 protein isoforms in both western blots of DP-3 transfected cells and bacterially expressed DP-3 proteins. The α7.2 antiserum recognised similar sized polypeptides in DP-3α transfected extracts (Figure 7.2b lane) as α7.6 antisera from both rabbits (Figure 7.2a lane 3, Figure 7.2b lane 11).

7.2.2.2 α7.6 antisera specifically recognises DP-3 E+ isoforms

The addition of peptide 7.6 to the binding reaction prevented detection of the DP-3 polypeptides (compare Figure 7.2a, lanes 3 and 4, 7 and 8, Figure 7.2b, lanes 11 and 12, 15 and 16), suggesting that the antisera raised specifically recognises an epitope common to both DP-3 E+ proteins and contained within peptide 7.6. The addition of a peptide of similar size and of unrelated sequence (peptide C) to peptide 7.6 ensured that the effects observed were due to competition for a specific peptide epitope rather than a non-specific effect. Similarly, when the 7.2 peptide was included in the α7.2 antisera binding reactions detection of the same polypeptides in DP-3α expressing cells was prevented (Figure 7.2b, lanes 17 and 18). Polypeptides that were apparent on blots both in the presence and absence of specific peptides were non-specific detection of polypeptides by the antisera, and thus deemed of little relevance to the analysis of DP-3.

7.2.2.3 α7.6 antisera specifically detects DP-3 E+ isoforms by immunostaining

The DP-3 E region plus several C-terminal residues found in all DP proteins can act as an NLS (de la Luna *et al.*, 1996). To study the cellular location of DP proteins, the antisera were tested by immunostaining of transfected cells. Immunostaining with α7.6 antisera revealed specific recognition of DP-3α, a nuclear DP (de la Luna *et al.*, 1996), and the addition of peptide 7.6 abolished the nuclear staining in expressing cells (Figure 7.3a and b). Mock transfected cells, and those expressing DP-3β showed slight background fluorescence (Figure 7.3d, e, g and h). Immunostaining with the α7.2 antisera, which recognises all DP-3 isoforms, revealed that the cells had been transfected, since both DP-3α and β were expressed. Extracts from mock transfected cells did not express DP-3 protein (Figure 7.2).

7.2.3 α7.7 antisera specifically detected DP-3 E+ isoforms by western blotting

The antisera raised to peptide 7.7 were tested by western blotting with extracts from COS-7 cells transfected with DP-3. Only one of the two rabbits immunised with peptide 7.7 produced antiserum that could be used in immunoblotting analysis. The α7.7 antisera recognised the 53 KDa DP-3α polypeptide and the 50 KDa DP-3δ polypeptide (Figure 7.4, lanes 3 and 4, lanes

7 and 8). The detection of polypeptides was specific, since the addition of 7.7 peptide abolished detection of these polypeptides. The same polypeptides were detected by the α7.6 and by α7.2 antisera (Figure 7.2). Likewise, α7.7 antisera failed to detect polypeptides in extracts from DP-3β transfected cells. The α7.2 antisera revealed that DP-3β transfected extracts were indeed expressing a 50 KDa band which was effectively competed by addition of 7.2 peptide (Figure 7.4, lanes 9 and 10).

7.2.4 Truncated DP-3 polypeptides are detected with α7.6 and α7.7 antisera

The results suggested that there were smaller DP-3 polypeptides since competition for the smaller polypeptides was apparent upon addition of specific peptide (Figure 7.2 and 7.4). The polypeptides were only observed in DP-3 transfected cell extracts and not in the mock transfected cells, indicating that they were likely to be DP-3 polypeptides. These polypeptides could possibly result from initiation of translation at internal methionines in the DP-3 ORF, as several likely methionines exist in DP-3, some being in a favourable context for initiating translation (Kozak, 1989). Alternatively the polypeptides may just be products of proteolytic degradation. Since there was only one possible initiating methionine before the E region, the array of smaller bands seen may be the combination of products of internal translation initiation and degradation.

7.3 Discussion

7.3.1 DP-3 anti-peptide antisera detect exogenously expressed DP-3 proteins, but not endogenous DP-3

The antisera raised to peptides 7.6 and 7.7 react specifically with exogenously expressed DP-3 E+ protein isoforms, both by western blotting and immunostaining analysis. However, the detection of endogenous DP-3 protein would have greatly advanced my studies into DP-3 expression and function. Although the anti-peptide antisera were tested with extracts from untransfected cells by western blotting (data not shown), I failed to detect any evidence that the antisera recognised endogenous DP-3. Hence, it has still not been possible to identify a polypeptide that reacts with two different DP-3 antisera, confirming that the polypeptide is indeed an endogenous DP-3 protein isoform.

In conclusion, the anti-peptide antisera produced specifically detected exogenous DP-3 E+ proteins expressed in transfected cells, but failed to identify polypeptides which could be precisely ascribed to endogenous DP-3.

7.3.2 DP-3 protein is expressed at low levels in cells

The lack of detection of endogenous DP-3 protein could be due to several factors. The levels of DP-3 protein in cells may be extremely low and all the antisera so far made may not be sensitive enough to detect them. It is possible, however, that the antisera can indeed identify DP-3 polypeptides, but these epitopes recognised by each antibody were not available on all DP-3 isoforms. Although this is a conceivable idea, is unlikely since some anti-peptide antisera, such as α7.2 were made to regions common to all DP-3 isoforms (Figure 7.1).

Although many cell lines have been tested, a cell line expressing detectable levels of DP-3 protein, via western blot, has still to be identified. Studies on hDP-2 protein expression has shown that ML-1 cells, a human myeloid leukemia cell line, express sufficient human DP-2 protein to be immunoprecipitated. Polypeptides of 110, 55, 48 and 43 kDa in size were immunopreciptated from metabolically labelled ML-1 cells using a human DP-2 specific antibody (Rogers *et al.* 1996). However, western blot analysis of ML1 cells extracts with the panel of anti-peptide DP-3 antisera did not reveal the detection of DP-3 proteins (data not shown).

7.3.3 DP-3 protein levels may be influenced by post-transcriptional regulation

DP-3 is regulated by RNA processing (Chapter 4) and by translational control (Chapter 5). Consequently, the levels of DP-3 proteins may be controlled at several levels. The translation of DP-3 appears to be regulated by the nature of the 5'UTR, some of which inhibit translation (Chapter 5), therefore, some DP-3 isoforms may be expressed at very low levels and consequently detection of these isoforms might be difficult.

7.3.4 DP-3 protein expression may be regulated by degradation

DP-3 protein levels could also be regulated at the level of degradation. If DP-3 proteins were to be rapidly degraded, the levels of protein required for detection by western blotting may be insufficient. Both E2F-1 and E2F-4 are unstable proteins

and are targeted for degradation by the ubiquitin-proteosome pathway (Hateboer *et al.*, 1996; Hofmann *et al.*, 1996). It is therefore possible that DP-3 protein expression may also be regulated by degradation. However, despite the degradation of E2F-1 and 4, several antisera have been developed which recognise the endogenous proteins by western analysis (Beijersbergen *et al.*, 1994; Ginsberg *et al.*, 1994; Vairo *et al.*, 1996; Leone *et al.*, 1998).

7.3.5 Regulation of DP-3 protein may impact on E2F activity

Analysis of E2F complexes by gel retardation assays has shown that DP-1 is the major DP component of the E2F heterodimer (Bandara *et al.*, 1993). It is possible that DP-3 has a very specific role in the context of the E2F DNA binding activity and possibly is expressed under certain physiological conditions, such as in early embryonal development or during specific cellular processes such as during particular stages of the mitotic cell cycle or during meiosis. Perhaps translation of DP-3 transcripts is induced under such conditions, and the protein is rapidly degraded once DP-3 has performed its biological functions.

Overall, these anti-peptide antisera specifically detected exogenously expressed DP-3 E+ isoforms in mammalian cells by western blotting and immunostaining analysis. However, the antisera did not appear to detect endogenous DP-3 proteins in either assay system, probably because DP-3 protein expression is likely to be regulated at several levels.

Figure 7.1

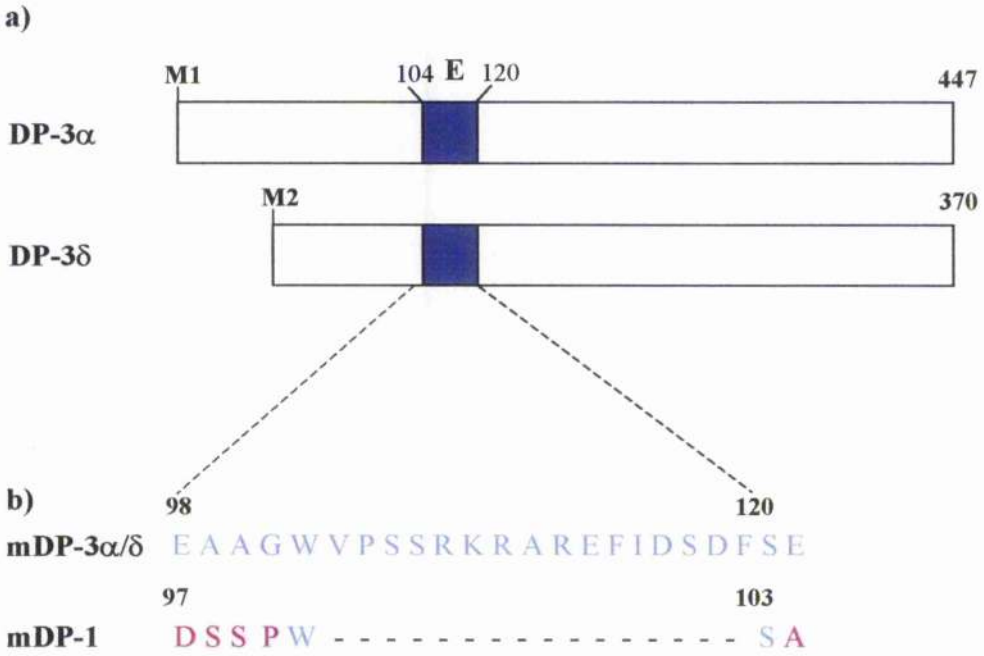
DP-3 peptides

a) Diagrammatic representation of DP-3α and DP-3δ protein isoforms.

b) The sequence of the E region of DP-3α and δ compared with the homologous region of DP-1

c) The sequences of peptides 7.6 and 7.7. The sequence of the control peptide C is also shown.

Figure 7.1



c)

7.6 S D R K R A E F I D S D F S E

7.7 E A A Q W V P - S D R K R A

C **D Y S T R R P L S P S N Q L Q E K H V**

Figure 7.2

Analysis of α7.6 antisera

a) Whole cell extracts from COS-7 cells transfected with expression vectors encoding the DP-3 isoforms, were immunoblotted with two different α7.6 antisera, with and without competing peptide, as indicated. M denotes mock transfected cell extracts. The positions of DP-3α and DP-3δ polypeptides are shown. Positions of molecular weight markers are shown in KDa. As a control for expression of DP-3α, α7.2 antisera, was used, as indicated (lanes 17 and 18).

Figure 7.2

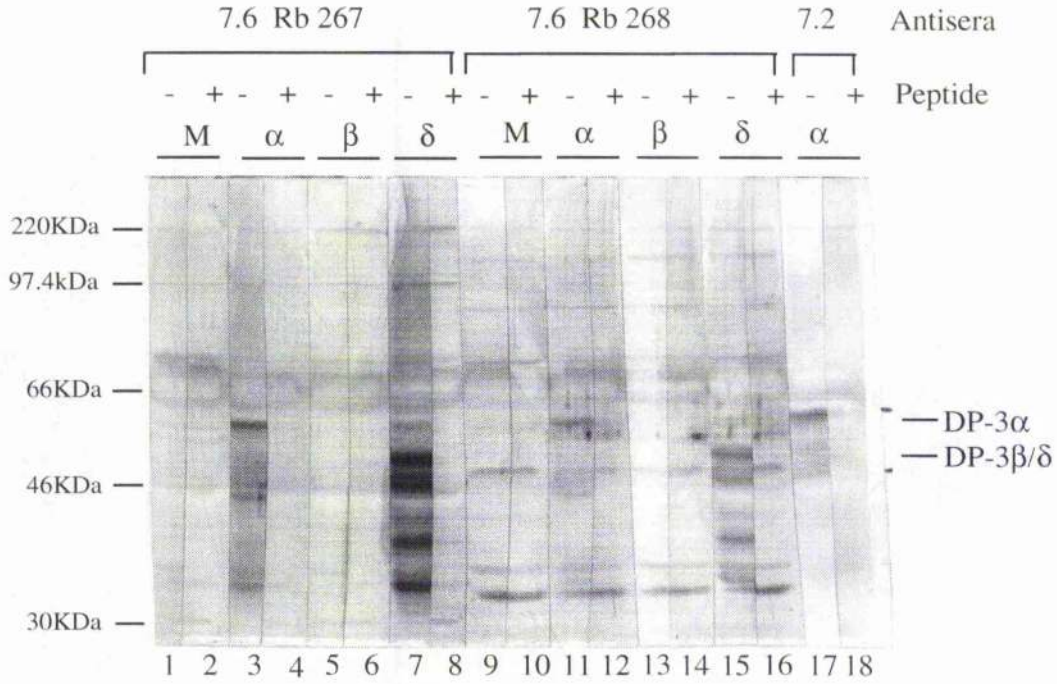

7.6 Rb 267
7.6 Rb 268
7.2
Antisera
- + - + - + - + - + - + - + - + - + Peptide
M α β δ M α β δ α
220KDa
97.4kDa
66KDa
46KDa
30KDa
DP-3α
DP-3β/δ
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

Figure 7.3

Immunostaining analysis with α7.6 antisera

U2OS cells were transfected with 2μg DP-3α and β expression vector. Immunostaining was performed with α7.6 antisera (rabbit 267), in the presence of either specific or non-specific peptide, with an anti-rabbit FITC-conjugated secondary antibody. DAPI staining of nuclei was performed.

a) exogenous DP-3α + α7.6 + peptide C, **b)** exogenous DP-3α + α7.6 + peptide 7.6 **c)** exogenous DP-3α + α7.2, **d),** DAPI stain of a), **e)** DAPI stain of b), **f)** DAPI stain of c), **g)** exogenous DP-3β + α7.6 + peptide C, **h)** exogenous DP-3β + α7.6 + peptide 7.6, **i)** exogenous DP-3α + α7.2, **j)** DAPI stain of g) **k)** DAPI stain of h), **l)** DAPI stain of i), **m)** Mock transfected +α7.6 + peptide C, **n)** Mock transfected +α7.6 + peptide 7.6, **o)** Mock transfected +α7.2, **p)** DAPI stain of m), **q)** DAPI stain of n), **r)** DAPI stain of o).

Figure 7.3

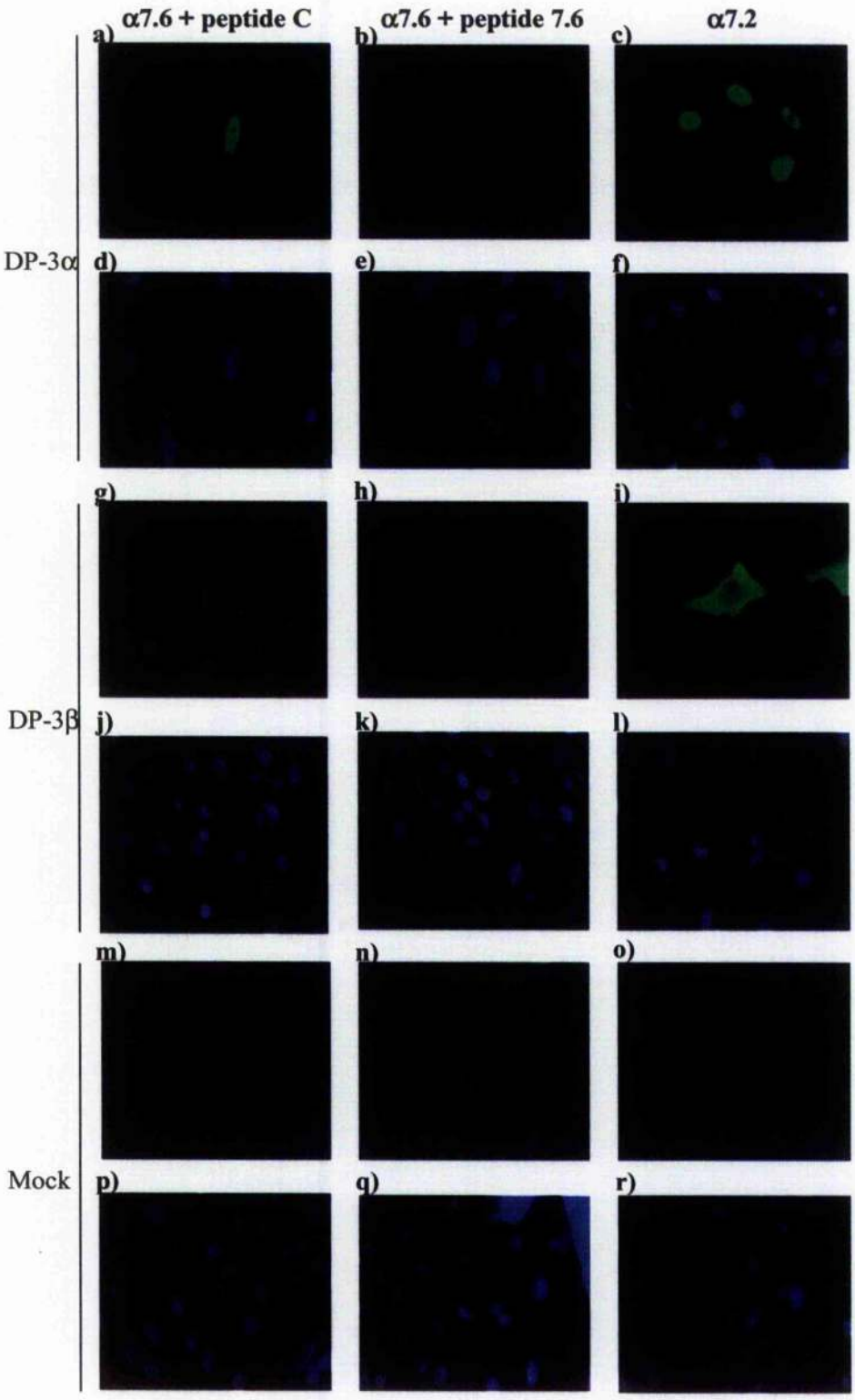
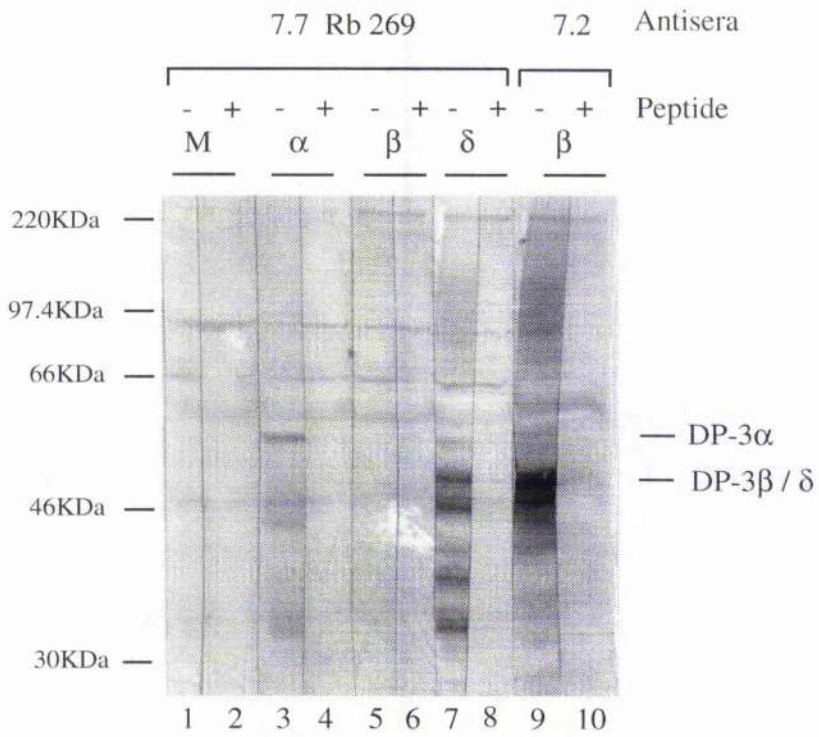

α7.6 + peptide C
α7.6 + peptide 7.6
α7.2
a)
b)
c)
DP-3α
d)
e)
f)
g)
h)
i)
DP-3β
j)
k)
l)
m)
n)
o)
Mock
p)
q)
r)

Figure 7.4

Analysis of α7.7 antisera

Whole cell extracts from COS-7 cells transfected with DP-3 expression vectors as indicated, were immunoblotted with α7.7 antisera, with and without competing peptide. M denotes mock transfected cell extracts. The positions of DP-3α and DP-3δ polypeptides are shown. Positions of molecular weight markers are shown in KDa. Control immunoblotting with DP-3 α7.2 antisera, with and without competing peptide was included (lanes 9 and 10).

Figure 7.4


7.7 Rb 269
7.2
Antisera
- + - + - + - + - +
Peptide
M
α
β
δ
β
220KDa
97.4KDa
66KDa
46KDa
30KDa
— DP-3α
— DP-3β / δ
1 2 3 4 5 6 7 8 9 10

Chapter 8

General Discussion

8.1 Implications of DP-3 alternative splicing

The isolation and characterisation of the murine DP-3 gene revealed the exon structure of the DP-3 gene which when compared to the cDNA sequences known for this gene suggested complex post-transcriptional regulation. Analysis of DP-3 mRNA sequences has suggested several splice variants existed. Variations in the open reading frame, combined with several different 5'UTR sequences and two possible initiating methionines, predicted four different protein isoforms, DP-3 α, β,γ and δ (Ormondroyd *et al.*, 1995).

The investigation of the genomic E region revealed that E+ DP-3 mRNAs arise via alternative splicing of a discrete 48 bp exon. The E region has been shown to form part of a NLS that can recruit E2F-4 and 5 to the nucleus and hence this splicing event has potentially important implications for E2F heterodimer function (de la Luna *et al.*, 1996). Additionally, the mechanism of heterodimer localisation to the nucleus can have different functional consequences on the cell cycle, as nuclear localisation of E2F-5 by co-expression of an E+ DP-3 resulted in promotion of cell cycle progression and growth, whereas localisation via pocket protein expression resulted in growth arrest (Allen *et al.*, 1997). The localisation of proteins, in particular transcription factors, to the nucleus is often regulated (Vandromme *et al.*, 1996). However most examples involve regulation at the protein level, either by interaction with other proteins that either promote or prevent nuclear accumulation or by modifications such as phosphorylation, an example is the localisation of the transcription factor NFκB. NFκB is retained in the cytoplasm and target genes are repressed, when bound to an inhibitor protein, I-κB. NFκB is only transported to the nucleus when I-κB is phosphorylated in response to signalling from mitogens and cytokines (Vandromme *et al.*, 1996). The regulation of nuclear localisation by alternative splicing of part of a nuclear localisation signal is therefore rare.

The alternative splicing of the E region of DP-3 transcripts, combined with translational regulation via the 5'UTRs may serve to allow fine control of both cell

cycle progression and the localisation of E2F family members. The regulation of translational, coupled with protein degradation processes, enables cells to rapidly alter levels of specific proteins, by both degrading existing proteins and translating new proteins. It may therefore be possible that when required the translation of E+ DP-3 transcripts is upregulated and E+ DP-3 isoforms are expressed, whilst at the same time E- DP-3s are degraded. Alternatively, by regulating transcription and splicing, E+ or E- transcripts are transcribed and translated as and when required. However, the ratios of E+ and E- DP-3 transcripts analysed by RNase protection suggested equal levels of E+ and E- transcripts, implying that either or even both mechanisms could have a role in regulating DP-3 and E2F cellular location.

The subnuclear location of the Wilm's tumour protein WT1 is regulated by alternative splicing (Larsson *et al.*, 1995). WT1 is a tumour suppresser protein involved in the formation of Wilm's tumour in the kidney and also in the generation of the Denys Drash and Frasier syndromes (Hastie, 1994; Barbaux *et al.*, 1997; Little and Wells, 1997). WT1 is considered to be a transcription factor that contains four Kruppel-type zinc fingers and has been implicated in growth regulation. Alternative splicing of WT1 results in numerous isoforms arising from the insertion of three amino acids (KTS) between the third and fourth zinc fingers and alternative splicing of the first exon. Additionally there are two alternative translational start sites and the pre-mRNA undergoes editing (Sharma *et al.*, 1994; Breuning and Pelletier, 1996). There are therefore several striking similarities between DP-3 and WT1. Analysis of the subcellular localisation of WT1 revealed that the localisation of the KTS- isoforms correlated with transcription factors, whereas the KTS+ isoforms associate with splicosomes and directly interact with U2AF65, a component of the splicing machinery (Larsson *et al.*, 1995; Davies *et al.*, 1998). Such data suggested that not all forms of WT1 functions as transcription factors. It is interesting to note that imbalance between the different isoforms is thought to be the basis of Frasier syndrome. This example suggests that although DP-3 isoforms can all bind E2F partners and bind to DNA, they do not necessarily have to have similar roles in cells (Ormondroyd *et al.*, 1995). Isoforms of WT1 which vary only by the insertion of three amino acids are implied to have very different and important roles in cells and in development.

The DP-3 γ isoform differs from the other DP-3 isoforms due to the insertion of a single glutamine (Q) residue between the DNA binding and dimerisation domains (Ormondroyd *et al.*, 1995). Analysis of the DP-3 gene revealed that this variation arises from alternative selection of two adjacent 3' splice acceptor sites in the intron between exons 8 and 9. This selection might arise from random selection between these two sites (named slippage) or a regulated process. Indeed, RNase protection analysis revealed that approximately 20% of DP-3 RNAs contain this glutamine residue. It is likely that if this process was a random event approximately equal amounts of Q+ and Q- RNAs might be predicted.

Examination of the amino acid sequence in the region of the insertion revealed theroretical phosphorylation sites. Therefore, this event may be regulated and suggests that DP-3 proteins could be regulated by post-translational modifications such as phosphorylation at this site. Insertion of the glutamine residue destroys a putative casein kinase II phosphorylation site and creates a hypothetical DNA PK site (Figure 8.1) (Bannister *et al.*, 1993; O'Reilly *et al.*, 1997). The implications of such regulation on E2F function have so far not been investigated. However, the regulation DP-3 γ by DNA-PK may have implications in processes such as the cellular response to DNA damage. Additionally, the influence of p53 on the translational regulation of DP-3 RNAs via the 5'UTRs may also prove to be important; since the analysis suggested that translation of DP-3γ is reduced when p53 is expressed. Thus, under conditions of DNA damage it may be necessary to reduce E2F activity to aid cell cycle arrest prior to DNA repair. It is possible that phosphorylation at the glutamine region by DNA-PK may act to reduce DP-3γ activity or perhaps target it for degradation.

The insertion or exclusion of a single glutamine has been observed in other genes, such as the human *brca 1* gene; two postions have been noted in which this insertion can occur. Analysis of the genomic organisation revealed that like DP-3, these insertions occur at exon/intron junctions (Miki *et al.*, 1994). The functional relevence of this event in BRCA 1 protein has not been determined. Likewise, the functional relevence of the insertion of a single glutamine residue in the mouse obese gene has also not been resolved (Zhang *et al.*, 1994).

8.2 DP-3 RNA expression, regulation and functions

The 5' regulatory regions of the DP-3 gene were not isolated and hence conclusions relating to the transcriptional regulation of DP-3 cannot be made. However, extensive analysis of RNA expression patterns via northern blotting revealed several transcripts of differing mobility, assumed to be alternatively spliced variants. Notably, differences in DP-3 and DP-1 RNA expression in adult mouse tissues were observed, suggesting that different tissues may have different E2F requirements.

The analysis of E2F-1 and 5 null mice revealed tissue specific abnormalities, suggesting particular E2F family members may be important for normal function in some tissues (Field *et al.*, 1996; Yamasaki *et al.*, 1996; Lindeman *et al.*, 1998). Analysis of E2F-5 mRNA levels in normal tissues showed that transcripts were highly expressed in some tissues, including the choroid plexus, skin and bowel, however E2F-5-null mice exhibited defects only in the choroid plexus, the other tissues appeared normal (Lindeman *et al.*, 1998). Such data implies that there may not be a simple correlation between level of mRNA and function in a particular cell type. Furthermore, although significant homology exists between the E2F family members, this gene knockout data indicates that E2F family members do not have degenerate roles. It is possible therefore that DP-1 and DP-3 have different roles and that DP-3 may have a specific, as yet undefined role, perhaps during specific cellular conditions such as the response to stress, DNA damage, apoptosis, at specific stages of the mitotic cell cycle or perhaps during meiosis. All of these are possible and further experiments including the generation and analysis of a DP-3 null mouse, are required to define the role of DP-3 in cells.

The intracellular location of specific mRNAs can vary in some cells and since mRNA are the templates for the translation of proteins; the localisation of mRNAs represents another level of regulation. RNA localisation enables the accumulation of high levels of proteins in specific locations and additionally, the regulation of the mRNA represents a more efficient way of localising proteins, rather than by protein targeting. For example, analysis of β-actin mRNA revealed localisation to the leading edge of migrating fibroblasts, the site in the cell where β-actin protein is required (Lawrence and Singer, 1986).

Many localised RNAs exist as complexes with components of the translation machinery, and are thought to be transported in a translationally repressed state, suggesting that translation only occurs at the specified location. The localisation and translation of specific mRNAs also prevents the expression of proteins in inappropriate regions of the cell, an example being myelin basic protein (MBP). MBP interacts strongly with plasma membranes, causing compaction of the membrane. MBP mRNA is therefore localised and translated where it is needed, preventing damage to other cellular membranes (Trapp *et al.*, 1987). Analysis of localised RNAs revealed *cis*-acting signals, named zipcodes, which target RNAs to different cellular domains (Oleynikov and Singer, 1998). The RNA zipcodes are usually situated in the 3'UTR, although they can occur in the 5'UTR of transcripts where they are predicted to have strong secondary structure and may be linked to sequences that regulate translation, (St. Johnston, 1995; Hazelrigg, 1998). The *Drosophila bicoid* mRNA is an example, as it is localised to the anterior pole of the developing oocyte via *cis*-acting elements in the 3'UTR and furthermore, *trans*-acting factors are involved in this localisation (Oleynikov and Singer, 1998).

DP-3 RNAs have a complex structure, as several 5'UTRs exist and these influence translation. The 5'UTRs of DP-3 may contain *cis*-acting elements that target the different mRNAs to different cellular destinations and novel proteins may bind these sequences and regulate localisation and/or translation. Alternative 3'UTRs have been noted for DP-3 and hDP-2 mRNA (Zhang and Chellapan, 1995; unpublished data) and the influence of these 3'UTRs has yet to be investigated. The translation of *Drosophila oskar* mRNA is localisation-dependant and requires a functional interaction between elements in the 5' and 3'UTRs. Once *oskar* RNA is localised to the posterior pole of the oocyte, translation is derepressed by a process requiring an element in the 5'UTR, a derepressor element. Activation of *oskar* translation requires an interaction between the derepressor element of the 5'UTR and the repressor element of the 3'UTR and additionally, RNA binding proteins are also thought to be involved (Gunkel *et al.*, 1998). Perhaps the different 5'UTRs or 3'UTRs of DP-3 mRNA contain zipcodes or other localisation elements. The two alternative 3'UTRs are largely uncharacterised and at present it is not known whether these contain potential localisation elements.

It is conceivable that the multiple DP-3 RNAs are located in specific regions of the cell and that function of the DP-3 protein isoforms is related to cellular location. In the developing nervous system of *Drosophila melanogaster*, neuroblasts undergo an asymmetric division into neuroblasts and ganglion mother cells (GMC), the localisation of the prospero transcription factor and *prospero* mRNA to the GMC is important in the determination of cell fate during this cell division. Staufen, a double stranded RNA binding protein is required for localisation of *prospero* mRNA and prospero protein to the GMC, after which prospero enters the nucleus (Oleynikov and Singer, 1998). Analysis of DP-3 RNA suggested that most DP-3 transcripts are cytoplasmic, but DP-3 RNAs may have specific locations within the cytosol. To date, the analysis of endogenous DP-3 protein by immunostaining has been unsuccessful. Analysis of exogenously expressed DP-3 protein isoforms revealed E+ DP-3s (α and δ) to be nuclear, as would be expected for a member of a transcription factor family, and that the E- DP-3s (β and γ) to be cytoplasmic (de la Luna *et al.*, 1996). However these proteins were translated from transcripts that do not contain UTR sequences and therefore RNA localisation-dependent expression patterns would not be observed.

8.3 DP-3 translation maybe regulated by RNA/protein interactions

There are several mechanisms for the regulated translation of mRNA in eukaryotes (Kozak, 1992; Pain, 1996). Many of these result in modification of translation factors that have global impact on translation in the cell. However, a few instances of regulation of specific mRNAs do exist. In mammalian cells the synthesis of ferritin is regulated by the binding of a repressor protein, the iron regulatory factor (IRF) to the iron response element (IRE) in the 5'UTR of the ferritin mRNA, repressing ferritin translation. An increase in cellular iron levels causes a change in the affinity or availability of the IRF for the IRE and translation is de-repressed (Melefors and Hentze, 1993). In contrast few protein activators of translation have been characterised in eukaryotes, although activators of translation have been described for viral translation these are not thought to be via a direct mechanism (Kozak, 1992). It is possible that DP-3 expression could be regulated by RNA/protein interactions. DP-35'UTRs are translated differently *in vivo*, but *in vitro* translation is unregulated suggesting that specific cellular factors may be required

for the differences in translation observed in transfected mammalian cells (Chapter 5). Possibly, repressor proteins bind to type II and III 5'UTR and inhibit translation, these proteins may be unable to bind to type I or IV RNA. Conversely, activators may bind to type I 5'UTR and not to the other 5'UTRs. Therefore, the variation in the level of type IV translation could be via altered affinity of a repressor or activator protein for type IV 5'UTR, perhaps in response to growth signals. The regulation of translation by RNA/protein interactions provides a system whereby a rapid translational response to changes in cellular environment can occur.

The observation that p53 can influence the translation of DP-3 type IV transcripts, coupled with the evidence that p53 can bind it RNA *in vitro* (Mosner *et al.*, 1995), suggests that p53 may influence translation of DP-3 directly (Chapter 6; Section 8.5). In addition, other proteins may also interact with DP-3 RNA and influence translation.

8.4 The potential regulation of DP-3 translation by reinitiation

Translation can be regulated by reinitiation. The presence of uORFs and uAUG in the 5'UTR of an mRNA can influence translation at downstream methionines. Usually uORFs have a suppressive influence on translation of a downstream cistron, although this suppression of translation can be regulated (Geballe and Morris, 1994). An example of an mRNA with suppressive uORFs is the 5'UTR of yeast GCN4 mRNA, which contains four short uORFs. GCN4 is a transcription factor required for amino acid biosynthesis. The suppressive influence of the uORFs is reduced during amino acid starvation and GCN4 translation is up regulated. Many cells limit the overall level of translation during nutrient deprivation by phosphorylating the α subunit of eukaryotic initiation factor 2 (eIF2). The phosphorylation of eIF2 has been shown to reduce the suppressive influence of the uORFs of GCN4 mRNA, and GCN4 is translated even though the overall level of translation has been reduced (Hinnebusch, 1996). uORFs can also have a positive effect on downstream translation. Indeed it is uORF1 of GCN4, which under low nutrient conditions enables the ribosomes to bypass the strongly repressive uORF4 and translate GCN4 protein (Hinnebusch, 1996).

DP-3 5'UTRs contain uORFs that may influence translation. Type I, II and IV transcripts all contain M1 and therefore the translation of type I and IV

transcripts may result in translation of short peptides which terminate before M2, allowing reinitiation at M2 and translation of DP-3 M2 products. The distance between the stop signal of these short peptides and the uAUG of M2 is different in type I and type IV and this intercistronic distance may influence the likelihood of reinitiation at M2 and could account for the differences observed in translation from transcripts with these 5'UTRs. It is possible therefore that the regulation of splicing of the 5'UTRs may determine the probability of reinitiation at M2.

The 5'UTR of DP-3 type II contains two in frame initiation signals, M1 and M2 and since type II 5'UTR is only associated with an E+ Q- ORF, translation at M1 or M2 would result in DP-3α or δ respectively. The differential use of in frame initiation codons has been noted in several genes including the mRNA of LAP protein, a liver-enriched transcription factor. uAUG in-frame codons in the 5'UTR of the lap mRNA result in synthesis of LAP protein and an inhibitor of LAP called LIP (Descombes and Schibler, 1991). A switch in the use of AUG codons during development produces a change in the ratio of LAP to LIP, causing an alteration in transcription. Similarly, it is possible that initiation at M1 and M2 of type II may also be regulated in particular tissues or in response to changes in cell conditions, such as stress, and could possibly have dramatic influence on E2F heterodimer formation and influence E2F-dependent gene expression.

The N terminus of DP-3α has been shown to interact with a DP interacting protein (DIP), a POZ domain containing protein that influences the cell cycle, additionally DP-3δ, which lacks the N terminus of DP-3α, also weakly interacts with DIP (de la Luna *et al.*, 1999). Consequently, it is possible that the interaction between DP-3 isoforms and DIP could be influenced by translational initiation, selection of M2 of a type II 5'UTR transcript would result in translation of DP-3δ which only weakly interacts with DIP. Transfection data has suggested that translational initiation occurs at M1 rather than at M2, however it may be possible that a switch to translation initiation at M2 may occur under as yet undefined cellular conditions and that this could influence the interaction of DP-3 and DIP and the functional outcome.

The translational regulation of DP-3 expression via the 5'UTRs may therefore influence E2F formation and function and additionally interactions with

other cellular proteins. Translational regulation of protein expression is often associated with genes that are required rapidly under specific conditions, such as nutrient deprivation. Additionally, many genes with a role in cell growth or proto-oncogenes often have elements such as uORFs and uAUGs within the 5'UTR and also may have a poor initiator context or highly structured 5'UTRs and that expression is tightly regulated (Kozak, 1991, Pain, 1996). Likewise, it is therefore possible that DP-3 is required under specific conditions and that protein expression is also tightly controlled under normal cellular conditions. As discussed (Chapter 6; Section 8.5), the potential influence of p53 on DP-3 translation may provide an indication of the conditions under which DP-3 is required and ultimately, its physiological role.

8.5 The role of DP-3 during activation of p53

The p53 tumour suppresser protein is an important regulator of cellular proliferation and is induced in response to many different stimuli, including DNA damage and other genotoxic stress (Ko and Prives, 1996). Activation of p53 results in cell cycle arrest and in some cases cell death by apoptosis (Ko and Prives, 1996). The data presented suggests that p53 may influence the expression of DP-3 isoforms via the different 5'UTRs of DP-3 transcripts (Chapter 6). This data revealed the specific translational inhibition of transcripts with a type IV 5'UTR. DP-3 transcripts with a type IV 5'UTR have only been observed in association with an M2 E- Q+ ORF (DP-3γ). It is interesting to note that the DP-3γ isoform also contains a putative DNA-PK site, providing a second possible link with DNA damage. It can be speculated that DP-3γ may be actively targeted during the cellular response to DNA damage, via a translational mechanism influenced by p53 and also by phosphorylation by DNA-PK (Figure 8.3). Since p53 inhibits translation of DP-3 type IV transcripts, it is possible that phosphorylation by DNA-PK could correspondingly, have a negative influence on DP-3γ function. Phosphorylation by DNA-PK in the glutamine region may prevent dimerisation with E2F partners or interaction with other cellular factors or perhaps influences transcription functions (Figure 8.3). Alternatively, phosphorylation by DNA-PK may act as a signal for rapid degradation of DP-3γ protein.

The activation of p53 results in cell cycle arrest and sometimes apoptosis, the inhibition of translation by p53 implies that DP-3γ may have a role in promoting cell cycle progression and need to be rapidly inactivated to allow cell cycle arrest and DNA repair. Although at present one can only speculate the role of each different DP-3 isoform, this data does suggest a link between the DP-3γ isoform and activation of the p53 pathway during DNA damage and the targeting of this particular isoform may be important in some circumstances. Additionally, this isoform may be targeted at two levels; p53 inhibits the translation of new DP-3γ protein and DNA-PK may alter DP-3γ function by post-translational modification. If both events do indeed occur *in vivo*, such targeting would imply an important role for DP-3γ in growth regulation.

In contrast to type IV transcripts, this study suggested that p53 has no effect on the translation of the type I 5'UTR and that the other DP-3 isoforms may have different or even opposing roles during p53 activation, DNA damage or the cell cycle. The translation of type III transcripts, which correspond to an M2 E- Q- ORF (DP-3β), was enhanced in the presence of p53, suggesting a role during DNA damage and repair and perhaps in cell cycle arrest. It is conceivable that DP-3β has a negative influence on E2F transcription, perhaps forming repressive E2F heterodimers that cannot bind to DNA or activate transcription (Figure 8.2). Moreover, the formation of heterodimers such as E2F-5/DP-3β, are not able to enter the nucleus without binding to pocket proteins and are observed to have a growth suppressing function (Allen *et al.*, 1997, Figure 1.10). It is also possible that DP-3β may be important in the activation of genes involved with processes such as DNA repair.

It is notable that the only difference between DP-β and DP-3γ is the presence of the extra glutamine residue in DP-3γ that destroys the putative casein kinase II phosphorylation site and creates a theoretical DNA-PK site. The potential for post-translational modification of DP-3 isoforms by two different kinases and the many implications for regulation of protein activity by phosphorylation, combined with the likely translational regulation by an important tumour suppresser protein, emphasises the potential functional importance of DP-3 in regulating cell growth.

8.6 A second DP family member, why?

Analysis of the genomic organisation of murine DP-1 and DP-3 revealed striking similarities in exon organisation, suggesting a common evolutionary origin for these genes (Chapter 3). Analysis has shown that DP-1 is the major DP component of the E2F heterodimer in cells and that DP-3 is difficult to detect in cells (Bandara *et al.*, 1993; Ormondroyd *et al.*, 1995; Chapter 7). Additionally, other organisms such as flies function with a single DP protein, which although highly related to mammalian DP proteins performs functions similar to DP-1 (Dynlacht *et al.*, 1994; Hao *et al.*, 1995). This suggests conservation of not only the E2F regulatory pathway, but also the functions of DP-1 in different species. Hence, it can be speculated that a gene encoding a DP-1-like protein has been conserved through evolution and that via duplication and subsequent functional divergence of this first gene, a second DP protein evolved and was maintained in the genome. The differences between DP-1 and DP-3 might suggest that they may have different roles in cells, but perhaps maintain some level of functional degeneracy. Further analysis of the differences in function of DP-1 and DP-3 is required to answer these questions.

Many genes are found to be members of gene families with similar characteristics, some have distinct functions but family members can also have related functions. An example is the p53 gene family, which has several similarities with the DP family; indeed both p53 and DP-1 are important components of growth control pathways. RNA analysis of the p53 family members, p73 and p51 revealed obvious similarities with DP-3; multiple transcripts were observed, arising from alternative splicing and resulting in different protein isoforms (Ormondroyd *et al.*, 1995; Kaghad *et al.*, 1997; Kaelin, 1999). Additionally, comparison of the p53 and p73 genes, like those presented for DP-1 and DP-3, revealed similar genomic organisations and suggested a common evolutionary origin (Kaghad *et al.*, 1997; Chapter 3). Both DP-1 and p53, are ubiquitously expressed, however p73 and p51, like DP-3, exhibit tissue specific distribution of both RNA and protein (Ormondroyd *et al.*, 1995; Kaelin, 1999; Chapter 4). Functional similarities may exist for family members; *in vitro* assays have shown that like p53, p73 and p51 can bind to canonical p53 DNA binding sites, likewise, all DP-3 isoforms form heterodimers with E2F family members and bind to E2F sites (Ormondroyd *et al.*, 1995; Kaelin,

1999). Further analysis of p73 and p51 has suggested a different role in cells from that of p53 and importantly, p73 and p51 may not act as tumour suppressers (Kaelin, 1999). Similarly, DP-3 does not appear to have the same functions as DP-1 and may also function in a tissue specific manner. Therefore, clear similarities exist between these two families of proteins. The obvious question is why have these second family members evolved? p53 has important functions in cells, however, the p53 family members are not thought to have overlapping functions. Nevertheless, analysis of p53-like DNA binding activities purified from p53-null cells have suggested that different family members may activate different p53-responsive promoters (Kaelin, 1999). It is also likely therefore, that p73 and p51 have evolved to have a more specialised function. The same may be true for DP family members; DP-1 and each of the DP-3 isoforms may modulate the activity of different E2F-responsive promoters, perhaps during different cellular conditions, such as during the cell cycle or conditions of stress. Moreover, different E2F family members may further influence DP activity and adding another level of complexity to the regulation of E2F function.

Comparisons of DP-3 with other genes have also suggested that distinct isoforms may have a non-transcriptional role, as observed for particular WT1 isoforms. Some WT1 isoforms are thought to have a transcriptional role and others have been shown to interact with components of the splicing machinery and have a non-transcriptional role (Larsson *et al.*, 1995; Davies *et al.*, 1998). It is therefore possible that DP-3 isoforms may have very distinct, as yet unknown functions in cells.

8.7 Future Work

Although at present no definite functions have been assigned for DP-3 isoforms, the data presented begins to provide some direction for future research.

8.7.1 Translational regulation of DP-3 expression

Further work is required to confirm the results presented (Chapters 5 and 6). Additionally, an investigation into the mechanism of translational regulation is required. Preliminary RNA binding assays suggested that cellular proteins may bind to the 5'UTRs of DP-3 (data not shown), however these experiments must repeated and extended before firm conclusions can be made. If protein factors are shown to directly interact with DP-3 5'UTR, several techniques are currently available to clone RNA binding proteins allowing further examination of the regulation of DP-3 expression (Harada *et al.*, 1996; Putz *et al.*, 1996; Wang *et al.*, 1996; Sägesser *et al.*, 1997). Additionally, RNA binding assays can be employed to determine whether p53 directly interacts with DP-3 5'UTRs. Further experiments are required to investigate both the mode and functional importance of the translational regulation of DP-3 by p53.

8.7.2 The generation of DP-3 null mice

The generation of both DP-1 and DP-3-null mice and comparisons of the phenotypes would reveal valuable new data relating both to the role of DP-3 *in vivo* and the functional differences between DP-1 and DP-3. The isolation of the DP-3 gene enables the construction of a DP-3 targeting construct and in theory, a DP-3 null mouse could be generated. In addition, mice could be generated that can only express particular DP-3 isoforms, such as E+ or E- isoforms, enabling the study of the influence of DP-3 on subcellular localisation of E2F heterodimers *in vivo* and perhaps some indication of the roles of the individual DP-3 isoforms may be obtained.

It is possible that the loss of DP-1 or DP-3 may result in a lethal phenotype. In such a case, the generation of mice in which the inactivation of the DP-3 gene can be induced in different cell types could be attempted. The use of the Cre/*loxP* recombination system would enable the homozygous deletion of DP-3 in specific cell types only (Gu, *et al.*, 1994; Kühn, *et al.*, 1995; Rossant and Nagy, 1995). In

this system, the gene targeting construct would contain the DP-3 gene flanked by recombination sites (*loxP* sites). Mice containing the DP-3 gene with the recombination sites are generated and then crossed with mice expressing the Cre recombinase under the control of cell type specific promoters. Thus, the deletion of the DP-3 gene can be restricted to the cells expressing Cre only. Using this system it may be possible to study the function of DP-3 in specific tissues or during development.

Figure 8.1

The insertion of a glutamine residue disrupts a potential DP-3 phoshorylation site

a) The nucleotide and amino acid sequence of DP-3α, β and δ in the region of the glutamine insertion. The cascin kinase II consensus sequence is given and the corresponding DP-3 residues are highlighted.

b) The nucleotide and amino acid sequence of DP-3γ in the same region as a), showing the DNA-PK phosphorylation consensus sequence and the corresponding DP-3 residues. The insertion of a glutamine residue in the DP-3 γ isoform disrupts the potential casein kinase II phosphorylation site and creates a theoretical DNA-PK phosphorylation site.

Figure 8.1

a)

DP-3 Q-
α, β, γ

G C T G A T T C G G C T T A T G A T
A D S A Y D

S/D X X D/E

Casein kinase II
consensus site

b)

DP-3 Q+
γ

G C T G A T T C G C A G G C T T A T G A T
A D S Q A Y D

S Q

DNA-PK
consensus site

Figure 8.2

The composition of E2F heterodimers may influence E2F activity

The formation of E2F heterodimers composed of different E2F and DP partners may influence E2F transcriptional activity. The creation of E2F complexes may result in E2F that can bind to E2F sites and initiate E2F-dependent transcription. Alternatively, some combinations of E2F family members with DP family members may result in the formation of E2F heterodimers that can bind to DNA but cannot activate transcription and thereby block E2F-dependent transcription. The formation of complexes that cannot bind to DNA prevents the activation of E2F target genes and may sequester E2F components and prevent the creation of other E2F complexes.

Figure 8.2

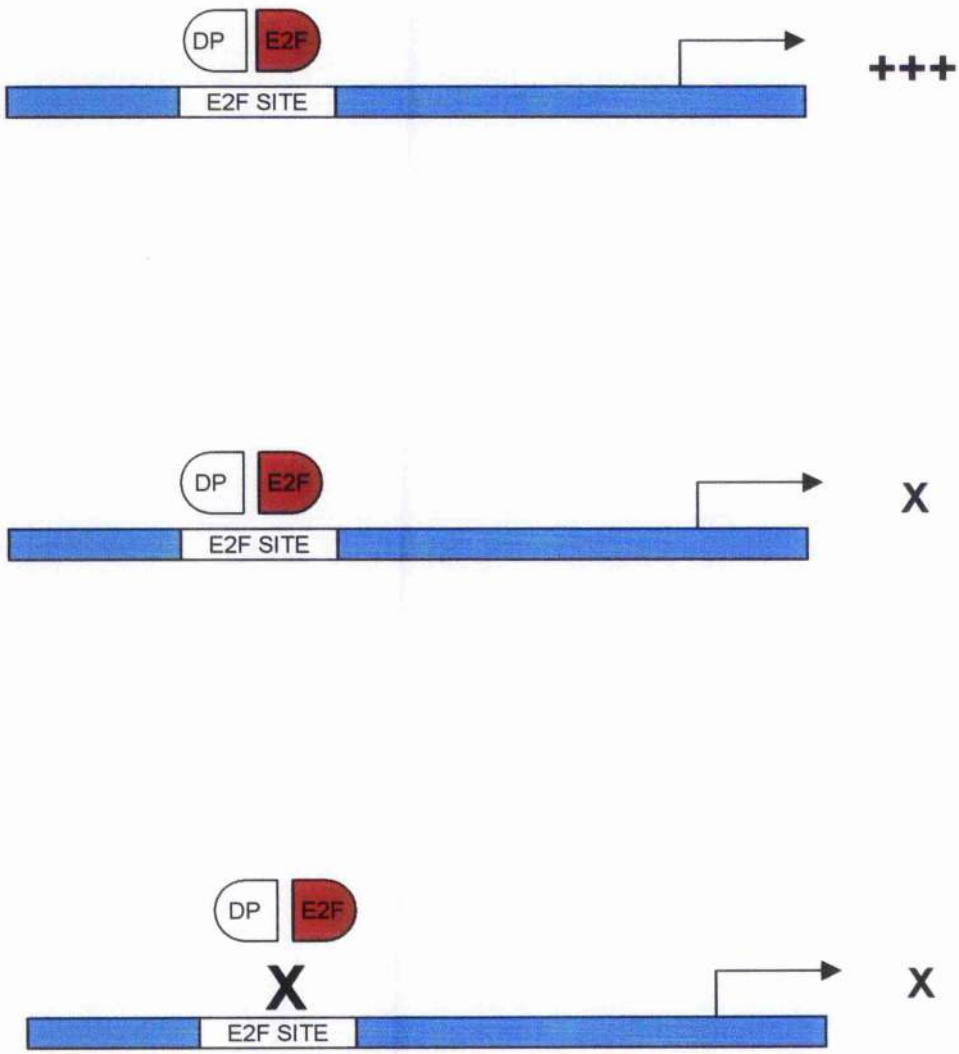
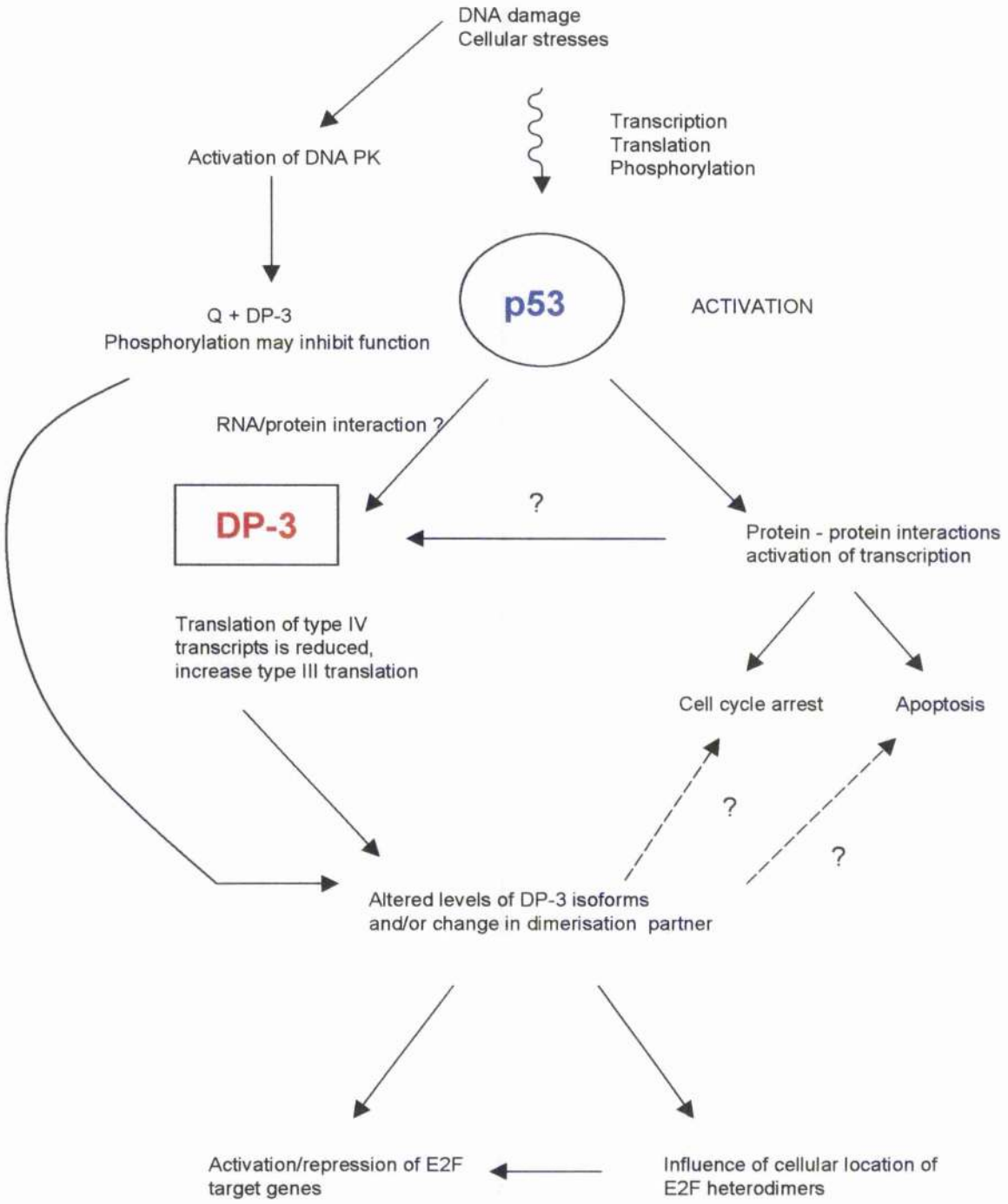


Figure 8.3

Summary of the potential influence of p53 on DP-3

A schematic representation of how p53 may influence the expression of DP-3 isoforms via the 5'UTRs. p53 influences the translation of DP-3 type IV 5'UTR transcripts. This influence may be exerted through a direct RNA/protein interaction. Alternatively, an indirect mechnism may result in the effects observed. p53 may interact with other proteins to exert its effects on type IV translation. p53 has several transcriptional targets and one of these may influence translation of DP-3. Additionally. Q+ DP-3 isoforms contain a DNA-PK site and may therefore be modified by DNA-PK in a DNA damage dependent manner. DP-3 isoforms are therefore potentially targeted by two members of the DNA damage pathway. Such targeting could result in an alteration in the levels of DP-3 isoforms and this may modify the levels of E2F heterodimers in the cell. A change in E2F heterodimers formation may in turn alter E2F activity in the cell and modify the expression of target genes.

Figure 8.3


DNA damage
Cellular stresses
Activation of DNA PK
Transcription
Translation
Phosphorylation
p53
ACTIVATION
Q + DP-3
Phosphorylation may inhibit function
RNA/protein interaction ?
DP-3
?
Protein - protein interactions
activation of transcription
Translation of type IV
transcripts is reduced,
increase type III translation
Cell cycle arrest
Apoptosis
?
?
Altered levels of DP-3 isoforms
and/or change in dimerisation partner
Activation/repression of E2F
target genes
Influence of cellular location of
E2F heterodimers

Acknowledgements

Firstly, I would like to thank Nick La Thangue for his expert supervision and for the opportunity to work in his lab. Secondly I must thank Susana de la Luna for all the practical advice and endless discussions throughout my time in the lab. I would also like to thank everyone in the Cathcart lab in Glasgow for making my time in Glasgow so enjoyable. I would particularly like to thank Marija Krstic-Demonacos and Oonagh Loughran for helpful suggestions and Noriko Shikama for all her help and friendship throughout. Thanks must also go to all the members of EKG-R at Mill Hill for advice during my first year. A big thank you to Allison Chudleigh and Louise Graham for being such good friends when I really needed it.

Thanks to Helen Luetchford just for being my best friend and never letting me down and also to Peter Luetchford for computing support and paying Helen's phone bill!

I would also like to thank all the members of my family for support, both financial and otherwise, over the last ten years, in particular my parents, my grandparents and my brother. Special thanks to Mum for proofreading and for a boot up the backside when I needed it! I would like to dedicate this thesis to Granny Burden, who died in April 1999, I know she would have loved to see it completed.

Final thanks have to go to my husband Lan for everything; you're the best!

References

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

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D.J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nuc. Acids. Res.***25**, 3389-3402.

Anderson, C.W. and Carter, T.H. (1996). The DNA-activated protein kinase-DNA-PK. *Curr. Op. Microbiol.Immunol.* **217**, 91-111.

Bagchi, S., Weinmann, R. and Raychaudhuri, P. (1991). The retinoblastoma protein co-purifies with E2F-I, an E1a-regulated inhibitor of the transcription factor E2F. *Cell* **65**, 1063-1072.

Bandara, L.R., Adamczewski, J.P., Hunt, T. and La Thangue, N.B. (1991). Cyclin-A and the Retinoblastoma gene-product complex with a common transcription factor. *Nature* 352, 249-251.

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

Bandara, L.R., Lam, E.W.F., Sørensen, T.S., Zamanian, M., Girling, R. and La Thangue, N.B. (1994). DP-1-a cell-cycle-regulated and phosphorylated component of transcription factor DRTF1/E2F which is functionally important for recognition by pRb and the adenovirus E4-Orf-6/7 protein. *EMBO J.*, **13**, 3104-3114.

Bandara, L.R. and La Thangue, N.B. (1991). Adenovirus E1a prevents the retinoblastoma gene product from complexing with a cellular transcription factor. *Nature* **351**, 494-497.

Banin, S., Moyal, L., Shieh, S.Y., Taya, Y., Anderson, C.W., Chessa, L., Smorodinsky, N.I., Prives, C., Reiss, Y., Shiloh, Y. and Ziv, Y. (1998). Enhanced phosphorylation of p53 by ATM in response to DNA damage. *Science* **281**, 1674-1677.

Bannister, A.J., Gottlieb, T.M., Kouzarides, T. and Jackson, S.P. (1993). c-Jun is phosphorylated by the DNA-dependent protein kinase *in vitro*; definition of the minimal kinase recognition motif. *Nuc. Acids Res.* **21** (5), 1289-1295.

Barbaux, S., Niaudet, P., Gubler, C., Grünfield, J-P., Jaubert, F., Kutten, F., Fékété, C.N., Souleyreau-Therville, N., Thibaud, E., Fellous, M. and McElreavey, K. (1997). Donor splice site mutations in WT1 are responsible for Fraier syndrome. *Nat. Genet.* **17**, 467-470.

Bates, S., Bonetta, L., MacAllan, D., Parry, D., Holder, A., Dickson, C. and Peters, G. (1994). CDK6 (PLSTIRE) and CDK4 (PSK-J3) are a distinct subset of the cyclin-dependent kinases that associate with cyclin D1. *Oncogene*, **9**, 71-79.

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

Beijersbergen, R.L., Carlee, L., Kerkhoven, R.M. and Bernards, R. (1995). Regulation of the Retinoblastoma protein-related p107 By G1 cyclin complexes. *Genes Dev.* **9**, 1340-1353.

Breuning, W. and Pelletier, J. (1996). A non-AUG translational initiation event generates novel WT1 isoforms. *J. Biol. Chem.* **271**, 8646-8654.

Brugarolas, J., Chandrasekaran, C., Gordon, J.I., Beach, D., Jacks T. and Hannon, G.J. (1995). Radiation-induced cell cycle arrest compromised by p21 deficiency. *Nature* **377**, 552-557.

Buchkovich, K., Duffy, L.A. and Harlow, E. (1989). The retinoblastoma protein is phosphorylated during specific phases of the cell cycle. (1989) *Cell* **58**, 1097-1105.

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

Buckbinder, L., Talbott, R., Velasco-Miguel, S., Takenaka, I., Faha, B., Seizinger, B.R. and Kley, N. (1995). Induction of the growth inhibitor IGF-binding protein 3 by p53. *Nature* **377**, 646-649.

Buratowski, S., Hahn, S., Guarente, L. and Sharp, P.A. (1989). Five intermediate complexes in transcription initiation by RNA polymerase II. *Cell* **56**, 549-561.

Burley, S.K. and Roeder, R.G. (1996). Biochemistry and structural biology of transcription factor IID (TFIID). *Annu. Rev. Biochem.* **65**, 769-799.

Bookstein, R., Shew, J.-Y., Chen, P.-L., Scully, P. and Lee, W.-H. (1989). Suppression of tumourigenicity of human prostate carcinoma cells by replacing a mutated RB gene. *Science* **247**, 712-715.

Canman, C.E., Lim, D.S., Cimprich, K.A., Taya, Y., Tamai, K., Sakaguchi, K., Appella, E., Kastan, M.B. and Siliciano, J.D. (1998). Activation of the ATM kinase by ionising radiation and phosphorylation of p53. *Science* **281**, 1677-1679.

Cao, L., Faha, B., Dembski, M., Tsai, L.-H., Harlow, E. and Dyson, N. (1992). Independent binding of the retinoblastoma gene product and p107 to the transcription factor E2F. *Nature* **355**, 176-179.

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

Chan, F.K.M., Zhang, J., Cheng, L., Shapio, D.N. and Winoto, A. (1995). Identification of human and mouse p19, a novel CDK4 and CDK6 inhibitor with homology to $p16^{Ink4}$. *Mol. Cell. Biol.*, **15**, 2682-2688.

Chellappan, S.P., Hiebert, S., Mudryj, M., Horowitz, J.M. and Nevins, J.R. (1991) The E2F transcription factor is a cellular target for the Rb protein. *Cell* **65**, 1053-1061.

Chen, C. and Okayama, H. (1987). High-efficiency transformation of mammalian cells by plasmid DNA. *Mol.Cell.Biol.* **7**, (8) 2745-2752.

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

Clarke, A.R., Purdie, C.A., Harrison, D.J., Morris, R.G., Bird, C.C., Hooper, M.L and Wyllie, A.H. (1993). Thymocyte apoptosis induced by p53-dependent and independent pathways. *Nature*, **362**, 849-852.

Cobrink, D., Le, M-H., Hannon, G., Mulligan, G., Bronson, R.T., Dyson, N., Harlow, E., Beach, D., Wenberg, R.A. and Jacks, T. (1996). Shared role of the pRB-related p130 and p107 proteins in limb development. *Genes Dev.* **10**, 1633-1644.

Cobrinik, D., Whyte, P., Peeper, D.S., Jacks, T. and Weinberg, R.A. (1993). Cell cycle-specific association of E2F with the p130 E1a-binding protein. *Genes. Dev.* **7**, 2392-2404.

Dagnino, L., Fry, C.J., Bartley, S.M., Farnham, P., Gallie, B.L. and Phillips, R.A. (1997). Expression patterns of the E2F family of transcription factors during murine epithelial development. *Cell Growth Diff.* **8**, 553-563.

Davies, R.C., Calvio, C., Bratt, E., Larsson, S.H., Lamond, A.I. and Hastie, N.D.

(1998). WT1 interacts with the splicing factor U2AF65 in an isoform-dependent manner and can be incorporated into spliceosomes. *Genes. Dev.* **12**, 3217-3225.

DeBondt, H.L., Rosenblatt, J., Jancarik, J., Jones, H.D., Morgan, D.O. and Kim, S.-H. (1993). Crystal structure of cyclin dependent kinase 2. *Nature*, **363**, 595-602.

Deng, C., Zhang, P., Harper, J.W., Elledge, S.J and Leder, P. (1995). Mice lacking p21 (CIP1/WAF1) undergo normal development, but are defective in G1 checkpoint control. *Cell*, **82**, 675-684.

DeGregori, J., Leone, G., Ohtani, K., Miron, A. and Nevins, J.R. (1995). E2F-1 accumulation bypasses a G(1) arrest resulting from the inhibition of G(1) cyclin-dependent kinase-activity. *Genes. Dev.* **9**, 2873-2887.

Descombes, P. and Schibler, U. (1991). A liver-enriched transcriptional activator protein, LAP, and a transcriptional inhibitory protein,LIP, are translated from the same mRNA. *Cell* **67**, 569-579.

Donehower, L.A. and Bradley, A. (1993). The tumour suppressor p53. *Biochim. Biophys. Acta*, **1155**, 181-205.

Donehower, L.A., Harvey, M., Slagle, B.L., McArthur, M.J., Montgomery Jr, C.A., Butel, J.S. and Bradley, A. (1992). Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. *Nature* **356**, 215-221.

Dowdy, S.F., Hinds, P.W., Louie, K., Reed, S.I., Arnold, A. and Weinberg, R.A. (1993). Physical interaction of the retinoblastoma protein with human D cyclins. *Cell* **73**, 499-511.

Dutta, A. and Stillman, B. (1992). cdc2 family kinases phosphorylate a human cell DNA replication factor, RPA, and activate DNA replication. *EMBO J.*, **11**, 2189-2199.

Dulic, V., Kaufmann, W.K., Wilson, S.J., Tlsty, T.D., Lees, E., Harper, J.W., Elledge, S.J. and Reed, S.I. (1994). p53-dependent inhibition of cyclin-dependent kinase activities in human fibroblasts during radiation-induced G1 arrest. *Cell* **76**, 1013-1023.

Dynlacht, B.D., Brook, A., Dembski, M., Yenush, L. and Dyson, N. (1994). DNA-binding and trans-activation properties of *Drosophila* E2F and DP proteins. *Proc. Natl. Acad. Sci. USA*. **91**, 6359-6363.

Dynlacht, B.D., Flores, O., Lees, J.A. and Harlow, E. (1994). Differential regulation of E2F transactivation by cyclin-CDK2 complexes. *Genes Dev.* **8**, 1772-1786.

Dyson, N. (1998). The regulation of E2F by the pRB-family proteins. *Genes. Dev.* **12**, 2245-2262.

Dyson, N. Howley, P.M., Munger, K. and Harlow, E. (1989). The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. *Science* **243**, 934-936.

Eckner, R., Yao, T.P., Oldread, E., and Livingston, D.M. (1996). Interaction and functional collaborationof p300/CBP and bHLH proteins in muscle and B-cell differentiation. *Genes Dev.* **10**, 2478-2490.

El-Deiry, W.S., Tokino, T., Velculescu, V.E., Levy, D.B., Parsons, R., Trent, J.M., Lin, D., Mercer, W.E., Kinzler, K.W. and Vogelstein, B. (1993). Waf1, a potential mediator of p53 tumor suppression. *Cell*, **75**, 817-825.

Elenbaas, B., Dobbelstein, M., Roth, J., Shenk, T. and Levine, A.J. (1996). The MDM2 oncoprotein binds specifically to RNA through its RING finger domain. *Mol. Med.* **2** (4), 439-451.

Ewen, M.E., Oliver, C.J., Sluss, H.K., Miller, S.J., and Peeper, D.S. (1995). p53-dependent repression of CDK4 translation in TGF-β-induced G_1 cell-cycle arrest. *Genes Dev.* **9**, 204-217.

Ewen, M.E., Sluss, H.K., Sherr, C.J., Matsushime, H., Kato, J. and Livingston, D.M. (1993a). Functional interactions of the retinoblastoma protein with mammalian D-type cyclins. *Cell* **73**,487-497.

Fagan, R., Flint, K. and Jones, N. (1994) Phosphorylation of E2F-1 modulates its interaction with the Retinoblastoma gene product and the adenoviral E4 19 kDa protein. *Cell* **78**, 799-811.

Faha, B., Ewen, M.E., Tsai, L.-H., Livingston, D.M. and Harlow, E. (1992). Interaction between human cyclin A and the adenovirus E1a-associated p107 protein. *Science* **255**, 87-90.

Fero, M.L., Rivkin, M., Tasch, M., Porter, P., Carow, C.E., Firpo, E., Polyak, K., Tsai, L.H., Broudy,, V., Perlmutter, R.M., Kaushansky, K. and Roberts, J.M. (1996). A syndrome of multiorgan hyperplasia with features of gigantism, tumorigenesis, and female sterility in $p27^{KIP1}$- deficient mice. *Cell* **85**, 733-744.

Fesquet, D., Labbe, J.C., Derancourt, J., Capony, J.P., Galas, S., Girad, F., Lorca, T., Shuttleworth, J., Doree, M. and Cavadore, J.C. (1993). The MO15 gene encodes the catalytic subunit of a protein kinase that activates cdc2 and other cyclin-dependent kinases (CDKs) through phosphorylation of Thr161 and its homologues. *EMBO J.*, **12**, 3111-3121.

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

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

Fu, L. and Benchimol, S. (1997). Participation of the human p53 3'UTR in translational repression and activation following γ-irradiation. *EMBO.J.* **16** (13), 4117-4125.

Fu, L., Minden, M.D. and Benchimol, S. (1996). Translational regulation of human p53 gene expression. *EMBO. J.* **15** (16), 4392-4401.

Geballe,A.P. and Morris, D.R. (1994). Initiation codons within 5'-leaders of mRNAs as regulators of translation. *TIBS* **19**, 159-164.

Ginsberg, D., Vairo, G., Chittenden, T., Xiao, Z.X., Xu, G.F., Wydner, K.L., Decaprio, J.A., Lawrence, J.B. and Livingston, D.M. (1994). E2F-4, a new member of the E2F transcription factor family, interacts with p107. *Genes Dev.* **8**, 2665-2679.

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

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

Girard, F., Stausfeld, U., Fernandez, A. and Lamb, N.J.C. (1991). Cyclin A is required for the onset of DNA replication in mammalian fibroblasts. *Cell* **67**,1169-1179.

Goodrich, J.A. and Tjian, R. (1994). Transcription factors IIE, IIH and ATP hydrolysis direct promoter clearance by RNA polymerase II. *Cell*, 77, 145-156.

Gopalkrishnan, R.V., Dollé, P., Mattei, M-G., La Thangue, N.B. and Kedinger, C. (1996). Genomic structure and developmental expression of the mouse cell cycle regulatory transcription factor DP1. *Oncogene* **13**, 2671-2680.

Gopalkrishnan, R.V., Lam, E.W-F. and Kedinger, C. (1998).The p53 tumor suppressor inhibits transcription of the TATA-less mouse DP1 promoter. *J. Biol. Chem.* **273** (18), 10972-10978.

Grossman, S.R., Perez, M., Kung, A.L., Joseph, M., Mansur, C., Xiao, Z.-X., Kumar, S., Howley, P.M. and Livingston, D.M. (1998). p300/MDM2 complexes participate in MDM2-mediated p53 degradation. *Molecular Cell* **2**, 405-415.

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

Gu, H., Marth, J.D., Orban, P.C., Mossmann, H. and Rajewsky, K. (1994). Deletion of a DNA polymerase β gene segment in T cells using cell type-specific gene targeting. *Science* **265**, 103-106.

Guan, K.-L., Jenkins, C.W., Li, Y., Nichols, M.A., Wu, X., O'Keefe, C.L., Matera, A.G. and Xiong, Y. (1994). Growth suppression by p18, a p16INK4/MTS1 and p14INK4B/MTS2-related CDK6 inhibitor correlates with wild-type pRb function. *Genes Dev.*, **8**, 2939-2952.

Gulbis, J.M., Kelman, Z., Hurwitz, J., O'Donnell, M. and Kuriyan, J. (1996). Structure of the C-terminal region of p21 (WAF1/CIP1) complexed with human PCNA. *Cell* **87**, 297-306.

Gunkel, N., Yano, T., Markussen, F-H., Olsen, L.C. and Ephrussi, A. (1998). Localisation-dependent translation requires a functional interaction between the 5' and 3' ends of *oskar* mRNA. *Genes. Dev.* **12**, 1652-1664.

Ha, I., Roberts, S., Maldonado, E., Sun, X., Kim, L., Green, M. and Reinberg, D. (1993). Multiple functional domains of human transcription factor IIB: distinct interactions with two general transcription factors and RNA polymerase II. *Genes Dev.*, **7**, 1021-1032.

Hagemeier, C., Cook, A. and Kouzarides, T. (1993). The Retinoblastoma protein binds E2F residues required for activation *in-vivo* and TBP binding *in-vitro*. *Nuc. Acids Res.* **21**, 4998-5004.

Hampsey, M. (1997). A SAGA of histone acetylation and gene expression. *Trends. Genet.* **13** (11), 427-429.

Hannon, G.J., Demetrick, D. and Beach, D. (1993). Isolation of the RB-related p130 through its interaction with cdk2 and cyclins. *Genes Dev.* **7**, 2378-2391.

Hannon, G.J. and Beach, D. (1994). p15INK4B is a potential effector of TGF-beta-induced cell cycle arrest. *Nature*, **371**, 257-261.

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

Harada, K., Martin S.S. and Frankel, A.D. (1996). Selection of RNA-binding peptides *in vivo*. *Nature* **380**, 175-179.

Harper, J.W., Adami, G.R., Wei, N., Keyomarsi, R. and Elledge, S.J. (1993). The p21 cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. Cell 75, 805-816.

Harper, J.W., Elledge, S.J., Keyomarsi, K., Dynlacht, B., Tsai, L.-H., Zhang, P., Dobrowolski, S., Bai, C., Connell-Crowley, L., Swindell, E., Fox, M.P. and Wei, N. (1995). Inhibition of cyclin-dependent kinases by p21. *Mol. Biol. Cell*, **6**, 387-400.

Hassig, C.A. and Schrieber, S.L. (1997). Nuclear histone acetylases and deacetylases and transcriptional regulation: HATs off to HDACs. *Curr. Op. Chem. Biol.* **1**, 300-308.

Hastie, N.D. (1994). The genetics of Wilm's tumour-A case of disrupted development. *Ann. Rev. Biochem.* **28**, 523-558.

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

Haupt, Y., Maya, R., Kazaz, A. and Oren, M. (1997). MDM2 promotes the rapid degradation of p53. *Nature* **387**, 296-303.

Hazelrigg, T. (1999). The destinies and destinations of RNAs. *Cell* **95**, 451-460.

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

Helin, K., Lees, J.A., Vidal, M., Dyson, N., Harlow, E. and Fattaey, A. (1992). A cDNA encoding properties of the transcription factor E2F. *Cell* **70**, 337-350.

Helin, K., Wu, C-L., Fattaey, A.R., Lees, J.A., Dynlacht, B.D., Ngwu, C. and Harlow, E. (1993). Heterodimerisation of the transcription factors E2F-1 and DP-1 leads to cooperative *trans*-activation. *Genes Dev.* **7**, 1850-1861.

Hengst, L. and Reed, S.I. (1996). Translational control of $p27^{Kip1}$ accumulation during the cell cycle. *Science* **271**, 1861-1864.

Herwig, S. and Strauss, M. (1997). The retinoblastoma protein: a master regulator of cell cycle, differentiation and apoptosis. *Eur. J. Biochem.* **246**, 581-601.

Hijmans, E.M., Voorhoeve, P.M., Beijersbergen, R.L., Vantveer, L.J. and Bernards, R. (1995). E2F-5, a new E2F family member that interacts with p130 *in vivo*. *Mol. Cell. Biol.*, **15**, 3082-3089.

Hinnebusch, A.G. (1996). Translational control of GCN4: gene-specific regulation by phoshorylation of eIF2. In Translational Control. J.W.B. Hershey, M.B. Mathews, and N. Sonenburg, eds. (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press), pp. 199-244.

Hirai, H., Roussel, M.F., Kato, J.-Y., Ashmun, R.A. and Sherr, C.J. (1995). Novel INK4 proteins, p19 and p18, are specific inhibitors of the cyclin D-dependent kinases CDK4 and CDK6. *Mol. Cell. Biol.*, **15**, 2672-2681.

Hocy, T., Weinzierl, R.O.J., Gill, G., Chen, J.-L., Dynlacht, B.D. and Tjian, R. (1993). Molecular cloning and functional analysis of *Drosophila* TAF110 reveals properties expected of coactivators. *Cell* **72**,247-260.

Hofmann, F. and Livingston, D.M. (1996). Differential effects of cdk2 and cdk3 on the control of pRb and E2F function during G_1 exit. *Genes Dev.* **10**, 851-861.

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

Honda, R. and Yasuda, H. (1999). Association of $p19^{ARF}$ with MDM2 inhibits ubiquitin ligase activity of MDM2 for tumour suppressor p53. *EMBO.J.* **18** (1), 22-27.

Horikoshi, N., Usheva, A., Chen, J., Levine, A.J., Weinmann, R. and Shenk, T. (1995). Two domains of p53 interact with the TATA-binding protein and the adenovirus 13S E1A protein disrupts the association, relieving p53-mediated transcriptional repression. *Mol. Cell. Biol.*, **15**, 227-234.

Hsiao, K-M., McMahon, S.L. and Farnham, P.J. (1994). Multiple DNA elements are required for the growth regulation of the mouse E2F1 promoter. *Genes Dev.* **8**, 1526-1537.

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

Huang, H.-J.S., Yee, J.-K., Shew, J.-Y., Chen, P.-L., Bookstein, R., Freidmann, T.,Lee,E.Y.-H.P. and Lee, W.-H. (1988). Suppression of the neoplastic phenotype by replacement of the Rb gene in human cancer cells. *Science* **242**, 1563-1566.

Huang, Y. and Carmichael, C.G. (1996). Role of polyadenylation in nucleocytoplasmic transport of mRNA. *Mol. Cell Biol.* **16** (4), 1534-1542.

Hupp, T.R., Meek, D.W., Midgley,C.A. and Lane, D.P. (1992). Regulation of the specific DNA binding functions of p53. *Cell* **71**, 875-886.

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

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

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

Jackson, R.J. and Wickens, M. (1997). Translational controls impinging on the 5'-untranslated region and initiation factor proteins. *Curr. Op. Genet. Dev.* 7, 233-241.

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

Johnson, D.G., Schwarz, J.K., Cress, W.D. and Nevins, J.R. (1993). Expression of transcription factor E2F1 induces quiescent cells to enter S phase. *Nature* **365**, 349-352.

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

Kaelin, W.G. Jr. (1999). The emerging p53 gene family. *J. Nat. Cancer. Inst.* **91** (7), 594-598.

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

Kaghad, M., Bonnet, H., Yang, A., Creancier, L., Biscan, J.C., Valent, A. (1997). Monoallelelically expressed gene related to p53 at 1p36, a region frequently deleted in neuroblastomas and other human cancers. *Cell* **90**, 809-819.

Kamb, A., Gruis, N.A., Weaver, F.J., Liu, Q., Harshman, K., Tavtigian, S.V., Stockert, E., Day, R.I., Johnson, B.E. and Skolnick, M.H. (1994). A cell cycle regulator potentially involved in genesis of many tumour types. *Science*, **264**, 436-440.

Kamijo, T., Zindy, F., Roussel, M.F., Quelle, D.E., Downing, J.R., Ashmun, R.A., Grosveld, G. and Sherr, C.J. (1997). Tumor suppression at the mouse *INK4a* locus mediated by the alternative reading frame product $p19^{ARF}$. *Cell* **91**, 649-659.

Kamijo, T., Weber, J.D., Zambetti, G., Zindy, F., Roussel, M.F. and Sherr, CJ. (1998). Functional and physical interactions of the ARF tumor suppressor with p53 and Mdm2. *Proc. Natl. Acad. Sci. USA*. **95**, 8292-8297.

Karlseder, J., Rotheneder, H. and Wintersberger, E. (1996). Interaction of Sp1 with the growth- and cell cycle-regulated transcription factor E2F. *Mol Cell Biol.* **16** (4), 1659-1667.

Kastan, M.B., Onyekwere, O., Sidransky, D., Vogelstein, B. and Craig, R.W. (1991). Participation of p53 protein in the cellular-response to DNA damage. *Cancer Research*, **51**, 6304-6311.

Kastan, M.B., Zhan, Q., El-Deiry, W.S., Carrier, F., Jacks, T., Walsh, W.V., Plunkett, B.S., Vogelstein, B. and Fornace, A.J. (1992). A mammalian cell cycle checkpoint pathway utilizing p53 and GADD45 is defective in ataxia-telangiectasia. *Cell* **71**, 587-597.

Kiyokawa, H., Kineman, R.D., Manova-Todorova, K.O., Soares, V.C., Hoffman, E.S., Ono, M., Khanam, D., Hayday, A.C., Frohman, L.A. and Koff, A. (1996). Enhanced growth of mice lacking the cyclin-dependent kinase inhibitor $p27^{Kip}$. *Cell 85*, 721-732.

Ko, L. and Prives, C. (1996). p53: Puzzle and Paradigm. *Genes. Dev.* **10**, 1054-1072.

Kovesdi, I., Reichel, R. and Nevins, J.R. (1987). Role of an adenovirus E2 promoter binding factor in E1a-mediated coordinate gene control. *Proc.Natl.Acad.Sci.USA* **84**, 2180-2184.

Kozak, M. (1989) Circumstances and mechanisms of inhibition of translation by secondary structure in eucaryotic mRNAs. *Mol. Cell. Biol.* **9** (11), 5134-5142.

Kozak, M. (1991). An analysis of vertebrate mRNA sequences: intimations of translational control. *J. Cell Biol.* **115** (4), 887-903.

Kozak, M. (1992). Regulation of translation in eukaryotic systems. *Ann. Rev. Cell Biol.* **8**, 197-225.

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

Kubbutat, M.H.G., Lones, S.N. and Vousden, K.H. (1997). Regulation of p53 stability by Mdm2. *Nature* **387**, 299-303.

Kühn, R., Schwenk, F., Aguet, M. ad Rajewsky, K. (1995). Inducible gene targeting in mice. *Science* **269**, 1427-1429.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

La Thangue, N.B. (1994). DRTF1/E2F: An expanding family of heterodimeric transcription factors implicated in cell cycle control. *Trends Biol. Sci.* **19**, 108-114.

La Thangue, N.B. and Rigby, P.W.J. (1987). An adenovirus E1a-like transcription factor is regulated during the differentiation of murine embryonal carcinoma stem cells. *Cell* **49**, 507-513.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680-685.

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

Lam, E. W-F. and Watson, R.J. (1993). An E2F-binding site mediates cell-cycle regulated repression of mouse B-*myb* transcription. *EMBO.J.* **12** (7), 2705-2713.

Larsson, S.H., Charlieu, J-P., Miyagawa, K., Engelkamp, D., Rassoulzadegan, M., Ross, A., Cuzin, F., van Heyningen, V. and Hastie, N.D. (1995). Subnuclear lacaisation of WT1 in splicing or transcription factor domains is regulated by alternative splicing. *Cell* **81**, 391-401.

Lawence, J.B. and Singer, R.H. (1986). Intracellular localisation of messenger RNAs for cytoskeletal proteins. *Cell* **45**, 407-415.

Lee, C-W., Sørensen, T.S., Shikama, N. and La Thangue, N.B. (1996). Functional interplay between p53 and E2F through co-activator p300. *Oncogene* **16**, 2696-2710.

Lee, EY-HP., Chang, C-Y., Hu, N., Wang, Y-CJ., Lai, C-C., Herrup, K., Lee, W-H. and Bradley, A. (1992). Mice deficient for Rb are non-viable and show defects in neurogenesis and haematopoiesis. *Nature* **359**, 288-294.

Lee, M.-H., Reynisdottir, I. and Massague, J. (1995). Cloning of $p57^{KIP2}$, a cyclin-dependent kinase inhibitor with unique domain structure and tissue distribution. *Genes Dev.*, **9**, 639-649.

Lee, M-H., Williams, B.O., Mulligan, G., Mukai, S., Bronson, R.T., Dyson, N., Harlow, E. and Jacks, T. (1996). Targeted disruption of *p107*: functional overlap between *p107* and *Rb*. *Genes Dev.* **10**, 1621-1632.

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

Lees-Miller, S.P., Sakaguchi, K., Ullrich, S.J., Appella, E. and Anderson, C.W. (1992). Human DNA-activated protein kinase phosphorylates serines 15 and 37 in

the amino-terminal transactivation domain of human p53. *Mol. Cell. Biol.* **12**, 5041-5049.

Leone, G., DeGregori, J., Yan, Z., Jakoi, L., Ishida, S., Williams, R.S. and Nevins, J.R. (1998). E2F3 activity is regulated during the cell cycle and is required for the induction of S phase. *Genes Dev.* **12**, 2120-2130.

Leveillard, T., Andera, L., Bissonette, N., Schaeffer, L., Bracco, L., Egly, J-M. and Wasylyk, B. (1996). Functional interactions between p53 and the TFIIH complex are affected by tumour-assiciated mutations. *EMBO. J.* **15** (7), 1615-1624.

Levine, AJ. (1997). p53, the cellular gatekeeper for growth and division. *Cell* **88**, 323-331.

Lew,D.J., Dulic,V. and Reed,S.I. (1991). Isolation of three novel human cyclins by rescue of G1 cyclin (CLN) function in yeast. *Cell* **66**,1197-1206.

Li, Y., Slansky, J.E., Myers, D.J., Drinkwater, N.R., Kaelin, W.G. and Farnham, P.J. (1994). Cloning, chromosomal location and characterisation of mouse E2F-1. *Mol. Cell Biol.* **14**, 1861-1869.

Li, Y., Graham, C., Lacy, S., Duncan, A.M.V. and Whyte, P. (1993). The adenovirus-E1a-associated 130-Kd protein is encoded by a member of the Retinoblastoma gene family and physically interacts with cyclin-A and cyclin-E. *Genes Dev.* **7**, 2366-2377.

Lill, N., Grossman, S., Ginsberg, D., DeCaprio, J., and Livingston, D. (1997). Binding and modulation of p53 by p300/CBP coactivators. *Nature* **387**, 823-827.

Lin, S-Y., Black, A.R., Kostic, D., Pajovic, S., Hoover, C.N. and Azizkhan, J.C. (1996). Cell cycle-regulated association of E2F1 and Sp1 is related to their functional interaction. *Mol. Cell Biol.* **16** (4), 1668-1675.

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

Little, M. and Wells, C. (1997). A clinical overview of WT1 gene mutations. *Hum. Mut.***9**, 209-225.

Lui, N., Lucibello, F.C., Engeland, K. and Muller, R. (1998). A new model of cell cycle-regulated transcription: repression of the cyclin A promoter by CDF-1 and anti-repression by E2F. *Oncogene* **16** (23), 2957-2963.

Lu, X. and Lane, D.P. (1993). Differential induction of transcriptionally active p53 following UV or ionizing radiation: defects in chromosome instability syndromes? *Cell* **75**, 765-778.

Lu, H. and Levine, A.J. (1995). Human TAF(II)31 protein is a transcriptional coactivator of the p53 protein. *Proc. Natl Acad. Sci. U.S.A.* **93**, 5154-5158.

Lucibello, F.C., Truss, M., Zwicker, J., Ehlert, F., Beato, M. and Muller, R. (1995a) Periodic *cdc25C* transcription is mediated by a novel cell cycle-regulated repressor element (CDE). *EMBO. J.* **14** (1), 132-142.

Lucibello, F.C., Liu, N., Zwicker, J., Gross, C. and Muller, R. (1995b). The differential binding of E2F and CDF repressor complexes contributes to the timing of cell cycle-regulated transcription. *Nuc. Acids. Res.* **25** (24), 4921-4925.

Lukas, J., Bartkova, J., Rohde, M., Strauss, M. and Bartek, J. (1995a). Cyclin D1 is dispensable for G1 control in Retinoblastoma gene- deficient cells independently of cdk4 activity. *Mol. Cell. Biol.* **15**, 2600-2611.

Lukas, J., Muller, H., Bartkova, J., Spitkovsky, D., Kjeruff, A.A., Jansen-Durr, P, Strauss, M. and Bartek, J. (1994). DNA tumour virus oncoproteins and

retinoblastoma gene mutations share the ability to relieve the cells requirements for cyclin D1 function in G1. *J. Cell, Biol.* **125**, 625-638.

Lukas, J., Parry, D., Aagaard, L., Mann, D.J., Bartkova, J.,Strauss, M., Peters, G. and Bartek, J. (1995b). Retinoblastoma-protein-dependent cell-cycle inhibition by tumour suppressor p16. *Nature* **375**, 503-506.

Lukas, J., Petersen, B.O., Holm, K., Bartek, J. and Helin, K. (1996). Deregulated expression of E2F family members induces S-phase entry and overcomes $p16^{INK4A}$-mediated growth suppression. *Mol. Cell Biol.* **16**, 1047-1057.

de la Luna, S., Allen, K. E., Mason, S. L. and La Thangue, N. B. (1999). Intergration of a growth-suppressing BTB/POZ domain protein with the DP component of the E2F transcription factor. *EMBO. J.* **18** (1), 212-228.

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

Macleod, K.F., Hu, Y. and Jacks, T. (1996). Loss of *Rb* activates both *p53*-dependent and independent cell death pathways in the developing muse nervous system. *EMBO. J.* **15** (22), 6178-6188.

Macleod, K.F., Sherry, N., Hannon, G., Beach, D., Tokino, T., Kinzler, K., Vogelstein, B. and Jacks, T. (1995). p53-dependent and independent expression of p21 during cell-growth, differentiation, and DNA-damage. *Genes. Dev.* **9**, 935-944.

Maccjak, D.G and Sarnow, P. (1991). Internal initiation of translation mediated by the 5' leader of a cellular mRNA. *Nature* **353**, 90-94.

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

Maniatis, T., E. F. Fritsch and J. Sambrook (1989). Molecular cloning: A laboratory manual, 2nd Ed. New York, Cold Spring Harbour Laboratory Press.

Manley, J.L. and Proudfoot, N.J. (1994). RNA 3' ends: formation and function-meeting review. *Genes Dev.* **8**, 259-264.

Marechal, V., Elenbaas, B., Piette, J., Nicolas, J-C. and Levine, A.J. (1994). The ribosomal L5 protein is associated with mdm-2 and mdm-2-p53 complexes. *Mol. Cell Biol.* **14** (11), 7414-7420.

Martin, K., Trouche, D., Hagemeier, C., Sørensen, T.S., La Thangue, N.B. and Kouzarides, T. (1995). Stimulation of E2F-1/DP-1 transcriptional activity by mdm2 oncoprotein. *Nature*, **375**, 691-694.

Matsushime, H., Roussel, M.F., Ashmun, R.A. and Sherr, C.J. (1991). Colony-stimulating factor 1 regulates novel cyclins during the G1 phase of the cell cycle. *Cell* **65**,701-713.

Matsuoka, S., Edwards, M.C., Bai, C., Parker, S., Zhang, P., Baldini, A., Harper, J.W. and Elledge, S.J. (1995). p57KIP2, a structurally distinct member of the p21KIP1 cdk inhibitor family, is a candidate tumor suppressor gene. *Genes Dev.*, **9**, 650-662.

Mayol, X., Garriga, J. & Grana, X. (1995). Cell cycle-dependent phosphorylation of the Retinoblastoma-related protein p130. *Oncogene* **11**, 801-808.

Means, A.L., Slansky, J.E., McMahon, S.L., Knuth, M.W. and Farnham, P.J. (1992). The HIP1 binding site is required for growth regulation of the dihydorfolate reductase gene promoter. *Mol.Cell.Biol.* **12**, 1054-1063.

Melefors, O. and Hentze, M.W. (1993). Translational regulation by mRNA/protein interactions in eukaryotic cells; Ferritin and beyond. *BioEssays* **15** (2), 85-90.

Merrick, W.C. and Hershey, J.W.B. (1996). The pathway and mechanism of eukaryotic protein synthesis. In Translational Control. J.W.B. Hershey, M.B. Mathews, and N. Sonenburg, eds. (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press), pp. 31-69.

Miki, Y., Swensen, J., Shattuck-Eidens, P., Futreal, A., Harshman, K., Tavtigan, S., Liu, Q., Cochran, C., Bennett, L.M., Ding, W., Bell, R., Rosenthal, J., Hussey, C., Tran, T., McClure, M., Frye, C., Hattier, T., Phelps, R., Haugen-Strano, A., Katcher, H., Yakumo, K., Ghoami, Z., Shaffer, D., Stone, S., Bayer, S., Wray, C., Bogden, R., Dayananth, P., Ward, J., Tonin, P., Narod, S., Bristow, P.K., Norris, F.H., Helvering, L., Morrison, P., Rosteck, P., Lai, M., Barrett, J.C., Lewis, C., Neuhausen, S., Cannon-Albright, L., Goldgar, D., Wiseman, R., Kamb, A. and Skolnick, M.H. (1994). A strong candidate for the breast and ovarian cancer susceptibility gene *BRCA1*. *Science* **266**, 66-71.

Malkin, D. (1994). p53 and the Li-Fraumeni syndrome. *Biochem Biophys Acta.* **1198**, 197-213.

Mitchell, P.J. and Tjian, R. (1989). Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. *Science* **245**,371-378.

Mittnacht, S. (1998). Control of pRB phosphorylation. *Curr. Op. Genet. Dev.* **8**, 21-27.

Mizzen, C., Yang, X., Kokubo, T., Brownwell, J., Bannister, A., Owen-Hughes, T., Workman, J., Wang, L., Berger, S., Kouzarides, T., Nakatani, Y., and Allis, C.D. (1996). The $TAF_{II}250$ subunit of TFIID has histone acetyltransferase activity. *Cell* **87**, 1261-1270.

Miyashita, T. and Reed, J.C. (1995). Tumor suppressor p53 is a direct transcriptional activator of the human *bax* gene. *Cell* **80**, 293-299.

Moberg, K., Starz, M.A. and Lees, J.A. (1996). E2F-4 switches from p130 to p107 and pRb in response to cell cycle reentry. *Mol. Cell Biol.* **16** (4), 1436-1449.

Momand, J., Zambetti, G.P., Olson, D.C., George, D. and Levine, A.J. (1992). The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. *Cell* **69**, 1237-1245.

Morgenbesser, S.D., Williams, B.O., Jacks, T. and DePinho, R.A. (1994). *p53*-dependent apoptosis produced by *Rb*-deficiency in the developing mouse lens. *Nature* **371**, 72-74.

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

Mosner, J., Mummenbrauer, T., Bauer, C., Sczakial, G., Grosse, F. and Deppart, W. (1995). Negative feedback regulation of wild-type p53 biosynthesis. *EMBO. J.* **14** (18), 4442-4449.

Mountford, P.S. and Smith, A.G. (1995). Internal ribosome entry sites and dicistronic RNAs in mammalian transgenesis. *Trends Genet.* **11** (5), 179-184.

Müller, H., Moroni, M.C., Vigo, E., Petersen, B.O., Bartek, J. and Helin, K. (1997). Induction of S-phase entry by E2F transcription factors depends on their nuclear localisation. *Mol. Cell. Biol.* **17** (9), 5508-5520.

Murre, C., McCaw, P.S., Vaessin, H., Caudy, M., Jan, L.Y., Jan, Y.N., Cabrera, C.V., Buskin, J.N., Hauschka, S.D., Lasser, A.B., Weintraub, H. and Baltimore, D., (1989). Interaction between heterologous helix-loop-helix proteins generate complexes that bind specifically to a common DNA sequence. *Cell* **58**,537-544.

Nakayama, K., Ishida, N., Shirane, M., Inomata, A., Inoue, T., Shishido, N., Horii, I. and Loh, D.Y. (1996). Mice lacking $p27^{KIP1}$ display increased body size, multiple organ hyperplasia, retinal dysplasia, and pituitary tumors. *Cell* **85**, 707-720.

Neuberg, M., Schuermann, M., Hunter, J.B. and Müller, R. (1989). Two functionally different regions in fos are required for the sequence-specific DNA interaction of the fos/jun protein complex. *Nature* **338**,589-590.

Neuman, E., Flemington, E.K., Sellers, W.R. and Kaelin, W.G. (1994). Transcription of the E2F1 gene is rendered cell cycle-dependent by E2F DNA binding sites within its promoter. *Mol. Cell Biol.* **14**, 6607-6615.

O'Conner, D.J., Lam, E.W.-F., Griffin, S., Zhong, S., Leighton, L.C., Burbidge, S.A. and Lu, X. (1995). Physical and functional interactions between p53 and cell cycle co-operating transcription factors, E2F1 and DP1. *EMBO J.* **14**,6184-6192.

Ohtani, K. and Nevins, J.R. (1994). Functional properties of a Drosophila homolog of the E2F-1 gene. *Mol. Cell Biol.* **14** (3), 1603-1612.

Ohtsubo, M. and Roberts, J.M. (1993). Cyclin-dependent regulation of G1 in mammalian fibroblasts. *Science* **259**,1908-1912.

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

Okamoto, K. and Beach, D. (1994). Cyclin G is a transcriptional target of the p53 tumor suppressor protein. *EMBO.J.* **13**, 4816-4822.

Oleynikov, Y. and Singer, R.H. (1998). RNA localisation: different zipcodes, same postman?. *Trends. Cell Biol.* **8**, 381-383.

Oliner, J.D., Peitenpol, J.A., Thiagalingham, S., Gyuris, J., Kinzler, K.W. and Vogelstein, B. (1993). Oncoprotein MDM2 conceals the activation domain of tumour suppresser p53. *Nature* **362**, 857-860.

Oliner, J.D., Kinzler, K.W., Meltzer, P.S., George, D.L. and Vogelstein, B. (1992). Amplification of a gene encoding a p53-associated protein in human sarcomas. *Nature* **358**, 80-86.

O'Reilly, D., Hanscombe, O. and O'Hare, P. (1997). A single serine reside at position 375 of VP16 is critical for complex assembly with Oct-1 and HCF and is a targt of phosphorylation by casein kinase II. *EMBO.J.* **16** (9), 2420-2430.

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

Padgett, R.A., Grabowski, P.J., Seiler, S. and Sharp, P.A. (1986). Splicing of messenger RNA precursors. *Annu Rev. Biochem.* **55**, 1119-1150.

Pagano, M., Pepperkok, P., Verde, F., Ansorge, W. and Draetta, G. (1992). Cyclin A is required at two points in the human cell cycle. *EMBO J.* **11**,961-971.

Pain, V.M. (1996). Initiation of protein synthesis in eukaryotic cells. *Eur. J. Biochem.* **236**, 747-771.

Pasteau, S., Loiseau, L., Arnaud, L., Trembleau, A., Brun, G. (1995). Isolation and characterisation of achicken homologue of the E2F-1 transcription factor. *Oncogene* **11**, 1475-1486.

Pazin, M.J. and Kadonaga, J.T. (1997). What's up and down with histone deactylation and transcription. *Cell* **?**, 325-328.

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

Peeper, D.S., Keblusek, P., Helin, K., Toebes, M., van der Eb, A.J. and Zantema, A. (1995). Phosphorylation of a specific cdk site in E2F-1 affects its electrophoretic mobility and promotes pRB-binding *in vitro*. *Oncogene* **10**, 39-48.

Pelletier, J. and Sonenberg, N. (1988). Internal initiation of translation of eukaryotic mRNA directed by a sequence derived from poliovirus RNA. *Nature* **334**, 320-325.

Phillips, A.C., Bates, S., Ryan, K.M., Helin, K. and Vousden, K.H. (1997).Induction of DNA synthesis and apoptosis are separate functions of E2F-1. *Genes Dev.* **11**, 1853-1863.

Philpott, A. and Friend, S.H. (1994). E2F and its developmental regulation in *Xenopus laevis*. *Mol. Cell Biol.* **14** (7), 5000-5009.

Pines, J. (1995). Cyclins and cyclin-dependent kinases: a biochemical review. *Biochem. J.* **308**, 697-711.

Polyak, K., Lee, M.H., Erdjument, B.H., Koff, A., Roberts, J.M., Tempst, P. and Massague, J. (1994). Cloning of p27Kip1, a cyclin-dependent kinase inhibitor and a potential mediator of extracellular antimitogenic signals. *Cell*, **78**, 59-66.

Pomerantz, J., Schriber-Angus, N., Liegeois, N.J., Silverman, A., Alland, L., Chin, L., Potes, J., Chen, K., Orlow, I., Lee, H.-W., Cordon-Cardo, C. and DePinho, R.A. (1998). The *Ink4a* tumour suppressor gene product, $p19^{ARF}$, interacts with MDM2 and neutralises MdM2's inhibition of p53. *Cell* **92**, 713-723.

Poon, R.Y., Yamashita, K., Adamczewski, J.P., Hunt, T. and Shuttleworth, J. (1993). The cdc2-related protein p40MO15 is the catalytic subunit of a protein kinase that can activate p33cdk2 and p34cdc2. *EMBO J.*, **12**, 3123-3132.

Proud, C.G. and Denton, R.M. (1997). Molecular mechanisms for the control of translation by insulin. *Biochem. J.* **328**, 329-341.

Pugh,B.F. and Tjian,R. (1990). Mechanisms of transcriptional activation by Sp1: evidence for coactivators. *Cell* **62**,1187-1197.

Putz, U., Skehel, P. and Kuhl, D. (1996). A tri-hybrid ystem for the analysis and detection of RNA-protein interaction. *Nucleic Acids Res.* **24** (23), 4838-4840.

Resnitzky, D., Gossen, M., Bujard, H. and Reed, S.I. (1994). Acceleration of the G1/S phase transition by expression of cyclins D1 and E with an inducible system. *Mol. Cell. Biol.*, **14**, 1669-1679.

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

Rossant, J. and Nagy, A. (1995). Genome engineering: the new mouse genetics. *Nature Med.* **1** (6), 592-594.

Roth, J., Dobbelstein, M., Freedman, D.A., Shenk, T. and Levine, A.J. (1998). Nucleo-cytoplamic shuttling of the hdm2 oncoprotein regulates the levels of the p53 protein via a pathway used by the human immunodeficiency virus rev protein. *EMBO. J.* **17** (2), 554-564.

Roy, R., Adamczewski, J.P., Seroz, T., Vermeulen, W., Tassan, J.P., Scaeffer, L., Nigg, E.A., Hoeijmakers, J.H.J. and Egly, J.M. (1994). The MO15 cell cycle kinase is associated with the TFIIH transcription-DNA repair factor. *Cell*, **79**, 1093-1101.

Russo, A.A., Jeffery, P.D., Patten, A.K., Massague, J. and Pavletich, N.P. (1996). Crystal structure of the p27Kip1 cyclin-dependent kinase inhibitor bound to the cyclin A-cdk2 complex. *Nature* **382**, 325-331.

Sabbatini, P., Lin, J.Y., Levine, A.J. and White, E. (1995). Essential role for p53-mediated transcription in E1a-induced apoptosis. *Genes. Dev.* **9**, 2184-2192.

Sachs, A.B., Sarnow, P. and Hentze, M.W. (1997). Starting at the beginning, middle, and end: Translation initiation in eukaryotes. *Cell* **89**, 831-838.

Sears, R., Ohtani, K. and Nevins, J.R. (1997). Identification of positively and negatively acting elements regulating expression of the E2F2 gene in response to cell growth signals. *Mol Cell Biol.* **17** (9), 5227-5235.

Sägesser, R., Martinez, E., Tsagris, M. and Tabler, M. (1997). Detection and isolation of RNA-binding proteins by RNA-ligand screening of a cDNA expression library. *Nucleic Acids Res.* **25** (19), 3816-3822.

Sakaguchi, K., Herrera, J.E, Saito, S., Miki, T., Bustin, M., Vassilev,A., Anderson, C.W. and Appella, E. (1998). DNA damage activates p53 through a phosphorylation-acetylation cascade. *Genes Dev* **12**, 2831-2841.

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

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

Schwarz, J.K., Devoto, S.H., Smith, E.J., Chellappan, S.P., Jakoi, L. and Nevins, J.R. (1993). Interactions of the p107 and Rb proteins with E2F during the cell proliferation response. *EMBO J.* **12**, 1013-1020.

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

Serrano, M., Hannon, G.J. and Beach, D. (1993). A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. *Nature*, **366**, 704-707.

Serrano, M., Lee, H., Chin, L., Cordon-Cardo, C., Beach, D. and DePinho, R.A. (1996). Role of the *INK4a* locus in tumor suppression and cell mortality. *Cell* **85**, 27-37.

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

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

Sharma, P.M., Bowman, M., Madden, S.L., Rauscher, F.J. and Sukumar, S. (1994). RNA editing in the Wilm's tumor susceptibility gene, WT1. *Genes. Dev.* **8**, 720-731.

Shen, Y. and Shenk, T. (1994). Relief of p53-mediated transcriptional repression by the adenovirus E1B 19-kDa protein or the cellular Bcl-2 protein. *Proc.Natl.Acad.Sci.* **91**, 8940-8944.

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

Shew, J.Y., Lin, B.T.Y., Chen, P.L., Tseng, B.Y., Yang-Feng, T.L. and Lee, W.H. (1990). C-terminal truncation of the retinoblastoma gene product leads to functional inactivation. Proc. *Natl. Acad. Sci. USA*. **87**, 6-10.

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

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

Singh, P., Wong, S.H. and Hong, W. (1994). Overexpression of E2F-1 in rat embryo fibroblasts leads to neoplastic transformation. *EMBO.J.* **13** (14), 3329-3338.

Smale, S. and Baltimore, D. (1989). The initiator as a transcription control element. *Cell*, **57**, 103-113.

Smith, M.L., Chen, I-T., Zhan, Q., Bae, I., Chen, C-Y., Gilmer, T.M., Kastan, P.M., O'Connor, P.M. and Fornace, A.J. (1994). Interaction of the p53-regulated protein Gadd45 with proliferating cell nuclear antigen. *Science* **266**, 1376-1380.

Sørensen, T.S., Girling, R., Lee, C-W., Gannon, J., Bandara, L.R. and La Thangue, N.B. (1996). Functional interaction between DP-1 and p53. *Mol. Cell Biol.* **16** (10), 5888-5895.

St Johnston, D. (1995). The intracellular localisation of messenger RNAs. *Cell* **81**, 161-170.

Stott, F.J., Bates, S., James, M.C., McConnell, B.B., Starborg, M., Brookes, S., Palmero, I., Ryan, K., Hara, E., Vousden, K.H. and Peters, G. (1998). The alternative product from the human CDKN2A locus, $p14^{ARF}$, participates in a regulatory feedback loop with p53 and MDM2. *EMBO. J.* **17** (17), 5001-5014.

Strickland, S., Smith, K.K. and Marotti, K.R. (1980). Hormonal induction of differentiation in teratocarcinoma stem cells: generation of parietal endoderm by retinoic acid and dibutyryl cAMP. *Cell* **21**, 347-355.

Tao, Y., Kassatly, R.F., Cress, W.D. and Horowitz, J.M. (1997). subunit composition determines E2F DNA-binding site specificity. *Mol Cell Biol.* **17** (12), 6994-7007.

Tassio, P.T. and La Thangue, N.B. (1990). A multiplicity of differentiation-regulated ATF site-binding activities in embryonal carcinoma cells with distinct sequence and promoter specificities. *New Biol.* **2** (12), 1123-1134.

Tevosian, S.G., Paulson, K.E., Bronson, R. and Yee, A.S. (1996). Expression of the E2F-1/DP-1 transcription factor in murine development. *Cell Growth Diff.* **7**, 43-52.

Thut, C.J., Chen, J-L., Klemm, R. and Tijan, R. (1995). p53 transcriptional activation mediated by coactivators $TAF_{II}40$ and $TAF_{II}60$. *Science* **267**, 100-104.

Tibbetts, R.S., Brumbaugh, K.M., Williams, J.M., Sarkaria, J.N., Cliby, W.A., Shieh, S-Y., Taya, Y., Prives, C. and Abraham, R.T. (1999). A role for ATR in the DNA damage-induced phosphorylation of p53. *Genes. Dev.* **13**, 152-157.

Trapp, B.D., Moench, T., Pulley, M., Barbosa, E., Tennekoon, G. and Griffin, J. (1987). Spatial segregation of mRNA encoding myelin-specific proteins. *Proc. Natl. Acad. Sci. USA*. **84**, 7773-7777.

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

Trouche, D., Cook, A. and Kouzarides, T. (1996). The CBP co-activator stimulates E2F1/DP1 activity. *Nuc Acids Res.* **24** (21), 4139-4145.

Tschochner, H., Sayre, M.H., Flanagan, P.M., Feaver, W.J. and Kornberg, R.D. (1992). Yeast RNA polymerase II initiation factors: isolation and identification as the functional counterpart of human transcription factor IIB. *Proc. Natl. Acad. Sci. U.S.A.*, **89**, 11291-11296.

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

Vandromme, M., Gauthier-Rouviere, C., Lamb, N. and Fernandez, A. (1996). Regulation of transcription factor localisation: fine-tuning of gene expression. *TIBS.* **21**, 59-64.

Verona, R., Moberg, K., Estes, S., Sartz, M., Vernon, J.P. and Lees, J.A. (1997). E2F activity is regulated by cell cycle-dependent changes in subcellular localisation. *Mol. Cell Biol.* **17** (12), 7268-7282.

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

Wang, Z-F., Whitfield, M.L., Ingledue III, T.C., Dominski, Z. and Marluff, W.F. (1996). The protein that binds the 3' end of histone mRNA: a novel RNA-binding protein required for histone pre-mRNA processing. *Genes Dev.* **10**, 3028-3040.

Waterman, M.J., Starvirdi, E.S., Waterman, J.L. and Halazonetis, T.D. (1998). ATM-dependent activation of p53 involves dephosphorylation and association with 14-3-3 proteins. *Nat. Genet.* **19**, 175-178.

Wickens, M., Anderson, P. and Jackson, R.J. (1997). Life and death in the cytoplasm: messages from the 3' end. *Curr. Op. Genet. Dev.* 7, 220-232.

Woo, R.A., McLure, K.G., Lees-Miller, S.P., Rancourt, D.E. and Lee, P.W.K. (1998). DNA-dependent protein kinase acts upstream of p53 in response to DNA damage. *Nature* **394**, 700-704.

Whyte, P., Buchkovich, K.J., Horowitz, J.M., Friend, S.H., Raybuck, M., Weinberg, R.A. and Harlow, E. (1988). Association between an oncogene and an anti-oncogene: the adenovirus E1a proteins bind to the retinoblastoma gene product. *Nature* **334**,124-129.

Wu, X.W. and Levine, A.J. (1994). p53 and E2F-1 cooperate to mediate apoptosis. *Proc. Natl Acad. Sci. U.S.A.* **91**, 3602-3606.

Wu, C-L., Zukerberg, L.R., Ngwu, C., Harlow, E. and Lees, J.A. (1995). *In vivo* association of E2F and DP family proteins. *Mol. Cell Biol.* **15** (5), 2536-2546.

Wu, X., Bayle, J.H., Olson, D. and Levine, A.J. (1993). The p53-mdm2 autoregulatory feedback loop. *Genes Dev.* **7**, 1126-1132.

Xing, Y., Johnson, C.V., Dobner, PR. and Lawrence, J.B. (1993). Higher level organisation of individual gene transcription and RNA splicing. *Science* **259**, 1326-1330.

Xiong, Y., Zhang, H. and Beach, D. (1992). D-type cyclins associate with multiple protein kinases and the DNA replication and repair factor PCNA. *Cell*, **71**, 504-514.

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

Yamasaki, L., Jacks, T., Bronson, R., Goillot, E., Harlow, E. and Dyson, N.J. (1996). Tumor induction and tissue atrophy in mice lacking E2F-1. *Cell* **85**, 537-548.

Zamanian, M. and La Thangue, N.B. (1992). Adenovirus E1a prevents the retinoblastoma gene product from repressing the activity of a cellular transcription factor. *EMBO. J.* **11** (7), 2603-2610.

Zamanian, M., and La Thangue, N.B. (1993). Transcriptional repression by the Rb-related protein p107. *Mol.Biol.Cell* **4**, 389-396.

Zawel, L. and Reinberg, D. (1992). Advances in RNA polymerase II transcription. *Curr.Opin.Cell Biol.* **4**,488-495.

Zhang, Y. and Chellappan, S.P. (1995). Cloning and characterisation of human DP-2, a novel dimerisation partner of E2F. *Oncogene* **10**, 2085-2093.

Zhang, Y. and Chellappan, S.P. (1996). Transcriptional activation and expression of DP transcription factors during cell cycle and TPA-induced U937 differentiation. *Mol Cell Diff.* **4** (4), 297-316.

Zhang, Y., Xiong, Y. and Yarbrough, W.G. (1998). AFR promotes MDM2 degradation and stabilises p53: *ARF-INK4a* locus deletion impairs both the Rb and p53 tumour suppression pathways. *Cell* **92**, 725-734.

Zhang, L., Proenca, R., Maffei, M., Barone, M., Leoplod, L. and Friedman, J.M. (1994). Positional cloning of the mouse *obese* gene and its human homologue. *Nature* **372**, 425-432.

Zhang, Y., Vankatraj, V.S., Fischer, S.G., Warburton, D. and Chellapan, S.P. (1997). Genomic cloning and chromosomal assignment of the E2F dimerisation partner *TFDP* gene family. *Genomics* **39**, 95-98.

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

Zhu, L., Harlow, E. and Dynlacht, B.D. (1995). p107 uses a $p21^{CIP11}$-related domain to bind cyclin/cdk2 and regulate interactions with E2F. *Genes Dev.* **9**, 1740-1752.

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

Zwicker, J., Lucibello, F.C., Wolfman, L.A., Gross, C., Truss, M., Engeland, K. and Muller, R. (1995). Cell cycle regulation of the *cyclinA*, *cdc25C* and *cdc2* genes is

based on a common mechanism of transcriptional repression. *EMBO. J.* **14** (18), 4514-4522.

Zwicker, J. and Muller, R. (1997). Cell-cycle regulation of gene expression by transcriptional repression. *TIG.* **13** (1), 3-6.