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A thesis entitled

Regulation of RNA polymerase III transcription by the tumour suppressor p53

Presented by

Diane Crighton

to

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Division of Biochemistry and Molecular Biology

Institute of Biomedical and Life Science

University of Glasgow

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Abstract

The tumour suppressor protein p53 is inactivated in a large proportion of human cancers. p53 controls growth and proliferation, through multiple mechanisms, including the ability to regulate transcription. p53 can function as a general repressor of RNA polymerase (pol) III transcription. Pol III is responsible for transcribing a variety of small stable RNAs including tRNA, 5S rRNA, and the adenoviral VA1 RNA. p53 targets TFIIIB, a TBP-containing factor that is essential for recruitment of pol III to its templates. This study investigates the TFIIIB-p53 interaction, and how it serves to regulate pol III-transcribed genes. p53 does not disrupt the interaction between the TFIIIB subunits, TBP and Brf1. It does, however, prevent association of TFIIIB with the pol III specific factor TFIIIC2, and pol III itself. Immobilised template and chromatin immunoprecipitation analysis show that p53 prevents the recruitment of TFIIIB, but not TFIIIC2, to the tRNA promoter. Pol III repression cannot be attributed to one clearly defined region of p53. Sequence within both the N- and C-termini are essential, and the central core domain is also implicated in playing a role. Evidence is provided here that p53-mediated repression of tRNA genes occurs via a trichostatin A-sensitive histone deacetylase independent mechanism.

p53 is deregulated or mutated in the vast majority of human cancers. Individuals who inherit mutant forms of p53 can suffer from Li-Fraumeni Syndrome (LFS), a familial cancer syndrome associated with a range of malignancies. Here is shown that pol III transcriptional activity is often highly elevated in primary fibroblasts from Li-Fraumeni patients, especially if the germline p53 mutation is followed by loss of the remaining allele. Deregulation of p53 function through the action of various

oncoproteins can also contribute to carcinogenesis. E6 from human papilloma virus can bind to p53 and neutralise its function and E6 releases pol III from p53-mediated repression. Induction of the Mdm2 regulating protein $p14^{ARF}$ results in a p53-mediated repression of pol III activity. p53 does not interfere with normal cellular growth and development; it is, however, rapidly induced in response to cellular stress. Here it is shown that the DNA-damaging agent MMS provokes a p53 response that correlates with a dramatic pol III transcriptional repression.

Collectively the data presented here support the idea that p53 can directly repress pol III transcription through interactions with the basal pol III machinery. p53 status can have a profound effect upon pol III activity; the precise circumstances under which such control becomes physiologically important however, remains to be determined.

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Abbreviations

ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia and Rad-3-related
Brf1	TFIIIB-related factor
Bp	Base pair
CDK	Cyclin-dependent kinase
CK2	Casien kinase 2
DMEM	Dulbecco's modified Eagle's mediun
DNA-PK	DNA-dependent protein kinase
DSE	Distal sequence element
DTT	Dithiothreitol
EBV	Epstein Barr virus
EDTA	Ethylene diamine tetra-acetic acid
EMSA	Electrophoretic mobility shift assay
GST	Glutathione-S-transferase
HAT	Histone acetyltrasferase
HDAC	Histone deacetyltrasferase
Hepes	*N*-2-hydroxyethylpiperazine-*N*'-2-ethane-sulphonic acid
His	Histidine
HPV	Human papillomavirus
HTML-1	Human T-cell leukaemia virus 1
ICR	Internal control region
IPTG	Isopropyl-β-D-thiogalactopyranoside
kDa	Kilodalton
Leu	Leucine

LFS	Li-Fraumeni syndrome
Mdm2	Mouse double minute clone 2
MMS	Methyl methane sulfonate
PAGE	Polyacrylamide gel elecrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PIC	Preinitiation complex
PMSF	Phemylmethylsulfonyl fluoride
PNK	polynucleotide kinase
Pol III	RNA polymerase III
PSE	Proximal sequence element
RB	Retinoblastoma protein
SDS	Sodium dodecyl sulphate
Ser	Serine
SINE	Short interspersed repeat
SV40	Simian virus 40
TBP	TATA-box binding protein
TBS	Tris buffered saline
Thr	Threonine
Tris	Tris(hydroxymethyl)methylamine
TSA	Trichostatin A
Tween 20	Polyoxyethylene sorbitan monolaurate
Tyr	Tyrosine

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Firstly I would like to thank my supervisor, Bob White, for his enormous support and encouragement over the past three years. I am especially thankful for his help with presentations, meticulous reading of this manuscript and his never wavering enthusiasm. I'd also like to thank the other members of the lab, past and present, for there endless support, help and practical advise. I am also especially thankful to our collaborators at the Paterson Institute and the University of York, particularly Lorna who has become a good friend over the course of my PhD.

I would like to thank my mum and dad, without whom none of this would have been possible, for their belief in me, and their financial and emotional support throughout my years of study.

Finally, thanks to Iain who has unconditionally given his love, and support, provided me with perspective, and kept me smiling.

Declaration

I am the sole author of this thesis. All the work presented in this thesis was performed by myself, unless otherwise acknowledged.

Diane Crighton, October 2002

Publications

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Stein, T., D. Crighton, L.J. Warnock, J. Milner, and R.J. White. 2002b. Several regions of p53 are involved in repression of RNA polymerase III transcription. *Oncogene* **21**: 5540-7.

Chapter 1

Introduction

1.1 p53-'Guardian of the Genome'

The tumour suppressor protein, p53, aptly named the 'Guardian of the Genome' (Lane 1992), is mutated in over 50% of all human cancers (Hollstein et al. 1994). Furthermore, in many tumours which do not harbour a p53 mutation, deregulation events occur frequently at other points in the p53 activation or response pathways (Sherr and Weber 2000). Deregulation of p53 provides a selective advantage for the clonal expansion of genetically damaged cells, ultimately leading to tumour progression (Hollstein et al. 1991). The discovery of p53, in 1979, was the culmination of two studies. Initially, studies of simian virus 40 (SV40) transformed cells demonstrated that a 54kDa protein coprecipitated with the large T-antigen of SV40 (Chang et al. 1979;Kress et al. 1979; Lane and Crawford 1979; Linzer and Levine 1979). In addition, it was shown that the 54kDa protein was over expressed in a wide variety of murine SV40 transformed cells and uninfected embryonal carcinoma cells (Linzer and Levine 1979). At the same time, but independently from the above studies, the p53 protein was identified due to its high expression level in chemically induced tumours (DeLeo et al. 1979).

Early observations suggested that p53 might function as an oncogene, since overexpression of p53 appeared to cause oncogenic transformation of cells (Jenkins et al. 1984; Parada et al. 1984; Eliyahu et al. 1985). However, once p53 cDNA was

cloned (Zakut-Houri et al. 1985), a variety of studies demonstrated the normal function of p53 to be anti-oncogenic. This conclusion was also based on the findings that rearrangement and functional inactivation of the p53 gene was observed at high frequency in mouse spleen tumours induced by the Friend erythroleukaemia virus (Mowat et al. 1985; Chow et al. 1987; Rovinski et al. 1987; Munroe et al. 1988). Furthermore introduction of wild-type p53 was found to be growth suppressive (Mercer et al. 1990; Michalovitz et al. 1990; Martinez et al. 1991; Crook et al. 1994). p53 is not an essential gene; mice that are homozygously null for the p53 gene develop normally; they do, however, display a strong disposition to cancer such that 74% develop tumours by the age of 6 months (Donehower et al. 1992). The importance of p53 is also displayed in individuals with Li-Fraumeni syndrome (LFS), a rare familial cancer disorder (Li 1969).

The genetic basis of LFS is an inherited mutation in the gene encoding p53 (Malkin et al. 1990). Unusual characteristics of this syndrome are the young age at which individuals develop cancer, the fact that individuals often develop multiple primary tumours, and the overall high frequency of cancer in the family. Many different types of cancer develop in families with LFS; the most common of which are sarcomas, breast cancer, leukaemia, brain tumours and adrenocortical carcinoma (Li 1969; Kleihues and al 1997). In LFS, an infected individual is heterozygous for the TP53 mutant allele, having one wild-type and one defective TP53 gene. The TP53 germline mutation and the cancer phenotype caused by the mutation are inherited in an autosomal-dominant fashion (Lustbader and al 1992); therefore, an individual with cancer inheriting the TP53 mutation will have a 50% chance of producing offspring with cancer. The penetrance of cancer is also high in LFS; an individual

who inherits a TP53 mutation has a very high risk of developing cancer (Lustbader and al 1992).

1.2 p53 structure

The p53 protein can be divided roughly into thirds (Figure 1.1). The acidic activation domain at the amino terminus, mediates transcriptional activation. This domain, which constitutes the first 42 amino acids of p53, is capable of interacting with several proteins. It binds directly to the TBP-TAF complex in TFIID (Martin et al. 1993; Thut et al. 1995b), directly regulating pol II gene expression. Amino acids F19, L22 and W23 have been shown to be required for transcriptional activation *in vivo* (Lin et al. 1995). These amino acids make contacts with the TATA-associated factors $TAF_{II}70$ and $TAF_{II}31$ (Lu and Levine 1995; Thut et al. 1995a). The adenovirus E1B-55kD protein and the human Mdm2 protein negatively regulate transcriptional activation. In both cases, amino acids 22 and 23 play a key role in binding of p53 to E1B-55Kd or Mdm2 (Lin et al. 1995).

The central core domain encompasses amino acids 102-292, and contains the sequence specific DNA-binding domain. This portion of the protein forms a protease-resistant and independently folded domain containing an essential Zn^{++} ion required for sequence specific DNA binding activity. Tetrameric p53 binds to four repeats of a consensus DNA sequence 5'-PuPuPuC(A/Y)-3'. This sequence is repeated as a pair of inverted repeats. An extensive analysis of thousands of tumours and tumour cell lines has revealed the extraordinary extent to which p53 mutations are found (Hollstein et al. 1994). Approximately half the major forms of cancer

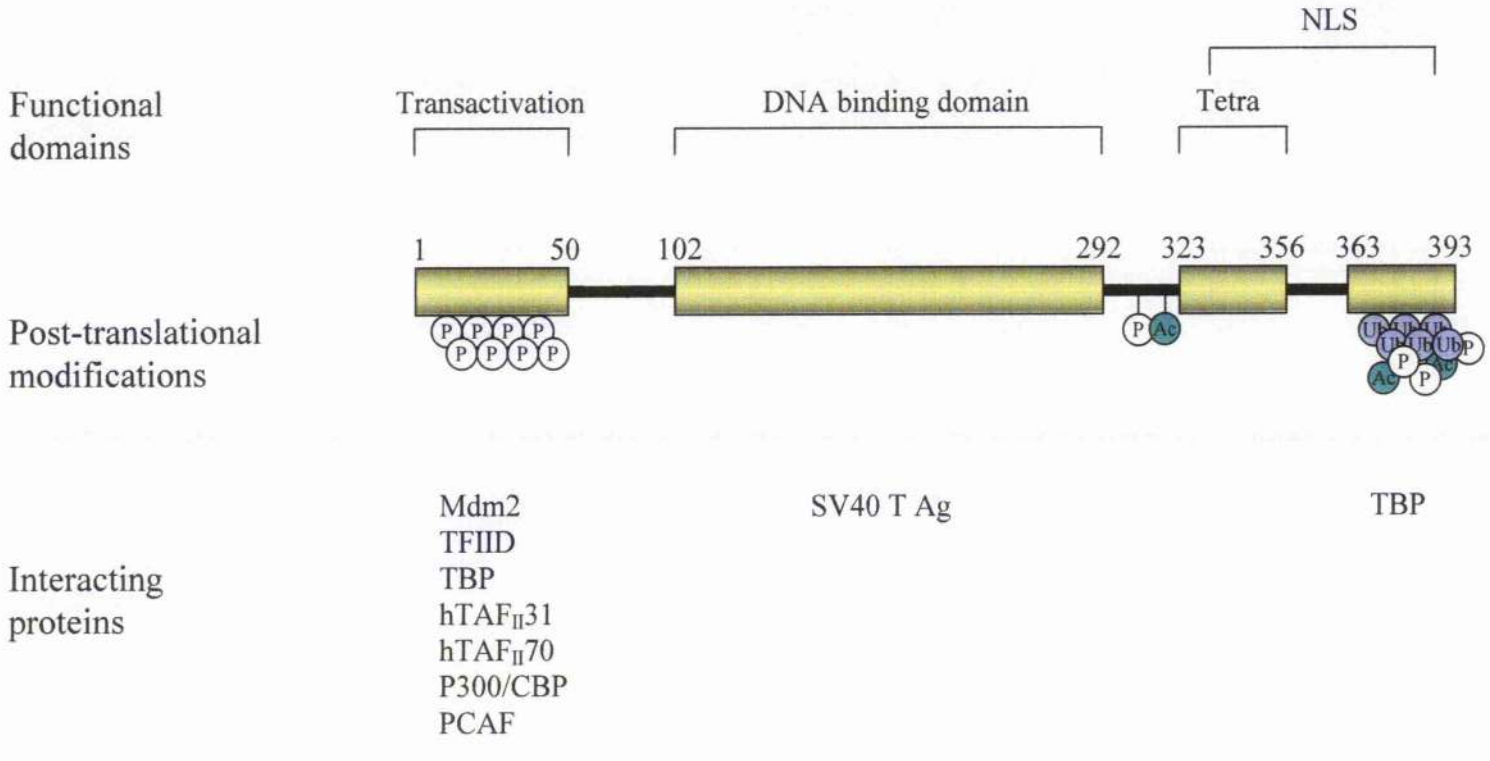


Figure 1.1. Major structural domains of human p53. The major functional domains of p53 are shown along with the sites of interaction with several other proteins. The sites of post-translational modifications within the N- and C-termini are also indicated. Tetra, tetramerisation domain; NLS, nuclear localisation signals; P, phosphorylation site; Ac, acetylation site; Ub, ubiquitination site.

contain p53 missense mutations, 90% of which reside in the sequence specific DNA binding domain, and more than 40% of these mutations are localised to residues R175, G245, R249, R273 and R282 (Figure 1.2). These are known as the 'hot-spot' mutation sites (Cho et al. 1994). Mutations generally fall into two classes. Mutation at amino acid residues such as R248 or R273, result in defective contacts with the DNA and consequently loss in the ability of p53 to function as a transcription factor. The second class of mutation disrupts the structural integrity of the protein. The resultant altered conformation produces a protein that is reactive with the monoclonal antibody pAb240. The epitope recognised by this antibody is not accessible in the native or wild-type p53 conformation (Cho et al. 1994; Hollstein et al. 1994).

The carboxy terminus is a multifunctional domain and comprises the remainder or the protein (Ko and Prives 1996). Native p53 exists as a tetramer in solution, and amino acids 324-356 are required for oligomerisation of the protein. The 26 extreme C-terminal residues form a protease-sensitive domain rich in basic amino acids. There is substantial evidence that the p53 protein requires a structural change to activate it for sequence-specific DNA binding. This latent, non-DNA binding, form of p53 can be regulated by the C-terminal amino acids. Deletion of this domain, phosphorylation at residue S378 by protein kinase C or residue S392 by casein kinase II, or binding of antibody pAb421 all activate the specific DNA binding activity of the central core domain (Hupp et al. 1992; Hupp and Lane 1994). Acetylation of sites within the C-terminus or the adjacent linking region has also been shown to stimulate the ability of p53 to bind DNA (Gu and Roeder 1997; Sakaguchi et al. 1998). Based on these observations it was assumed that the C-terminus of p53 serves as a negative regulator of the central core domain's sequence-

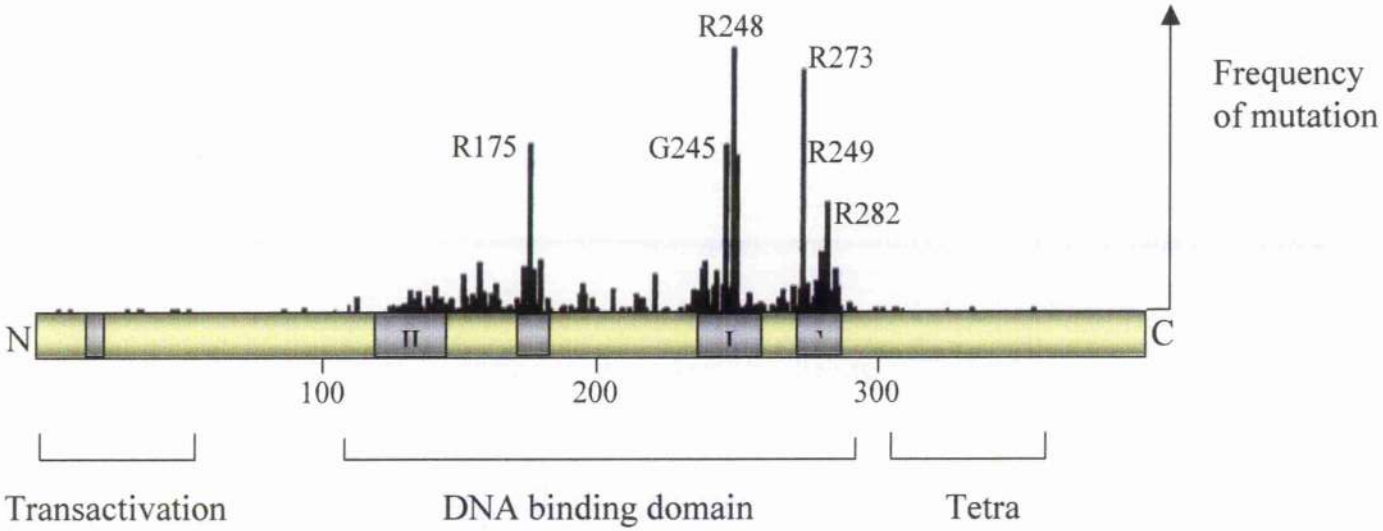


Figure 1.2. p53 mutations found in human cancer. Grey boxes represent evolutionarily conserved regions. Vertical lines above represent the frequency at which mutations are found at each particular residue. Several 'hot spots' for mutation are also indicated.

specific DNA binding activity. This hypothesis was, however, challenged recently by Espinosa and Emerson. The authors showed that while acetylation of p53 by p300 can stimulate its ability to bind to short oligonucleotides containing a binding site from the p21 promoter, it had no effect on the ability of p53 to bind its sites within the p21 promoter when they are present in a much longer DNA molecule. It was also shown that a p53 protein deleted of its C-terminus is actually less effective in interacting with the p21 promoter when present in naked or chromatinised DNA (Espinosa and Emerson 2001). These experiments therefore challenge the notion that the p53 C-terminus serves in an autoinhibitory role. They also provide evidence that acetylation has no significant effect on the interaction of p53 with DNA. These findings are supported by chromatin immunoprecipitation (ChIP) assays demonstrating that wild-type p53, or p53 containing several mutated acetylation sites, bind similarly to the p21 promoter in U2OS cells (Barlev et al. 2001).

1.3 Activation of p53

Normally in a cell, the p53 protein is kept at low levels, in a latent inactive form. Consequently, it does not interfere with cell cycle progression or cell survival. A variety of conditions can lead to the rapid induction of p53 activity (Figure 1.3). Each condition represents a different form of cellular stress. Several different type of DNA damage can activate p53. These include double DNA stand breaks, produced by γ-irradiation, the presence of DNA repair intermediates, after ultraviolet irradiation, or chemical damage to DNA. It has been predicted that even a single double-stranded break is enough to induce p53 (DiLeonardo et al. 1994). Damage to

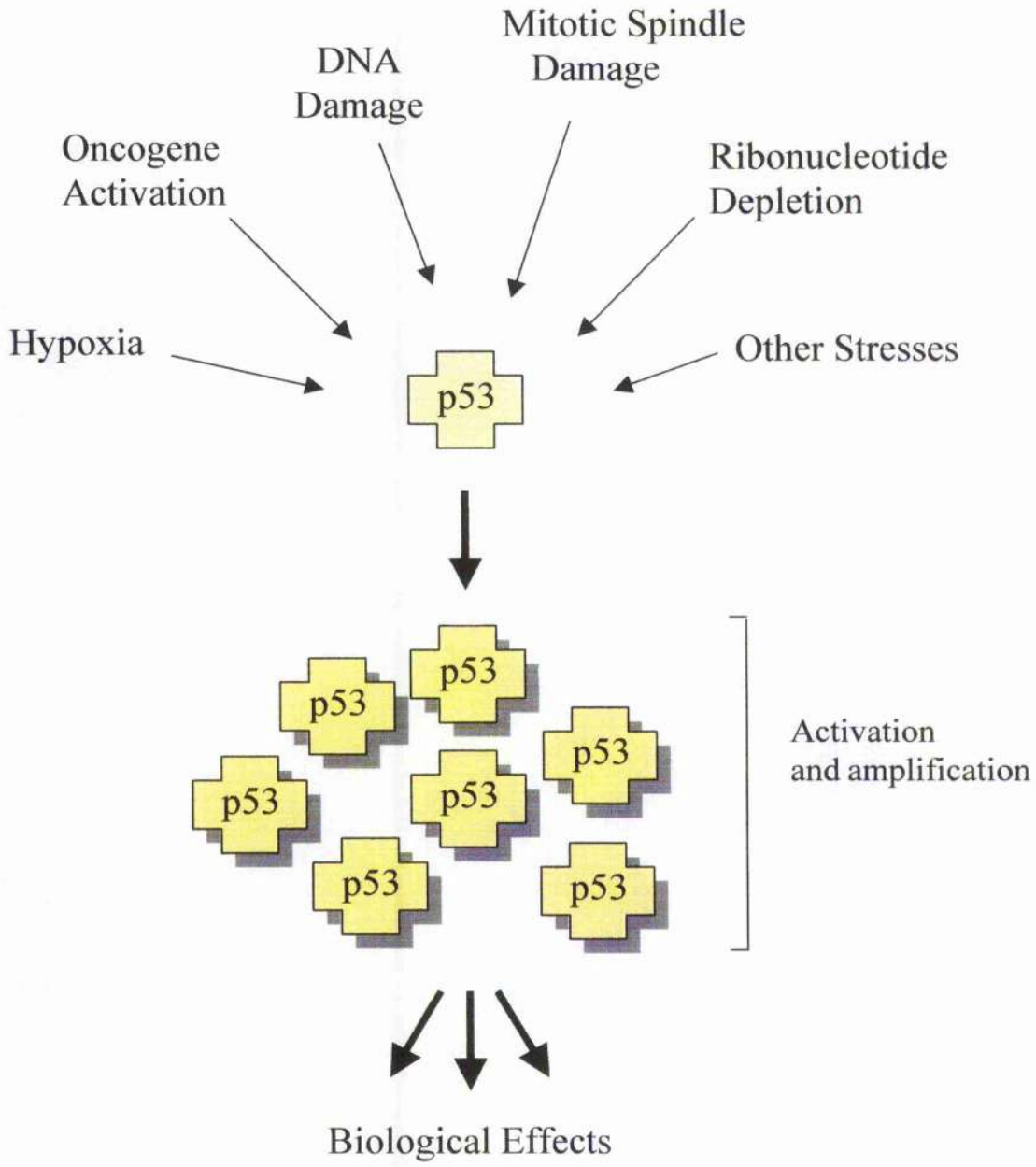


Figure 1.3. Signals that activate p53. Multiple stress induced signals can activate p53. These include DNA damage, hypoxia, ribonucleotide depletion, oncogenic stresses and mitotic spindle damage. Both p53 activity and protein levels. Are increased. Activated p53 can then elicit its biological effects.

cellular components such as the mitotic spindles can also induce a p53 response (Cross et al. 1995).

In addition to cellular damage, exposure of cells to suboptimal growth conditions such as hypoxia (Graeber et al. 1994) can stimulate p53 levels and activate the p53 protein. This process may represent another way that p53 may act as a gatekeeper against the formation of cancers. Many tumours begin to replicate and reach a critical size when the blood supply becomes limiting. The resultant hypoxia might trigger p53 activity and kill such cells (Levine 1997). Another signal reported to activate p53 is ribonucleotide depletion (Linke et al. 1996). Oncogenic proteins such as Myc, Ras, adenovirus E1A and β-catenin can also induce a p53 response (Debbas and White 1993; Hermeking and Eick 1994; Serrano et al. 1997; Damalas et al. 1999).

1.4 Regulation of p53 activity

Induction of p53 through stress occurs via several mechanisms. Stress-induced increases in the transcription of p53 have been described (Oren 1999). Furthermore, an increase in p53 mRNA translation may play a minor role in activating p53 in response to stress. There is evidence that this may be achieved through relief of translational repression, operating through the 3'-untranslated region of the p53 mRNA (Fu et al. 1996). Despite these observations, it is generally accepted that accumulation of active p53, in response to stress, occurs mainly through post-translational mechanisms.

p53 generally exists in an inactive, labile state, with a very short half-life. In response to stress the p53 protein is markedly stabilised (Kastan et al. 1991). Additionally, there is also a conversion of p53 from the latent to active form. (Hupp et al. 1992; Bayle et al. 1995; Lambert 1998). It is becoming increasingly apparent that post-translational modifications such as phosphorylation and acetylation play a major role in the rapid stabilisation and activation of the p53 protein.

1.4.1 Regulation of p53 by post-translational modifications

Phosphorylations within the N-terminus of p53 are clearly involved in the regulation of p53 stability. Such modifications can interfere with the ability of p53 to bind Mdm2. By phosphorylating serine-15 and serine-37, both ATM and ATR kinases can activate p53 transcriptional activity, in response to DNA damage (Dumaz and Meek 1999). ATM can also phosphorylate Chk2, a kinase that phosphorylates p53 directly within the Mdm2 binding domain (Chehab et al. 2000; Hirao et al. 2000; Shieh et al. 2000). ATM also induces dephosphorylation of the C-terminus of p53 (Waterman et al. 1998). Many other kinases have been shown to be capable of phosphorylating the N-terminus of p53 *in vitro*. These include DNA-PK, JNK, p38, CKI and CAK (Ljungman 2000), although in many cases the physiological relevance of such modifications remains to be clarified (Woods and Vousden 2000).

A series of C-terminal residues undergo phosphorylation resulting in the activation of the p53 protein. Phosphorylation of serine-392, by CK2 or PDK can efficiently activate DNA binding and transcriptional activity (Hupp et al. 1992; Blaydes and Hupp 1998; Cuddihy et al. 1999). A similar effect has been described for phosphorylation of serine-315, by cyclin-dependent kinases (Wang and Prives 1995;

Blaydes et al. 2000). It has also been suggested that subcellular localisation of p53 might be regulated through the coordinate effect of these two kinases, with CK2 phosphorylation favouring nuclear localisation and CDK phosphorylation driving nuclear export (Ljungman 2000).

Acetylation of lysine residues within p53 has been demonstrated to modulate its function. p300/CBP can enhance the transcriptional function of p53 by interacting with the N-terminus and acetylating lysine-382 located in the C-terminus (Sakaguchi et al. 1998). The precise function of p53 acetylation/deacetylation unfortunately remains unclear at present (Prives and Manley 2001). Acetylation of this site was believed to activate the DNA-binding activity of p53 in a manner similar to that of phosphorylation of the C-terminus (Gu and Roeder 1997). This idea has, however, been challenged (Barlev et al. 2001; Espinosa and Emerson 2001), and it seems unlikely that acetylation of p53 has a significant effect on its ability to bind to sites within promoters of target genes. Possible roles for p53 acetylation include transcriptional activation, mediated through the recruitment of the coactivators p300/CBP, and the PCAF complex (Barlev et al. 2001). Acetylation of p53 may also serve to recruit deacetylases inorder to down regulate gene expression. There are several lines of evidence that interactions of p53 with deacetylases play a role in p53 regulation (Murphy et al. 1999; Juan et al. 2000).

Phosphorylation of p53 within the N-terminus has been shown to enhance p300 binding and p53 acetylation of lysine 382 (Sakaguchi et al. 1998). This finding is supported by reports showing that phosphorylation of p53 at N-terminal residues indeed increases its interactions with p300 (Dumaz and Meek 1999; Dornan and

Hupp 2001). A similar stress-induced phosphorylation-dependent interaction with PCAF has been shown to result in acetylation of lysine 320 (Sakaguchi et al. 1998; Liu et al. 1999). In contrast to phosphorylation, Mdm2 blocks the ability of p300 to acetylate p53 (Kobet et al. 2000; Ito et al. 2001).

1.4.2 Regulation of p53 by Mdm2

One of the central components in regulating p53 is the p53-interacting protein, Mdm2 (Lohrum and Vousden 1999). Mdm2 is a transcriptional target of p53. In this feedback loop, p53 drives the expression of its own negative regulator (Figure 1.4) (Wu et al. 1993). This mechanism may serve to keep check on p53 activity in normal unstressed cells or may contribute to limiting or reversing the p53 response following removal of the initiating stress (Bar-Or et al. 2000). Mdm2 is essential in the prevention of p53 activation during development. This is exemplified by the consequences of deleting the Mdm2 gene in mice. Homozygous deletion of Mdm2 results in a very early embryonic lethality, resulting from excessive apoptosis (de Rozieres et al. 2000). This lethality is completely rescued however by the simultaneous deletion of p53, allowing entirely normal growth and development (Jones et al. 1995; Montes de Oca Luna et al. 1995).

Mdm2 interacts with the N-terminal transcriptional activation domain of p53 (Momand et al. 1992) Binding of Mdm2 can therefore inhibit p53's transcriptional activity by interfering with the recruitment of coactivators such as p300/CBP (Oliner et al. 1993; Wadgaonkar and Collins 1999). Mdm2 also regulates the stability of p53 (Haupt et al. 1997; Kubbutat et al. 1997a). Mdm2 is a RING-finger protein that functions as a ubiquitin ligase for both p53 and itself

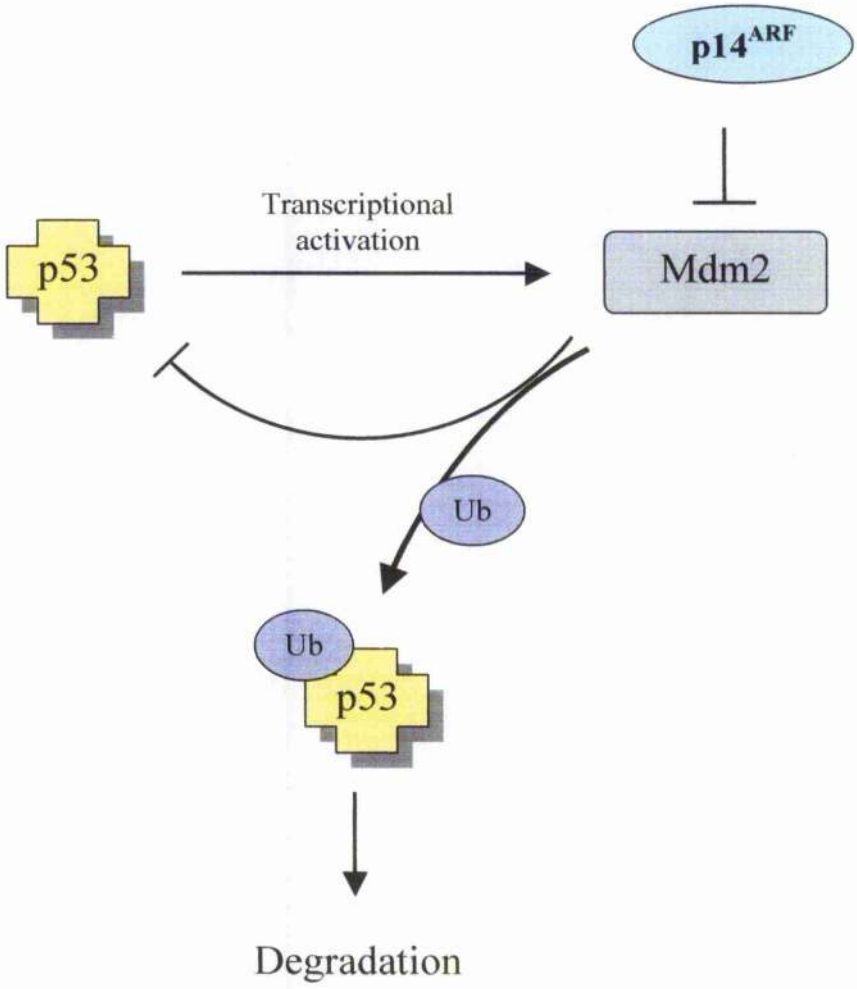


Figure 1.4. The p53-Mdm2 auroregulatory loop. Activated p53 protein binds to the *Mdm2* gene and activates its expression. The resultant Mdm2 protein binds to p53 and blocks its activity. Binding of Mdm2 to p53 promotes the ubiquitination of p53 and its subsequent degradation. Deregulated oncogenic signals can also induce the synthesis of $p14^{ARF}$, which binds to Mdm2 and blocks its action. Ub, ubiquitin.

(Honda et al. 1997; Fang et al. 2000; Midgley et al. 2000). Mutations within the RING-fingers domain of Mdm2 inhibit ubiquitination and result in stabilization of both proteins. The ability of Mdm2 to regulate p53 activity can be modulated by other factors. A role for p300 has been described in supporting efficient degradation of p53 (Grossman et al. 1998).

p53 activation may also involve a change in subcellular localisation. Both nuclear import and export are tightly regulated (Vousden and Woude 2000). Nuclear import of p53 depends on its interaction with the microtubule network (Giannakakou et al. 2000), although nuclear localisation signals located on the C-terminus of p53 (Figure 1.1) mediate this response. p53 also contains a nuclear export signal in the carboxy terminus (Stommel et al. 1999); however, efficient export of p53 to the cytoplasm depends on Mdm2 function. Studies have shown that the ubiquitin ligase function of Mdm2 is essential for p53 export (Boyde et al. 2000; Geyer et al. 2000). Some studies have suggested that efficient degradation of p53 requires nuclear export (Freedman and Levine 1998; Boyde et al. 2000; Geyer et al. 2000).

Like p53, Mdm2 is subject to extensive regulation. Mdm2 expression can be directly inhibited in response to various stress signals (Freedman et al. 1999; Ashcroft et al. 2000). Mdm2 is subject to post-translational modifications (Hay and Meek 2000). Phosphorylation within the N-terminal p53-binding region of Mdm2, reduces the interaction between p53 and Mdm2 (Mayo et al. 1997). Sumoylation and ubiquitination of Mdm2 has also been reported to modulate its function. Stress-induced loss of SUMO-1 modification allows autoubiquitination of lysine 446 and subsequent degradation of Mdm2 (Buschmann et al. 2000).

Disruption of Mdm2 function can also be achieved by $p14^{ARF}$. This small protein arises through the translation of an alternate reading frame derived from the INK4A tumour suppressor gene (Kamijo et al. 1997). $p14^{ARF}$ can act directly to inhibit the p53-directed ubiquitin ligase activity of Mdm2 (Honda and Yasuda 1999; Midgley et al. 2000). Abnormal oncogenic signals, such as E1A and Myc, have been shown to lead to the activation of p53 through induction of $p14^{ARF}$ (Weber et al. 1999; Lohrum et al. 2000). In some cells, the expression of $p14^{ARF}$ also alters the subcellular localisation of Mdm2, targeting itself and Mdm2 to the nucleolus (Weber et al. 1999; Lohrum et al. 2000). Under these circumstance, p53 remains in the nucleoplasm, releasing p53 from the inhibitory effect of Mdm2 interaction.

1.5 Oncogenic deregulation of p53

Mechanisms other than point mutation can also functionally inactivate p53. Several oncoproteins can bind and neutralise p53 (Vousden 1995; Levine 1997). Although over 50% of all human cancers harbour p53 mutations, this is not the case in cancers of the cervix. More than 90% of cervical carcinomas contain DNA from a high risk Human Papillomavirus (HPV), most notably HPV-16 and 18. HPV is associated with a range of clinical conditions, the most serious of which is cervical carcinoma. The E6 oncoprotein from HPV-16 and 18 has been shown to complex with and target p53 for rapid proteosome-mediated degradation (Scheffner et al. 1990). As a consequence, the functions of p53 are abrogated (White et al. 1994a). Since p53 is frequently wild-type in cervical cancers, it is proposed that the activity of E6 is

equivalent to an inactivating p53 mutation found in many other tumours (Crook et al. 1992).

Other small DNA tumour viruses can target and inactivate p53. Adenovirus E1B protein binds directly to the N-terminal activation domain of p53 (Kao et al. 1990), blocking its transcriptional activation function (Yew et al. 1994). Simian virus 40 (SV40) can also inactivate p53 function. SV40 Large T antigen interacts with the central core domain of p53, inhibiting sequence specific DNA binding activity (Marston et al. 1994).

Some tumours inactivate p53 by amplification of the Mdm2 gene. Currently it is estimated that Mdm2 is over expressed in 30-40% of human sarcomas (Oliner et al. 1992). Conversely, p14 ARF, which binds to and functionally inactivates Mdm2 (Figure 1.4), is deleted or methylated in many human cancers (Ruas and Peters 1998). Several other mechanisms exist to compromise p53 function. For example, in a small number of breast tumours and neuroblastomas, p53 is inactivated by localisation of the protein in the cytoplasm. (Levine 1997).

1.6 p53 function

The p53 protein is a key regulator in a wide range of cellular processes including cell cycle control, DNA repair, genome stability, programmed cell death, differentiation, senescence and angiogenesis (Figure 1.5) (Ko and Prives 1996; Levine 1997). p53 mediates many of these effects through its ability to regulate transcription (Figure 1.5) (Cox and Lane 1995; Haffner and Oren 1995; Ko and Prives 1996;

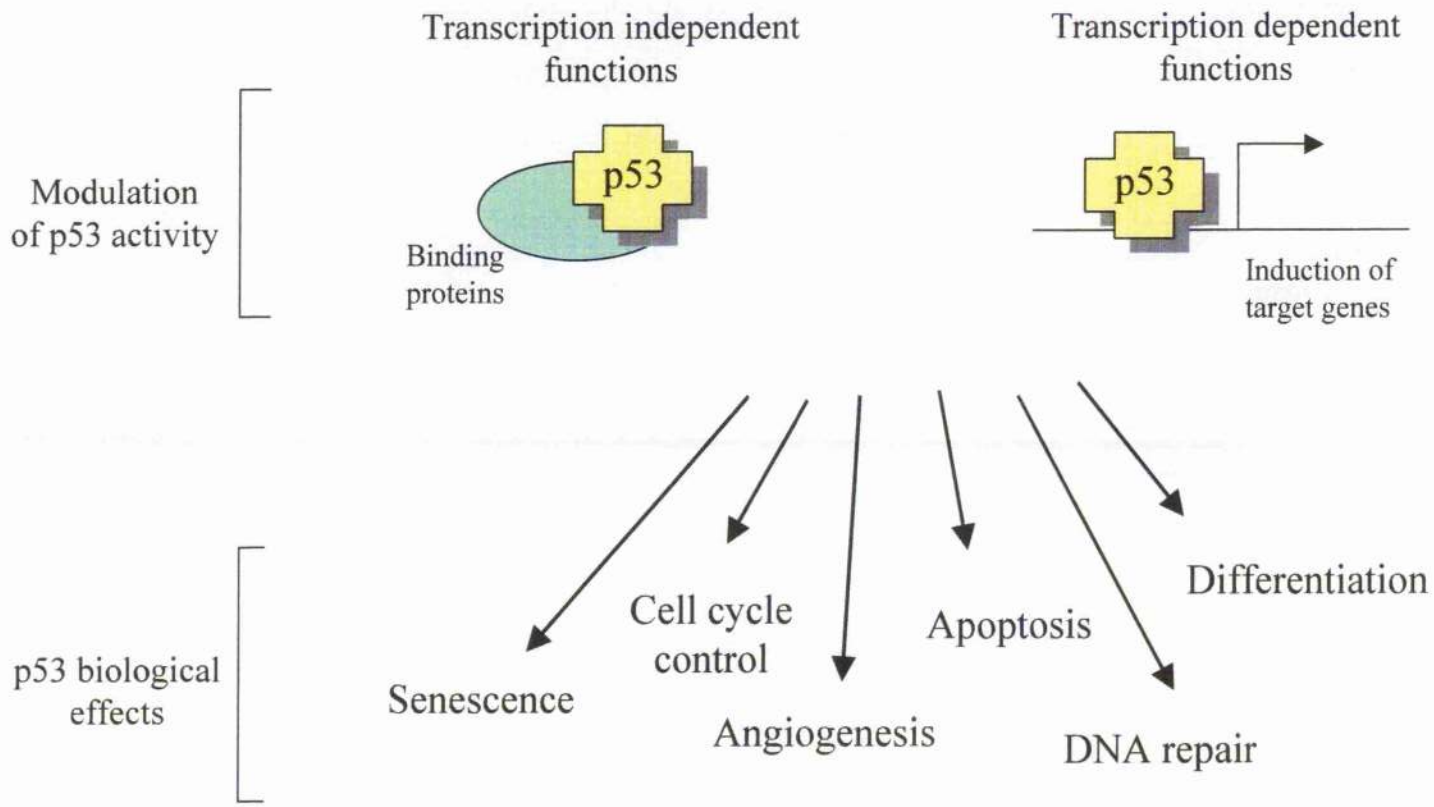


Figure 1.5. Cellular functions of p53. p53 activity is modulated through the induction of p53 target genes or through association with p53 binding proteins. Subsequent effects include cell cycle arrest, apoptosis, cellular senescence, inhibition of angiogenesis, differentiation and DNA repair.

Levine 1997). p53 can bind to DNA, as a tetramer, in a sequence-specific fashion, and stimulate expression of genes carrying p53 recognition elements in their promoters (Bargonetti et al. 1991; Kern et al. 1991; Farmer et al. 1992; Zambetti et al. 1992). A huge array of p53 target genes have been described, and the coordinate expression of sub sets of these genes, depending on cell type, environment and stimulating signal, is likely to determine the outcome in response to cellular stress (Woods and Vousden 2000).

The p21/WAF1/Cip1 gene (El-Deiry et al. 1993; Harper et al. 1993; Xiong et al. 1993a) is one of the most studied p53 response genes (El-Deiry et al. 1993). Deletion of this gene significantly reduces the cell cycle response to p53 (El-Deiry 1998). The encoded protein forms part of a quaternary complex found in normal cells along with cyclin/CDKs and the DNA polymerase processivity factor PCNA (Xiong et al. 1993a). In response to irradiation, G1 arrest is mediated, at least in part, through induction of p21/WAF1/Cip1 by p53 (El-Deiry et al. 1994), which at high protein concentrations, inhibits the function of CDKs (Gu et al. 1993; Harper et al. 1993; Xiong et al. 1993b). Recently, it has been demonstrated that p53 can directly participate in DNA repair through induction of a ribonucleotide reductase, p53R2 (Nakano et al. 2000; Tanaka et al. 2000).

Induction of apoptosis is one of the most extensively studied functions of p53. p53 has been shown to induce the expression of several genes that participate in the apoptotic response (Vousden 2000). Bax, a protein with homology to the survival factor Bcl-2, is upregulated in response to p53 (Miyashita and Reed 1995). Bax, in cooperation with other p53 inducible genes such as NOXA (Oda et al. 2000a),

PUMA (Nakano and Vousden 2001) and p53AIP1 (Oda et al. 2000b), trigger cytochrome c release from the mitochondria and induce activation of the Apaf-1/caspase9 apoptosome. p53 also induces expression of death receptors, such as Fas and Killer/DR5 and the death domain containing protein PIDD (Lin et al. 2000).

Control of gene expression is clearly an important function of p53, indeed, a recent study indicated that substitution of a gene encoding a transcriptionally inactive mutant p53 for the wild-type gene in mice, lead to loss of growth inhibitory activity (Chao et al. 2000; Jimenez et al. 2000). Despite this, transcriptionally independent activities of p53 play a role in mediating some of its downstream effects (Figure 1.5). For example, p53 has been implicated in the relocalisation of death receptors to the cell surface (Bennett et al. 1998).

1.7 Transcriptional repression by p53

In addition to activating genes whose promoters contain p53 binding sites, p53 can also repress expression of an ever increasing array of genes (Ginsberg et al. 1991; Mercer et al. 1991; Santhanam et al. 1991; Seto et al. 1992; Subler et al. 1992; Mack et al. 1993; Ragimov et al. 1993; Crook et al. 1994; Horikoshi et al. 1995; Chesnokov et al. 1996; Cairns and White 1998). A microarray analysis of ~6000 RNA polymerase II-dependent genes, revealed that 1.8% were induced by p53 whereas 0.9% were repressed (Zhao et al. 2000). Genes repressed by p53 include *c-fos*, IL-6, PCNA, cyclin A and Bcl2. Many of the gene products suppressed by p53 are involved in promoting cell cycle progression. It has therefore, been proposed that the transcriptional repression function of p53, may contribute to its tumour

suppression function (Ko and Prives 1996). This is underscored by the fact that many tumour derived p53 mutants have lost the capacity to inhibit transcription (Ginsberg et al. 1991; Mercer et al. 1991; Subler et al. 1992; Ragimov et al. 1993; Crook et al. 1994). In addition, two oncoproteins have been shown to block p53-mediated repression without affecting activation (Crook et al. 1994).

The requirements for repression by p53 are not well established. p53 has been shown to associate with histone deacetylase complexes (HDACs). HDAC complexes function by deacetylating histone tails. Such modifications generally produce chromatin structures that are not conducive to transcription. By utilising this mechanism, p53 was shown to inhibit the expression of Map4, a gene linked to the control of apoptosis (Murphy et al. 1996). Other proposed methods of transcriptional repression by p53 include p53 interacting with TBP-associated factors (TAFs) (Farmer et al. 1996b). Interestingly, a $p53_{gln22,ser23}$ mutant, which retains the ability to interact with TBP but is unable to bind to TAFs is unable to repress transcription (Sabbatini et al. 1995). Therefore, although p53 has been shown to interact with TBP *in vitro* and *in vivo* (Seto et al. 1992; Liu et al. 1993; Truant et al. 1993a; Chang et al. 1995; Horikoshi et al. 1995; Farmer et al. 1996a), the biological relevance of this interaction has not yet been fully established.

In one case transcriptional repression by p53 was shown to act through interaction with a transcriptional activator. Repression of *hsp70* by p53 is mediated through interaction with CCAAT binding factor (CBF), a transcriptional activator of the hsp70 promoter (Agoff et al. 1993). All the genes mentioned so far are transcribed by the RNA polymerase II. There are however three nuclear RNA polymerases in

eukaryotic cells, and recently, it was demonstrated that p53 can also repress pol I and pol III transcribed genes (Chesnokov et al. 1996; Cairns and White 1998; Budde and Grummt 1999; Zhai and Comai 2000).

1.8 RNA polymerase III

RNA pol III is the largest and most complex of the three nuclear RNA polymerases. It has an aggregate mass of 600-700 kDa and contains 17 subunits in both humans and yeast. Pol III has been purified form a variety of organisms, including humans (Jaehning et al. 1977; Wang and Roeder 1997), mouse (Sklar and Roeder 1976), frog (Engelke et al. 1983), silkworm (Sklar and Roeder 1976), fruitfly (Gundelfinger et al. 1980), wheat (Jendrisak 1981) and yeast (Valenzuela et al. 1976), and there has proved to be significant similarity in composition of pol III subunits between these species.

Each of the three nuclear RNA polymerases consist of two large subunits and a complex array of smaller components, which range in size from 10kDa to 90kDa. All three polymerases share five common subunits, ABC10α, ABC10β, ABC14.5, ABC23 and ABC27: A denotes a subunit found in pol I, B a subunit in pol II and C a subunit in pol III and the number indicates its size in kDa. Pols I and III additionally share subunits AC19 and AC40 (Mann et al. 1987). These subunits are not found in pol II, but B12.5 and B44 are functionally equivalent (Martindale 1990). The two largest polypeptides of each polymerase are homologous to the equivalent polypeptides of the other enzymes (Breant et al. 1983). Furthermore, these subunits are also homologous to the β' and β subunits of prokaryotic polymerases (Allison et

al. 1985; Sweetser et al. 1987). The presence of common subunits may offer the opportunity for coordinate regulation (Paule 1998), although evidence for such a mechanism has remained elusive.

Although pol I, II and III share several common subunits, they do not display functional redundancy. Each polymerase is responsible for transcribing its own specific set of templates. These are commonly termed class I, II and III genes, respectively. Within the nucleus, pol I synthesises only a single RNA species, the large ribosomal RNA (rRNA) precursor molecule. This large rRNA precursor is processed into the mature 5.8S, 18S and 28S rRNA components of the ribosomes. Although pol I synthesises only one product, it is estimated that pol I is responsible for 70% of all nuclear transcription.

Pol II alone is responsible for synthesising the majority of genes. These include all messenger RNAs (mRNAs), which encode protein, and most small nuclear RNAs (snRNA). Incredibly, however, only 20% of total nuclear transcription is ascribed to pol II. The remaining 10% of nuclear transcription is carried out by pol III. The products synthesised by pol III encode a variety of untranslated small stable RNAs, including 5S rRNA, transfer RNAs (tRNA) and U6 snRNA.

The three polymerases are localised to distinct sites within the nucleus (Shaw et al. 1995; Pombo et al. 1999). Pol I transcription is localised to discrete sites called nucleoli. These 'ribosome factories' are the sites at which rRNA is synthesised, processed and assembled into ribosomes (Shaw et al. 1995). Confocal and electron microscopy revealed that pol II and pol III function at their own spatially discrete

sites. Each nucleus was estimated to contain approximately 8000 pol II sites per HeLa cell. Furthermore, pol III transcription was localised to around 2000 discrete sites within the nucleoplasm, with each site containing on average 5 molecules of active pol III (Pombo et al. 1999).

Ribosome synthesis requires equimolar amounts of ~80 ribosomal proteins and 28S, 18S, 5.8S and 5S rRNAs. It would therefore seem logical that the activity of all three nuclear RNA polymerases, used to synthesise these products, is co-ordinately controlled. To a certain extent this does appear to be the case. In particular the rates of ribosome biogenesis and transcription by pols I and III are closely coupled to cell growth. An obvious way to coordinate transcription by pols I, II and III would be to target shared components. These three polymerases have five common subunits, but there is no evidence at present that these are used to coregulate activity. The TATA-binding protein, TBP, is shared by all three transcription systems. By targeting TBP, a repressor protein called Dr1 has been shown to coregulate pols II and III (White et al. 1994b). Additional regulatory proteins have also been shown to control the activity of other combinations of polymerases. For example, the retinoblastoma protein, RB, can repress pols I and III transcription, and to a lesser extent control pol II transcription. Factors such as these may provide a network of control mechanisms used to regulate and coordinate the activity of the three nuclear RNA polymerases.

1.9 Pol III transcripts

The genes transcribed by pol III encode a variety of small stable RNA molecules that do not get translated into protein. Many of these have essential functions in cellular

metabolism. The tRNAs range between 70 and 90 nucleotides in length and are essential in the process of translation. tRNAs serve as adaptor molecules, translating the genetic information contained within mRNA into a specific sequence of amino acid residues. A three-residue anticodon sequence of a particular tRNA is specific for a particular amino acid. By base pairing of the tRNA anticodon with the complementary codon of the mRNA nucleotide sequences facilitates accurate synthesis of a polypeptide chain. The human genome contains around 500 tRNA genes, which give rise to 60 to 90 tRNA species (Lander 2001; Venter 2001; Hatlen and Attardi 1971). This considerable redundancy results in an average copy number of 10 to 20 genes per amino acid adaptor molecule.

5S rRNA is the smallest (120 nucleotides) of all the ribosomal RNAs. It is the only rRNA transcribed by pol III, and like tRNAs has an essential role in translation. 5S rRNA is found associated with the large ribosomal subunit in all eukaryotic organisms. The haploid genome contains 200 to 300 copies of the 5S gene, many of which occur in clusters of tandem repeats (Sorensen and Frederiksen 1991).

The U6, H1 and MRP RNAs, which also fall into the class III gene family, play important roles in the processing of RNA transcripts. The 106 nucleotide U6 transcript is the most highly conserved of the spliceosomal RNAs (Brow and Guthrie 1988). It is also the only spliceosomal RNA transcribed by pol III. U6 is the smallest of five snRNA species that make up a ribonucleprotein (RNP) complex termed a spliceosome (Kunkel et al. 1986; Moenne et al. 1990). The essential function of the spliceosome, is to post-transcriptionally modify pre-mRNA molecules (Maniatis and Reed 1987). Intronic sequences are removed and regions encoded by exons are

religated to form mature mRNA. H1 is a 369 nucleotide RNA which forms part of RNase P, an endoribonuclease involved in processing of the 5'-termini of pre-tRNA (Bartkiewicz et al. 1989; Lee and Engelke 1989; Morrissey and Tollervey 1995). MRP, a 265 nucleotide transcript, which shares several regions of sequence homology with H1 (Gold et al. 1989), forms part of RNase MRP, another endoribonuclease. Rnase MRP serves an important role in the endonucleolytic processing of pre-rRNA (Morrissey and Tollervey 1995).

7SL RNA forms the scaffold of the signal recognition particle (SRP), which plays an essential role in the intracellular localisation of proteins (Walter and Blobel 1982). There are four 7SL genes, all of which are transcribed by pol III. Each gene encodes a highly conserved, 300 nucleotide transcript (Ullu and Tschudi 1984; Ullu and Weiner 1984).

Two recent studies have shown that the evolutionarily conserved 7SK RNA acts as a negative regulator of the RNA polymerase II elongation factor P-TEFb (Nguyen et al. 2001; Yang et al. 2001). The transcription-dependent interaction of P-TEFb with 7SK may well contribute to an important feedback loop modulating the activity of Pol II (Nguyen et al. 2001; Yang et al. 2001).

The VA RNAs encoded by adenovirus are also synthesised by pol III. These serve to divert the translational machinery of an infected cell towards the more effective production of viral proteins. Many other class III genes encode transcripts of unknown function. This category includes various gene families of repetitive short interspersed elements (SINEs). SINE DNA accounts for a substantial proportion of

the mammalian genome and therefore constitutes a quantitively important class of pol III template (Jelinek and Schmid 1982; Singer 1982). The principle SINE in primates is the Alu family. It is estimated that Alu sequences comprise around 10% of the total human genome and that there are in the region of one million copies (Jelinek et al. 1980; Rubin et al. 1980; Britten 1994). Alu genes consist of two imperfect repeats separated by an 18bp spacer (Rubin et al. 1980; Deininger et al. 1981) with a functional pol III promoter located in the upstream repeat (Paolella et al. 1983).

The B1 and B2 SINEs are the most abundant SINE family found in rodents. There are estimated to be approximately 100 000 and 80 000 copies of B1 and B2 per haploid mouse genome, respectively (Krayev et al. 1980; Bennett et al. 1984; Rogers 1985). B1 genes show 80% homology with human Alu genes, while the B2 family is specific to rodents and alone constitutes ~0.7% of total mouse genomic DNA. SINEs are frequently clustered together and Alu or B1 genes are found immediately downstream of 7SK, H1 and MRP genes (Murphy et al. 1986; Chang and Clayton 1989; Baer et al. 1990).

Retrotransposition, where pol III transcripts are reverse transcribed into DNA and subsequently integrated into new genomic sites is thought to allow the dispersal and amplification of SINESs (Weiner et al. 1986). SINEs do not have a clearly defined function (Howard and Sakamoto 1990). The principle SINE families appear to be derived from class III genes of known physiological significance. The B2 family seems to have evolved from tRNA genes (Daniels and Deininger 1985). It is also likely that the B1 and Alu families evolved from the 7SL genes (Ullu and Tschudi

1984). It is possible that the SINEs exist as pseudogenes of no functional significance, but during the course of evolution some SINE transcripts may have acquired roles. Such roles include the regulation of adjacent gene expression (Britten and Davidson 1969), splicing (Krayev et al. 1982), translation (Chang et al. 1994), DNA replication (Anachkova et al. 1984; Ariga 1984; Anachkova et al. 1985), cell stress response (Fornace and Mitchell 1986; Liu et al. 1995) and regulation of growth (Sakamoto et al. 1991) or the turnover of specific mRNAs (Clemens 1987).

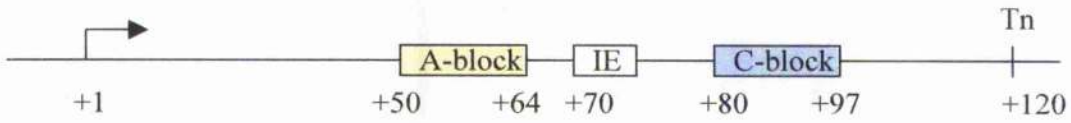
Despite possessing no clear functional role, the insertion of SINEs into new genomic locations has a significant impact on the structure and evolution of the genome. SINEs have a huge capacity to disrupt genomic sequences through multiple recombination events, however, since SINEs are generally expressed at low levels, this mutagenic potential is limited.

1.10 Pol III promoters

There are three promoter types used by pol III, namely types I, II and III, which are presented in Figure 1.6. The most unusual and striking feature of pol III promoters is that the majority require sequence elements down stream of the transcriptional start site. These control regions, which lie within the transcribed region, are discontinuous structures composed of essential blocks of sequence separated by non-essential regions (Paule and White 2000). A small minority of pol III promoters, namely those of type III, lack these intragenic control regions. These promoters are similar to those utilized by pol I and pol II in that they rely on 5' flanking sequences to direct transcription (Murphy et al. 1987).

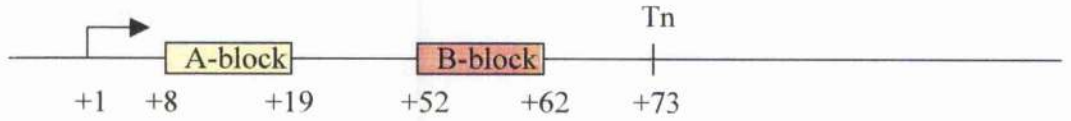
Type I promoter

e.g. *Xenopus* somatic 5S rRNA genes



Type II promoter

e.g. *Saccharomyces* SUP4 tRNA gene



Type III promoter

e.g. Human U6 snRNA gene

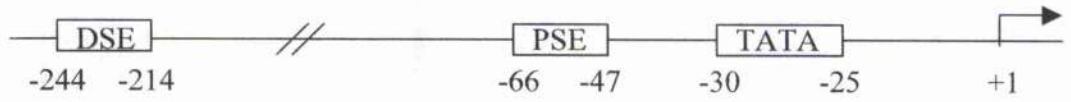


Figure 1.6. Class III genes utilize three types of promoter arrangement. The sites of transcription initiation are indicated by +1 and the sites of termination are indicated by Tn. Promoter elements are shown as boxes. A-block, yellow; B-block, orange; C-block, blue.

1.10.1 Type I promoters

The *Xenopus laevis* somatic 5S rRNA gene serves as a classical type I promoter. It requires three internal elements for efficient transcription. An A block is situated between +50 and +64, an intermediate element at +67 to +72 and a C block from +80 to +97 (Pieler et al. 1987). Although the sequence separating these elements does not influence transcription efficiency, variation in distance between the essential blocks of sequence is poorly tolerated (Pieler et al. 1987). Studies have also demonstrated that the region between the A-block and the transcriptional start site is an important determinant of 5S rRNA expression (Fradkin et al. 1989; Keller et al. 1990).

1.10.2 Type II promoters

The most common promoter arrangement used by pol III is found in the tRNA genes, the adenovirus VA genes and many middle repetitive gene families. It is called the type II promoter and consists of two highly conserved sequence blocks, A and B. Each block is ~10bp and they are separated by 30-40 bp (White 1998a). The A blocks of type I and II promoters are homologous and sometimes interchangeable, although they differ in their location relative to the start site of transcription (Ciliberto et al. 1983b). The A-block of the type II promoter is located at approximately +10 to +20, whereas on type II promoters it is typically found ~40bp further upstream (Galli et al. 1981). The location of the B-block is extremely variable. This is in part due to the fact that some tRNA genes possess short introns within their coding region. A distance of ~30-60 bp between the A and B-blocks is optimal for transcription, although a separation of 365 bp can be tolerated (Baker et

al. 1987; Fabrizio et al. 1987). This flexibility is particularly remarkable since a single transcription factor, TFIIIC, binds simultaneously to both the A- and B-blocks (Schultz et al. 1989).

The A- and B-block have the consensus sequences TGGCNNAGTGG and GGTTCGANNCC, respectively. Both these elements are essential for transcription and point mutations within the A- and B-blocks have been found to have a substantial effect on transcription efficiency (Newman et al. 1983; Traboni et al. 1984; Nichols et al. 1989). Additional internal or flanking sequences often confer modulatory effects. For example, although the start site at which transcription can commence is dictated primarily in relation to the A-block (Ciliberto et al. 1983a; Baker et al. 1987), the precise start site is determined by local sequence. Pol III favours initiation at a purine preceded by a pyrimidine (Ciliberto et al. 1983b; Fruscoloni et al. 1995). In addition, the upstream flanking region can be influential. In most cases, the 5' flanking sequences have an overall stimulatory influence upon transcription, although repressive effects can also occur (DeFranco et al. 1981; Dingermann et al. 1982; Hipskind and Clarkson 1983). Despite their modulatory effects, 5' flanking regions display little or no homology even between different genes encoding the same tRNA isoacceptor (White 1998a). This variation may, however, provide a mechanism for differential regulation of tRNA genes in response to differing codon and amino acid demands in various cell types (White 1998a). It is also believed that internal block and external sequences may account for the differential promoter strengths of distinct class III genes with type II promoters.

1.10.3 Type III promoters

A small proportion of vertebrate pol III templates lack any requirement for intragenic promoter elements, these are referred to as type III promoters. Human and mouse U6 snRNA promoters have been identified that retain full activity following deletion of all sequence downstream of the transcription start site (Das et al. 1988; Kunkel and Pederson 1989; Lobo and Hernandez 1989). This was also found to be the case for human 7SK and MRP RNA genes (Murphy et al. 1987; Yuan and Reddy 1991). Although this feature of type III promoters is an anomaly for class III genes, it is the normal occurrence for class I and II genes as well as bacteria.

The best characterised type III promoter belongs to a human U6 gene. Efficient expression relies upon a TATA box between –30 and –25, a proximal sequence element (PSE) between –66 and –47 and a distal sequence element (DSE) between –244 and –214 (Bark et al. 1987; Carbon et al. 1987; Das et al. 1988; Kunkel and Pederson 1988). These control regions display considerable homology to regions within the promoters of the U2 snRNA gene, transcribed by pol II. Indeed, the PSE and DSE sequence elements are interchangeable between the U6 and U2 snRNA promoters (Bark et al. 1987; Carbon et al. 1987; Kunkel and Pederson 1988; Lobo and Hernandez 1989). Although TATA sequences are a classical feature of class II genes, the U2 gene does not contain this element. Indeed, the fact that the U6 snRNA promoter contains a TATA box presents a curious anomaly, since TATA sequences are not a normal occurrence in class III genes. Paradoxically, insertion of a TATA box can convert U2 it into a pol III template, while inactivation of the U6 TATA box allows U6 to be transcribed by pol II (Mattaj et al. 1988; Lobo and Hernandez 1989)

1.10.4 Other promoter types

Several of the class III genes do not fall neatly into any of the three promoter types. For example the EBER2 gene of Epstein-Barr virus has A and B blocks that are typical of type II promoters and are essential for its transcription (Howe and Shu 1989). However, deletion of sequence upstream reduces expression (Howe and Shu 1989). Upstream binding site for Sp1 and ATF are thought to be responsible for this effect (Howe and Shu 1989). The EBER2 promoter also has a TATA box located between –28 and –23 that can stimulate transcriptional activity 5-fold (Howe and Shu 1989).

Transcription of the human 7SL gene also depends on internal and external promoter elements. It possesses A- and B-blocks, although they do display sequence variation to those of type II promoters (Allison et al. 1983). Like the EBER2 promoter, efficient expression requires upstream sequences including a ATF binding site at –51 to -44 and a putative TATA box between –28 to –24 (Bredow et al. 1990; Howe and Shu 1993). Other examples of pol III promoters that rely on both internal and upstream sequences for efficient expression include silkworm $tRNA^{Ala}$ genes (Sprague et al. 1980), the Xenopus $tRNA^{Sec}$ gene (Carbon and Krol 1991) and the rat vault RNA gene (Vilalta et al. 1994).

1.11 Transcription factors and complex assembly on class III genes

The process of transcription by pol III involves a complex array of trans-acting factors that work in concert with cis-acting DNA elements. Multiple transcription factors cooperate in order to recruit pol III to the start site of transcription on class III

genes (Parker and Roeder 1977). Without these factors, pol III will initiate randomly (Weil et al. 1979; Cozzarelli et al. 1983). Many of these essential transcription factors are common to all pol III templates, but some are required for the transcription of certain promoter sub types.

1.11.1 Type I and II promoters

The A- and B-blocks in type II promoters are initially recognised by TFIIIC (Figure 1.7A). TFIIIC is one of the largest and most complex transcription factors known. In *S. cerevisiae*, TFIIIC consists of two globular domains, each approximately 10nm in diameter and ~300 kDa (Schultz et al. 1989). TFIIIC is composed six subunits, none of which are capable of binding DNA specifically on their own. Photocrosslinking studies have revealed that the various subunits of TFIIIC extend across the entire length of the $tRNA^{Tyr}$ gene (Figure 1.8) (Bartholomew et al. 1990; Braun et al. 1992). TFIIIC contacts both the A and B blocks, although the B block is the major determinant of binding affinity (Baker et al. 1986). The relative helical orientation of the A and B blocks is not important for transcription efficiency (Baker et al. 1987). Electron microscopic studies suggest that the two domains of TFIIIC can stretch, giving the protein the appearance of a dumb-bell when bound to DNA (Schultz et al. 1989). This finding can account partially for the fact that the distance between the A and B block can vary substantially. On promoters with very long inter block separations, the intervening DNA is looped out so that both sequence elements can be contacted efficiently by TFIIIC (Schultz et al. 1989).

Human TFIIIC varies significantly from the yeast factor. It can be resolved by ion exchange chromatography into two components, TFIIIC1 and TFIIIC2 (Yoshinaga et

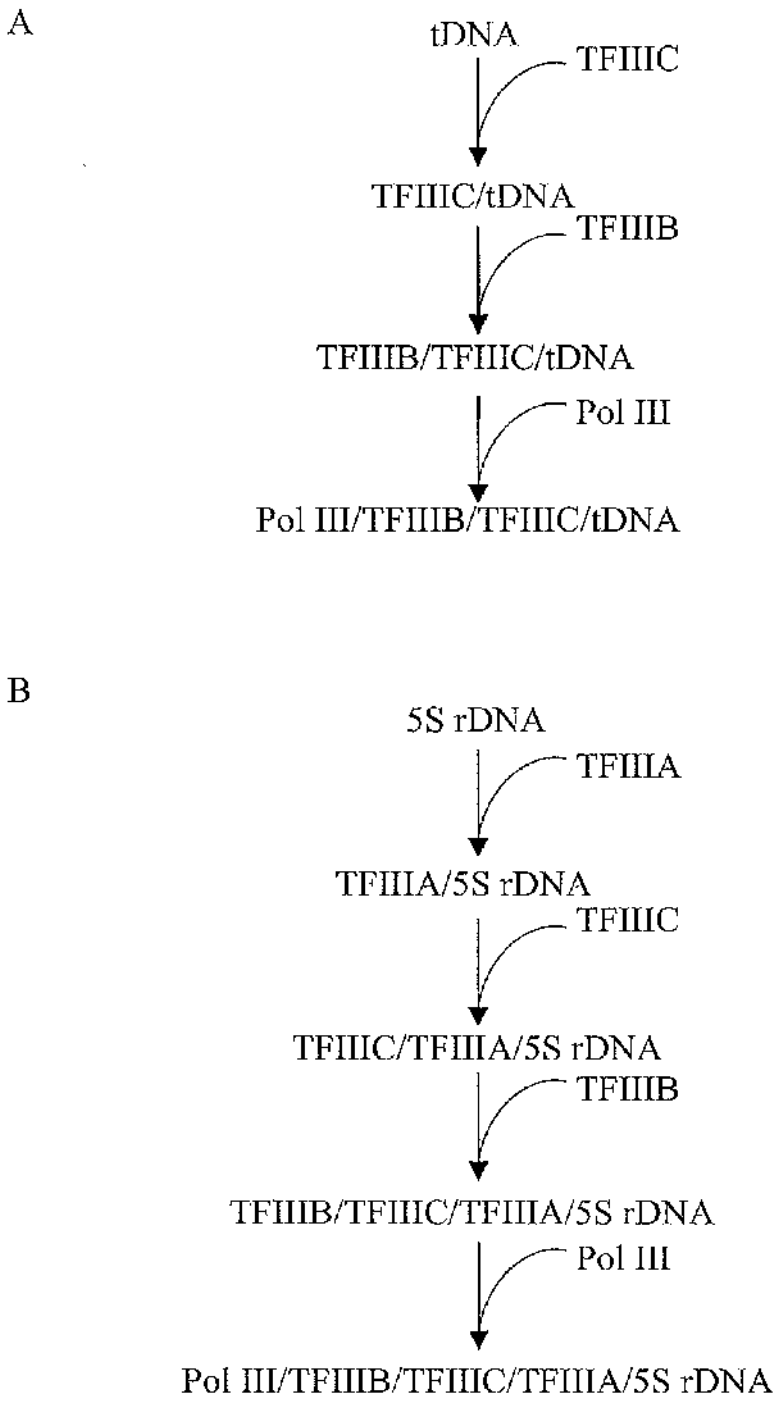


Figure 1.7. Order of transcription factor assembly on type I and II pol III promoters. Flow chart indicates the order of interaction of factors and polymerase on (A) a typical type II promoter such as a tRNA gene. (B) the promoter of a 5S rRNA gene.

al. 1987; Wang and Roeder 1996; Oettel et al. 1997). Both components are required for the transcription of 5S rRNA, VA1 and tRNA genes, but U6 and 7SK transcription requires only TFIIIC1 (Lagna et al. 1994; Yoon et al. 1995; Oettel et al. 1997). Sedimentation analysis has suggested that TFIIIC1 has a mass of up to 200 kDa (Yoshinaga et al. 1987). However, little progress has been made to further characterise this factor. On the other hand, TFIIIC2 has been purified and shown to consist of five polypeptides of 220, 110, 102, 90 and 63 kDa, with a total mass of ~600kDa (Dean and Berk 1988; Yoshinaga et al. 1989; Wang and Roeder 1996). TFIIIC2 can be isolated in two forms designated TFIIIC2a and TFIIIC2b (Hoeffler et al. 1988; Sinn et al. 1995). Both forms share the 220, 102, 90 and 63kDa subunits, and can bind DNA producing identical footprints on VA1. Transcriptional activity is, however, only associated with TFIIIC2a, which contains the 110kDa subunit (Hoeffler et al. 1988).

All five of the TFIIIC2 subunits have been cloned (Lagna et al. 1994; L'Etoile et al. 1994; Sinn et al. 1995; Hsieh et al. 1999a; Hsieh et al. 1999b). HTFIIIC220, hTFIIIC110 and hTFIIIC90 show no significant homology to any of the subunits of yeast TFIIIC. HTFIIIC102 and hTFIIIC63 display only a weak homology to τ131 and τ95 respectively (Hsieh et al. 1999b). This apparent lack of homology is particularly surprising since A and B blocks, contacted by TFIIIC220 in humans, are well conserved between mammals and yeast (Yoshinaga et al. 1987)

Productive recruitment of TFIIIC to the 5S rRNA promoters requires TFIIIA (Figure 1.7B). TFIIIA serves as an adaptor, providing a platform that allows TFIIIC to be recruited onto the 5S rRNA genes for which it has little affinity. *Xenopus laevis*

TFIIIA was the first eukaryotic transcription factor to be purified to homogeneity (Engelke et al. 1980) and the first to have its cDNA cloned (Ginsberg et al. 1984). TFIIIA is a gene-specific factor that is only required for the expression of 5S genes (Engelke et al. 1980). The 334 amino acid sequence of TFIIIA is predominantly composed of nine tandem, zinc-dependant DNA binding protein domains (Miller et al. 1985). NMR and crystal structure analysis has revealed that the C block on type I promoters is recognised by the three N-terminal fingers. These fingers wrap smoothly around the DNA major groove, and constitute 95% of the total binding energy of full length TFIIIA (Clemens et al. 1992; Foster et al. 1997; Nolte et al. 1998). The C-terminal three fingers are thought to contact the A block in a similar manner, albeit with a lower affinity (Clemens et al. 1992). The middle three fingers span the intermediate region. This region is twice as long as the regions bound by fingers 1-3 and 7-9, and these fingers adopt a completely different configuration in order to cover this area. Finger 5 makes base contacts in the major groove, at the intermediate element, whereas finger 4 and 6 straddle the neighbouring minor grooves and function primarily as spacer elements (Nolte et al. 1998).

Photocrosslinking studies have revealed that the positioning of the various TFIIIC subunits relative to each other, and to the initiation site, is similar on both a yeast 5S rRNA gene and a tRNA gene (Figure 1.8) (Braun et al. 1992). This finding is quite remarkable since, although, type I and II promoters both contain an A-block, it is bound by TFIIIA in the former case and by TFIIIC in the later case. Regardless of promoter type, the primary function of TFIIIC is to recruit TFIIIB (Figure 1.7).

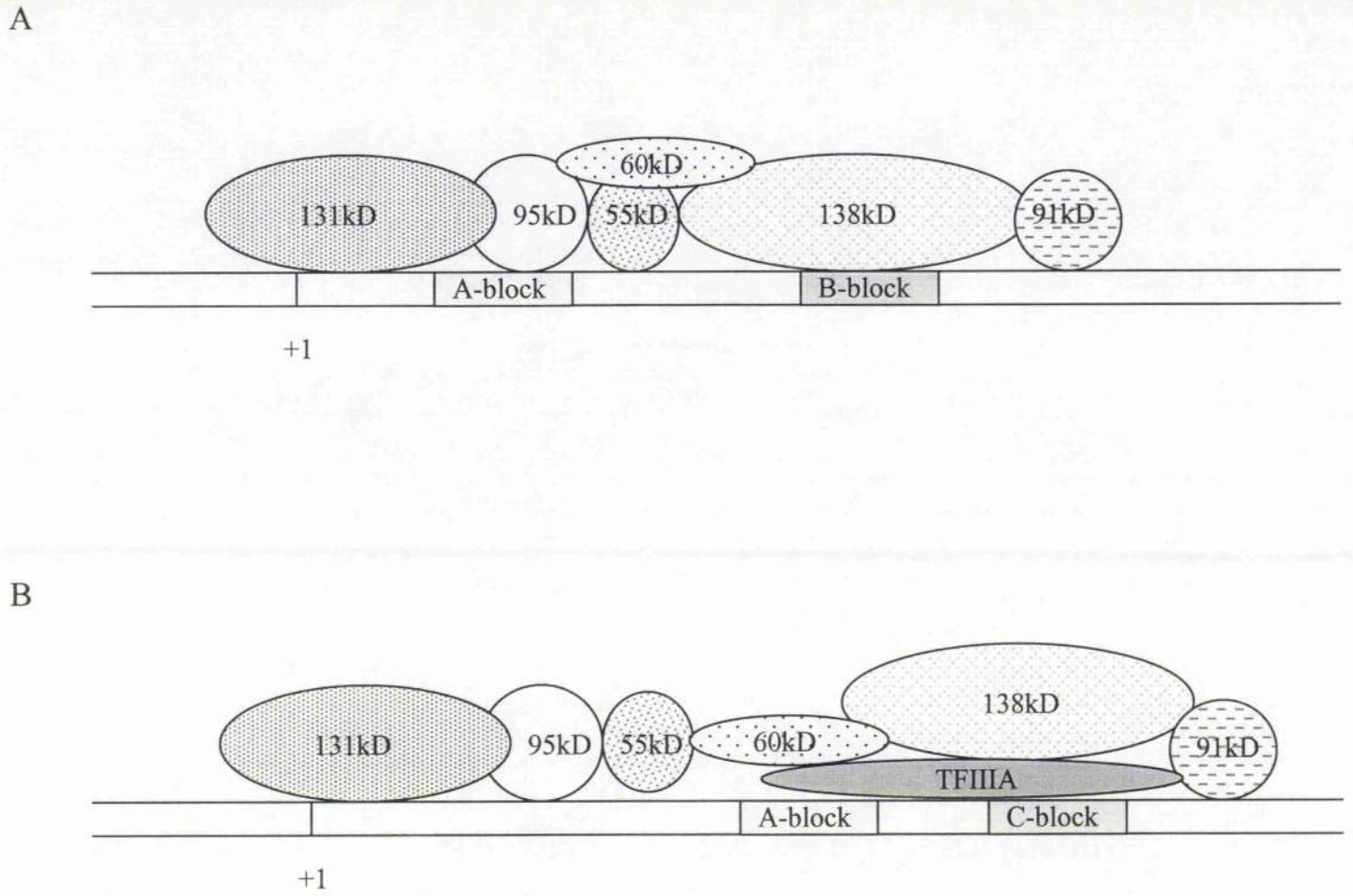


Figure 1.8. Relative position of TFIIIC on type I and II promoter subtypes. (A) Relative positions of the various subunits of *s. cerevisiae* TFIIIC when bound to a tRNA gene. (B) Relative positions of TFIIIA and the various subunits of TFIIIC when bound to a 5S rRNA promoter in *s. cerevisiae*.

TFIIIB has been characterised extensively (Kassavetis et al. 1995; Roberts et al. 1996; Ruth et al. 1996). It is a complex of three components, one of which is the TATA-binding protein, TBP. TBP is a central transcription factor utilized by all three nuclear RNA polymerases (White and Jackson 1992b; Hernandez 1993; Rigby 1993). Although the majority of pol III templates lack TATA-like sequences (White and Jackson 1992b; White et al. 1992b), TBP forms an integral part of the transcription initiation complexes formed on these genes.

TBP is the most conserved eukaryotic transcription factor known. All TBP genes encode a small (27-38kDa) polypeptide. The DNA-binding domain of TBP is 180 residues long and displays an extraordinary level of evolutionary conservation. This C-terminally located domain is identical between man and mouse, and is 81% identical between humans and the yeast S. *cerevisiae*. In contrast, the N-terminal region of TBP varies through evolution, in both its length and sequence. It has been suggested that it may modulate the activity of the highly conserved C-terminus (Kuddus and Schmidt 1993; Lescure et al. 1994; Mittal and Hernandez 1997; Zhao and Herr 2002). The DNA-binding domain of TBP is composed of two directly repeated halves. Although these halves are only 31% identical in sequence, they are virtually identical topologically (Reddy and Hahn 1991; Strubin and Struhl 1992; Heard et al. 1993). The α-carbon atoms of each polypeptide chain follow the same path through space in the two regions, forming a saddle structure that sits astride the DNA. The convex upper surface of the saddle consists of four α-helices, which make contact with other transcription factors (Nikolov et al. 1992). The concave under side contacts the DNA. This curved face is composed of ten antiparallel β-sheets, which forms a surface wide enough to accommodate the DNA double helix (Nikolov et al.

1992). TBP severely bends the DNA, creating an angle of approximately 80°. The minor grove is widened and flattenened, making extensive contacts with the underside of TBP through many hydrophobic and some hydrophylic interactions (Nikolov et al. 1992).

TBP interacts with other polypeptides, termed TBP-associated factors (TAFs). The different TBP-TAF complexes provide the basis of polymerase specificity. These complexes, in conjunction with appropriate initiation factors, form class-specific complexes at promoters and facilitate recruitment of the correct RNA polymerase (White and Jackson 1992b; Hernandez 1993; Rigby 1993). Pol I is recruited via the TBP-containing complex SL1 (Comai et al. 1992; Eberhard et al. 1993; Zomerdijk et al. 1994), and the equivalent complex in the pol II system, is TFIID (Greenblatt 1991).

TFIIIB, the pol III TBP-containing complex, has two TAFs. The largest of these is a 90kDa polypeptide called Bdp1. Bdp1 displays little homology to other known proteins except for a putative SANT domain (Kassavetis et al. 1995; Aasland et al. 1996; Ruth et al. 1996). This subunit is unusually resistant to truncation and will continue to support U6 transcription after all but 176 of its 594 residues have been deleted (Kumar et al. 1997). Two distinct domains (residues 270-305 and 390-460) are required for tRNA synthesis, whereas either alone is sufficient in the case of U6 (Kumar et al. 1997). Footprinting suggested that Bdp1 is folded such that these two domains are in close proximity when TFIIIB is assembled onto DNA (Kumar et al. 1997). Bdp1 makes multiple contacts with the transcription complex (Bartholomew et al. 1991; Kassavetis et al. 1991; Joazeiro et al. 1994; Roberts et al. 1996). This

may possibly account for its resilience to deletion mutagenesis. If an individual contact is lost, it may be compensated by other interactions made by the protein.

The other component of TFIIIB is a 70 kDa subunit that displays in its N-terminal 320 residues 23% identity and 44% similarity to the pol II transcription factor TFIIB (Buratowski and Zhou 1992a; Colbert and Hahn 1992; Lopez-de-Leon et al. 1992). Because of this homology, it is often referred to as TFIIB-related factor, or Brf1, although it has also been called TDS4, PCF4 and TFIIIB70. The majority of the homologous region is made up of a zinc ribbon at the extreme N-terminus and two imperfect repeats of 76 amino acids within the remainder of the amino-terminal half (Buratowski and Zhou 1992b; Colbert and Hahn 1992; Lopez-de-Leon et al. 1992). The C-terminal half of yeast Brf1 displays no obvious homology to any other know protein, and in contrast to the N-terminal half, is poorly conserved. However, three discrete regions of significant conservation have been identified, these are termed yeast homology regions HI, HII and HIII (Khoo et al. 1994).

Extensive mutagenesis has revealed that Brf1 contains two distinct TBP-binding domains, which interact with opposite faces of the TBP-DNA complex (Colbert et al. 1998; Kassavetis et al. 1998). The HII domain of the C-terminal half of Brf1 is thought to mediate one interaction with TBP (Khoo et al. 1994; Colbert et al. 1998; Andrau et al. 1999). The second TBP-binding site is located in the conserved direct repeat region in the N-terminal half of Brf1. This region is also believed to directly contact pol III (Khoo et al. 1994) and interact with the largest subunit of yeast TFIIIC (Chaussivert et al. 1995). A remarkable feature of Brf1 is that it can be split

down the middle to give two separate halves that continue to function when recombined (Kassavetis et al. 1998).

TFIIIB, reconstituted from recombinant TBP, Brf1 and Bdp1, is able to support TFIIIC-dependent and TATA-dependent DNA binding and transcription (Roberts et al. 1996; Ruth et al. 1996). Nevertheless, this reconstituted factor was found to be less active than native TFIIIB (Kassavetis et al. 1995; Ruth et al. 1996). Although this could be due to mis-folding of recombinant polypeptides, or the lack of important post-transcriptional modifications, the possibility exists that a non-essential, but stimulatory factor(s) is missing from the reconstituted TFIIIB preparation.

Human TFIIIB also contains TBP. Using anti-TBP antibodies, a TAF of 88-90 kDa was immunoprecipitated from TFIIIB fractions (Wang and Roeder 1995; Mital et al. 1996). The N-terminal 280 residues of this 677 amino acid protein, share 24% identity to human TFIIB, and 41% identity with *S. cerevisiae* Brf1. On the basis of this it was termed human TFIIB-related factor (hBrf1) (Mital et al. 1996). The N-terminal half of hBrf1 also shares regions of extensive homology with other Brf species; these include a zinc ribbon motif and two direct repeats (Mital et al. 1996). In contrast, with the exception of yeast homology region II (HII), the C-terminal half of hBrf1 displays little homology to yBrfs (Mital et al. 1996).

Like yBrf1, hBrf1 possesses two TBP-binding sites. One is located in the N-terminal half, with a stronger site associated with the conserved HII domain in the C-terminal domain of the protein (Wang and Roeder 1995). Transcription of VA and tRNA

genes was found to be severely inhibited following immunodepletion of hBrf1 and was restored by addition of recombinant TBP and hBrf1, proving it to be essential in human pol III transcription (Wang and Roeder 1995; Mital et al. 1996).

Recently a human homologue of yeast Bdp1 was cloned (Schramm et al. 2000). It is significantly larger that yBdp1, with a calculated mass of 156 kDa. It contains three principle regions of sequence conservation. A putative SANT domain exists between residues 415-472, which shows 43% identity to that of yeast Bdp1. In addition, a 131 amino acid region immediately upstream, and 151 amino acids downstream of the SANT domain, share 21% and 17% identity with yBdp1, respectively (Schramm et al. 2000). A striking feature of hBdp1 is a region of 19 repeats of 26-28 amino acids in the C-terminal domain, which is absent from yBdp1. Immunodepletion of hBdp1 from cell extracts severely impaired pol III gene expression. Addition of recombinant hBdp1 could restore this activity, confirming the functional significance of hBdp1 in pol III transcription (Schramm et al. 2000).

Since TFIIIB contains TBP, TFIIIB can bind independently to a TATA box (Joazeiro et al. 1994). However, since most type I and II promoters lack TATA sequences, they can not be recognised directly by TFIIIB (Kassavetis et al. 1989; Kassavetis et al. 1992; White and Jackson 1992b; Huet and Sentenac 1993). In these cases, TFIIIB is recruited by protein-protein contacts with DNA-bound TFIIIC (Lassar et al. 1983; Huet and Sentenac 1993). *S. cerevisiae* Brf1 has been shown to interact with τ131, the subunit of TFIIIC located furthest upstream along the promoter (Chaussivert et al. 1995; Kassavetis et al. 1998). It has been suggested that the TFIIIB-binding site may be masked prior to conformational rearrangements, since certain mutation in

τ131 can stimulate transcription complex assembly (Ramcau et al. 1994; Chaussivert et al. 1995; Moir et al. 1997). Indeed τ131 becomes reoriented during TFIIIB assembly, indicated by changes in the efficiency of photocrosslinking (Kassavetis et al. 1992). Once recruited, yeast TFIIIB is situated approximately 40 bp upstream from the transcription start site (Kassavetis et al. 1989; Kassavetis et al. 1990; Braun et al. 1992; Kassavetis et al. 1998; Persinger et al. 1999). From this position, TFIIIB can efficiently recruit pol III to the initiation region (Kassavetis et al. 1990). Although all three subunits are required for pol III recruitment, direct interaction have only been identified in the case of Brf1 (Bartholomew et al. 1993; Werner et al. 1993; Khoo et al. 1994).

Once assembled, transcription complexes on class III genes display considerable stability towards high salt concentrations that would prevent their formation entirely (Setzer and Brown 1985; Carey et al. 1986; Kassavetis et al. 1989; Kassavetis et al. 1990). For example, after a 6.5 min exposure of the preformed human complex to 1M KCl, ~45% of VA1 transcriptional activity remains (Carey et al. 1986). In yeast, the interaction between TFIIIB and DNA is the most resistant to salt, whereas TFIIIC and TFIIIA are dissociated more readily (Kassavetis et al. 1989; Kassavetis et al. 1990). Once recruited, TFIIIB will remain stably bound, even in 1M KCl (Kassavetis et al. 1990). This is remarkable, since TFIIIB alone is incapable of recognising TATA-less class III genes. It seems probable that interaction with TFIIIC unmasks a cryptic DNA-binding capacity that locks TFIIIB onto the promoter (Huet et al. 1997). TFIIIA and TFIIIC can be regarded as assembly factors, as they can be stripped from fully assembled transcription complexes on tRNA and 5S rRNA promoters without compromising the efficiency of transcription (Kassavetis et al.

1990). Under these circumstances, TFIIIB is sufficient on its own to recruit pol III and direct multiple rounds of accurately initiated transcription (Kassavetis et al. 1990). This exceptional salt stability is not a feature of vertebrate pol III systems. Where the dissociation pathway is the reverse of the assembly pathway (Setzer and Brown 1985; Carey et al. 1986; Jahn et al. 1987).

1.11.2 Type III promoters

The assembly of transcription complexes on type III promoters differs due to the lack of ICRs. This consequently eliminates the need for TFIIIA and TFIIIC2 (Bernues et al. 1993; Lagna et al. 1994; Yoon et al. 1995). Although these promoters still utilize TFIIIC1, they employ a form of TFIIIB chromatographyically separable from the form used by type I and II promoters (Lobo et al. 1992; Teichmann and Seifart 1995). Recent analysis has demonstrated that a Brf1-like factor, Brf2 (BRFU) is required for transcription of the U6 snRNA genes instead of Brf1 (Schramm et al. 2000). The N-terminal domain of Brf2 is related to both Brf1 and TFIIB, whereas the C-terminal region is divergent.

The PSE of type III promoters is recognised by SNAPc/PTF (Figure 1.9). SNAPc/PTF is essential for transcription of 7SK and some vertebrate U6 genes (Yoon et al. 1995). It associates with the PSE of type III promoters and can greatly enhance the recruitment of TFIIIB to the TATA box (Yoon et al. 1995). SNAPc/PTF is a complex of five subunits, with a native mass of approximately 350 kDa (Sadowski et al. 1993; Henry et al. 1995). The largest subunit, SNAP190, contains a Myb DNA-binding domain, and can be cross-linked to the PSE (Yoon et al. 1995; Wong et al. 1998). The second largest subunit, SNAP50, also associates with the

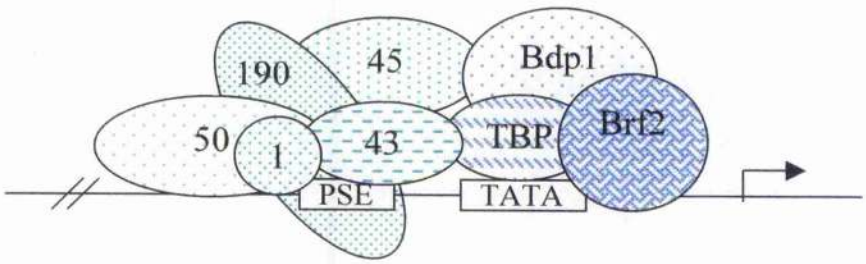


Figure 1.9. PTF/SNAPc interactions with TFIIIB on the U6 promoter. Schematic diagram illustrates the interactions between PTF/SNAPc and TFIIIB that may occur on a vertebrate U6 promoter.

DNA (Henry et al. 1996). In contrast, the other subunits, SNAP45, 43 and 19, do not bind directly to the DNA (Yoon and Roeder 1996), but are found associated with the PSE-binding complex. Immunodepletion of SNAP50, SNAP43 or SNAP45 specifically repressed 7SK and U6 transcription, whilst having no effect on VA1 and AdML expression (Henry et al. 1996; Sadowski et al. 1996; Yoon and Roeder 1996). Transcription of the pol II transcribed U1 and U2 genes was also inhibited by immunodepletion of these factors. This repressive effect could be restored by addition of highly purified SNAPc/PTF, indicating that, SNAPc/PTF is required for transcription of both pol II and pol III U snRNA genes (Henry et al. 1995; Bai et al. 1996; Henry et al. 1996; Sadowski et al. 1996; Yoon and Roeder 1996). The distance separating the PSE and the TATA box is constrained very precisely on type III promoters (Lescure et al. 1991; Goomer and Kunkel 1992). This suggests that PTF/SNAPc interacts with the TATA-bound TBP or TFIIIB. Both PSE and TATA recognition are relatively slow (Wanandi et al. 1993; Mittal et al. 1996). Transcription assembly can, however, be stimulated by Oct-1, which binds to the upstream DSE. Oct-1 interacts directly with SNAPc190, and thereby stimulates PSE occupancy (Murphy et al. 1992; Mittal et al. 1996; Ford et al. 1998). Occupancy of the PSE is of primary importance for stable complex formation on the human U6 promoter (Kunkel and Danzeiser 1992). However, SNAPc/PTF and TBP enhance each other's recruitment (Mittal and Hernandez 1997). Figure 1.9 illustrates the composition of TFIIIB on type III promoters. It remains to be determined how TFIIIC1 is recruited, but this may be a late step in complex assembly on type III promoters (Paule and White 2000).

1.12 TFIIIB; the p53 target

Expression of p53 has been shown to repress transcription of a wide range of pol III transcripts including Alu, U6 (Chesnokov et al. 1996; Cairns and White 1998), tRNA, 5S rRNA, VA, B2 and EBER (Cairns and White 1998) and thus functions as a general regulator of pol III transcription. As yet, the mechanistic basis of pol III transcriptional repression by p53 has not been established.

It has been shown that an N-terminal fragment of p53, overexpressed as a GST fusion protein, can bind to TFIIIB in a 'pull-down' assay (Chesnokov et al. 1996). In addition, it has been demonstrated by immunoprecipitation and cofractionation experiments that endogenous p53 and TFIIIB associate at physiological ratios. It is proposed that TFIIIB is a specific target for repression by p53. This is supported by the finding that when p53 is present in excess, adding more purified TFIIIB can efficiently relieve VA1 gene expression from repression by p53. It is well documented that TBP, one of the components of TFIIIB can bind to p53 (Seto et al. 1992; Liu et al. 1993; Martin et al. 1993; Truant et al. 1993b; Horikoshi et al. 1995; Tansey and Herr 1995; Chesnokov et al. 1996; Farmer et al. 1996a). It appears that this interaction is required for repression of TFIIIB, since heat inactivation of TBP abolishes the ability of TFIIIB to restore transcription in the presence of p53.

Fibroblasts derived from p53 knock out mice transcribe pol III templates at elevated levels, this is due to a specific rise in TFIIIB activity (Cairns and White 1998). It is proposed that TFIIIB is a target for repression by endogenous p53 and that this regulation may contribute to the proper control of pol III transcription in untransformed cells (Cairns and White 1998). Although it has been demonstrated

that p53 can inactivate TFIIIB specifically, once TFIIIB has been assembled into a preinitiation complex it becomes far less susceptible to repression (Cairns and White 1998).

1.13 Regulation of Pol III transcription

Synthesis of pol III products is clearly an essential component of cellular metabolism and consequently pol III transcription is subject to a wide range of regulatory signals (Brown et al. 2000). It is tightly linked to growth conditions, increasing in response to mitogenic signals and falling when serum factors or nutrients are limiting. It is also subject to cell cycle control in vertebrates, being activated at the G1/S transition and repressed at mitosis. In addition, many viruses have potent effects upon the rate of pol III transcription. Furthermore, the majority of transformed and tumour cell types display abnormally elevated pol III products (Kramerov et al. 1982; Brickell et al. 1983; Scott et al. 1983; Majello et al. 1985; Ryskov et al. 1985; Lania et al. 1987; Kramerov et al. 1990). In each case, regulation is mediated through changes in activities of the pol III specific transcription factors, and not through the polymerase itself (Brown et al. 2000).

p53 is not alone in its ability to target TFIIIB (Figure 1.10). This is perhaps not surprising, given the essential central role TFIIIB plays in mediating pol III transcription (White 1998a). TFIIIB is bound and regulated by the tumour suppressor RB, the product of the retinoblastoma susceptibility gene (Chu et al. 1997; Larminie et al. 1997; Larminie et al. 1999; Sutcliffe et al. 1999; Sutcliffe et al. 2000). Overexpressing RB in transfected cells produces a significant decrease in pol III

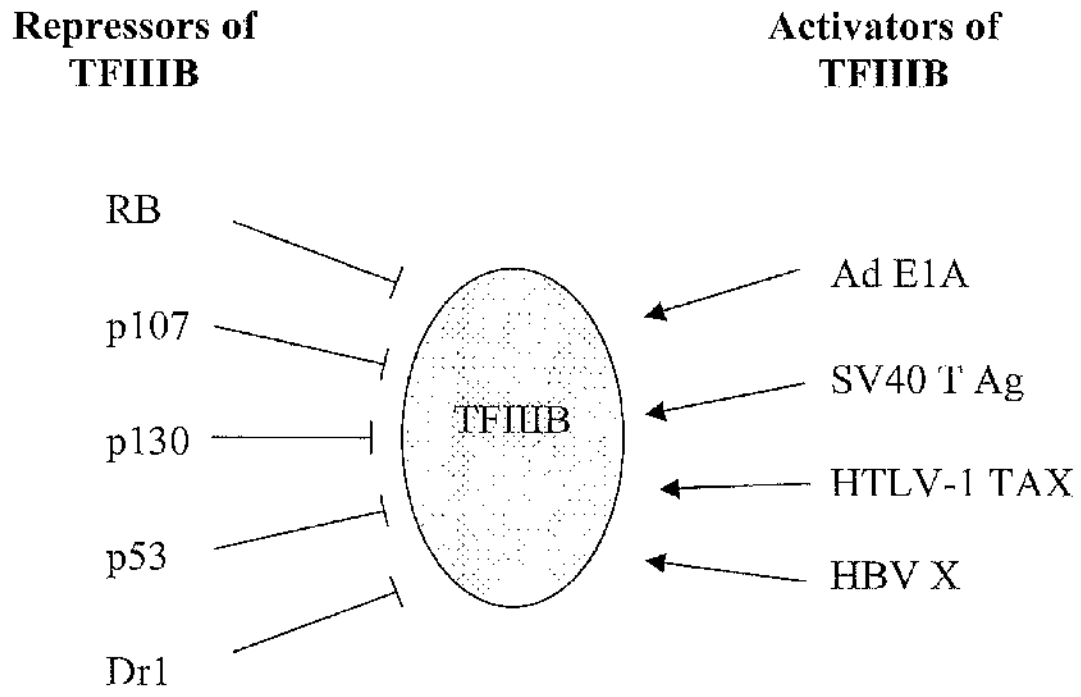


Figure 1.10. TFIIIB antagonistic influences. The growth suppressors RB, p53 and Dr1 can all repress TFIIIB. In contrast a variety of oncoproteins can stimulate TFIIIB activity. Activators of TFIIIB include the E1A protein of adenovirus, SV40 large T antigen, the Tax protein of HTLV-1, the X protein of HBV.

activity (White et al. 1996; Chu et al. 1997). Recombinant RB also represses pol III transcription that has been reconstituted from partially purified factors (White et al. 1996; Chu et al. 1997). Additionally, inactivation of endogenous RB results in a fivefold increase in the synthesis of tRNA and 5S rRNA (White et al. 1996). Extracts prepared from RB-knockout mice show a specific increase in TFIIIB activity (White et al. 1996). RB coimmunoprecipitates with endogenous TFIIIB (Larminie et al. 1997). Indeed, such an association has been demonstrated to result in disruption of TFIIIB interactions with TFIIIC2 and pol III (Sutcliffe et al. 2000). p107 and p130, two RB relatives, also bind to TFIIIB (Sutcliffe et al. 1999). Like RB, recombinant p107 and p130 repress transcription of a wide range of pol III templates, both *in vitro* and in transfected cells (Sutcliffe et al. 1999). Collectively these three proteins are referred to as the pocket protein, because most of their homology lies within a bipartite region called the pocket domain.

The functions of the pocket proteins are subject to cell cycle control (Figure 1.11) (Brown et al. 2000). They are found to be underphosphorylated, and hence in an active state, during G0 and early G1 phases. As cells pass the restriction point in mid G1, the pocket proteins are inactivated through hyperphosphorylation by the cyclin D- and cyclin E-dependent kinases (Herwig and Strauss 1997; Taya 1997; Grana et al. 1998; Mittnacht 1998; Mulligan and Jacks 1998). For much of the mammalian cell cycle, there is an inverse correlation between the activity of the pocket proteins and the level of pol III transcription (Johnson et al. 1974; Mauck and Green 1974; White et al. 1995a). TFIIIB activity is also regulated strongly in cycling cells (White et al. 1995b; White et al. 1995a). During mitosis the activity of TFIIIB is severely limiting. This is achieved through phosphorylation and inactivation of TFIIIB

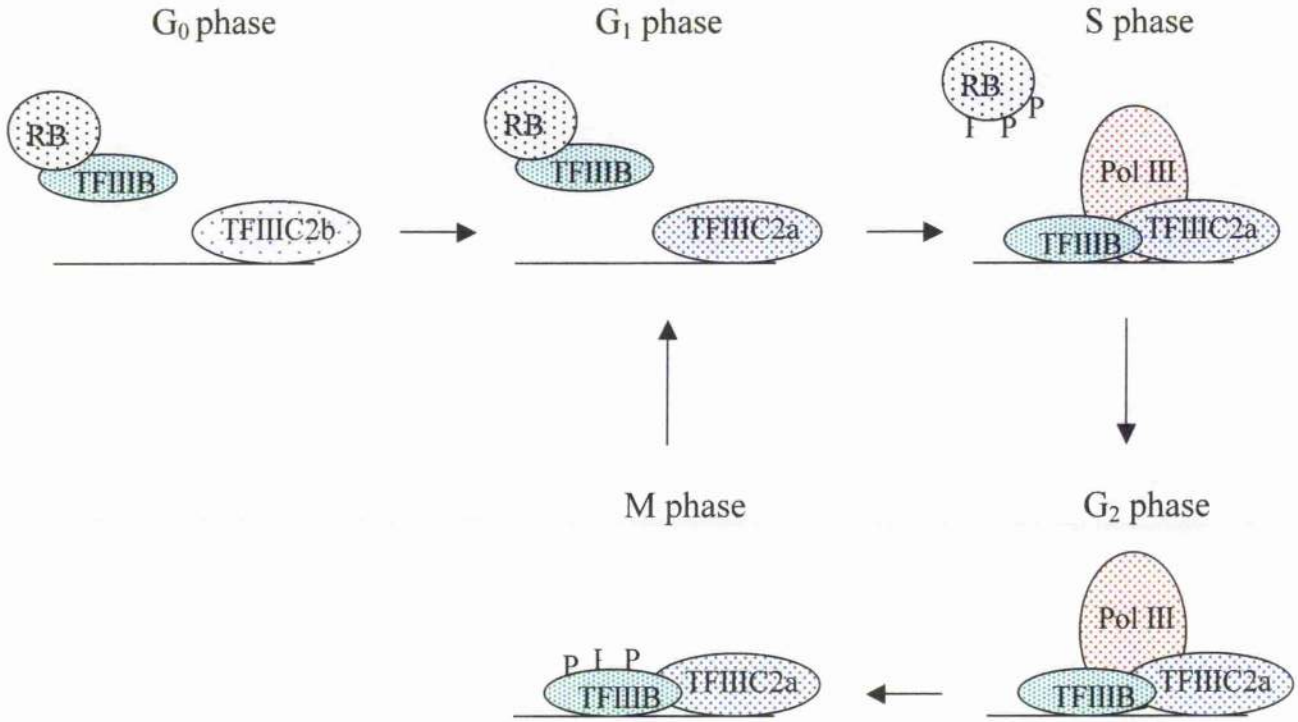


Figure 1.11. Cell cycle control of pol III transcription. Model illustrating the regulatory mechanisms that contribute to the cell cycle control of pol III transcription in mammalian systems. During quiescence (G_0), when expression of pol III transcripts is low, TFIIIC may be present in its transcriptionally inactive TFIIIC2b form and TFIIIB activity is subject to repression by RB. In cycling cells TFIIIC predominates in its active TFIIIC2a form, however, TFIIIB remains associated with RB, sustaining low transcription levels. Following the G_1/S transition, transcription levels are significantly elevated until the end of G_2 phase. Transcription is repressed again during mitosis through the phosphorylation-mediated inactivation of TFIIIB.

(Gottesfeld and Forbes 1997). As cells exit mitosis, TFIIIB hyperphosphorylation is rapidly reversed. The rate of pol III transcription increases gradually as cells pass through the G1 phase (White et al. 1995a; Scott 2001). RB binds and represses TFIIIB during G0 and early G1, but this interaction decreases as cells approach S phase. Full induction of pol III coincides with mid- to late G1 phase, when RB becomes phosphorylated by cyclin D- and E-dependent kinases. TFIIIB only associates with the underphosphorylated form of RB, and overexpression of cyclins D and E stimulate pol III transcription in vivo (Scott et al. 2001). Pol III transcription is maximal throughout S and G2 phases, where TFIIIB activity is in relative excess (White et al. 1995b; White et al. 1995a).

The 19 kDa phosphoprotein Dr1 can also bind to and inhibit the activity of TFIIIB (White et al. 1994b). Dr1 was found to inactivate TFIIIB by binding to TBP and disrupting its interaction with Brf1 (White et al. 1994b; Kim et al. 1997).

A variety of viruses have been shown to stimulate pol III transcription. This is most likely necessary to meet an increase in biosynthetic demands. Multiple mechanisms contribute to the regulation of pol III transcription in response to adenovirus infection. Adenovirus encodes two pol III products, VAI and VAII, which are synthesised at high levels during the late stages of infection (Weinmann et al. 1974; Soderlund et al. 1976). These short RNAs are involved in subverting the host's cells translational apparatus, in order to ensure the synthesis of viral proteins (Thimmappaya et al. 1982). Cells infected with wild-type adenovirus display a marked increase in TFIIIC activity (Hoeffler and Roeder 1985; Yoshinaga et al. 1986; Hoeffler et al. 1988; Sinn et al. 1995). Additionally, the E1A transforming

protein from adenovirus can also relieve TFIIIB from repression by RB (White et al. 1996). SV40, human T-cell leukaemia virus type I and hepatitis B virus also all possess the capacity to stimulate pol III transcription, which is mediated at least in part through their action on TFIIIB (White 1998b).

1.14 Pol III transcription and cellular transformation

Many transformed cell lines overexpress pol III products (Brickell et al. 1983; Scott et al. 1983; Majello et al. 1985; Ryskov et al. 1985; Lania et al. 1987; Kramerov et al. 1990). Pol III responds to a variety of transforming agents, including DNA tumour viruses, RNA tumour viruses and chemical carcinogens (White 1998b). *In situ* hybridisation has also revealed this to be the case *in vivo* (Chen et al. 1997a). Elevated pol III transcripts were found in the neoplastic cells of breast carcinoma, colon adenocarcinoma and skin fibrosarcoma samples, relative to the surrounding tissue (Chen et al. 1997a).

In many cases the mechanistic basis of the elevation in pol III transcript levels has yet to be determined (White 1998b). It however seems likely that release of TFIIIB from repression by RB is a pivotal feature of tumour cells. RB function has been shown to be compromised in a broad range of human cancers (Strauss et al. 1995; Weinberg 1995; Whyte 1995). Most RB mutations that occur naturally in tumours lie within the pocket domain, the same region that is required for regulation of pol III transcription (White et al. 1996; Chu et al. 1997).

Approximately half the major forms of human cancer contain p53 mutations (Hollstein et al. 1991). Several such mutations can abrogate the ability of p53 to repress pol III transcription (Stein et al. 2002a). For example, in SAOS2 cells, transient transfection of a mutant p53 in which arginine 175 is substituted to proline (R175P), originally derived from an anogenital tumour, was severely compromised in its ability to repress pol III transcription (Stein et al. 2002a). Mutation R175H, in which arginine 175 was substituted to histidine, is one of the most common substitutions associated with human cancers. This mutation is also associated with an extremely poor prognosis in cancer patients (Goh et al. 1995). R175H was unable to repress pol III transcription in transient transfection assays (Stein et al. 2002a). Remarkably, this mutant actually activates pol III transcription in a dose-dependent manner (Stein et al. 2002a). The mechanistic basis of these effects are, however, still to be determined.

A considerable weight of evidence indicates that the regulation of protein synthesis is an important aspect of growth control. It has been shown that a 50% reduction in the rate of protein synthesis is sufficient to cause proliferating cells to withdraw from the cell cycle and quiesce (Brooks 1977; Ronning et al. 1981).

There are many documented examples in which the translation machinery has become deregulated following transformation (Rosenwald 1996), providing strong support for the belief that the control of protein synthesis is an important aspect of growth regulation. Translation is clearly dependent on the availability of adequate supplies of tRNA and rRNA and thus active pol III transcription. These facts may

therefore explain why pol III transcription is subject to deregulation by viruses, and frequent activation in transformed and tumour cells (White 1998b).

1.15 Aims and objectives

Clearly, p53 can repress pol III transcription. The mechanistic basis of this however, remains elusive. The principal aim of this project was to establish the means by which p53 can repress pol III transcription. Many lines of evidence indicate that TFIIIB is targeted by p53, the biochemical and functional consequences of this interaction were therefore investigated both *in vitro* and *in vivo*.

p53 is deregulated or mutated in a majority of human cancers; the consequence of such deregulation was investigated with respect to pol III transcriptional control. Additionally, it was investigated whether DNA damage, a cellular stress known to activate p53, could stimulate endogenous p53 into repressing pol III gene expression.

Chapter 2

Materials and methods

2.1 Cell culture

HeLa, primary mouse embryo fibroblasts (MEFs) and SAOS2 cells were cultured in DMEM (Dulbecco's Modified Eagle Medium) containing 10% fetal calf serum (FCS, Sigma), 2mM L-glutamine, 100U/ml penicillin and 100μg/ml streptomycin. CHO cells, stably transfected with pcDNA3HABrf1, expressing Haemaglutinin (HA) tagged Brf1 (Johnston et al. 2002), were grown in Ham's F-12 medium supplemented with 10% FCS 100U/ml penicillin and 100μg/ml streptomycin. NARF2 cells stably expressing inducible $p14^{ARF}$ (Stott et al. 1998) were maintained in DMEM containing 10% fetal calf serum, 150μg/ml hygromycin, 300μg/ml geneticin, 2mM L-glutamine, 100U/ml penicillin and 100μg/ml streptomycin. For Methanesulfonic Acid Methyl Ester (MMS) treatment of HeLa cells, cells were grown to 80% confluency then treated with 0.04% MMS for 2 hours. Cells were routinely grown in a humidified atmosphere containing 5% CO_2 at 37°C. Cell culture was performed in a class II hood, using aseptic technique and sterile equipment and reagents.

Cells were passaged when subconfluent; approximately every 2 to 3 days. After media was aspirated from the flask, 2ml of buffered trypsin-EDTA (0.05% trypsin, 0.02% EDTA) was added to the cells, then aspirated immediately. A further 2ml was added and left for approximately 2 minutes at 37°C. Following trypsinisation, fresh media was immediately added to the dissociated cells in order to neutralise the

trypsin. Cells were centrifuged at 1200g for 2 minutes at 4°C and the media removed. The pelleted cells were then resuspended in fresh media at a ratio of 1:4 for the NARF2 or wild-type MEFs or 1:8 for the HeLa or p53 knockout MEFs.

Cyro-freezing was used for storage of all cell lines. Cells were trypsinised as described and, following pelleting by centrifugation, cells were resuspended in a solution of 82% DMEM or Ham's F12 (10% FCS), 10% neat FCS and 8% dimethylsulphoxide (DMSO). Cells were aliquotted into cyro-tubes and frozen at –80°C overnight then transferred to liquid nitrogen. Thawing of cells was performed rapidly by placing cyro-tubes in a waterbath at 37°C until just thawed. Cells were then mixed with fresh media, centrifuged and the supernatant aspirated off to ensure removal of DMSO prior to resuspension in 10% FCS DMEM.

2.2 Transient transfection of SAOS2 cells by calcium phosphate precipitation

Approximately 6 x 10^5 cells were seeded onto 100mm tissue culture plates the day prior to transfection. Media was changed 2-3 hours preceding transfection. A total of 20μg plasmid DNA (4μg HSV-p53, 6μg pVA1, 6μg HSV-CAT and 4μg PJ4Ω16.E6 or pG4-p300) in 225μl of 0.1×TE was mixed with 25μl of 2.5M $CaCl_2$ then added dropwise to 250μl of 2×HBS (50mM HEPES, 250mM NaCl, 1.5mM $NaHPO_4$ pH 7.12) with vigorous mixing. DNA calcium phosphate precipitate was allowed to form for 30 minutes at RT then added dropwise to tissue culture plates. The precipitate was removed after 16 hours and the cells washed 2× in PBS and fresh media added. Cells were harvested 48 hours later.

The plasmids used for transient transfection were as follows: pVA1 contains the adenovirus VA1 gene (White et al. 1989); HSV-CAT contains the CAT gene driven by the thymidine kinase promoter of Herpes Simplex Virus; HSV-p53 (Crook et al. 1994); pJ4Ω16.E6 (Storey et al. 1988); pG4-p300 was prepared by subcloning p300 from Gal4-p300 into pCDNA3 (Shikama et al. 2000) and pcDNA3 (Invitrogen).

2.3 Preparation of whole cell extracts

All whole cell extracts were prepared from cells grown in 10cm tissue culture dishes to facilitate scraping and were harvested at approximately 80% confluency. Preparation was performed on ice as rapidly as possible and all solutions and tubes were kept ice-cold to maintain cell activity. Cells were washed twice with 5ml of PBS before being scraped with a plastic spatula into 5ml of ice-cold PBS. Cells were collected in 50ml Falcon tubes and pelleted by centrifugation at 1200g for 8 minutes at 4°C. 1ml of fresh ice-cold PBS was used to resuspend the cell pellets and allow the cells to be transferred to eppendorf tubes. These were then microcentrifuged briefly at 4°C to re-pellet the cells and the PBS removed. The volumes of cell pellets were then measured by comparison with pre-measured volumes of water. Microextraction requires pellets to be between 50 - 150µl, giving approximately 0.5 – 3 x 10^7 cells; larger pellets were subdivided. An equal volume of freshly made cold microextraction buffer (450mM NaCl, 50mM NaF, 20mM Hepes pH 7.8, 25% glycerol, 1mM DTT, 0.5mM PMSF, 0.2mM EDTA, 40µg/ml bestatin, 1µg/ml trypsin inhibitor, 0.7µg/ml pepstatin, 0.5µg/ml aprotinin, 0.5µg/ml leupeptin) was added to the cells and, following resuspension, the cells were immediately snap-frozen on dry ice. Cells were then placed in a 30°C water bath until just thawed

before being immediately returned to dry-ice. This freeze-thaw procedure was performed a total of 3 times to ensure optimal cell lysis. Cells were then microcentrifuged at 7000g for 7 minutes at 4°C. The supernatant was removed, aliquoted and snap frozen. Whole cell extracts were stored at –70°C.

2.4 Preparation of total cellular RNA

Total cellular RNA was isolated from cells when approximately 80% confluent using TRI reagent (Sigma), in accordance with the manufacturer's instructions. Media was aspirated off cells grown in 10cm tissue culture dishes and residual media removed with two washes using 5ml ice-cold PBS. Cells from each dish were harvested by scraping in 1ml of TRI reagent per dish and transferred to a sterile eppendorf tube. Cells were left to stand for 5 minutes at room temperature to ensure complete dissociation of nucleoprotein complexes. 0.2ml of chloroform was then added to each tube and the samples vortexed for 15 seconds. The samples were then allowed to stand for a further 15 minutes at room temperature prior to being centrifuged at 13 000g for 15 minutes at 4°C. This resulted in separation of the samples into three phases: a lower red organic phase containing protein, a middle white interphase containing precipitated DNA and an upper colourless aqueous phase containing RNA. These upper phases were carefully removed, ensuring no contamination from the remaining phases and transferred to fresh eppendorf tubes. Isopropanol (500µl) was added to each of the tubes containing the aqueous RNA and thoroughly mixed by repeated inverting. Following 5 – 10 minutes incubation at room temperature to allow maximal precipitation of RNA, samples were centrifuged at 13 000g for 10

minutes at 4°C. The supernatant was then removed and the remaining RNA pellet was washed with 1ml of 75% ethanol made up with diethypyrocarbonate (DEPC)-treated dH_2O (0.1% DEPC), thoroughly mixed into solution, left overnight at room temperature and then autoclaved to inactivate the remaining DEPC. The samples were vortexed briefly, microcentrifuged at 7500g for 5 minutes at 4°C and the supernatant aspirated off. Residual supernatant was removed following pulse microcentrifugation. Appropriate volumes of DEPC-dH_2O, in the range of 10 - 30µl, were added to the RNA pellets and the samples were heated in a 65°C waterbath for 10 – 15 minutes to facilitate resuspension of the RNA. RNA was stored at –70°C.

RNA concentration was determined by UV spectrophotometry using the calculation: RNA concentration (µg/ml) = absorbance at 260nm x 40 x dilution factor. A ratio of absorbance at 260nm to 280nm in the range of 1.8 – 2 indicated the RNA samples were relatively free from contamination with DNA or protein.

2.5 Measuring protein concentration

The protein concentration of samples was determined using Bradford's reagent (Biorad). Quantification of the colour reaction produced upon mixing 1ml of diluted reagent (1:4 in distilled water) with a volume of sample containing protein in the range of ~1 –12µg gave an indication of protein concentration. This was achieved by measuring the absorbance of these samples at 595nm in a UV spectrophotometer, as absorbance in response to increasing amounts of protein under these conditions is approximately linear. Absorbance readings obtained were then compared to a set of standards of known protein concentration using bovine serum albumin (BSA)

measured at 595nm. A range of sample dilutions were measured and compared in this manner in order to obtain an average. Where sample absorbance readings fell outside the linear range of the standard set, appropriate dilutions were made and the samples re-measured.

2.6 Preparation of cDNAs

cDNAs were prepared from 3μg of RNA. Primer annealing was carried out in a final volume of 24μl with 0.67 x hexanucleotide mix (Roche) (diluted in DEPC-dH_20) and allowed to proceed for 10 minutes before being transfered to ice. 8μl of 5 x First Strand Buffer (Life Technologies), 4μl of 0.1M DTT, 2μl of 10mM dNTP mix (made up in DEPC-dH_20) and 1μl (200U) of Superscript II Reverse Transcriptase (Life Technologies) was added to initiate reverse transcription, which was performed for 1hour at 47°C before the reaction was stopped by heating at 70°C for 15 minutes. cDNAs were stored at –20°C

2.7 Reverse transcriptase – Polymerase chain reaction (RT-PCR)

PCRs were carried out using a Proteus II thermal controller (MJ Research, Helena BioScience). 2μl of cDNA was amplified with 20pmol of primer, 0.5U of *Taq* DNA polymerase (Promega) in 20μl of 1 x *Taq* DNA polymerase buffer (Promega) containing 1.5mM $MgCl_2$, 0.2mM of each dNTP and 1.8μCi of [α^{32}P] dCTP (10mCi/ml, 3000Ci/mmol).

The following forward (F) and reverse (R) primers were used:

ARPP P0:	(F) 5'-GCA CTG GAA GTC CAA CTA CTT C-3'
	(R) 5'-TGA GGT CCT CCT TGG TGA ACA C-3'
$tRNA^{Leu}$:	(F) 5'-GTC AGG ATG GCC GAG TGG TCT AAG-3'
	(R) 5'-CCA CGC CTC CAT ACG GAG ACC AGA AGA CCC-3'
TFIIIC220:	(F) 5'-TCC AGC GAG ACC TTC ACA CC-3'
	(R) 5'-GGA TTG AGT GTT GCT GGG CT-3'
$tRNA^{Tyr}$:	(F) 5'-CCT TCG ATA GCT CAG CTG GTA GAG CGG AGG-3'
	(R) 5'-CGG AAT TGA ACC AGC GAC CTA AGG ATG TCC-3'
p21 gene:	(F) 5'-CAC CTC CTC ATG TAC ATA CC-3'
	(R) 5'-AG GTC TGA GTG TCC AGG AA-3'
p21 promoter:	(F) 5'-CCA GCC CTT GGA TGG TTT-3'
	(R) 5'-GCC TCC TTT CTG TGC CTG A-3'

PCR was performed under the following cycling parameters:

ARPP P0: 95°C for 2 minutes, 18 cycles of [95°C for 1 minute, 58°C for 30 seconds, 72°C for 1 minute], 72°C for 3 minutes.

$tRNA^{Leu}$: 95°C for 2 minutes, 30 seconds, 25 cycles of [95°C for 30 seconds, 68°C for 30 seconds, 72°C for 20 seconds], 72°C for 5 minutes.

$TRNA^{Tyr}$: 95°C for 3 minutes, 24 cycles of [95°C for 1 minute, 65°C for 30 seconds, 72°C for 15 seconds], 72°C for 5 minutes.

TFIIIC220: 95°C for 3 minutes, 20 cycles of [94°C for 20 seconds, 62°C for 30 seconds, 72°C for 30 seconds], 72°C for 10 minutes.

p21 gene: 95°C for 2 minutes, 30 cycles of [95°C for 30 seconds, 59°C for 30 seconds, 72°C for 30 seconds], 72°C for 3 minutes.

p21 promoter: 95°C for 2 minutes, 35 cycles of [95°C for 1 minute, 62°C for 1 minute, 72°C for 1 minute], 72°C for 5 minutes.

Reaction products were resolved on 7% polyacrylamide sequencing gels containing 7M urea and 0.5 x TBE (45mM Tris, 45mM boric acid, 0.625mM EDTA pH 8.0). Gels were pre-run for 30 minutes at 40W in 0.5 x TBE and 2µl of each sample was loaded after being boiled at 95°C for 2 minutes and quenched on ice. Electrophoresis was carried out for a further 1hour at 40W and the gel subsequently vacuum-dried at 80°C for 1hour before being exposed to autoradiography film in order to detect the radiolabelled products. Quantification of results was achieved by phosphoimaging (Fujix Bas 1000).

2.8 Transformation of competent cells

E.coli XL-1 Blue supercompetent cells (Stratagene) were transformed for plasmid storage and propagation. Cells, which were stored at –80°C and highly temperature sensitive, were thawed on ice to prevent loss of transformation ability. 0.4µl of β-mercaptoethanol, which enhances transformation efficiency, was added to the 50µl of cells that were required per transformation reaction to give a final concentration of 25mM. Typically 10 – 20ng of plasmid DNA was then gently mixed into the chilled cells. The contents were gently tapped occasionally during a 30 minute incubation on ice, before being heat shocked at 42°C for exactly 45 seconds and then transferred

to ice for a further 2 minutes. Cells were incubated at 37°C for 1hour on an orbital shaker (225 - 250rpm) following the addition of 450µl of preheated (42°C) SOC medium (LB broth, 0.04% glucose, 10mM $MgSO_4$, 10mM $MgCl_2$). Typically 150µl of the transformation mixture was then plated on LB agar (2% LB, 2% agar) plates containing 50µg/ml ampicillin (Amp) and the plates were incubated at 37°C overnight to allow growth and colony-formation of the transformed cells.

2.9 Preparation of plasmid DNA

For large scale plasmid DNA preparation, a single isolated bacterial colony was selected from a freshly-streaked plate and used to inoculate 4ml of LB medium containing the selective antibiotic (50µg/ml ampicillin). This was allowed to incubate with vigorous shaking at 37°C for ~6 hours to form a mini-culture and was subsequently used to inoculate 250ml of LB medium containing 50µg/ml ampicillin. Following an overnight incubation at 37°C on an orbital shaker (~300rpm), cells were harvested by centrifugation at 6000g for 15 minutes at 4°C and plasmid DNA retrieved using the QIAGEN Plasmid Maxi Kit.

The bacterial pellet was resuspended in 10ml of Buffer P1 (50mM Tris pH 8.0, 10mM EDTA, 100µg/ml RNase A) and then gently but thoroughly mixed with 10ml of Buffer P2 (200mM NaOH, 1% SDS) to initiate an alkaline lysis reaction. This reaction was allowed to proceed for 5 minutes at room temperature before neutralising the lysate by the addition of 10ml of chilled Buffer P3 (3M potassium acetate, pH 5.5) which subsequently resulted in formation of a precipitate of potassium dodecyl sulphate. The SDS-denatured proteins and chromosomal DNA

were co-precipitated with the detergent whilst the plasmid DNA remained in solution due to a lack of close protein associations. Precipitation was enhanced by a 20 minute incubation on ice and the precipitate pelleted by centrifugation at 20 000g for 30 minutes at 4°C. The supernatant containing plasmid DNA was promptly removed and applied to a QIAGEN-tip 500 pre-equilibrated with 10ml of Buffer QBT (750mM NaCl, 50mM MOPS pH 7.0, 15% isopropanol, 0.15% Triton X-100). Gravity flow allowed the supernatant to pass through the anion-exchange resin to which plasmid DNA is able to tightly bind. The resin was then washed twice with 30ml of Buffer QC (1M NaCl, 50mM MOPS pH 7.0, 15% isopropanol) and the purified plasmid DNA was subsequently eluted with 15ml of Buffer QF (1.25M NaCl, 50mM Tris pH 8.5, 15% isopropanol) and precipitated with 10.5ml (0.7 volume) of room-temperature isopropanol. This was immediately followed with a 15 000g centrifugation at 4°C for 30 minutes. The plasmid DNA pellet was then washed with 70% ethanol, dried at room temperature for 5 – 10 minutes and resuspended in an appropriate volume of sterile water or TE buffer, pH 8.0 (10mM Tris pH 8.0, 1mM EDTA).

2.10 RNA pol III *in vitro* transcription assay

In vitro transcription of class III genes was reconstituted using 15-20µg of cell extracts to provide the basal pol III transcription components. 0.3-1.2µg His-p53 was preincubated with cell extracts for 10 mins at 4°C. 250ng of plasmid DNA was then added to supply a specific pol III template and reactions were carried out in a 25µl volume with a final concentration of 12mM HEPES pH 7.9, 60mM KCl, 7.2mM $MgCl_2$, 0.28mM EDTA, 1.2mM DTT, 10% (v/v) glycerol, 1mM creatine

phosphate, 0.5mM each of rATP, rCTP and rGTP and 10μCi [α-^{32}P] UTP (400mCi/mmol) (Amersham). Transcription components were assembled on ice and the reaction was performed at 30°C for 1hour. Transcription was terminated by the addition of 250μl of 1M ammonium acetate/0.1% SDS containing 20μg of yeast tRNA which acts as a stabiliser for the synthesised RNA. Phenol-chloroform extraction of the samples was performed to remove protein and DNA by adding 250μl of a 25:24:1 ratio solution of PhOH/$CHCl_3$/IAA. The samples were vortexed, microcentrifuged at 13 000g for 5 minutes and 200μl of the upper aqueous layer was then transferred to a fresh eppendorf tube containing 750μl of 96% ethanol in order to precipitate the RNA. The samples were thoroughly mixed by repeated inversion, left at --20°C overnight before being microcentrifuged at 13 000g for 20 minutes to pellet the precipitated RNA. The supernatant was carefully removed and 750μl of 70% ethanol was added to each sample to wash the pellet. This was also carefully removed to avoid dislodging the pellet and the samples were heated at 47°C for 5 – 10 minutes to dry. 4μl of formamide loading buffer (98% formamide, 10mM EDTA pH 8.0, 0.025% bromophenol blue, 0.025% xylene cyanol FF) was added to each sample, which was then vortexed for 20 minutes to ensure the RNA was fully redissolved. 2μl of each sample was loaded on a pre-run 7% polyacrylamide sequencing gel containing 7M urea and 0.5 x TBE (45mM Tris, 45mM boric acid, 0.625mM EDTA pH 8.0) after being boiled at 95°C for 2 minutes and quenched on ice. Electrophoresis was performed at 40W for 1hour in 0.5 x TBE before being dried and exposed to autoradiography film in order to detect the radiolabelled transcripts. Quantification of results was achieved by phosphoimaging (Fujix Bas 1000).

The plasmid templates used for *in vitro* transcription assays were as follows: pVA_1 is a 221bp *SalI-BalI* fragment of adenovirus 2 DNA containing the VA_1 gene subcloned into pUC18; pleu is a 240bp EcoRI-HindIII fragment of human genomic DNA carrying a $tRNA^{Leu}$ gene, subcloned into pAT153 (White et al. 1989); and pRH5.7 containing the Alu sequence (White and Jackson 1992a).

2.11 Immunoprecipition

Antibodies for immunoprecipitation experiments were coupled to protein A-Sepharose beads. 20µl of packed beads was used per sample and beads were washed twice with 1 x LDB (20mM HEPES-KOH [pH7.9], 17% glycerol, 100mM KCl, 12mM $MgCl_2$, 0.1mM EDTA, 2mM DTT) prior to incubation with the appropriate antibody on a shaker for 1hour at 4°C. The beads were then washed twice with 2 x LDB to ensure removal of unbound antibody.

For coimmunoprecipitation reactions, typically 200 - 500µg of cell extract (made up to a total volume of ~300µl with 1 x LDB) and/or 5-10µl of *in vitro* translated protein was incubated with the prepared protein A-Sepharose beads at 4°C for 3 hours on an orbital shaker. The beads were gently pelleted by pulse microcentrifugation and the supernatants carefully removed. The beads were then washed with 3 x LDB before the bound material was released by the addition of an equal volume of 2 x protein sample buffer (125mM Tris pH 6.8, 1% SDS, 10% β-mercaptoethanol, 20% glycerol, 0.25% bromophenol blue) and analysed by SDS-PAGE and either western blotting or autoradiography.

2.12 *In vitro* transcription-translation

Proteins were synthesized *in vitro* using the Single Tube Protein System 3 (STP3)-T7 kit (Novagen) following the manufacturers protocol. Reactions were assembled on ice. 8 µl of STP3 T7 Transcription Mix and 1µg plasmid DNA to a final reaction volume of 10µl made up with nuclease-free water were incubated at 30°C for 20 min. Translation was carried out by adding 30µl STP3 Translation Mix, 40µCi ^{35}S-labelled Cys/Met, final reaction volume was 50µl made up with nuclease-free water. Reactions were gently mixed with the pipette tip and incubated at 30°C for 60 min. 2µl of each sample was analysed via separation by SDS-PAGE. The gel was dried and visualized by autoradiography

The plasmids used for in *in vitro* transcription-translation were: pCITE.Brf1; pTBP; and pSBET.Bdp1 (Schramm et al. 2000).

2.13 Separation of proteins by polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were resolved on denaturing polyacrylamide gels according to molecular weight by electrophoresis. Typically, 7.8% polyacrylamide resolving minigels (375mM Tris pH 8.8, 0.1% SDS) were used with a stacking layer comprised of 4% polyacrylamide gel (125mM Tris pH 6.8, 0.1% SDS) based on the discontinuous buffer system described by Laemmli (Laemmli 1970). Samples were boiled for 2 minutes in 1 x protein sample buffer (62.5mM Tris pH 6.8, 0.5% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.125% bromophenol blue) prior to loading. Electrophoresis was performed in 1 x SDS running buffer (0.1% SDS, 76.8mM

glycine, 10mM Tris, pH 8.3) at an initial voltage of 70V while the bromophenol blue dye front moved through the stacking gel and a subsequent voltage of 140V after reaching the resolving gel. Electrophoresis was allowed to proceed until the dye front had reached the bottom of the gel, approximately 1 – 1.5 hours.

2.14 Western blotting

Electrophoretic transfer of proteins resolved by SDS-PAGE to PVDF membrane was achieved using the BioRad Mini Trans-Blot Electrophoretic Transfer Cell system. Transfer was carried out in 1 x transfer buffer (76.8mM glycine, 10mM Tris, pH 8.3, 16.5% methanol) at 50V for 1 hour. Following transfer, the membrane was blocked in milk buffer (32.5mM Tris, 150mM NaCl, 0.2% Tween-20, 4% skimmed milk powder (Marvel)) for 1 hour at room temperature. Membranes were incubated with primary antibodies (typically a 1:1000 dilution in milk buffer) overnight at 4°C. Excess primary antibody was removed by washing the blot 3 times for 3 minutes in fresh milk buffer before incubating for 1hour at room temperature with the appropriate horseradish peroxidase-conjugated secondary antibody (DAKO) (1:1000 dilution in milk buffer). To ensure removal of excess secondary antibody, the blot was sequentially washed in batches of fresh milk buffer, 3 times for 3 minutes, followed by 2 washes for 15 minutes. After one further 5 minute wash using 1 x TBS (2.5mM Tris-HCl pH 7.6, 15mM NaCl), the blot was developed using the enhanced chemiluminescence method (ECL, Amersham), as directed by the manufacturers.

2.15 Antibodies

DO-1	anti-p53 (Pharmingen)
4286-4	anti-TFIIIC110 (Sutcliffe et al., 2000)
4c826	anti-TBP (Sutcliffe et al. 2000)
C11	anti-actin (Santa Cruz)
M19	anti-TAF_I48 (Santa Cruz)
113	anti-BN51 (Ittmann et al. 1993)
128-4	anti-Brf1 (Cairns and White 1998)
330-4	anti-Brf1 (Alzuherri and White 1998)
1663-4	anti-B" (Schramm et al. 2000)
F-7	anti-HA (Santa Cruz)
pAb421 (Ab1)	anti-p53 (Calbiochem)
pAb240 (Ab3)	anti-p53 (Calbiochem)
pAb1620 (Ab5)	anti-p53 (Calbiochem)
pAb108	anti-SV40 Tag (Santa Cruz)
N15/C20	anti-p300 (Santa Cruz)

2.16 Primer extension

Expression levels of the transfected pol III template VA_I and the CAT gene, which was co-transfected as an internal control for transfection efficiency, were analysed by primer extension. VA_I (5'-CACGCGGGCGGTAACCGCATG-3') or CAT (5'-CGA-TGCCATTGGGATATATCA-3') oligonucleotides were γ-^{32}P end-labelled using T4 polynucleotide kinase (PNK). For each primer extension reaction, 10μg of

total RNA (made up to 10µl with DEPC-dH_20) were incubated at 80°C for 10 minutes with 9µl of 5 x First Strand Buffer (Life Technologies) and 1µl (2.5ng) of the relevant probe to act as a primer. Samples were immediately transferred to a 50°C hotblock for a further 2 hours incubation. 30µl of an elongation mix (23µl DEPC-dH_20, 0.5µl 1M DTT, 5µl 5mM dNTP mix (5mM in DEPC-dH_2O), 0.5µl 4mg/ml actinomycin D, 0.5µl RNasin, 0.5µl (100U) of Superscript II Reverse Transcriptase (Life Technologies)) was then added to the samples to initiate reverse transcription and the reaction was allowed to proceed for 1hour at 42°C. Reaction products were precipitated overnight at –20°C by the addition of 5µl of 3M sodium acetate and 125µl of ethanol and subsequently pelleted by microcentrifugation at 13 000g for 15 minutes. Pellets were washed with 300µl of 75% ethanol and dried at 47°C for 5 minutes before being resuspended by vortexing for 10 minutes in 4µl of formamide loading buffer (98% formamide, 10mM EDTA pH 8.0, 0.025% bromophenol blue, 0.025% xylene cyanol FF). Electrophoresis through a 7M urea 7% polyacrylamide gel was used to resolve the samples The reaction products were detected by overnight exposure to autoradiography film at –80°C and quantitated by phosphoimaging (Fujix Bas 1000).

2.17 Electrophoretic mobility shift assay (EMSA)

TFIIIC2 DNA-binding activity was determined by EMSAs which were carried out using a γ-^{32}P labelled oligonucleotide containing a B-block consensus (5'-AGAGGT-CCTGAGTTCAAATCCCAG-3' (RJW1) annealed to the complementary 3' to 5' strand (RJW2)). For use in EMSAs, oligonucleotides were 5' end-labelled using T4

polynucleotide kinase (PNK). 40ng of RJW1 was assembled on ice with 10U of PNK in 1 x PNK buffer (Promega) and following the addition of 20μCi of [γ-^{32}P] dATP (10mCi/ml, 3000Ci/mmol) to give a total volume of 10μl, the reaction was performed at 37°C for 1hour. This was stopped by heating at 65°C for 10 minutes and was succeeded by phenol-chloroform extraction of the PNK enzyme; achieved by addition of 50μl of PhOH/$CHCl_3$/IAA (25:24:1) followed by vortexing and microcentrifugation at 13 000g for 5 minutes. The aqueous layer was transferred to a fresh eppendorf tube and 5μl of 3M sodium acetate and 125μl of 100% ethanol added. After a 30 minute incubation on dry ice, the precipitated oligonucleotide was pelleted by microcentrifugation at 13 000g for 10 minutes. The supernatant was removed and the pellet washed by sequential addition and removal of 100μl of 70% ethanol to ensure removal of unincorporated label. The pellet was then dried by heating at 47°C for 10 minutes before being redissolved by incubation at 30°C for 30 minutes in 20μl of TE buffer (10mM Tris pH 8.0, 1mM EDTA). This was followed by heating in a hotblock at 90°C for 2 minutes in the presence of unlabelled complementary oligonucleotide (RJW2') which was added in 2.5 fold excess to ensure that all labelled oligonucleotide was annealed. The hotblock was then turned off and the sample allowed to cool slowly overnight, after which, it was stored at 4°C until ready for use.

Each binding reaction was performed in a total volume of 10μl, with an optimal salt concentration of 60mM KCl and contained 1μg of poly(dI.dC) (2μl), 100ng of non-specific or specific competitor oligonucleotide (2μl), typically 2 - 4μl of cell extract and 0.25 – 0.5ng of labelled probe (2μl). A pre-incubation of 15 minutes at 30°C was carried out prior to addition of the probe, followed by a further 15 minutes at

30°C. Analysis of the formation of protein-DNA complexes was achieved by electrophoresis of samples on a pre-run 4% nondenaturing polyacrylamide gel in 1 x TAE buffer (40mM Tris acetate, 1mM EDTA pH 8.0) for 1.5 -2 hours at 4°C. The gel was dried for 1.5 hours at 80°C and exposed to autoradiography film overnight at –70°C.

2.18 Baculoviral expression and purification of His-p53

Human and murine p53-producing recombinant baculoviruses were constructed and amplified by L. Warnock at the Yorkshire Cancer Research Campaign P53 Research Group, Department of Biology, University of York. (Molinari et al. 1996). *Sf*9 insect cells at 80% confluency in TNM-FH medium (Pharmingene) were infected with high titre recombinant baculovirus and harvested 3 days later. After washing four times with phosphate-buffered saline (PBS) the cell pellet was resuspended and lysed for 30 min on ice in 5 ml lysis buffer (150 mM Tris-HCl, pH 9.0, 150 mM NaCl, 0.5% NP-40, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 50 μg/ml aprotinin, 50 μg/ml leupeptin, 10 μg/ml pepstatin A and 1 mM β-mercaptoethanol). The lysate was centrifuged at 20 000 r.p.m. in a Sorvall SS-34 rotor at 4°C for 30 min and loaded on a 0.5 ml Ni-NTA agarose column (Qiagen Inc.) prewashed with lysis buffer, pH 7.0. The column was washed with 10 ml lysis buffer, pH 9.0, and adjusted to pH 7.0 with 10 ml lysis buffer, pH 7.0. The column was step-eluted by one volume portions of elution buffer (50 mM NaCl, 10 mM Tris-HCl, pH 7.0, and 5 mM $MgCl_2$) containing increasing concentrations of imidazole (50, 100, 150 and 250 mM). The 100-150mM imidazole fractions were routinely used for analyses. Imidazole was removed using Centricon-10 cartridges (Amicon) by washing with 4 x LDB. p53

protein was quantitated by Bradford assay and purity checked by SDS-PAGE and silver staining. A single band corresponding to p53 was observed.

2.19 His-p53 binding assay

Binding assays were carried out with His-p53 wt and His-p53 core, comprising residues 98-303 (Okorokov and Milner 1999). Proteins were purified as described above; however, after the 50mM imidizole wash, the beads were removed from the column and 50µl aliquots were used in each assay. His-p53 bound to Ni-NTA agarose was incubated with either 5µl ^{35}S-labelled TBP, Brf1 or Bdp1 or CHO extract containing HA-Brf1. Binding was carried out in imidizole buffer (5mM MgCl2, 50mM NaCl, 10mM Tris-HCl pH7.0, 50mM imidizole) for 1 hour at 4°C with shaking in a final reaction volume of 250µl. Beads were then washed 5 x in TBS. Bound material was separated by electrophoresis and visualised by either autoradiography or western blotting.

2.20 Expression and purification of GST-p53

GST-p53 and GST were prepared using the GST Gene Fusion System (Stratagene) in E. coli. BL21 (DE3) (pLys). 40ml L-broth cultures containing 50µg/ml ampicillin were inoculated from glycerol stocks and grown overnight at 37°C with shaking. Cultures were then diluted into 400ml cultures and grown for 2 hours until an OD of 0.6 was reached. Cultures were then induced with 0.1mM IPTG for 16 hours. Expressed protein was purified from bacterial lysates by precipitation with

glutathione coated sepharose 4B beads (Pharmacia). Cultures were spun in the Sorval High Speed Centrifuge using the SLA-30 rotor at 3000rpm for 15 min at 4°C. Pelleted cells were then resuspended in 9ml ice cold PBS, 1% Triton X-100, 1% PMSF and sonicated 2 x 20 sec at 60-80W. Samples were centrifuged at 10 000rpm for 5 min at 4°C. The supernatant was bound to 500µl packed GST-sepharose beads for 30 min at 4°C, with mixing. After binding, the beads were washed 2 x with 50ml ice cold PBS, 1% Triton X-100, and 2 x with 50ml ice cold PBS. 20µl samples were analysed by SDS-PAGE and stained with Coomassie blue.

2.21 GST-pull down assays

Equal amounts of immobilised GST and GST-p53 (estimated by Coomassie staining) were used in the reactions. 20µl packed glutathione-sepharose beads bearing GST-p53 or GST alone were incubated with 5-10µl ^{35}S-labelled recombinant TBP or Brf1 in 50µl LDB. Samples were incubated on an orbital shaker for 3 hours at 4°C. Beads were then washed 5 x in 500µl LDB. Bound material was separation by SDS-PAGE and analysed by autoradiography.

2.22 Immobilised template assay

The biotinylated templates were synthesised by PCR as previously described (Panov et al. 2001). The plasmid pLeu was used as template. Primers used were forward, 5'-GCTTTGCCCCATTCCTTA-3'; reverse, 5'-CTTTCTGCATGTAAATAGGTGTAAC-3' which was biotinylated at the 5' end.

PCR was performed under the following cycling parameters: 95°C for 5 minutes, 35 cycles of [95°C for 1 minute, 58°C for 30 seconds, 72°C for 1 minute], 72°C for 3 minutes. A 240bp product was generated.

Immobilized template DNA (20µl) was incubated with gentle agitation with 200µg CHO extract stably transfected with HA tagged Brf1 for 20 mins at 30°C. Reactions were performed in the presence or absence of wild-type His-53 or His-p53 (24-392) (Okorokov and Milner 1999), in LDB (final salt concentration 60mM) and 10µg poly dIdC to a final reaction volume of 250ul. Using a magnetic stand, beads were washed 3 x with 2 reaction volumes of LDB (60mM) containing 0.003% NP-40, and resuspended in 20µl protein sample buffer. Bound material was separated by SDS-PAGE and analysed by Western blotting.

2.23 Chromatin immunoprecipitation assay

Cells were harvested, washed with ice-cold PBS, then crosslinked for 10 mins at 37°C in 10ml 0.5% NP-40/PBS containing 1% formaldehyde. After crosslinking, cells were washed with ice-cold 0.5% NP-40/PBS and incubated for 30 mins with 40ml high salt buffer (0.5% NP-40, PBS, 1M NaCl). Further washing with 0.5% NP-40/PBS was followed by hypotonic disruption for 30 mins in 40ml low salt buffer (0.1% NP-40, 10mM Tris-HCl pH 8.0, 1mM EDTA, 0.1M NaCl). After centrifugation at 500g, nuclei were obtained with 1 stroke through a 26 gauge needle and recentrifuged. Nuclei were resuspended in 2.7ml of low salt buffer and lysed with 300µl sarkosyl. Sample was transferred to a sucrose cushion (40ml low salt

buffer/100mM sucrose) and spun for 10 mins at 4000g. The pellet was resuspended in 3ml TE, and the process was repeated. The pellet was then resuspended in 2ml TE, and genomic DNA was sheared by sonication (Branson sonifier 250, 10 x 10sec, 30% duty cycle). Sonicated material was adjusted with 1/10 volume of 11 x NET (1.65M NaCl, 5.5mM EDTA, 5.5% NP-40. 550mM Tris-HCl pH 7.4) and immunoprecipitated overnight with 4µg of antibody. Protein A-sepharose beads were added for a further 2 hours and then recovered on polyprep columns (Biorad). The negative control was incubated with beads alone. After washing twice with 10ml RIPA buffer, 10ml of LiCl buffer (10mM Tris.HCl, 250mM LiCl, 0.5% NP-40, 0.5% deoxycholate, 1mM EDTA pH8.0) and 10ml TE, beads were transferred to 1.5ml tubes and immunoprecipitated material was eluted twice with 200µl of 1% SDS in TE. To isolate precipitated DNA, proteins and antibodies were degraded with proteinase K treatment overnight at 42°C. DNA was extracted twice using 400µl phenol/chloroform/isoamyalcohol and ethanol precipitated. Immunoprecipitated DNA was quantitated by PCR.

2.24 Li-Fraumeni Cells

Mutations in the p53 genes of Li-Fraumeni families were identified by direct genomic sequencing of all 11 exons plus the promoter region and all splice junctions (Varley et al, 1997a, b). Primary fibroblasts were cultured from skin biopsies obtained with informed consent from LFS patients and normal control individuals (Boyle et al. 1998). These steps were carried out at the Paterson Institute for Cancer Reseearch, Manchester.

Chapter 3

Several regions of p53 are involved in repression of RNA polymerase III transcription

3.1 Introduction

Previously it has been demonstrated that p53 can repress transcription of pol III transcription both *in vivo* and *in vitro* (Chesnokov et al. 1996; Cairns and White 1998), however currently the mechanism of transcriptional repression remains poorly understood. p53 is a complex molecule consisting of multiple domains (Ko and Prives 1996; Levine 1997). Transcriptional activation is mediated through the acidic activation domain located at the N-terminus of the protein (Unger et al. 1992), and the central portion of the molecule forms a sequence specific DNA-binding domain (Cho et al. 1994). A variety of functions have been mapped to the C-terminal domain; these include oligomerisation (Sturzbecher et al. 1992) and stabilization of DNA binding activity (Hupp et al. 1992). A minimal region of eight amino acids located at residues 339-346 was previously found to be essential for p53 to repress pol II transcription (Hong et al. 2001). In addition sequence at both the N- and C-termini of p53 have also been implicated in this function (Horikoshi et al. 1995), (Sang et al. 1994), (Subler et al. 1994). Investigation into which regions of p53 are involved in repressing pol III transcription may well provide clues as to the mechanism by which p53 can repress pol III transcription and therefore establishes the aim of this chapter.

3.2 Results

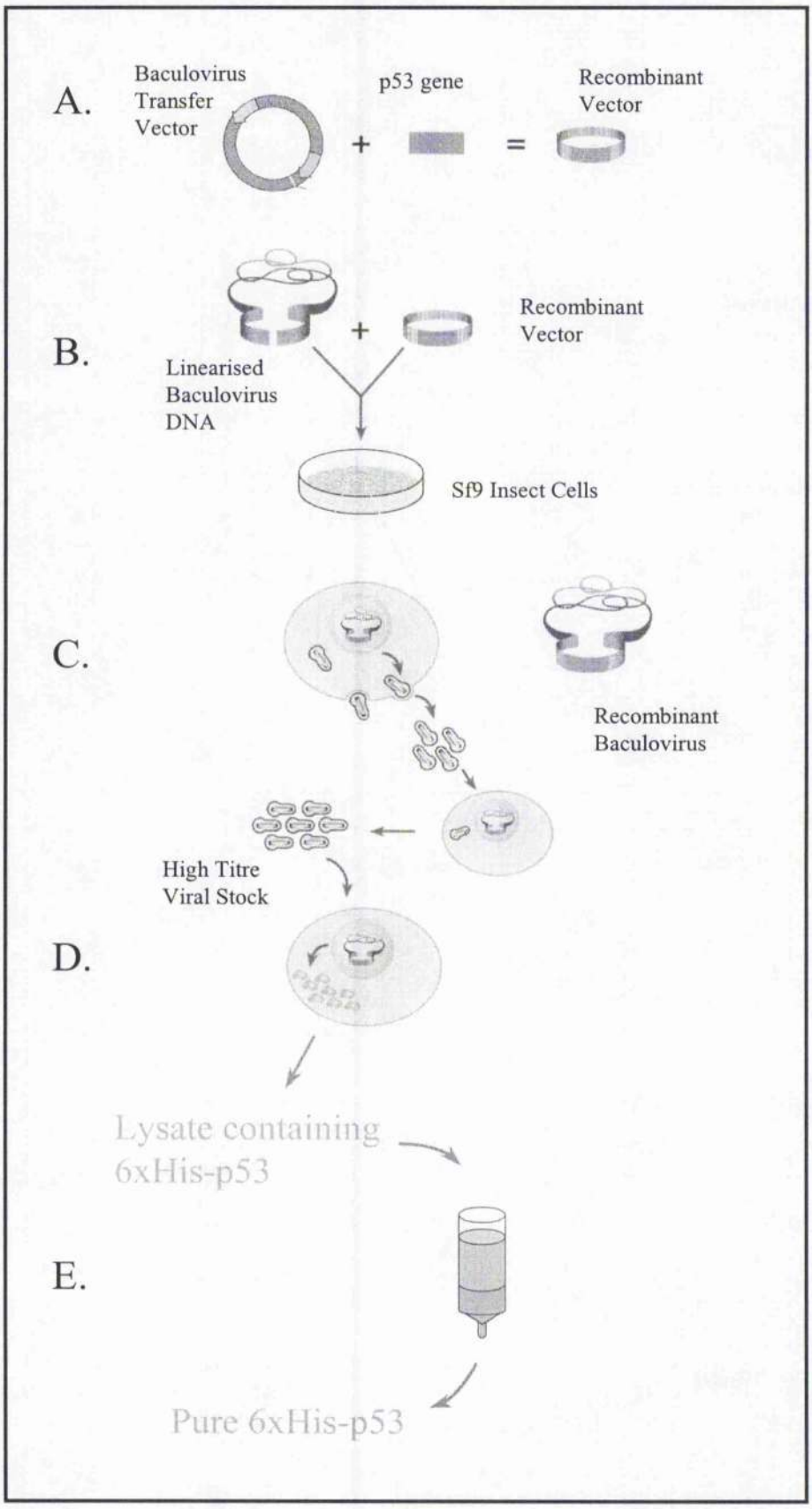
3.2.1 Expression purification and characterisation of recombinant His-p53

In order to study the mechanism by which p53 represses pol III transcription it was first essential to produce active recombinant p53. His-p53 was purified from Sf9 insect cells infected with high titre recombinant baculovirus encoding either murine or human His tagged p53. His-p53 was then purified to near homogeneity using Ni-NTA agarose beads. An overview of the process used to synthesise and purify His-p53 is shown in figure 3.1 and detailed methods are given in Chapter 2. In many instances, Lorna Warnock from the University of York carried out steps A-D (Okorokov and Milner 1999).

Purification of His tagged p53 using Ni-NTA agarose beads produced a single band migrating at approximately 53kDa on a Coomassie-stained 10% SDS-PAGE gel (Figure 3.2). Human His-p53 (Figure 3.2, upper panel) usually gave a greater yield of protein when compared to murine His-p53 (Figure 3.2, lower panel). The reason for this is unclear, but may reflect the stability of the two proteins.

There is evidence that wild-type p53 can both promote and suppress cell proliferation and it has been suggested that these opposing functions are achieved by alternative conformations of the p53 protein (Milner 1994). These forms of p53 are defined by reactivity with three monoclonal antibodies, pAb240, pAb421 and pAb1620 (Milner 1994). Normal cell proliferation is linked to two conformational variants of p53 both of which are reactive with pAb421 (Figure 3.3a). The form of p53 most commonly detected in proliferating cells is $240^0/1620^+$ (Figure 3.3a, left). This form correlates with the ability to suppress growth under appropriate conditions and is termed the

Figure 3.1. Schematic overview of the steps involved in producing recombinant Histidine tagged p53. (A) Generation of the recombinant baculovirus transfer vector (Molinari et al. 1996). (B) Co-transfection of BaculoGold (Pharmingen) and the linerised recombinant transfer vector into Sf9 insect cells. (C) Recombination between the vector and the viral DNA occurs within the cell and recombinant baculovirus is produced. (D) The recombinant virus is harvested and amplified to produce a high titre stock which can then be used to infect cells for recombinant His-p53 protein expression. (E) Recombinant His-p53 is purified to near homogeneity from the cellular lysate using Ni-NTA agarose beads (Qiagen). (Figure adapted from Baculovirus Expression System handbook, Pharmingen).


A.
Baculovirus
Transfer
Vector
p53 gene
Recombinant
Vector
B.
Linearised
Baculovirus
DNA
Recombinant
Vector
Sf9 Insect Cells
C.
Recombinant
Baculovirus
High Titre
Viral Stock
D.
Lysate containing
6xHis-p53
E.
Pure 6xHis-p53

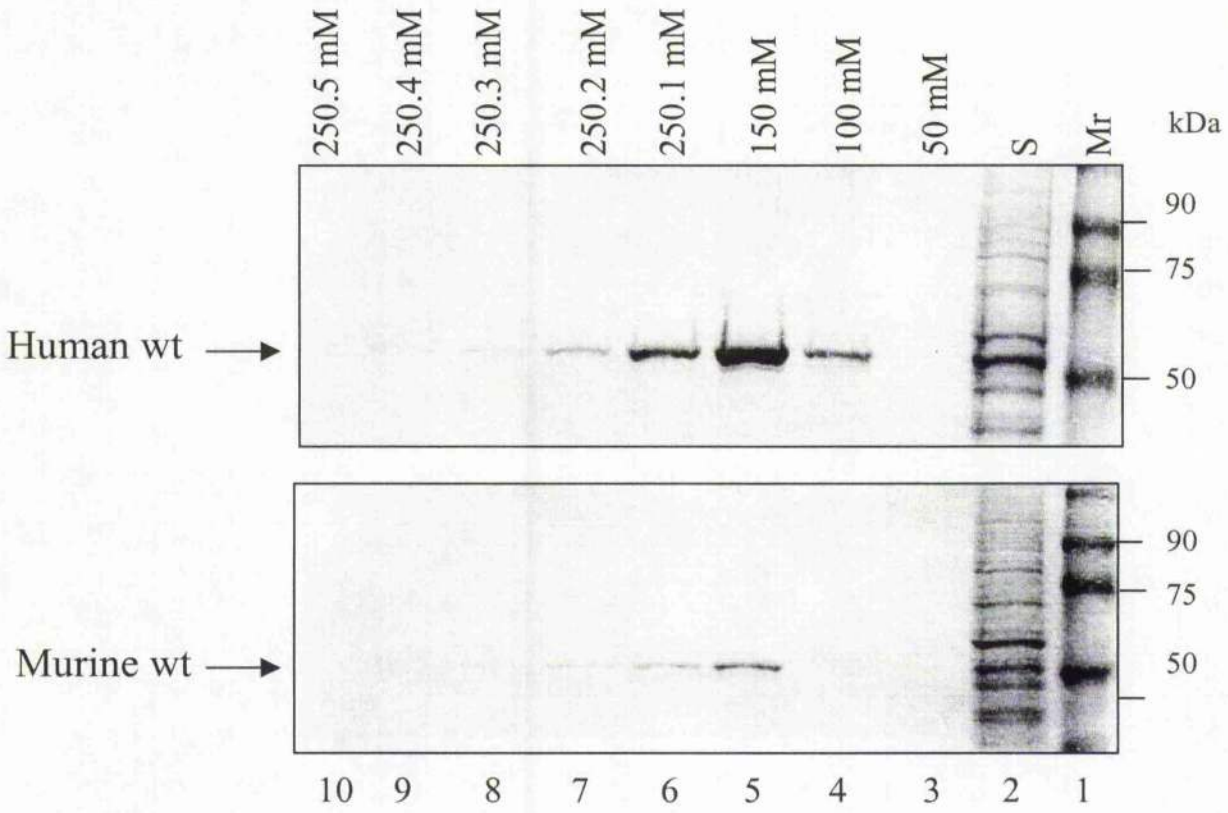
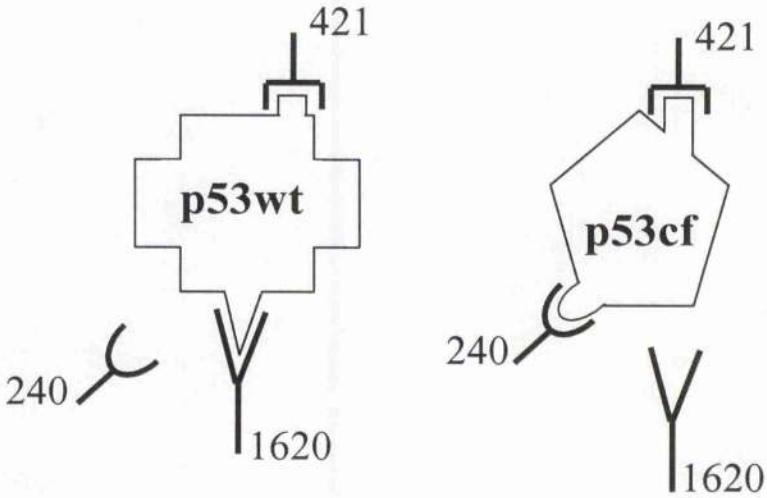


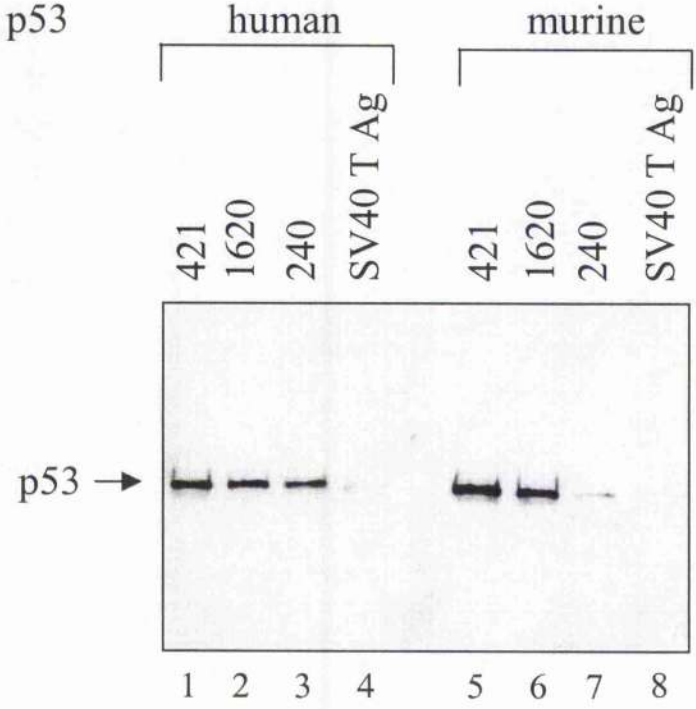
Figure 3.2. Purified human (panel A) and murine (panel B) recombinant His–p53. Molecular markers (lane 1) and 25µl samples either before purification (lane 2) of His-p53 or after elution with 50nM (lane 3), 100nM (lane 4), 150nM (lane 5), or 250nM (lanes 6-10) imidizole. Proteins were visualised by staining with Coomassie Blue. The 150mM fractions were generally used.

Figure 3.3. Recombinant p53 largely exists in an active form. (A) Schematic representation of the antibodies reactive with the active suppressor form of p53; $421^{+}/1620^{+}$ (left panel) and the conformational form of p53; $421^{+}/240^{+}$ (right panel). (B) A significant fraction of recombinant human, but not murine His-p53, exists in the mutant form. 500ng of recombinant human (lanes 1-4) and murine (lane 5-8) His-p53 protein was immunoprecipitated with anti p53 antibodies pAb421 (lanes 1 and 5), pAb1620 (lanes 2 and 6), pAb240 (lanes 3 and 7) or the antibody against SV40 large T antigen as a negative control (lanes 4 and 8). Immunoprecipitated material was elecrophoresed on a 7.8% SDS-polyacrylamide gel, transferred to nitrocellulose and detected via Western blotting with the anti p53 antibody DO-1.

A.


421
p53wt
240
1620
421
p53cf
240
1620

B.


p53
human
murine
421
1620
240
SV40 T Ag
421
1620
240
SV40 T Ag
p53
1 2 3 4 5 6 7 8

suppressor form (Milner 1994). The p53 epitope recognised by pAb240 is cryptic on the suppressor form of wild-type p53 but is frequently exposed on mutant p53 (Hupp et al. 1993).

Using immunoprecipitation it was investigated which of these three conformation specific antibodies were reactive with both human and murine recombinant His-p53 (Figure 3.3b). Both human and murine p53 was reactive with pAb421 (Figure 3.3b, lanes 1 and 5) and pAb1620 (Figure 3.3, lanes 2 and 6). In contrast to this, pAb240 recognised only significant levels of human p53 (Figure 3.3b, lane 3). Recognition by the antibodies was specific since the negative control antibody against SV40 T antigen immunoprecipitated undetectable levels of p53 in both cases (Figure 3.3b, lane 4 and 8). The p53 epitope recognised by pAb240 is frequently exposed on mutant p53 (Hupp et al. 1992), suggesting that a substantial portion of human but little murine His-p53 is present in this mutant conformation. For this reason, subsequent experiments were carried out using murine His-p53.

3.2.2 Recombinant His-p53 represses pol III transcription

Murine His-p53 was tested for its ability to repress pol III transcription (Figure 3.4). When titrated into nuclear extract, increasing amounts of His-p53 repress transcription of the pol III reporters Alu (lanes 2, 3 and 4, upper band) and $tRNA^{Leu}$ (lane 2, 3 and 4, lower band). This demonstrates that recombinant His-p53 can efficiently repress pol III transcription *in vitro*, thus providing a reliable tool for investigating the means by which p53 regulates pol III activity.

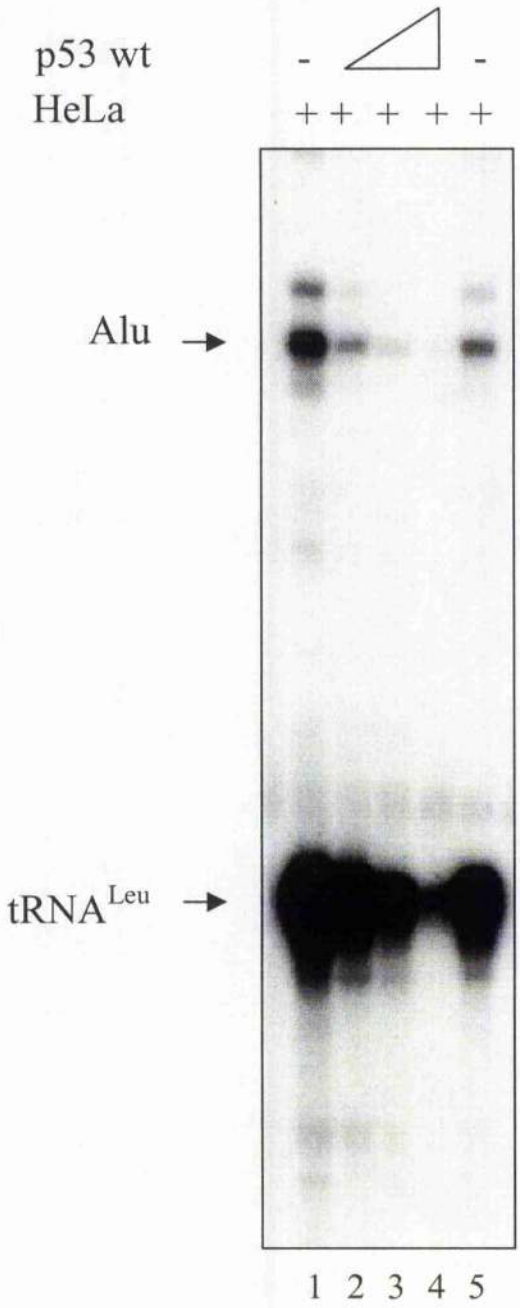


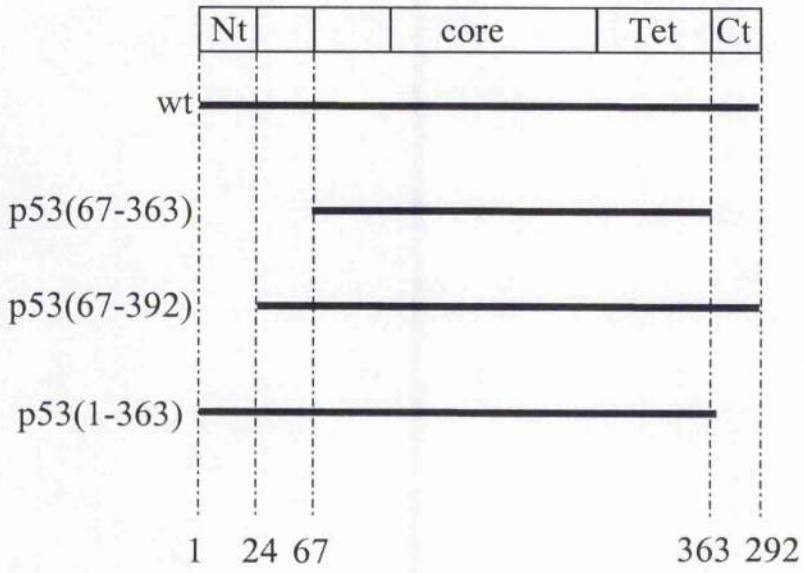
Figure 3.4. Recombinant His-p53 represses pol III transcription. pRH5.7 (250ng) and pleu (250ng) was transcribed with HeLa nuclear extract (15μg) that had been preincubated with 0.4μg (lane 2), 0.8μg (lane 3) or 1.2μg (lane 4) of murine His-p53.

3.2.3 The N-terminal 23 residues of p53 are important for repression of pol III transcription

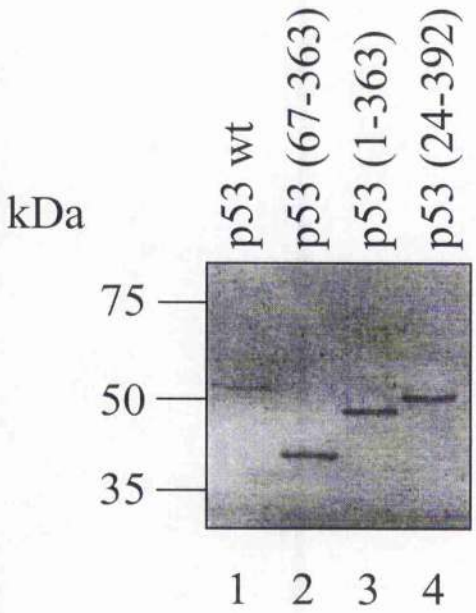
In order to address which regions of p53 are required to repress pol III transcription a series of previously characterised His-p53 mutants (Okorokov and Milner 1999) were employed (Figure 3.5a). Recombinant baculovirus encoding wild-type or mutant His-p53 was expressed in Sf9 cells, as described (Okorokov and Milner 1999) and purified to near homogeneity using the polyhistidine tag (Figure 3.5b). When titrated into nuclear extract, wild-type p53 produced a clear repression of VA1 (Figure 3.6, lane 3 and 4, upper panel) and Alu transcription (Figure 3.6, lane 3 and 4, lower panel). In contrast, no inhibition of VA1 or Alu was observed using an equal amount of mutant p53(67-363), which is missing the N-terminal 66 residues and the C-terminal 29 residues (Figure 3.6, lane 5 and 6). This demonstrates that the repression obtained with wild-type p53 is specific. It also suggests that regions outside the central domains of p53 contribute to repression of pol III transcription. A mutant p53(1-363) which is missing only the C-terminal 29 residues was found to retain a significant capacity to repress pol III transcription (Figure 3.6, lanes 8 and 9). Deletion of the N-terminal 13 residues p53(24-393), however, completely abolished the ability of p53 to repress either VA1 (upper panel) or Alu (lower panel) transcription (Figure 3.6, lane 11 and 12). These results suggest that N-terminal residues of p53 are important in mediating repression of pol III transcripts. It also suggests that the C-terminal 30 residues are unnecessary for this activity.

Figure 3.5. Schematic representation of the His-p53 truncated constructs. (A) The major domains of p53 are indicated. Nt, N-terminus; Ct, C-terminus; Tet, tetramerization domain. (B) Coomassie-stained gels of purified His-p53 proteins. 200ng of wild-type p53 (lane 1), p53(67-363) (lane 2), p53(1-363) (lane 3) and p53(24-392) (lane 4) were electrophoresed on a 10% SDS-polyacrylamide gel and visualised by staining with Coomassie Blue.

A.


Nt
core
Tet
Ct
wt
p53(67-363)
p53(67-392)
p53(1-363)
1
24
67
363
292

B.


kDa
p53 wt
p53 (67-363)
p53 (1-363)
p53 (24-392)
75
50
35
1
2
3
4

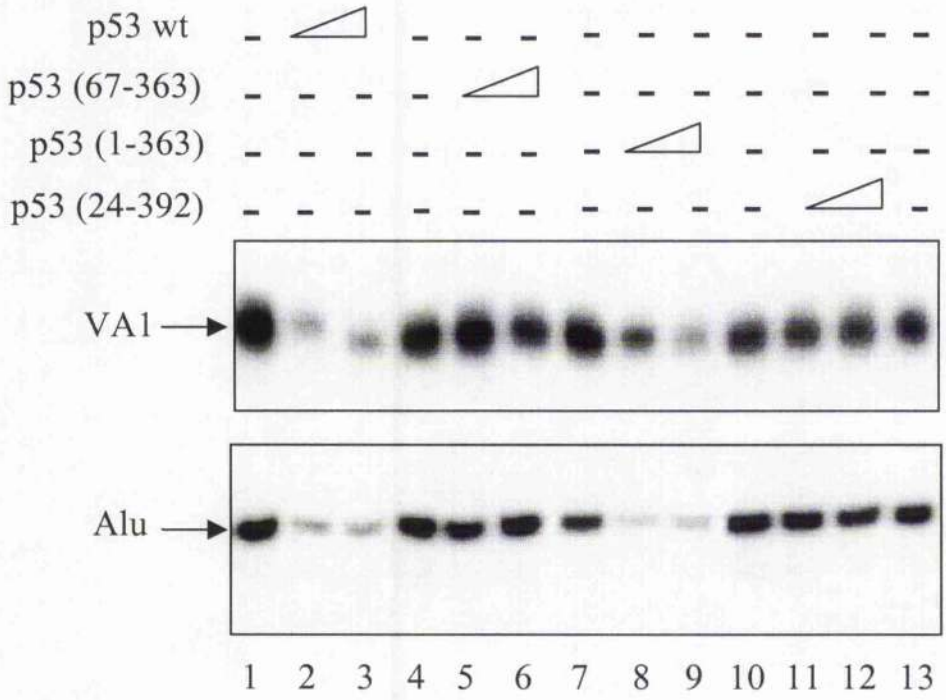


Figure 3.6. The N-terminal 23 residues of p53 are important for repression of pol III transcription. pVA1 (250ng) (top panel) and pRH5.7 (250ng) (bottom panel) were transcribed using HeLa nuclear extract (15μg) that had been preincubated for 10 min at 30°C in the absence (lanes 1, 4, 7, 10 and 13) or in the presence of 0.6μg or 1.2μg of wild-type p53 (lanes 2 and 3 respectively), 0.6μg or 1.2μg of p53(67-363) (lanes 5 and 6 respectively), 0.6μg or 1.2μg of p53(1-363) (lanes 8 and 9 respectively), or 6μg or 1.2μg of p53(24-392) (lanes 8 and 9 respectively).

3.2.4 Repression of pol III transcription by p53 can be blocked specifically using antibodies against the central core or C-terminal domains

Previously it has been demonstrated that preincubation of p53 with the monoclonal antibody DO-1 can specifically block p53-mediated repression of pol III transcription (Cairns and White 1998). Since DO-1 recognises residues 11-25, this result is consistent with the observation that the N-terminal domain of p53 is required for pol III repression (Figure 3.6). A similar approach using specific antibodies against p53 was used to further investigate the regions of p53 required for pol III regulation. Antibodies against the central core (pAb1620) or the C-terminus (pAb421) of p53 were preincubated with the protein prior to transcription. In the absence of antibody p53 clearly represses VA1 transcription (Figure 3.7, lane 2). pAb1801 recognises only human p53 and not the murine p53 used in this experiment. Since preincubation with pAb1801 does not perturb repression, it serves as a control vs non-specific effects of antibodies (Figure 3.7, lane 3). In contrast, preincubation with either pAb421 (Figure 3.7, lane 4) or pAb1620 (Figure 3.7, lane 5) blocks repression of VA1. This result suggests that both the core domain and the C-terminal residues of p53 may be involved in mediating repression of pol III transcription.

3.2.5 Double substitution at residues 340 and 344 does not affect the ability of p53 to repress pol III transcription

Native p53 forms a tetramer in solution and residues 325-356 are required for this oligomerisation (Jeffrey et al. 1995; Sturzbecher et al. 1992). A mutant version of full-length p53 that carries a double substitution at residues 340 and 344,

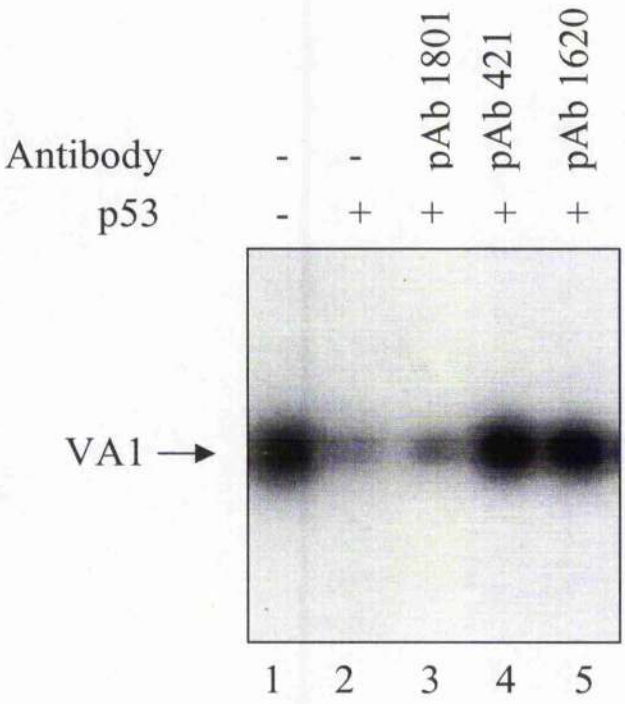


Figure 3.7. Repression of pol III transcription by p53 can be blocked specifically using antibodies against the central core or C-terminal domains. pVA1 (250ng) was transcribed using HeLa nuclear extract (15μg) that had been preincubated for 10 min at 30°C in the presence of buffer (lane 1) or 1μg of wild-type p53 (lanes 2-5). Prior to use, p53 was incubated at 4°C for 2 hours with buffer (lane 2) or 0.5μg of pAb1801 (lane 3), pAb421 (lane 4) or pAb1620 (lane 5).

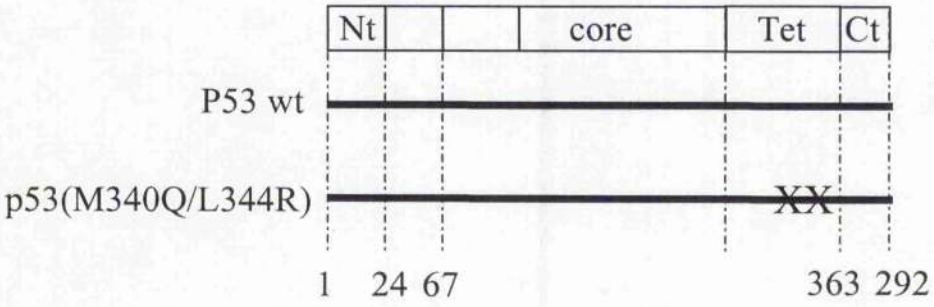
p53(M340Q/L344R) (Figure 3.8a) (Okorokov and Milner 1999) was used to investigate the involvement of this region in mediating pol III repression. This mutant can form dimmers, but is unable to tetramerise (Davison et al. 2001). p53(M340Q/L344R) was expressed in Sf9 insect cells and affinity purified (Figure 3.8b). When equal amounts were added to nuclear extract p53(M340Q/L344R) repressed Alu transcription as well as wild-type p53 (Figure 3.9 compare lanes 7-9 with 3-5). This suggests that tetramerisation of p53 is not essential in order for p53 to repress pol III transcription.

3.2.6 Substitution 341A prevents repression of pol III transcription

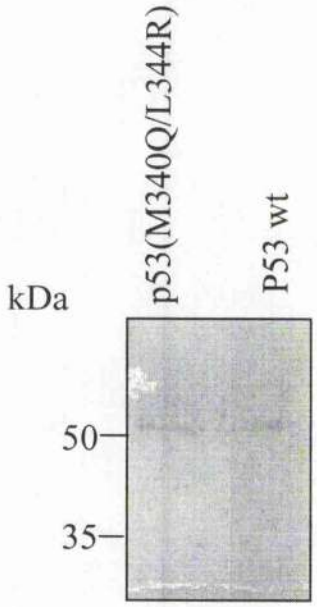
To further investigate the role of tetramerisation of p53 in mediating pol III transcriptional repression, two single residue substitution mutants were employed. The p53 mutant in which residue 344 has been substituted to Alanine (344A) is unable to form tetramers and largely exists in the dimeric form (McLure and Lee 1998); however, the mutant in which residue 341 is mutated to Alanine (341A) is unable to form dimers or tetramers, and therefore exists in a monomeric (Mateu and Fersht 1998). The p53 null, osteosarcoma cell line SAOS2 was transfected with the VA1 gene as a reporter for pol III activity and HSV-CAT, in which the chloroamphenicol acetyltransferase gene is transcribed from the basal thymidine kinase promoter of herpes simplex virus. This pol II transcribed gene serves as an internal control for transfection efficiency and RNA recovery. In addition, vectors encoding wild-type p53, p53(344A) or p53(341A) were cotransfected. Expression of the VA1 and HSV-CAT reporters were then monitored by quantitive primer extension assays.

Figure 3.8. Purification of wild-type p53 and p53 (M340Q/L334R). (A) Schematic representation of the p53 wild-type and p53(M340Q/L344R). The major domains of p53 are indicated. Nt, N-terminus; Ct, C-terminus; Tet, tetramerization domain. Sites of point mutations are indicated by crosses. (B) Coomassie-stained gels of purified His-p53 proteins. 200ng of wild-type p53 (lane 1) and p53(M340Q/L344R) (lane 2) were electrophoresed on a 10% SDS-polyacrylamide gel and visualised by staining with Coomassie Blue.

A.


Nt
core
Tet
Ct
P53 wt
p53(M340Q/L344R)
XX
1
24
67
363
292

B.


kDa
p53(M340Q/L344R)
P53 wt
50
35

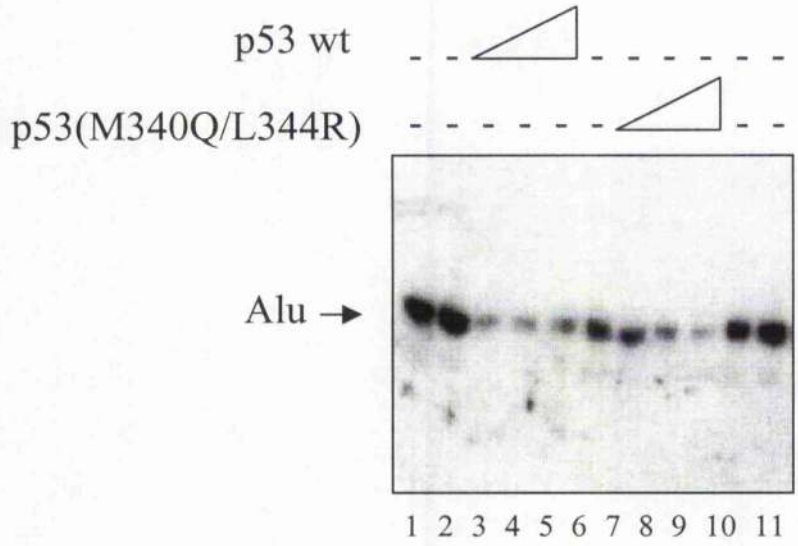


Figure 3.9. Double substitutions at residues 340 and 344 do not affect the ability of p53 to repress pol III transcription. pRH5.7 (250ng) was transcribed using HeLa nuclear extract (15μg) that had been preincubated for 10 mins at 30°C in the absence (lanes 1, 2, 6, 10 and 11) or presence of 0.3μg, 0.6μg or 0.9μg of wild-type p53 (lanes 3, 4 and 5 respectively) or p53(M340Q/L344R) (lanes 7, 8 and 9 respectively).

Wild-type p53 clearly represses pol III-dependent transcription of VA1 (Figure 3.10, lane 2, upper two panels) when compared to the empty vector control (Figure 3.10, lane 1, upper two panels). Transfection of p53(344A) also demonstrated a significant repression of VA1 (Figure 3.10, lane 3, upper two panels). In contrast to this, p53(341A) was unable to significantly repress pol III transcription. Western blotting confirmed that p53 was expressed in all transfection experiments (Figure 3.10, third panel). The levels of mutant p53 protein appear to be slightly higher than that of wild-type p53. This could be accounted for by an underloading of sample in lane 3, as suggested by the actin control (Figure 3.10, bottom panel). It is also possible that the mutations in p53 have disrupted the mechanisms normally used to control the levels of p53. This result demonstrates that a mutant 433A, which cannot tetramerise, can still repress pol III transcription *in vivo*; leading to the suggestion that tetramerisation of p53 is not an essential function to mediate pol III transcriptional repression. The monomeric form of p53 harbouring the substitution 341A was unable to repress pol III transcription, suggesting that monomeric p53 is insufficient to repress pol III transcription *in vivo*.

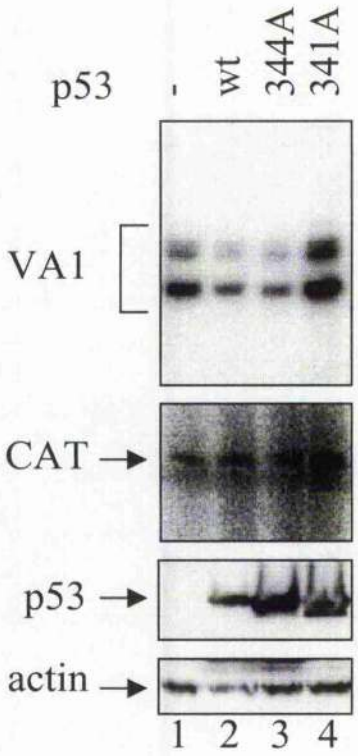


Figure 3.10. Mutation 341A prevents p53 from repressing pol III transcription. SAOS2 cells were cotransfected with pVA1 (3μg), HSV-CAT (3μg) and 2μg of empty vector (lane 1) or vectors encoding wild-type p53 (lane 2), p53(344A) (lane 3) or p53(341A). RNA and protein were harvested 48hrs after transfection. The upper two panels show primer extension analyses of extracted RNA using primers specific for VA1 (upper panel) and CAT (second panel). Extracts were analysed by Western blotting for the expression of p53 (third panel) and actin (bottom panel).

3.3 Discussion

Repression of pol III transcription by p53 is not attributed to one single region of p53. The N-terminal residues clearly play a role, since deletion of residues 1-23 abolished the ability of p53 to repress VA1 and Alu transcription *in vitro* (Figure 3.6). Stein et al. also demonstrated that residues 22 and 23 are specifically involved in mediating repression, since substitution of these residues abolished pol III repression in transient transfection assays (Stein et al. 2002b). These residues are important in binding TFIID, a basal transcription factor required for pol II transcription (Chang et al. 1995; Farmer et al. 1996b; Lin et al. 1994; Lu and Levine 1995; Thut et al. 1995b). In contrast to this, residues 13-19, which encompass conserved region I of p53, are not required for repression of VA1 transcription *in vivo* (Stein et al. 2002b).

Figure 3.7 demonstrated that preincubation with pAb1620 against the core domain of p53 abolished the ability of p53 to repress VA1 transcription *in vitro*. This suggests that the core domain may function in mediating repression of pol III transcripts. p53 consensus binding sites are not found within pol III genes, and it is believed that repression by p53 occurs not through DNA binding, but via protein-protein interactions. Individual deletion of conserved regions II-V, however, completely abolished the ability of p53 to repress pol III transcription *in vivo* (Stein et al. 2002b), suggesting that an intact central core domain is necessary for repression of pol III transcription. It is possible that the observed lack of repression could be attributed to a conformational change in p53 brought about by the deletions, however point mutations within the core domain that are not thought to affect p53 conformation were shown to block the ability of p53 to repress pol III transcription (Stein et al.

2002a). For example, a mutant in which arginine 273 had been substituted to cysteine was no longer able to repress pol III transcription *in vivo* (Stein et al. 2002a). This evidence further supports a role for the core domain of p53 in mediating a pol III transcriptional repression.

p53 largely exists as a tetramer in solution (Levine 1997), but tetramerisation of p53 has proved not to be essential for eliciting all of its cellular functions (Tarunina and Jenkins 1993; Shaulian et al. 1993; Slingerland et al. 1993). Here it is demonstrated that a mutant carrying the double substitution M340Q/L344R was able to repress pol III transcription *in vitro* (Figure 3.10). In addition transient transfection assays revealed that the single substitution p53 mutant 344A could still mediate repression. In contrast, the mutant 341A was unable to repress transcription. M340Q/L344R and 341A are reported to form dimers but not tetramers and 344A is unable to form dimers or tetramers. This suggests that dimerisation but not tetramerisation is essential for mediating repression of pol III transcription. p53 carrying an ALAL mutation at residues 365, 372, 379 and 387 was unable to repress pol III transcription in a transient transfection assay (Stein et al. 2002b). ALAL is unable to form tetramers but has been shown to allow dimerisation of p53 (Sturzbecher et al. 1992) providing a contradiction to the suggestion that tetramerisation is not essential for p53 to repress pol III transcription. Several distinct activities of p53 map to this region and at present is it not possible to draw any firm conclusions regarding the oligomerisation requirements of p53 in order to repress pol III transcription.

An inconsistency exists between the evidence implicating the C-terminal domain of p53 in pol III transcriptional control. Transcriptional repression by p53 could be

blocked by preincubation with antibody pAb421 that binds to residues 371-380 (Figure 3.7) however deletion of residues 364-392 (Figure 3.6) did not impair repression. An ALAL quadruple substitution at residues 365, 372, 379 and 387 was previously shown to diminish repression of pol III transcription *in vivo* (Stein et al. 2002b), as did deletion of residues 291-329 (Stein et al. 2002b). Both binding of pAb421 to the C-terminus of p53 and the ALAL substitution have been shown to cause a structural rearrangement within p53 that activates it for DNA binding (Hupp et al. 1992; Marston et al. 1998). It is possible that such a rearrangement interferes with repression of pol III transcription. Recently it was found that residues 339-346 within the C-terminus of p53 possess transcriptional repression activity with respect to pol II promoters (Hong et al. 2001). In addition, other sequences within the C-terminus have also been found to contribute to transcriptional repression of pol II genes (Horikoshi et al. 1995; Subler et al. 1994). On the basis of this, it is very likely that sequence within the C-terminus of p53 contributes to repression of pol III transcription.

p53 has been shown to inhibit a wide range of pol III templates (Cairns and White 1998). The only sequences shared by these genes are the promoter and terminator elements that are recognised by basal pol III transcription factors. p53 has been shown to bind to TFIIIB, a factor fundamental for the recruitment of polymerase to pol III templates. TFIIIB is a trimeric complex, an essential subunit of which is the TATA-box binding protein, TBP (Kassavetis et al. 1992; Lobo et al. 1992; Simmen et al. 1992; Taggart et al. 1992; White and Jackson 1992a). Previously it was demonstrated that functional TBP is mandatory for titration of TFIIIB to relieve p53 mediated repression of VA1 transcription *in vitro* (Cairns and White 1998). Both the

N- and C-terminal regions of p53 have been shown to interact with TBP (Horikoshi et al. 1995) and it is a possibility that the N- and C-terminal regions of p53 mediate repression of pol III transcription through interactions with TBP.

The N- and C-terminal regions of p53 have also been shown to function in mediating repression of pol II transcription (Horikoshi et al. 1995), (Sang et al. 1994), (Subler et al. 1994), and it has also been postulated that p53 mediates this repression through interactions with TBP (Horikoshi et al. 1995). It has recently been demonstrated that p53 represses pol II transcription through the recruitment of histone deacetylase complexes via the corepressor mSin3A (Murphy et al. 1999). mSin3A is thought to interact with the N- and C-terminal regions of p53, thus providing a further possible mechanism by which p53 can repress pol III transcription.

In conclusion, it is clear that from these analyses that repression of pol III transcription by p53 cannot be attributed to one single defined region. There is evidence implicating both the N- and C-termini of p53 and it appears that the core sequence-specific DNA binding domain also plays a role.

Chapter 4

Loss of p53 function allows increased pol III transcription

4.1 Introduction

Mutation of the p53 gene is observed in around half of all human cancers (Hollstein et al. 1991). In addition to this, it is becoming evident that many of the tumours that retain wild-type p53 frequently show defects in the ability to mount a p53 response (Vogelstein et al. 2000). This is often due to an aberration in a pathway responsible for the regulation of p53 itself. For example, the amplification of the Mdm2 gene has been described in some tumours types (Oliner et al. 1992) and in cervical carcinomas, a tumour associated with viral infection, p53 is directly targeted and inactivated by viral proteins (Vousden 1995).

The aim of this chapter was to investigate whether mutational events in the gene or deregulation of p53 itself can compromise its ability to regulate pol III transcription. Firstly it was examined what effect endogenous p53 has on pol III transcription by investigating the abundance of pol III transcripts in wild-type and knockout mouse embryonic fibroblasts (MEFs). It was then examined how the ability of p53 to regulate pol III transcription is influenced by inherited mutations in the p53 gene found in individuals with Li-Fraumeni syndrome (LFS). The consequence of deregulation of p53 on pol III transcription was also investigated. It was examined whether the viral E6 oncoprotein from Human Papillomavirus (HPV) affects the

ability of p53 to repress pol III transcription and what consequence induction of the $p14^{ARF}$ tumour suppressor has on pol III transcription.

4.2 Results

4.2.1 Primary p53 knockout mouse embryo fibroblasts display elevated levels of pol III products

Previously it was demonstrated that fibroblasts from p53 knockout mice synthesise pol III products at abnormally elevated levels (Cairns and White 1998). The cells used in this experiment were immortalised fibroblasts and therefore have almost certainly undergone multiple genetic changes both in the process of immortalisation and through multiple passages. Using untransformed primary MEFs RT-PCR analysis of cDNA prepared from wild-type (Figure 4.1, lane 1) or p53 knockout (Figure 4.1, lane2) cells demonstrate that $tRNA^{Tyr}$ (upper panel) and $tRNA^{Leu}$ (middle panel) are elevated in the knockout cells when compared with the wild-type controls. This effect is specific since the levels of the pol II transcribed gene encoding acidic ribosomal phospoprotein P0 (ARPP P0) remain constant (lower panel). This finding adds further evidence to the belief that endogenous p53 contributes to the control of pol III transcription.

4.2.2 Pol III transcriptional activity is often deregulated in primary fibroblasts from LFS patients

Sufferers of LFS, an inherited autosomal cancer syndrome, carry mutant forms of the p53 gene (Varley et al. 1997a). As with sporadic tumours, a variety of mutations are associated with this syndrome (Varley et al. 1997a). It was investigated whether the

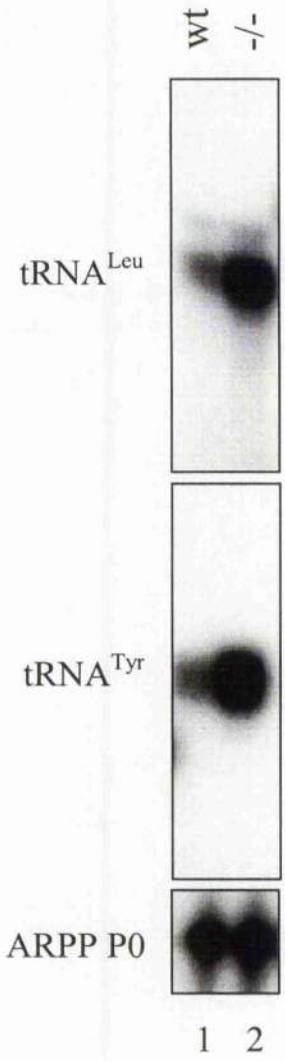


Figure 4.1. Primary p53 knockout mouse embryo fibroblasts (MEFs) display elevated levels of tRNA in vivo. RT-PCR analysis of cDNA prepared from RNAs extracted from wild-type MEFs (lane 1) or p53 knockout MEFs (lane 2) using primers for tRNA^{Leu} (top panel), tRNA^{Tyr} (middle panel) or ARPP P0 (bottom panel).

pol III machinery is deregulated in cells harbouring a variety of these mutations (Table 4.1). Protein extracts were prepared from primary human fibroblasts derived from healthy volunteers or patients carrying inherited mutations in the p53 gene. Transcription assays were then carried out using the VA1 (upper panel) and the $tRNA^{Leu}$ (second panel) genes as templates. A representative example of such experiments is shown in Figure 4.2. Lanes 1-4 show the pol III activity of cells obtained from healthy volunteers, all of which are presumed to carry only wild-type p53. The remaining lanes show samples from 11 Li-Fraumeni patients who have inherited a mutant copy of the p53 gene. Each patient carries a different mutation in p53, apart from 190MA and 19MA (lanes 13 and 14) who are unrelated but carry the same splicing mutation (Varley et al. 2001). When compared to the normal samples most of the Li-Fraumeni samples display elevated pol III activity. This however is not always the case, as samples from patients 109MA and 163MA show only low levels of pol III transcription (lanes 7 and 8). The $tRNA^{Leu}$ gene has a much weaker promoter than VA1 and gave little or no signal in the extracts from healthy volunteers (Figure 4.2, lane 1-4). In concurrence with the transcription assays carried out with the VA1 gene, many of the Li-Fraumeni cell extracts transcribed the tRNA gene at higher levels (Figure 4.2, lanes 5-15) with the relative levels of VA1 and $tRNA^{Leu}$ being similar. Again the lowest activity was observed in samples 109MA and 163MA (Figure 4.2, lanes 7 and 8). The exception is seen in sample 168MA (Figure 4.2, lane 10), where transcription of VA1 is relatively much higher than that of $tRNA^{Leu}$. The activation of pol III transcription is also much more pronounced with VA1 than $tRNA^{Leu}$ in sample 196MA (Figure 4.2, lane 9). The reason for this difference is unclear, since the tRNA and VA genes are believed to utilize the same set of transcription factors (White 1998a).

Lane	Patient	Mutation
1	Healthy volunteer	wt
2	Healthy volunteer	wt
3	Healthy volunteer	wt
4	Healthy volunteer	wt
5	194MA	P152L
6	138MA	R175H
7	109MA	E180K
8	163MA	R248W
9	196MA	T256A
10	168MA	R273H
11	193MA	Stop codon at R209
12	191MA	Mutation in splice acceptor of intron 3
13	190MA	Mutation in splice donor site of exon 3
14	19MA	Mutation in splice donor site of exon 3
15	161MA-F	L344P and loss of wt allele

Table 4.1. Li-Fraumeni mutations

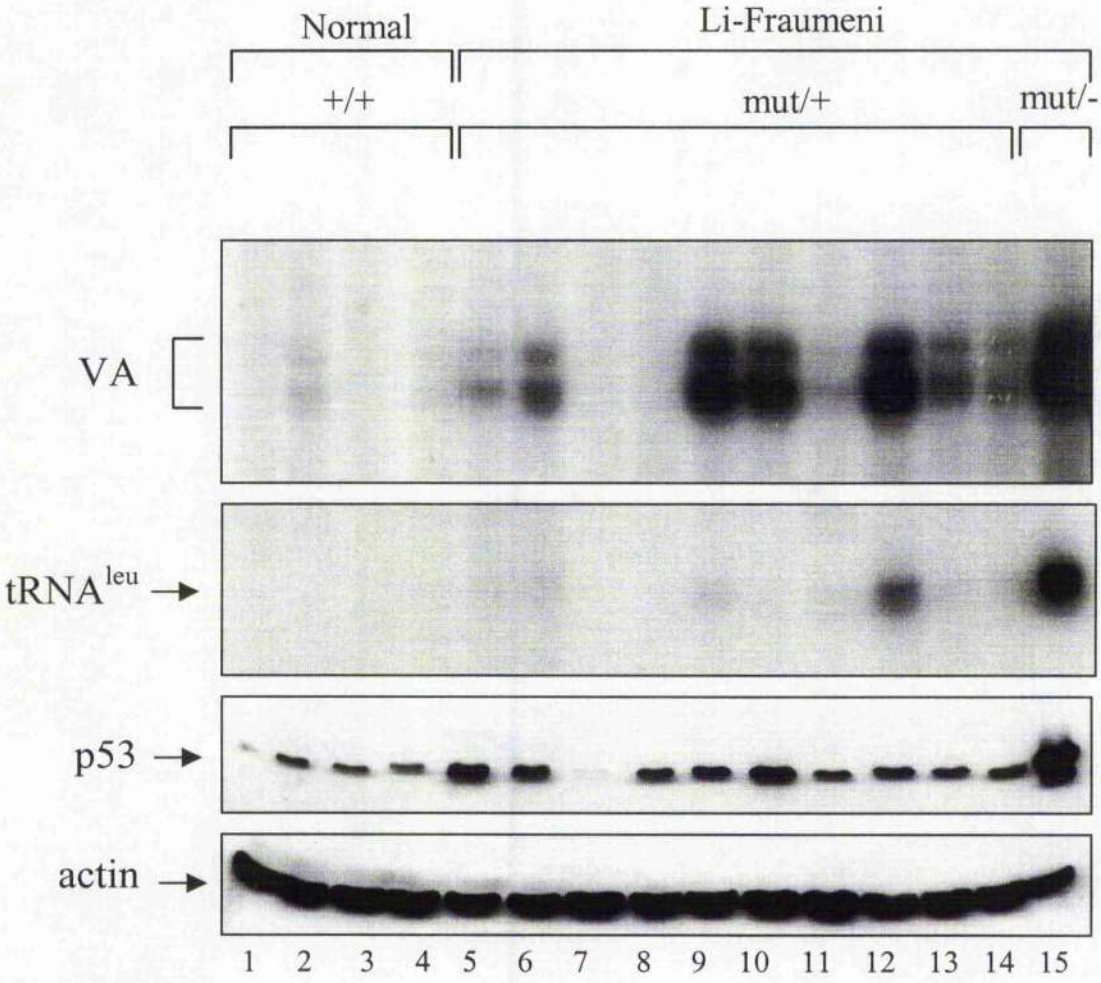


Figure 4.2. Pol III transcriptional activity is frequently elevated in extracts of primary fibroblasts from LFS patients. (Upper panel) pVA1 and (second panel) pleu were transcribed using whole cell extract (15μg) of primary fibroblasts from four healthy volunteers (lanes 1-4) or from 11 patients with LFS (194MA, lane 5; 138MA, lane 6; 109MA, lane 7; 163MA, lane 8; 196MA, lane 9; 168MA, lane 10; 193MA, lane 11; 191MA, lane 12; 190MA, lane 13; 19MA, lane 14; 161MA-F, lane 15). The same extracts were analysed by Western blotting for levels of p53 (third panel) and actin (bottom panel)

The p53-interacting protein mdm2 plays a critical role in regulating the level of wild-type p53 in healthy cells. This is by means of a negative feedback loop in which increased p53 drives the expression of mdm2 (Ko and Prives 1996). Inactive p53 mutants are expressed at elevated levels due to their inability to induce mdm2 (Prives 1998). Western blotting revealed that the p53 mutants are more abundant in the Li-Fraumeni samples than the normal controls (Figure 4.2, third panel). Blotting for actin confirmed equal loading in each case (Figure 4.2, lower panel). The exception is provided by patient 109MA (Figure 4.2, lane 7), where the levels of p53 are substantially lower than those of the other mutants. This suggests that the E180K mutant may retain some capacity to regulate transcription, resulting in down regulation of the protein. It is therefore significant that pol III activity also remains repressed in this sample. Despite this exception, it is clear that pol III transcription is elevated in the majority of Li-Fraumeni samples in despite of the high levels of mutant p53, suggesting that naturally occurring mutations can compromise the ability of p53 to repress pol III transcription.

4.2.3 Pol III transcription is further deregulated upon loss of wt p53

Patients with LFS who inherit mutant p53 invariably retain one wt copy of the gene. However fibroblasts from patient 161MA-F were found to have deleted their wild-type p53 allele (Varley et al. 1996). This may be explained by the fact that these cells were obtained during surgery to remove a tumour (Varley et al. 1996). Nevertheless these cells remained untransformed, like all the Li-Fraumeni fibroblasts examined in this study. This sample (Figure 4.2 lane 15) displayed exceptionally high pol III activity when compared with either the normal or heterozygous samples (Figure 4.1,

lanes 1-14). This observation raised the possibility that deletion of the wild-type copy of p53 may result in further deregulation of pol III transcription.

To test this, heterozygous cells taken from patient 19MA, were cultured through an additional nine passages, which resulted in spontaneous deletion of the wild-type allele. Pol III activity in this samples were found to be dramatically elevated when compared to the heterozygous sample (Figure 4.3, lanes 5 and 6). The same result was obtained with cells from patient 163MA (lanes 3 and 4). Clearly complete loss of wild-type p53 correlates with a substantial activation in pol III transcription. Western blotting confirmed that mutant p53 continues to be expressed in these cells (Figure 4.3, middle panel). Blotting for actin confirmed equal loading in each case (Figure 4.3, lower panel). The level of mutant p53 in the late passage sample 163MA is substantially higher than that of the heterozygous sample (Figure 4.3, lanes 3 and 4), indicating that regulation of mutant p53 is completely abolished as expected. In contrast to this, levels of mutant p53 in the late passage sample of 19MA remain comparable to those of sample 19MA (Figure 4.3, lanes 5 and 6). The reason for this is unclear, but may suggest degradation of p53.

4.2.4 Oncoprotein E6 abrogates the ability of p53 to repress pol III transcription

Apart from mutation, p53 function can also be compromised through the action of various oncoproteins. One such protein is the E6 oncoprotein from Human Papillomavirus (HPV) (Vousden 1995), which targets p53 for degradation. It was tested in transient transfection assays whether the repression of pol III transcription mediated by p53 was affected by cotransfection of E6 from HPV-16. The p53 null,

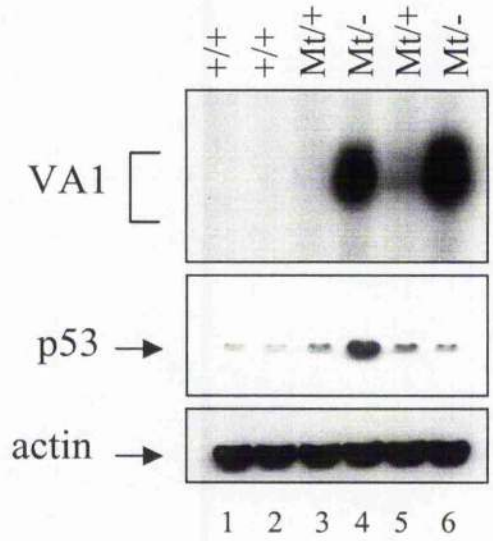


Figure 4.3. Deletion of wild-type p53 correlates with increased pol III transcriptional activity in extracts of primary fibroblasts from LFS patients. (Upper panel) pVA1 were transcribed using whole cell extract (15μg) of primary fibroblasts from two healthy volunteers (lane 1 and 2) or from LFS patients 163MA (lanes 3 and 4) and 19MA (lane 5 and 6). The cells used in lanes 3 and 5 have retained one wild-type copy of the p53 gene; however, the cells in lanes 4 and 6 were harvested at later passages and have consequently deleted the wild-type p53 allele. The same extracts were analysed by Western blotting for levels of p53 (middle panel) and actin (lower panel).

osteosarcoma cell line SAOS2 was transfected with the VA1 gene, as a reporter for pol III activity, and HSV-CAT, in which the chloroamphenicol acetyltransferase gene is transcribed from the basal thymidine kinase promoter of herpes simplex virus. This pol II transcribed gene serves as an internal control for transfection efficiency and RNA recovery. In addition, vectors encoding wild-type p53 and the E6 oncoprotein from HPV-16 were co-transfected. Expression of the VA1 and HSV-CAT reporters were then monitored by quantitive primer extension assays.

Wild-type p53 clearly represses pol III-dependent transcription of VA1 (Figure 4.4, lane 3, upper two panels) when compared to the empty vector control (Figure 4.4, lane 1, upper two panels). This effect was blocked by coexpression of E6 (Figure 4.4, lane 4, upper two panels). Western blotting confirmed that E6 expression reduced the level of p53 without affecting the level of actin (bottom two panels). From this it is concluded that pol III activity *in vivo* can be influenced by E6, a well-established oncoprotein that can counteract the function of p53 in human tumours.

4.2.5 $p14^{ARF}$ contributes to p53 mediated repression of pol III transcription

The alternative product of the human INK4a/ARF locus $p14^{ARF}$ has the potential to act as a tumour suppressor by binding to and inhibiting mdm2 (Stott et al. 1998), a key regulator of p53 activity. It was investigated whether an increase in endogenous p53 through the induction of $p14^{ARF}$ affected pol III transcription. The NARF2 cell line stably transfected with a plasmid encoding $p14^{ARF}$ was induced with IPTG. RT-PCR analysis (Figure 4.5a) of cDNAs prepared from RNA extracted from cells revealed that upon stabilization of p53 (Figure 4.5a, lane 2) through the induction of $p14^{ARF}$, levels of the pol III transcript $tRNA^{Tyr}$ (upper panel) were reduced. This

Figure 4.4. Repression of pol III transcription can be overcome by the E6 oncoprotein of HPV-16. SAOS2 cells were cotransfected with pVA1 (3μg), HSV-CAT (3μg) and 2μg of empty vector (lanes 1 and 2) or vector encoding wild-type p53 (lanes 3 and 4) in the presence (lanes 2 and 4) or absence (lanes 1 and 3) of 2μg pJ4Ω16.E6 encoding HPV-16 E6. RNA and protein were harvested 48hrs after transfection. The upper two panels show primer extension analyses of extracted RNA using primers specific for VA1 (upper panel) and CAT (second panel). Extracts were analysed by Western blotting for the expression of p53 (third panel) and actin (bottom panel).

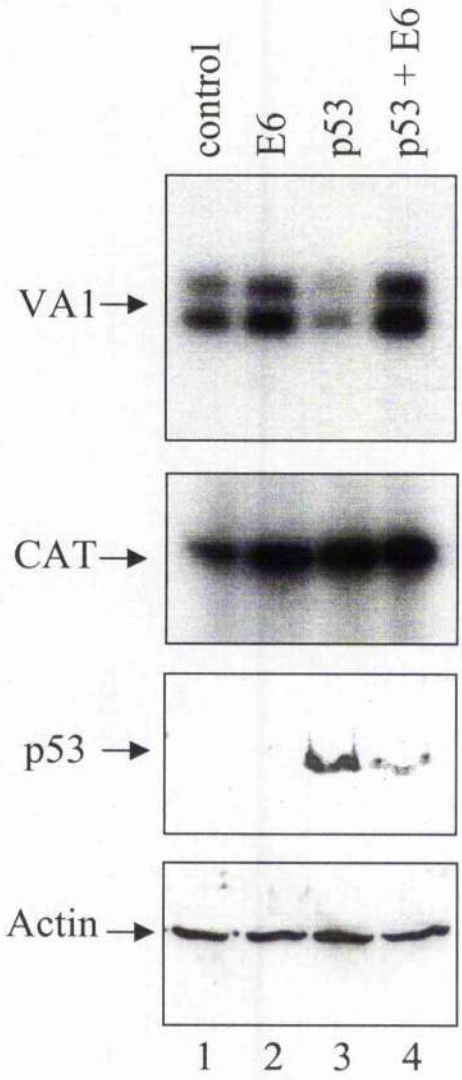
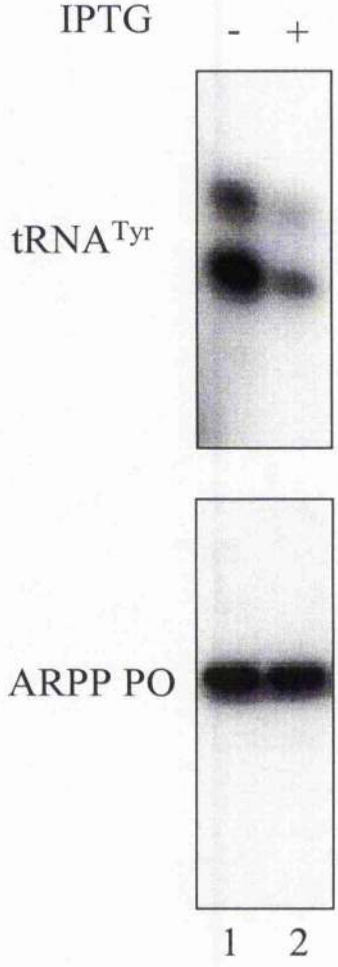

control
E6
p53
p53 + E6
VA1
CAT
p53
Actin
1
2
3
4

Figure 4.5. $p14^{ARF}$ induction results in decreased $tRNA^{Tyr}$. (A) RT-PCR analysis of cDNA prepared from RNAs extracted from uninduced NARF2 cells (lane 1) or $p14^{ARF}$ induced NARF2 cells (lane 2) using primers for $tRNA^{Tyr}$ (top panel) or ARPP P0 (bottom panel). (B) Western blot of uninduced (lane 1) and $p14^{ARF}$ induced (lane 2) NARF2 cells with DO-1 against p53.

A



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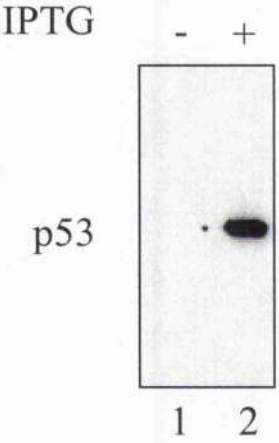


effect was specific since ARPP P0 remained constant (Figure 4.5a, lower panel). Western blotting confirmed that p53 protein levels were increased by the induction of $p14^{ARF}$ (Figure 4.5b, lane 2). It is unlikely that the reduction in pol III transcription is caused by $p14^{ARF}$ itself, since co-expression of E6 from HPV-16, which specifically targets p53, abrogates the observed decrease in pol III transcripts (J. Morten, unpublished results).

4.3 Discussion

Previously it was shown that established fibroblasts from knockout mice have significantly elevated pol III activity when compared with their wild-type counterparts (Cairns and White 1998). It has now been demonstrated that pol III transcripts are abnormally elevated in primary MEFs. The use of primary cells has the advantage over established cell lines since fewer genetic alterations will have occurred. This result clearly suggests that endogenous p53 contributes significantly towards the control of pol III transcription.

This study demonstrates that repression of pol III transcription can be compromised by mutations that commonly arise in untransformed cells from patients with LFS. In addition, it is shown that the oncoprotein E6 from HPV-16 can release pol III from repression by p53. In contrast, induction of the tumour suppressor $p14^{ARF}$, which leads to an increase in p53 levels, also causes a decrease in pol III transcripts.

E6 promotes the degradation of p53 by complexing and targeting it for ubiquitin-mediated proteolysis (Scheffner et al. 1993). It is therefore not surprising that E6 can compromise the ability of p53 to repress pol III transcription. However, it was important to confirm that oncoproteins such as E6 can stimulate pol III transcription. Mdm2, which also targets p53 for degradation, has been shown to elicit a similar effect on pol III transcription (Stein et al. 2002a). Where p53 produced a specific repression of pol III transcription, cotransfection of wild-type hdm2 blocked this effect. In contrast, an hdm2 mutant with a small deletion in its p53-binding domain was unable to overcome the repression mediated by p53. $p14^{ARF}$ targets mdm2 (Stott

et al. 1998). $p14^{ARF}$ can act directly to inhibit the p53-directed ubiquitin ligase activity of Mdm2 (Honda and Yasuda 1999; Midgley et al. 2000). Abnormal oncogenic signals, such as E1A and Myc, have been shown to lead to the activation of p53 through induction of $p14^{ARF}$ (Weber et al. 1999; Lohrum et al. 2000). In some cells the expression of $p14^{ARF}$ also alters the subcellular localisation of mdm2, targeting itself and mdm2 to the nucleolus (Weber et al. 1999; Lohrum et al. 2000). Under these circumstance, p53 remains in the nucleoplasm, releasing p53 from the inhibitory effect of mdm2 interaction. Here it is shown that stabilization of p53 through an induction of $p14^{ARF}$ results in a decrease of pol III transcription. Both E6, mdm2 are major instigators in the development of human cancers. In many cervical carcinomas p53 is bound and inactivated by the E6 product of the HPV, and amplification and overexpression of the gene encoding hdm2 is observed in many tumour types, especially osteosarcomas and soft tissue tumours (Momand et al. 1998). $p14^{ARF}$ has also been implicated in contributing to carcinogenesis, with the gene being to be deleted or methylated in many human cancers (Ruas and Peters 1998). Collectively, the capacity of E6, hdm2 and $p14^{ARF}$ to regulate pol III transcription, through their action on p53, may contribute to the ability of p53 to suppress tumour formation.

p53 is mutated in over 50% of all human cancers (Hollstein et al. 1991). Most of these mutations are mis-sense point substitutions, which lie within the sequence-specific DNA binding region of p53 (Bullock et al. 2000). Although it appears that p53 represses pol III transcription through disruption of protein-protein interactions (Chesnokov et al. 1996; Cairns and White 1998), certain mutations within this sequence-specific DNA binding domain can influence the effect of p53 on pol III

transcription (Stein et al. 2002a). For example in SAOS2 cells, transient transfection of a mutant p53 in which arginine 175 is substituted to proline (R175P), originally derived from an anogenital tumour, was severely compromised in its ability to repress pol III transcription. This mutant is also partially compromised in its ability to transactivate the mdm2 promoter, and repress the c-fos and SV40 early promoters (Crook et al. 1994). R175P, however, retains the capacity to suppress the proliferation of SAOS2 cells (Crook et al. 1994; Ryan and Vousden 1998), establishing the fact that the cell cycle arrest function by p53 is unable to account for the observed repression of pol III transcription (Stein et al. 2002a). The mutant R273C was also unable to repress pol III transcription (Stein et al. 2002a). The mutant R181L, which is compromised in its ability to repress various pol II transcribed genes (Crook et al. 1994), retains a significant capacity to repress pol III transcription (Stein et al. 2002a). Since the two systems respond differently to the R181L mutation, different structural and functional parameters may be required for p53 to repress pol II and pol III genes (Stein et al. 2002a).

The data from the Li-Fraumeni cells (Figures 4.1 and 4.2) support the finding that naturally occurring substitutions in p53 can influence the ability of p53 to repress pol III transcription. Furthermore, the Li-Fraumeni data have the advantage over the transient transfections that p53 is being expressed at physiological levels in a more normal cellular context. In several cases, such as 168MA, which carries a R273H substitution, pol III activity is elevated dramatically despite the presence of a wild-type allele. Other studies have also demonstrated dramatic changes resulting from the loss of a single wild-type allele. Heterozygous $p53^{+/-}$ mice have been shown to develop tumours at a higher rate than their wild-type littermates, with over 95% of

heterozygotes having died or developed tumours by the age of two years, compared with only 20% of the wild-type mice (Venkatachalam et al. 1998). Inactivation of a single allele clearly has dramatic effects on the regulation of pol III activity. However, as demonstrated in Figure 4.2, loss of the remaining wild-type allele can result in further deregulation.

Each patient with LFS comes from a different genetic background and it cannot be concluded that the increases in pol III transcription observed in fibroblasts from many of the patients can be attributed solely to the mutation of p53. Indeed, cells from different healthy volunteers show subtle variations in pol III activity. The results however, are striking and suggest that naturally occurring mutations in p53 can compromise its ability to control pol III transcription.

Chapter 5

Mechanism of pol III transcriptional repression by p53

5.1 Introduction

p53 represses a variety of genes (Ginsberg et al. 1991; Mercer et al. 1991; Santhanam et al. 1991; Kley et al. 1992; Seto et al. 1992; Subler et al. 1992; Mack et al. 1993; Ragimov et al. 1993; Crook et al. 1994; Horikoshi et al. 1995; Chesnokov et al. 1996; Farmer et al. 1996a; Werner et al. 1996; Cairns and White 1998; Venkatachalam et al. 1998; Yu et al. 1999; Sun et al. 2000; Zhao et al. 2000), although, at present the mechanism by which p53 elicits these effects is poorly understood. It is believed that p53-mediated repression of pol III transcription occurs via a direct mechanism. Assembly of a pol III transcription initiation complex on type two promoters involves a stepwise assembly of multiple transcription factors. The A- and B-block consensus sequences found in many of these promoters are recognised by the multisubunit complex TFIIIC2. TFIIIC1 and TFIIIB are then recruited. This DNA-TFIIIC-TFIIIB complex then serves to recruit pol III to the start site. It is believed that p53 targets TFIIIB (Chesnokov et al. 1996; Cairns and White 1998). It has been demonstrated by immunoprecipitation and cofractionation experiments that endogenous p53 and TFIIIB associate at physiological ratios. Furthermore when p53 is present in excess, adding purified TFIIIB can efficiently relieve VA1 gene expression from repression by p53. TFIIIB is a trimeric complex, consisting of the TATA-box binding protein, TBP, Brf1 and Bdp1. Previous analysis has demonstrated that both the N- and C-terminal domains of p53 are important in mediating pol III transcriptional repression (Stein et al. 2002b). p53 is a well-

established binding partner of TBP, and it is the N- and C-terminal domains of p53 that contact TBP. For repression of pol II-transcribed genes by p53, an interaction between TBP and p53 has been implicated (Seto et al. 1992; Liu et al. 1993; Martin et al. 1993; Farmer et al. 1996a; Farmer et al. 1996b; Tansey and Herr 1997); however, additional interactions between p53 and TAFs are also believed to contribute to the effect (Farmer et al. 1996b).

Here the functional consequences of p53-TFIIIB interactions are investigated. The involvement of histone acetylase transferase (HAT) and deacetylase (HDAC) complexes in p53-mediated pol III transcriptional repression is also examined. HATs and HDACs contribute to the control of transcription through multiple mechanisms (Kuo and Allis 1998). For example acetylation of the basal transcription factor SL1 has shown to play a major role in controlling expression of pol I transcription (Muth et al. 2001). p53 itself is subject to acetylation, a modification which results in structural changes within the protein and activation of intrinsic DNA-binding activity (Gu and Roeder 1997). Previous mechanistic analysis of pol II transcribed genes that are repressed by p53 revealed that the recruitment of HDAC complexes by p53, via the co-repressor complex mSin3A, was essential in mediating repression (Murphy et al. 1999).

5.2 Results

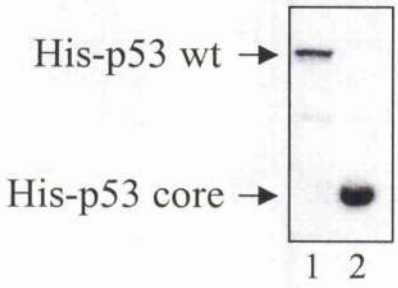
5.2.1 p53 associates with TBP and Brf1 but not Bdp1

Previously it was demonstrated that endogenous p53 cofractionates with TFIIIB (Cairns and White 1998). Cairns and White also showed that TFIIIB

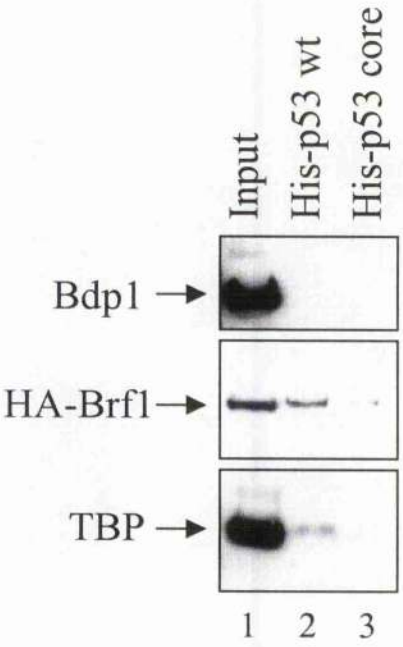
immunoprecipitates with p53 at physiological ratios (Cairns and White 1998). To further investigate the association of p53 with the individual subunits of TFIIIB, His-p53 pull down assays were performed. 50µl of purified wild-type His-p53 and His-p53 (98-303) (Figure 5.1A) was used in each binding assay. His-p53(98-303) lacks both TBP binding domains and is unable to repress pol III transcription (Stein et al. 2002b). The immobilised proteins were incubated with CHO cellular extract stably expressing HA tagged Brf1. Following incubation and extensive washing, Western blotting revealed that both TBP and HA-Brf1 associated with wild-type His-p53 (Figure 5.1B, bottom and middle panels). This interaction was specific since His-p53 (98-303), which encompasses only the core domain of p53, did not bind to either protein (Figure 5.1B, lane 3). Bdp1 did not associate with either wild-type His-p53 or His-p53 (98-303). This result may indicate that Bdp1 cannot associate with p53. In addition, it may further suggest that Bdp1 is unable to associate with TBP and Brf1 in the presence of p53. To further investigate these findings, wild-type and His-p53 (98-303) were incubated with reticulocyte containing Bdp1, Brf1 or TBP labelled with ^{35}S-Met and ^{35}S-Cys. As expected, TBP associated with wild-type His-p53 but not with His-p53 (98-303). Brf1 also bound to wild-type His-p53, whereas no significant association was observed between Brf1 and His-p53 (98-303). This result does not suggest unequivocally that Brf1 can bind directly to wild-type His-p53. It is possible that Brf1 interacts with wild-type His-p53 via another molecule contained in the reticulocyte. TBP may bridge the association between Brf1 and wild-type His-p53. ^{35}S-Bdp1 did again not interact with either wild-type His-p53 or His-p53 (98-303). This further suggests that Bdp1 is unable to associate with p53.

Figure 5.1. Wt His-p53 binds endogenous TBP and HA-Brf1, but not Bdp1. (A) Purification of p53 wt and p53(98-303). 50µl of packed beads binding His-p53 wt (lane 1) and His-p53(98-303) (lane 2) were boiled in SDS sample buffer, separated by SDS-PAGE and visualised by Coomassie Blue staining. (B) Wt His-p53 binds endogenous TBP and HA-Brf1, but not Bdp1. 200µg of CHO extract expressing HA tagged Brf1 was incubated with His-p53 wt (lane 2) or His-p53 (98-303) (lane 3) bound to Ni-NTA agarose beads for 1 hour at 4°C. After extensive washing, bound material was separated by gel electrophoresis and visualised by Western blotting with antibodies 58c9 against TBP, F7 against HA and 2663-4 against Bdp1. Lane 1 shows 10% CHO extract input. (C) Wt His-p53 binds ^{35}S-labelled TBP and Brf1, but not Bdp1. Reticulocyte lysate (5µl) containing in vitro-translated TBP (bottom panel), Brf1 (middle panel) or Bdp1 (top panel) was incubated with His-p53 wt (lane 2) or His-p53 (98-303) (lane 3) bound to Ni-NTA agarose beads. After extensive washing, bound material was separated by gel electrophoresis and visualised by autoradiography. Lane 1 shows 5% of the input reticulocyte lysate containing in vitro translated protein.

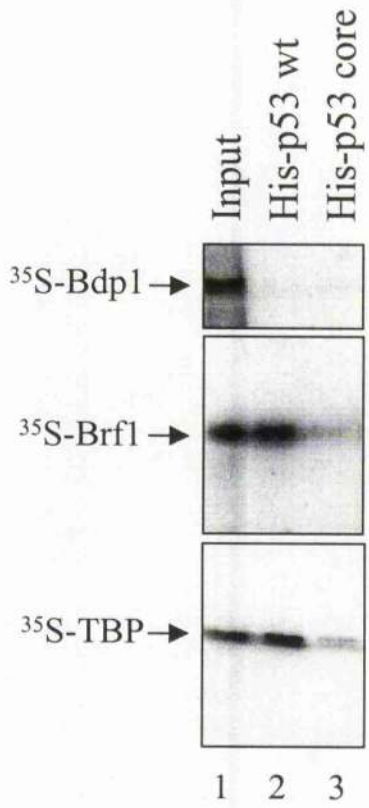
A


His-p53 wt
His-p53 core
1 2

B


Input
His-p53 wt
His-p53 core
Bdp1
HA-Brf1
TBP
1 2 3

C



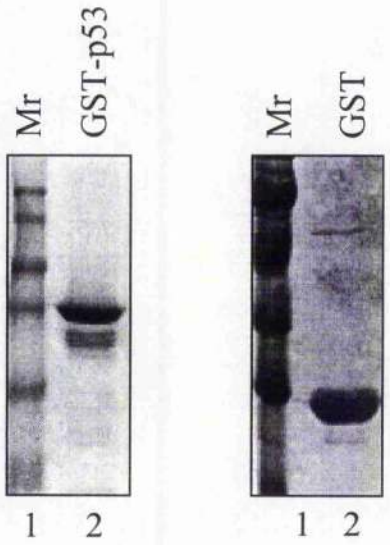
To further examine the binding between TFIIIB and p53, GST-pull down assays were performed. Equal amounts of GST-p53 and GST alone (Figure 5.2A) were incubated with reticulocyte containing ^{35}S-labelled Brf1 and TBP. The results shown demonstrate that significantly more TBP can associate with GST-p53 than GST alone (Figure 5.2B, lanes 2 and 3). In addition, substantially more Brf1 bound to GST-p53 when compared to GST alone (Figure 5.2B, lane 5 and 6). This adds further support to the suggestion that Brf1 can interact p53. These results demonstrate that recombinant wild-type p53 can associate with the TFIIIB subunits TBP and Brf1. No association was observed between wild-type His-p53 and Bdp1. The results also suggest that Bdp1 may be prevented from associating with TBP and Brf1 in the presence of p53.

5.2.2 TBP relieves p53 mediated repression of pol III transcription

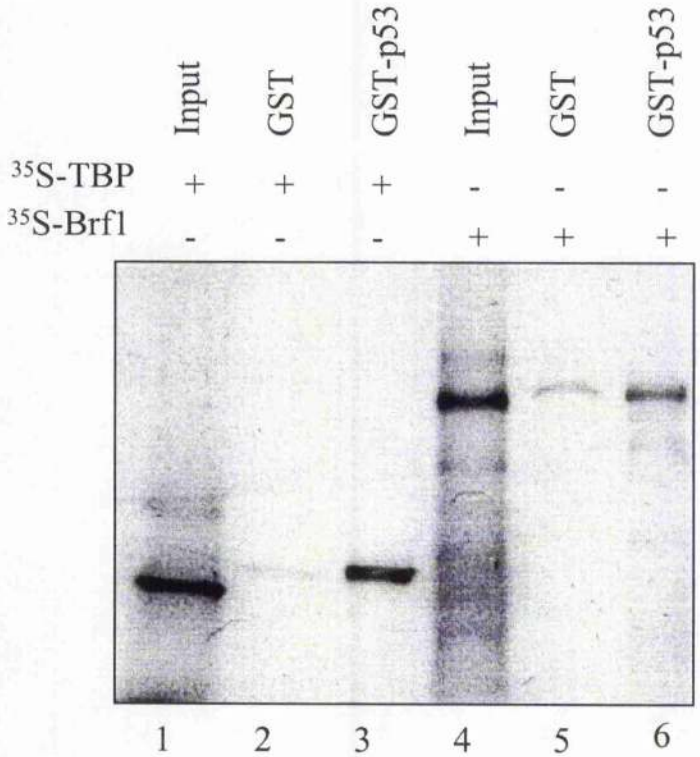
Since TFIIIB is targeted by p53 (Cairns and White 1998; Chesnokov et al. 1996), and an association exists between p53 and both TBP and Brf1, it was examined whether raising the concentration of TBP could reverse pol III transcriptional repression mediated by p53. In the absence of TBP, p53 mediates repression of Alu transcription *in vitro* (Figure 5.3, lanes 2 and 5). Titration of increasing amounts of TBP alleviated this repression (Figure 5.3, lane 3 and 4). In the absence of p53, increasing amounts of TBP had little effect on the level of Alu transcription (Figure 5.3, lanes 7 and 8). This result suggests that TBP becomes limiting for pol III transcription *in vitro* in response to p53. However, transcription was not fully reconstituted by TBP, which may suggest that additional TBP is required. It is also possible that another transcription factor becomes limiting. Both Brf1 and TBP can interact with p53, and it remains to be determined whether titration of Brf1 *in vitro*

Figure 5.2. ^{35}S-labelled TBP and Brf1 bind GST-p53. (A) Coomassie-stained gel of purified GST and GST-p53. Molecular markers (lane 1, left and right panel) and purified GST-p53 (1μg) (lane 2, left panel) or purified GST (2μg) (lane 2, right panel) were electrophoresed on a 7.8% SDS-polyacrylamide gel and then visualised by staining with Coomassie Blue. (B) ^{35}S-labelled TBP and Brf1 bind GST-p53. ^{35}S-labelled TBP (lanes 2 and 3) or Brf1 (lanes 5 and 6) were incubated with equal amounts of GST (lanes 2 and 5) or GST-p53 (lanes 3 and 6) for 3 hours at 4°C. After extensive washing with LDB, bound material was separated by gel electrophoresis and visualised by autoradiography. Lanes 1 and 4 show 10% ^{35}S-labelled TBP and ^{35}S-labelled Brf1.

A


Mr
GST-p53
Mr
GST
1 2
1 2

B


Input
GST
GST-p53
Input
GST
GST-p53
35S-TBP + + + - - -
35S-Brf1 - - - + + +
1 2 3 4 5 6

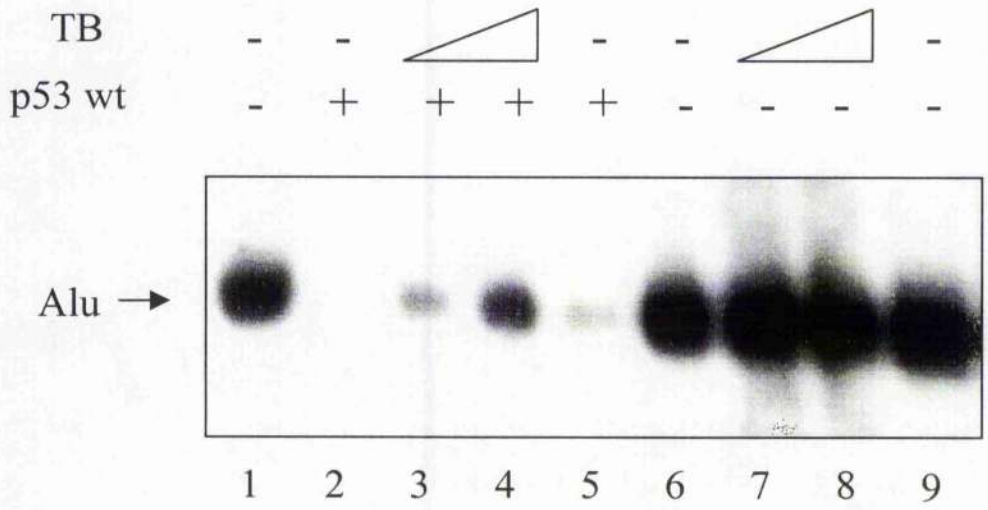


Figure 5.3. Titration of recombinant TBP relieves p53-mediated repression of Alu transcription. pRH5.7 (250ng) was transcribed using HeLa nuclear extract (15μg) that had been preincubated for 10 min at 30°C in the presence of 0.6μg of wild-type p53 (lanes 2-5) and 0.5μl (lanes 3 and 7) or 1μl (lanes 4 and 8) of recombinant TBP.

can also alleviate p53-mediated repression of pol III activity. It was not possible to test this, as we have been unable to express recombinant Brf1.

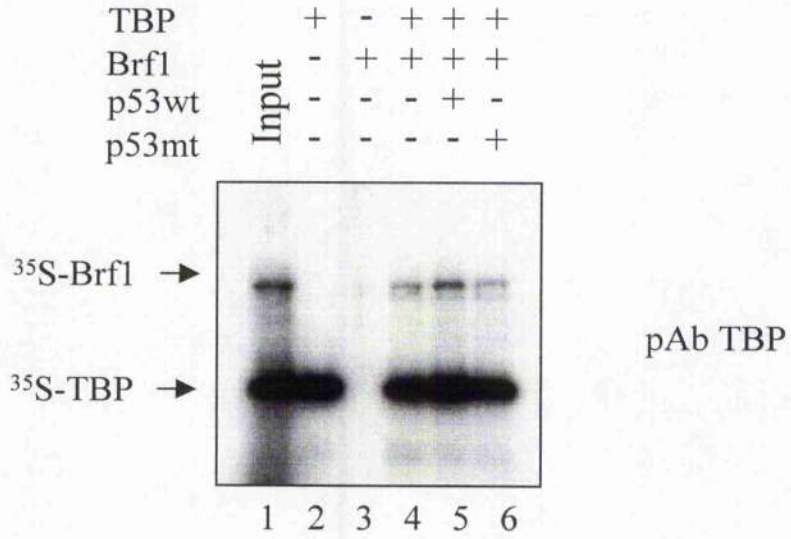
5.2.3 Association of p53 with TFIIIB results in disruption of essential pol III transcription complex interactions

It has been shown that TBP and Brf1 can associate with both wild-type GST-p53 and His-p53. From this it is unlikely that p53 prevents TBP-Brf1 interactions within the TFIIIB complex. To test this, reticulocyte containing TBP and Brf1, labelled with ^{35}S-Met and ^{35}S-Cys, were incubated together with recombinant wild-type His-p53 or His-p53 (98-303). Complexes were then immunoprecipitated using an antibody against TBP. After extensive washing, bound material was separated by elecrophoresis and visualised via autroradiography. Both ^{35}S-TBP and ^{35}S-Brf1 efficiently coprecipitated in the absence of p53 (Figure 5.4A, lanes 4). Coprecipitation of Brf1 was specific, since Brf1 was not precipitated in the absence of TBP (Figure 5.4A, lane 3). No disruption in the amount of Brf1 co-precipitating with TBP was observed in the presence of either wild-type His-p53 or His-p53 (98-303) (Figure 5.4A, lane 5 and 6). This result suggests that wild-type p53 does not disrupt the interactions formed between TBP and Brf1.

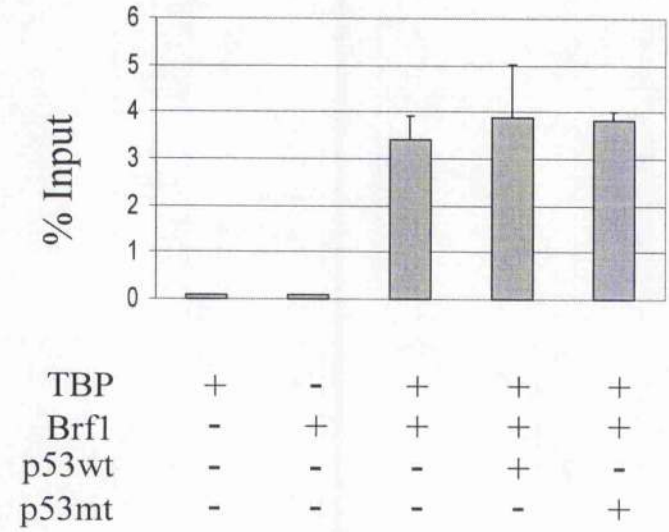
Multiple protein-protein interactions are formed between the basal pol III transcription factors during initiation of transcription (Paule 1998; Geiduschek and Kassavetis 2001). TFIIIB forms interactions with both TFIIIC2 and the polymerase itself. It was tested whether p53 could compromise the interactions between these factors. Using an antibody against RNA polymerase III, ^{35}S-labelled Brf1 efficiently co-precipitated in the presence but not in the absence of HeLa nuclear extract (Figure

Figure 5.4. p53 does not prevent Brf1 from binding to TBP. (A) Anti-TBP antibody 4c826 was used to immunoprecipitate reticulocyte containing in vitro translated TBP (2μl) and/or in vitro translated Brf1 (5μl). His-p53 wt (0.6μg) was added to lane 5 and His-p53 (98-303) (0.6μg) was added to lane 6. After extensive washing, bound material was separated by gel electrophoresis and visualised by autoradiography. Lane 1 shows 10% ^{35}S-labelled TBP and 35S-labelled Brf1. (B) Values shown represent the % of ^{35}S-Brf1 co-immunoprecipitating, compared to input ^{35}S-Brf1. Values represent the mean and standard deviation of three experiments.

A


TBP + - + + +
Brf1 - + + + +
p53wt - - - + -
p53mt - - - - +
Input
35S-Brf1
35S-TBP
pAb TBP
1 2 3 4 5 6

B


% Input
6
5
4
3
2
1
0
TBP + - + + +
Brf1 - + + + +
p53wt - - - + -
p53mt - - - - +

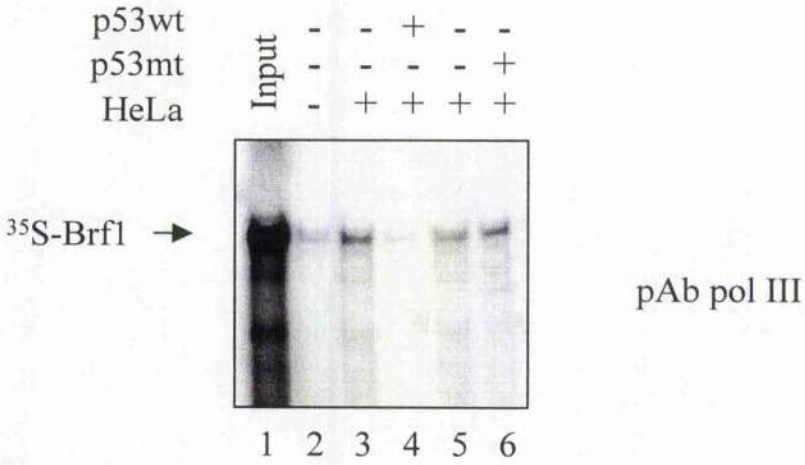
5.5, lanes 2 and 3). The amount of co-precipitating ^{35}S-Brf1 was dramatically reduced in the presence of wild-type His-p53 (Figure 5.5, lane 4). In contrast, binding of ^{35}S-Brf1 to pol III was unaffected by the addition of His-p53 that lacked both the N- and C-terminal regions (Figure 5.5, lane 6). Similarly ^{35}S-labelled Brf1 co-precipitated with an antibody against TFIIIC2 (Figure 5.6, lane 2). Addition of wild-type His-p53 resulted in a reduction in association between ^{35}S-Brf1 and TFIIIC2 (Figure 5.6, compare lanes 2 and 4). Addition of His-53 (98-303) had little effect on ^{35}S-Brf1 co-precipitating with TFIIIC2 (Figure 5.6, lane 6). These results suggest that the ability of TFIIIB to associate with TFIIIC2 and pol III is compromised by wild-type p53 *in vitro*.

5.2.4 TFIIIB but not TFIIIC2 is prevented from binding the tRNA^{Leu} promoter by wild-type p53 *in vitro*

Assembly of a functional pol III transcription complex on tRNA genes involves a stepwise assembly of three factors. TFIIIC2 recognises and binds to internal promoter elements; this then serves to recruit TFIIIB, which in turn associates with the polymerase, situating it at the start site of transcription (Paule and White 2000; Geiduschek and Kassavetis 2001). The effect on this recruitment process, and therefore the subsequent occupancy of pol III transcription machinery on the tRNA^{Leu} gene was investigated. An immobilised template assay was employed for this purpose (Figure 5.7A). The tRNA^{Leu} gene was amplified by PCR using specific primers. The reverse primer was covalently biotinylated at the 5' end. This allowed the immobilisation of the tRNA gene fragment using magnetic beads coated with streptavidin. The resultant 3' biotinylated fragment was separated by agarose gel

Figure 5.5. p53 prevents Brf1 from binding to pol III. (A) Anti-pol III antibody 114 was used to immunoprecipitate reticulocyte containing in vitro translated Brf1 (5μl) in the presence of 100μg HeLa NE preincubated with His-p53 wt (1μg) (lane 5) and His-p53 (98-303) (1μg) (lane 6). After extensive washing, bound material was separated by gel electrophoresis and visualised by autoradiography. Lane 1 shows 20% ^{35}S-labelled Brf1. (B) Values shown represent the % of 35S-Brf1 co-immunoprecipitating, compared to input ^{35}S-Brf1. Values represent the mean and standard deviation of three experiments.

A


p53wt
p53mt
HeLa
Input
- - + - -
- - - - +
- + + + +
35S-Brf1
pAb pol III
1 2 3 4 5 6

B

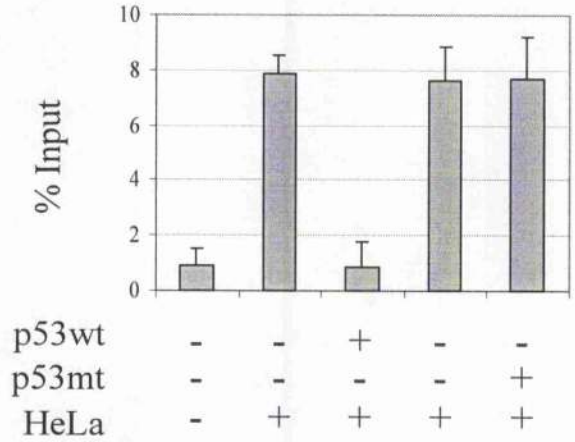
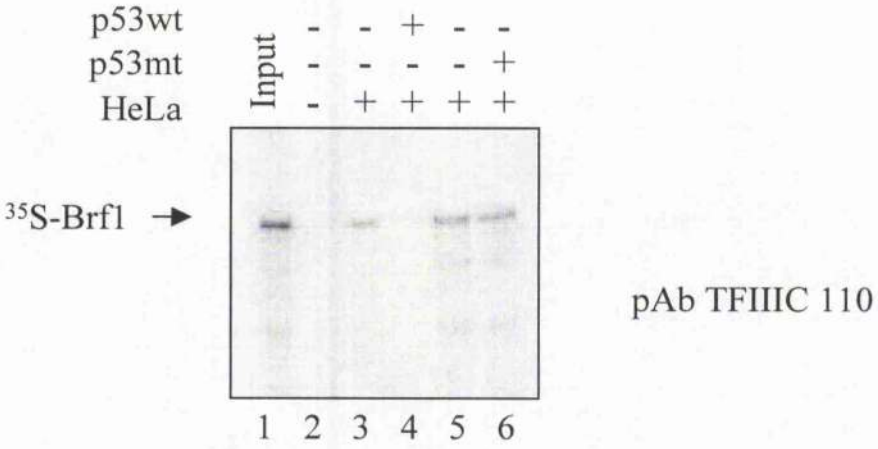

% Input
10
8
6
4
2
0
p53wt - - + - -
p53mt - - - - +
HeLa - + + + +

Figure 5.6. p53 prevents Brf1 from binding to TFIIIC2. (A) Anti-TFIIIC2 antibody 4286-4 was used to immunoprecipitate reticulocyte lysate containing in vitro translated Brf1 (5μl) in the presence of 100μg HeLa NE preincubated with His-p53 wt (1μg) (lane 5) and His-p53 (98-303) (1μg) (lane 6). After extensive washing, bound material was separated by gel electrophoresis and visualised by autoradiography. Lane 1 shows 10% ^{35}S-labelled Brf1. (B) Values shown represent the % of ^{35}S-Brf1 co-immunoprecipitating, compared to input 35S-Brf1. Values represent the mean and standard deviation of four experiments.

A


p53wt - - + - -
p53mt - - - - +
HeLa - + + + +
Input
35S-Brf1
pAb TFIIIC 110
1 2 3 4 5 6

B

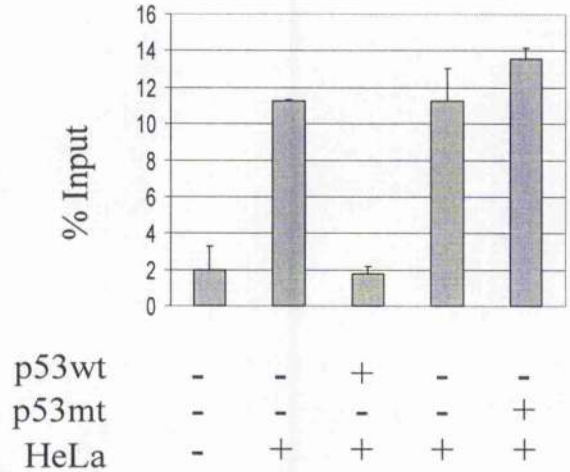
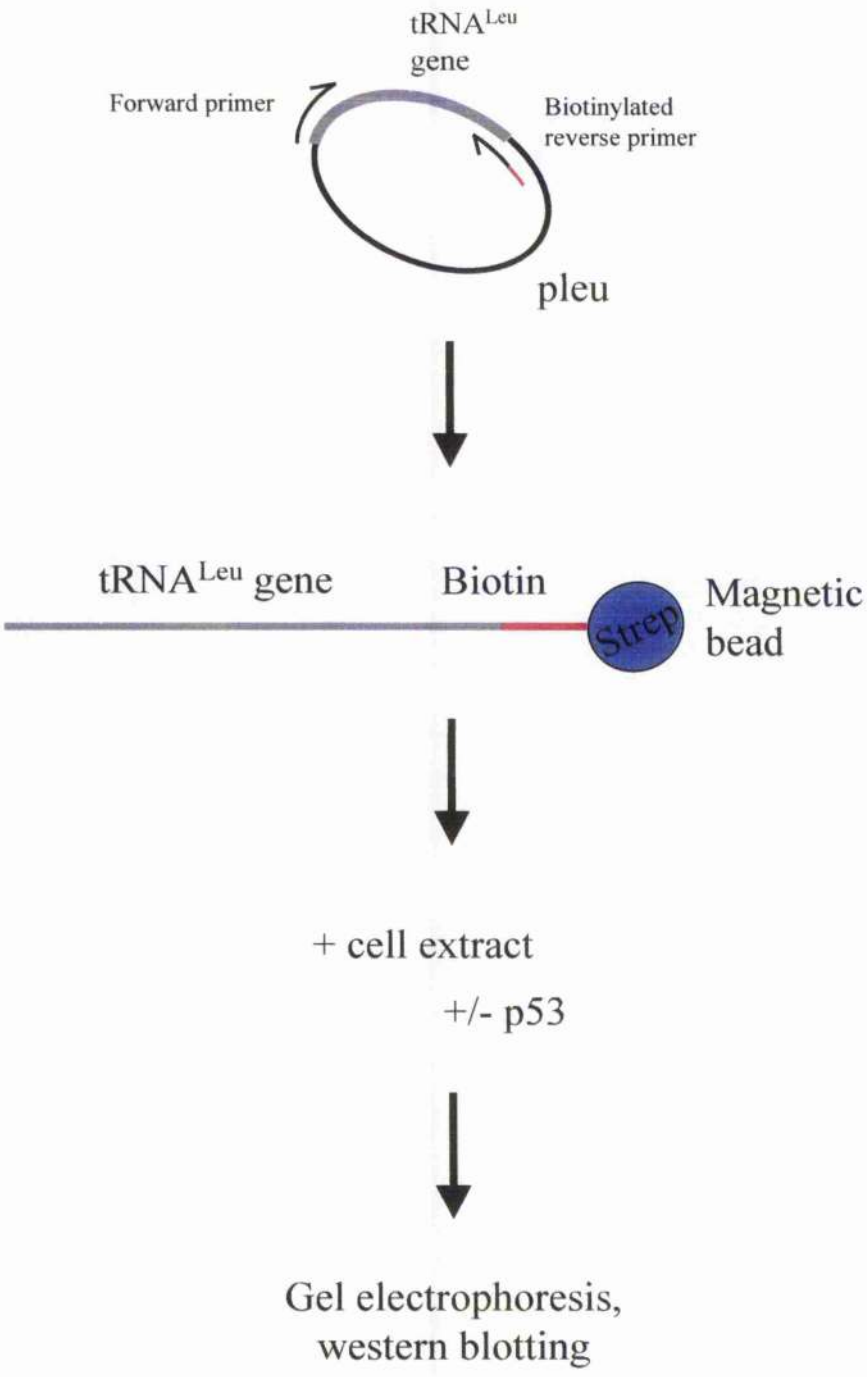

% Input
16
14
12
10
8
6
4
2
0
p53wt - - + - -
p53mt - - - - +
HeLa - + + + +

Figure 5.7. Biotinylated Template Assay. Schematic overview of the biotinylated template assay protocol. The biotinylated templates were synthesised by PCR with the reverse primer being biotinylated at the 5'end. Biotinylated fragments were purified then incubated with streptavidin coated magnetic beads. Immobilised DNA was incubated together with cellular extract, plus or minus recombinant p53. Bound material was washed extensively then separated by gel electrophoresis and visualised by Western blotting.


tRNA^{Leu}
gene
Forward primer
Biotinylated
reverse primer
pleu
tRNA^{Leu} gene
Biotin
Strep
Magnetic
bead
+ cell extract
+/- p53
Gel electrophoresis,
western blotting

electrophoresis. The DNA fragment was then purified and checked for its ability to support transcription *in vitro* (Figure 5.8).

The plasmid pleu (250ng) (Figure 5.8, lane 1) or 250ng (lane 2) or 500ng (lane 3) of the purified biotinylated $tRNA^{Leu}$ fragment were transcribed using HeLa nuclear extract. Although the tRNA fragment was transcribed at a lower level when compared to that obtained with the plasmid pleu (compare lanes 1 and 2), 500ng of fragment produced a signal approaching that obtained with 250ng of plasmid (compare lanes 1 and 3). This shows that the tRNA fragment can support active transcription *in vitro* and can therefore be used as a tool to investigate promoter occupancy.

The immobilised $tRNA^{Leu}$ template was incubated with extract prepared from CHO cells stably expressing HA tagged Brf1, either in the absence, or in the presence of wild-type p53 or p53 (24-392). Bound material was then separated by gel electrophoresis and detected via Western blotting. In the absence of p53, TFIIIC110 (top panel), HA-Brf1 (middle panel) and TBP (lower panel) could be detected bound to the $tRNA^{Leu}$ gene fragment (Figure 5.9, lane 2). Binding was specific, since no factor could be detected binding to the beads alone (Figure 5.9, lane 1). In the presence of increasing amounts of wild-type His-p53, the occupancy of the TFIIIB subunits TBP (lower panel) and HA-Brf1 (middle panel) were diminished. This decrease was specific, since adding equal amounts of His-p53 (24-392) resulted in no change in occupancy. TFIIIC110 (upper panel) levels remained constant in the presence of wild-type His-p53 (lanes 3 and 4). These results suggest that p53 interferes specifically with TFIIIB recruitment on tRNA genes *in vitro*.

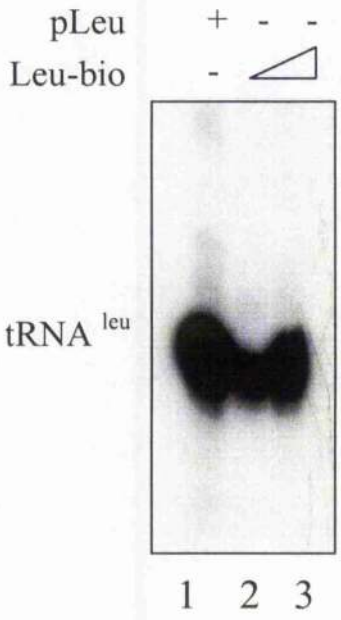
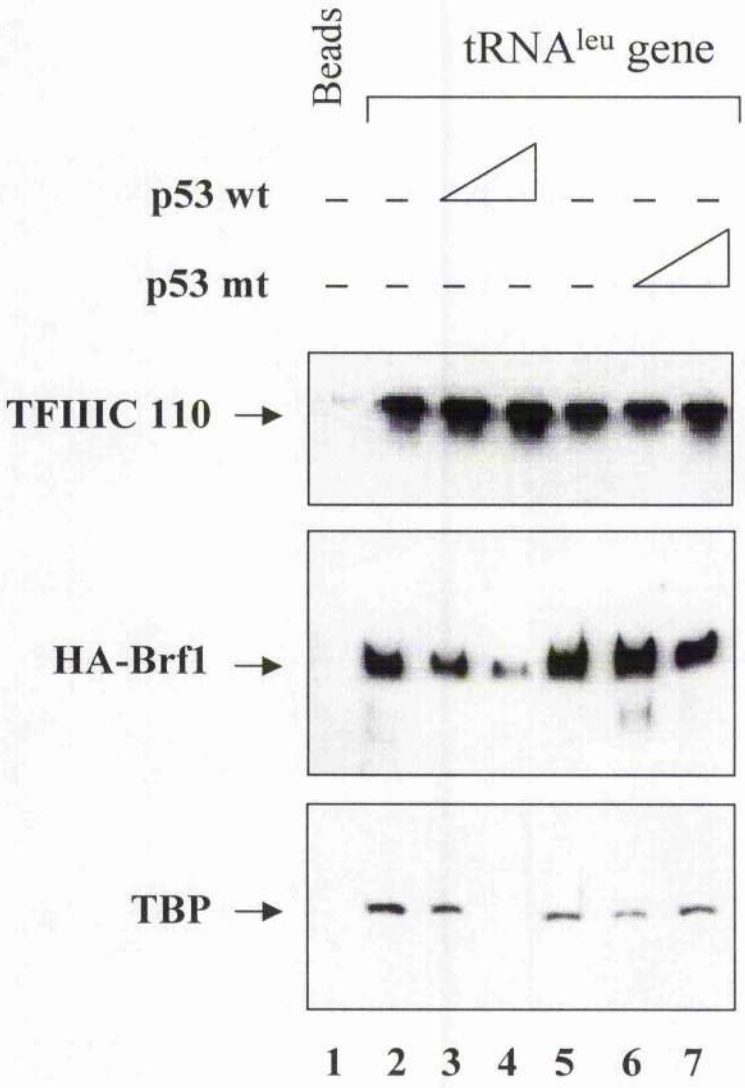


Figure 5.8. Biotinylated $tRNA^{Leu}$ gene fragment supports in vitro transcription. pLeu (250ng) (lane 1) or 250ng (lane 2) or 500ng (lane 3) of the biotinylated $tRNA^{Leu}$ gene fragment was transcribed in vitro using HeLa nuclear extract (15μg).

Figure 5.9. TFIIIB but not TFIIIC2 is prevented from binding the $tRNA^{Leu}$ promoter in the presence of wild-type His-p53. Immobilised biotinylated $tRNA^{Leu}$ gene fragments were incubated with CIIO cellular extract (200μg) stably expressing HA tagged Brf1 in the absence (lanes 2 and 5) or the presence of 2μg (lanes 3 and 6) or 4μg (lane 4 and 7) of wild-type His-p53 (lanes 3 and 4) or mutant p53 (p53(24-392)) (lanes 6 and 7). After incubation for 20 minutes at 30°C, beads were thoroughly washed, resuspended in protein sample buffer and separated by gel electrophoresis. Bound material was analysed by Western blotting with antibodies 58c9 against TBP, F7 against HA and 4286-4 against TFIIIC 110.


Beads
tRNA^leu gene
p53 wt
p53 mt
TFIIIC 110
HA-Brf1
TBP
1 2 3 4 5 6 7

5.2.5 TFIIIB and pol III occupancy at endogenous tRNA genes is elevated specifically in primary p53-knockout MEFs relative to wild-type control cells

Chromatin immunoprecipitation, or ChIP, refers to a procedure used to determine whether a given protein binds to a specific DNA sequence *in vivo* (Figure 5.10). This method was used to examine whether differences in promoter occupancy of pol III transcription complex subunits on tRNA genes *in vivo* could be observed between primary p53 knockout mouse embryo fibroblasts (MEFs) and matched wild-type control cells. RT-PCR analysis has confirmed that the levels of primary tRNA transcripts are elevated specifically in p53 knockout fibroblasts when compared to the wild-type controls (Figure 4.1). In addition nuclear run-on assays previously demonstrated a marked and specific increase in tRNA expression in p53-/- fibroblasts (Cairns and White 1998). First cells were grown to 80% confluency and DNA-binding proteins were then cross-linked to DNA with 1% formaldehyde. Cells were lysed by hypertonic shock and chromatin was isolated. DNA was then sheared into small fragments by sonication. Antibodies against TFIIIB, TFIIIC2 and pol III were then used to co-immunoprecipitate DNA bound to each of these factors. In order to assess whether changes in occupancy were apparent between the p53 knockout and wild-type MEFs, the DNA was amplified by semi-quantitive PCR using primers specific for tRNA genes. Various concentrations of DNA were used to ensure that the PCR reactions were in the linear range for each antibody used for ChIP.

ChIP analysis demonstrated that there was significantly more TBP present at $tRNA^{Leu}$ (upper panel) and $tRNA^{Tyr}$ (middle panel) promoters in p53 knockout MEFs compared to wild-type control cells (Figure 5.11, lanes 5 and 6). Promoter

Figure 5.10. Schematic overview of the Chromatin Immunoprecipitation assay (ChIP). (A) Cells are grown to 80% confluency then cross linked in a 1% formaldehyde solution. (B) Soluble sheared chromatin is prepared through a process of hypertonic disruption, purification and sonication. (C) Transcription complexes are immunoprecipitated overnight with antibodies against the factors of interest. (D) Immune complexes are extensively washed, and the cross-links between proteins and DNA are reversed by proteinase K treatment. DNA is purified by phenol-chloroform extraction and ethanol precipitation. (E) Amounts of co-precipitating purified DNA are analysed by semi-quantitive PCR and gel electrophoresis.

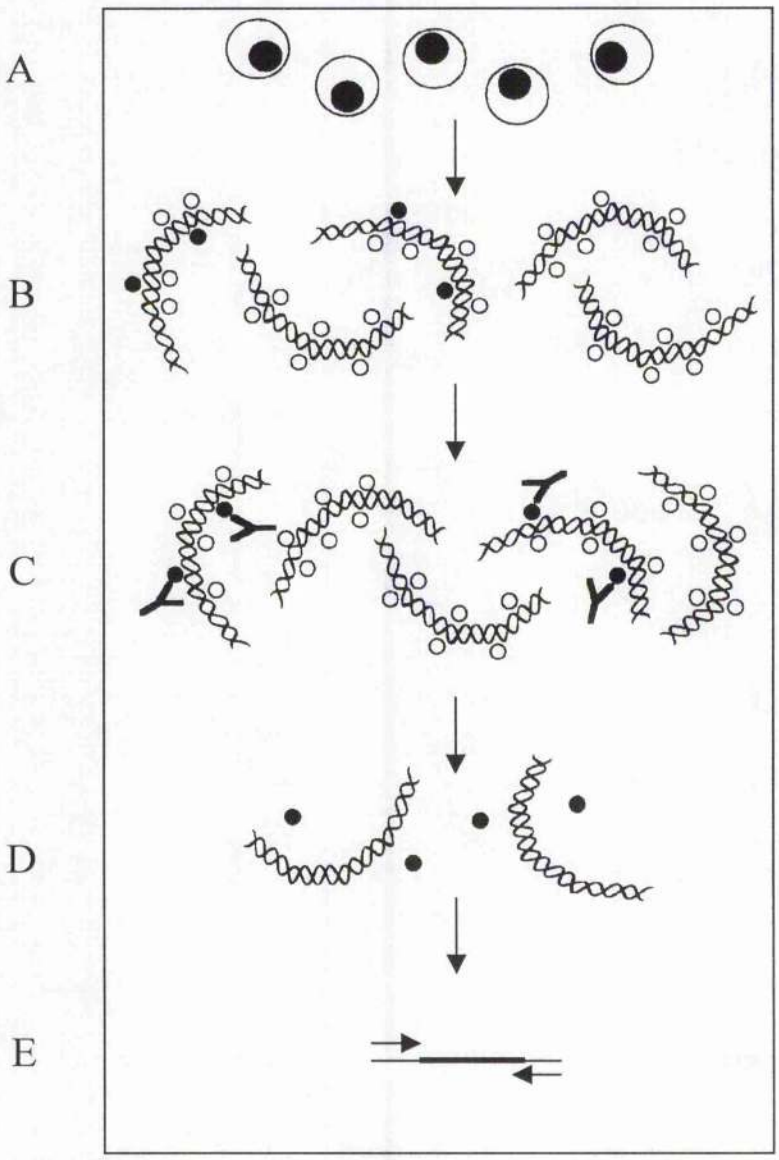
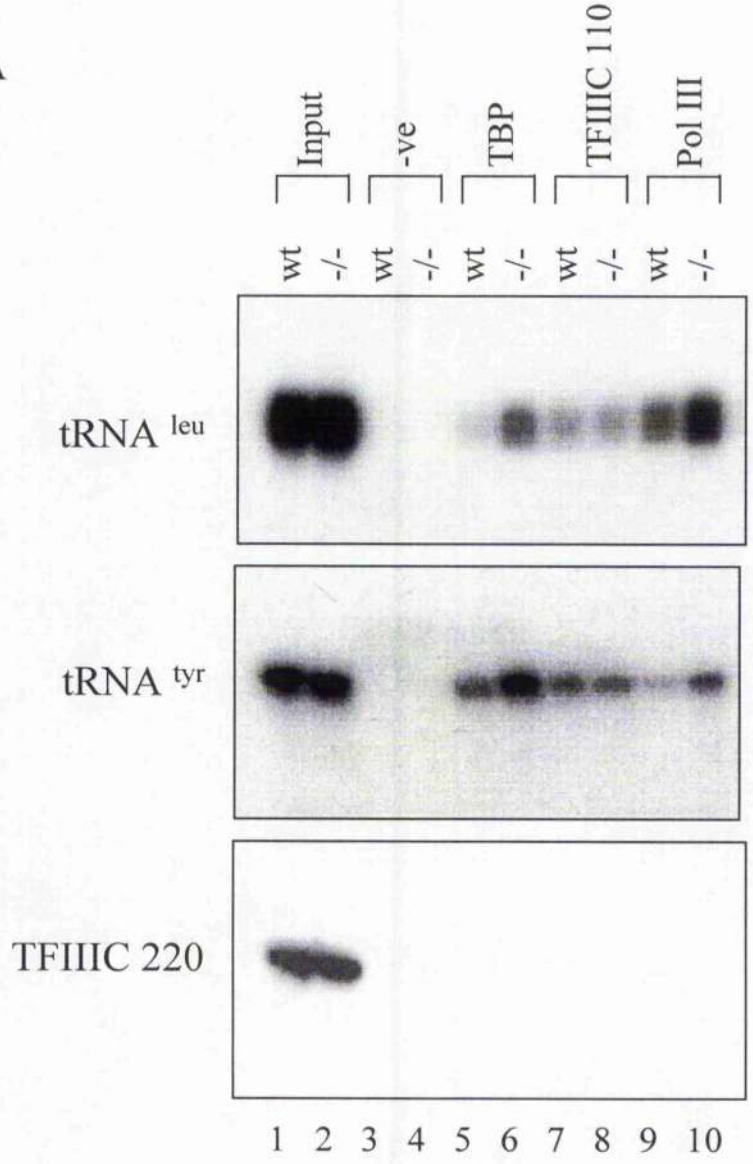
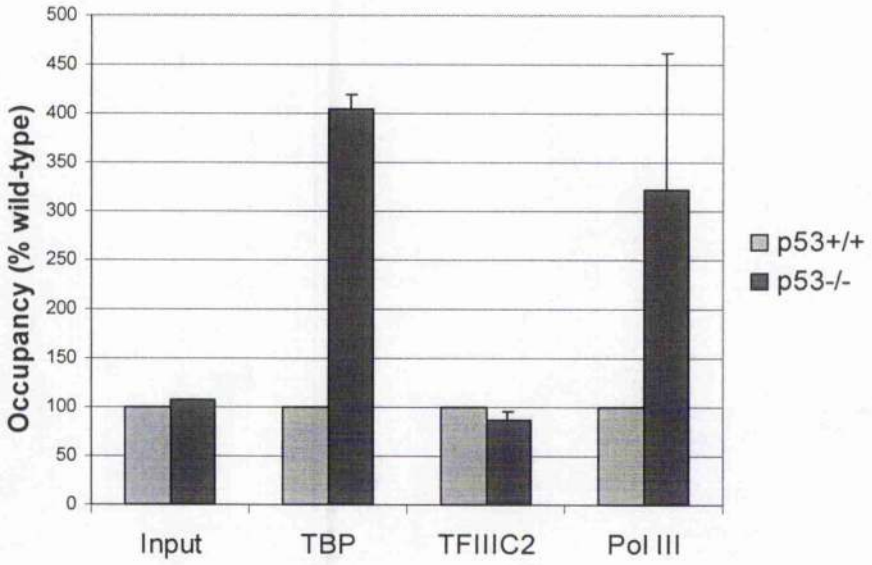

A
B
C
D
E

Figure 5.11. TFIIIB and pol III occupancy at tRNA genes is elevated specifically in primary MEFs from p53-knockout mice relative to wild-type controls. (A) ChIP was carried out with antibodies against TBP (lanes 5 and 6), TFIIIC2 (lanes 7 and 8) and RNA pol III (lanes 9 and 10) or beads alone as control (lane 3 and 4). Occupancy at $tRNA^{Leu}$ (upper panel), $tRNA^{Tyr}$ (second panel) and TFIIIC220 genes (lower panel) was analysed in primary MEFs from p53+/+ (odd lanes) and p53 -/- (even lanes) mice. Input DNA is shown in lanes 1 and 2. (B). Values shown represent the % of tRNA gene co-precipitating with factor in vivo, compared with the input DNA which is set at 100% for p53-/- and p53+/+ MEFs. Values represent the mean and standard deviation of three experiments. (C) Protein levels of TBP, TFIIIC110 and pol III are constant in p53-knockout and wild-type MEFs. Cellular extracts (50μg) obtained from wild-type (lane 1) and p53-knockout (lane 2) primary MEFs were separated by gel electrophoresis and analysed by Western blotting with antibodies 58c9, against TBP (top panel), 4286-4, against TFIIIC110 (middle panel) and BN51 against RNA pol III (bottom panel).

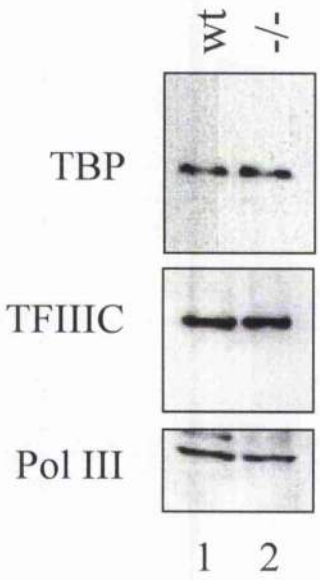
A


Input
-ve
TBP
TFIIIC 110
Pol III
wt
-/-
wt
-/-
wt
-/-
wt
-/-
wt
-/-
tRNA leu
tRNA tyr
TFIIIC 220
1 2 3 4 5 6 7 8 9 10

B


Occupancy (% wild-type)
500
450
400
350
300
250
200
150
100
50
0
Input
TBP
TFIIIC2
Pol III
p53+/+
p53-/-

C


wt
-/-
TBP
TFIIIC
Pol III
1
2

occupancy of pol III is also increased on both tRNA promoters in the knockout cells (Figure 5.11, lanes 9 and 10). An approximate 4-fold increase in TBP and pol III occupancy is observed in p53-knockout MEFs compared to the wild-type controls (Figure 5.11B). In contrast, equal amounts of TFIIIC2 were found to be associated with the tRNA genes (Figure 5.11, lanes 7 and 8). These effects were specific, since none of the factors tested were found to be associated with an internal fragment of the TFIIIC220 gene (Figure 5.11, lower panel). The immunoprecipitation control, in which no antibody was used, revealed no background binding of DNA (Figure 5.11, lane 3 and 4). Western blotting demonstrated that the abundance of all three factors is comparable in the two cell types (Figure 5.11C). This indicates that changes in tRNA gene occupancy in the two cells types are not due to differences in protein amounts. Overall these results suggest that the observed increase in tRNA gene transcription observed in p53-knockout MEFs is most probably due to the increased recruitment of TFIIIB and pol III to these promoters.

5.2.6 DNA-binding activity of TFIIIC2 is unaffected by p53

The previous results suggest that although TFIIIB and pol III occupancy on tRNA genes change in response to p53, TFIIIC2 binding activity does not. To further investigate this finding, eletrophoretic mobility shift assays (EMSAs) were carried out. A promoter fragment containing a B-block consensus sequence capable of binding TFIIIC2 was used. This B-block oligo was incubated with cellular extract obtained from NARF2 cells. As previously described, these cells can be induced with IPTG to express $p14^{ARF}$. Induction of $p14^{ARF}$ results in an increase in p53 levels and a correlative decrease in pol III transcription (Figure 3.5). Several protein-DNA complexes were detected with extracts from either uninduced NARF2 cells (Figure

5.12, lanes 1 and 2) or IPTG-treated NARF2 cells (Figure 5.12, lanes 3 and 4). Competition experiments suggested that many of these complexes result from sequence-specific binding and it cannot therefore be concluded without doubt which complex indicates TFIIIC2 binding to the probe. It is clear though, that upon induction of $p14^{ARF}$ there were no changes in complex association with the radiolabelled oligo. A positive control using partially purified TFIIIC2 may have helped distinguish which complex is TFIIIC2. The result, however, still suggests that the decrease in tRNA gene transcription observed upon $p14^{ARF}$ induction is not mediated by a change in TFIIIC2 binding activity.

5.2.7 HDAC activity is not required for p53 to repress pol III transcription.

Previously it has been shown that p53 can repress pol II transcription of *Map4* and *stathmin* genes by recruiting histone deacetylases (HDACs) (Murphy et al. 1999). In this study, the HDAC inhibitor trichostatin A (TSA) was used and was shown to abrogate p53-mediated repression of these genes (Murphy et al. 1999). HDAC complexes are recruited to chromatin, resulting in nucleosome deacetylation. The resulting compressed chromatin structure occludes transcription factors from the DNA, resulting in transcriptional repression (Knoepfler and Eisenman 1999). Such a mechanism is compatible with the discovery that TFIIIB is prevented from binding tRNA genes in the presence of p53 (Figures 5.9 and 5.11). Furthermore, it has been demonstrated that HDAC function contributes to the control of pol III transcription *in vivo* (Sutcliffe et al. 2000).

To test the possibility that HDACs contribute to pol III repression by p53, levels of tRNA synthesis between p53-knockout and wild-type MEFs were compared in the

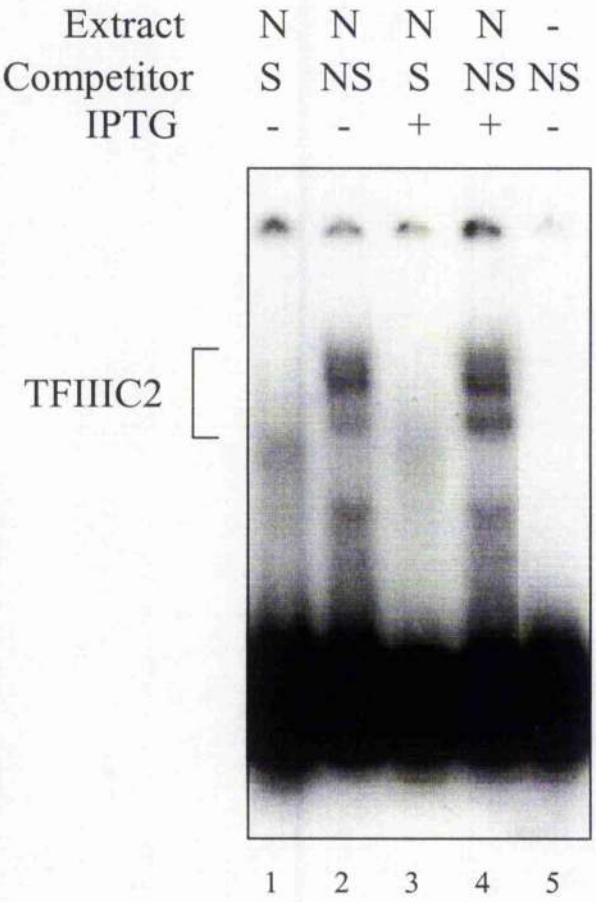
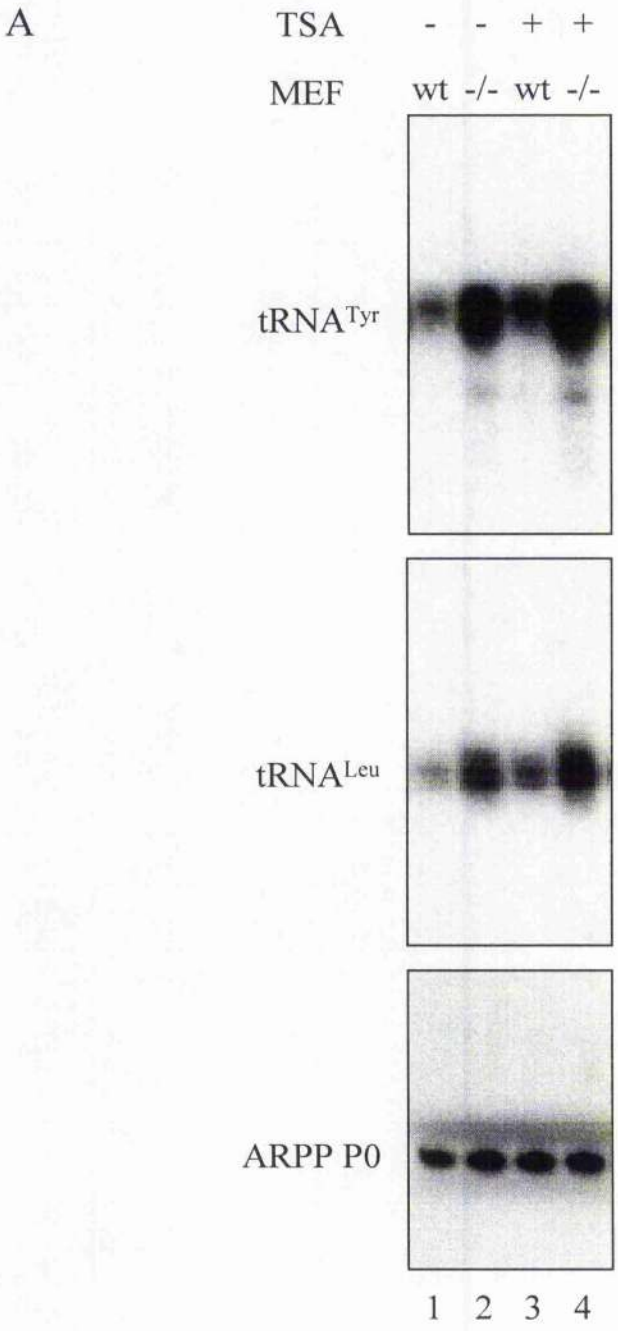


Figure 5.12. DNA-binding activity of TFIIIC2 is unchanged in the presence of p53. EMSA using 1ng of a B-block promoter fragment probe, 1μg polydI-dC, and no protein (lane 5) or 20μg of cellular extract from NARF2 cells (lanes 1 and 2) or $p14^{ARF}$ induced NARF2 cells (lanes 3 and 4) Lanes 1, 3 and 5 contained 50ng of unlabelled probe DNA whereas lanes 2 and 4 contained 20ng of non-specific DNA of a similar length.

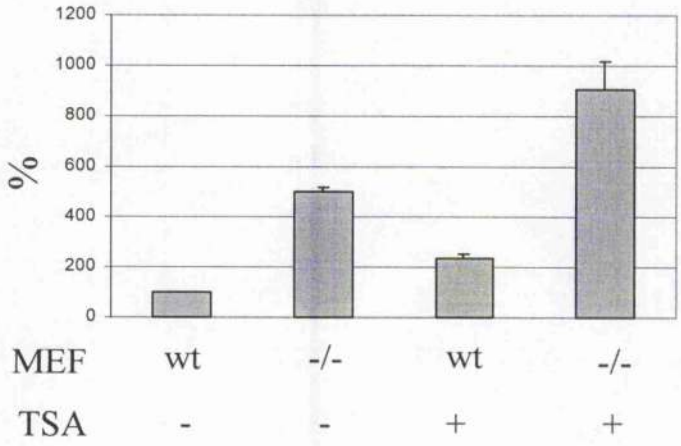
absence and presence of TSA. p53-knockout and wild-type control MEFs were grown to 70% confluency and the cells were treated with 300nM TSA for 24 hours. RT-PCR analysis was then performed on cDNA prepared from these cells. Pol III activity increased when wild-type MEFs were treated with TSA (Figure 5.15, lanes 1 and 3, upper two panels). This was a specific increase, since the levels of ARPP P0 remained constant (bottom panel). In p53-knockout MEFs, the levels of tRNA synthesis also changed in response to TSA (Figure 5.13, lane 2 and 4, upper two panels). However, the differences in tRNA synthesis, upon treatment with TSA, remained approximately five fold in both the p53 knockout and wild-type cells (Figure 5.13B). From this evidence, it is unlikely that TSA-sensitive HDAC activity is responsible for p53-mediated repression of pol III transcription in the wild-type fibroblasts.

In addition to modifying chromatin structure, HDACs have also been shown to regulate the function of transcription factors through direct deacetylation. To investigate this possibility, it was tested *in vitro*, using a naked DNA template, whether TSA might influence p53-mediated repression of pol III transcription. When added to cell extracts, wild-type His-p53 produced a strong repression of Alu transcription (Figure 5.14, lanes 6 and 9). This repression was maintained when TSA was included at concentrations of 100 and 300nM (Figure 5.14, lane 7 and 8). TSA had little effect on pol III transcription in the absence of p53 (Figure 5.14, lanes 3 and 4). It is therefore concluded from these experiments that p53 can repress pol III transcription efficiently via mechanisms that do not require HDAC activity.

Figure 5.13. Repression of tRNA by p53 is maintained in the presence of TSA. (A) RT-PCR analysis of cDNAs prepared from total RNA extracted from primary p53-/- MEFs (lanes 2 and 4) and matched wild-type MEFs (lanes 1 and 3) that were cultured in the presence (lanes 3 and 4) or absence (lanes 1 and 2) of 300nM TSA. The cDNAs were PCR amplified using specific primers against $tRNA^{Tyr}$ (upper panel), $tRNA^{Leu}$ (middle panel) or ARPP P0 (bottom panel). (B) Values shown represent the % of tRNA PCR product relative to the wild-type MEFs in the absence of TSA (100%).


A
TSA - - + +
MEF wt -/- wt -/-
tRNA^Tyr
tRNA^Leu
ARPP P0
1 2 3 4

B



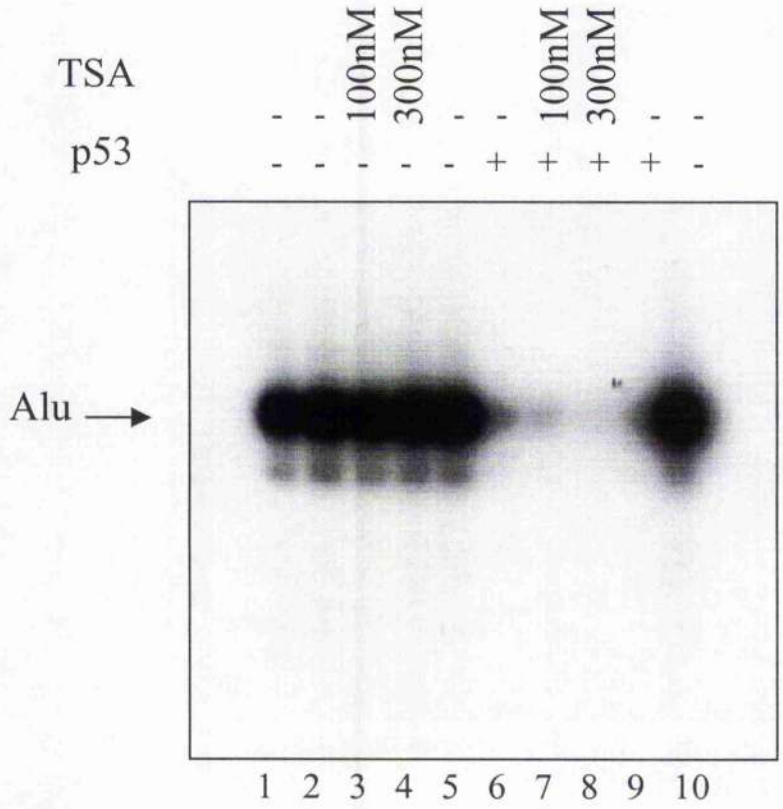


Figure 5.14. Recombinant His-p53 represses pol III transcription in vitro despite the presence of TSA. PVA1 (250ng) was transcribed using HeLa nuclear extract (15μg) that had been preincubated for 10 min at 30°C in the absence (lane 1-5 and 10) or in the presence of 0.6μg of wild-type p53 (lanes 6-9) and 100nM (lanes 3 and 7) or 300nM (lane 4 and 8) TSA.

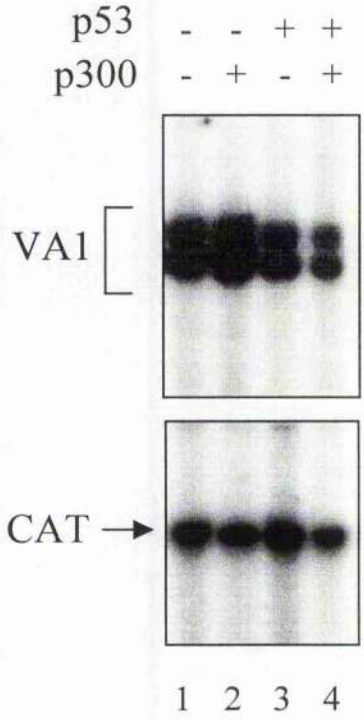
5.2.8 p300 stimulates pol III transcription through a p53-independent mechanism

As exemplified by the finding that tRNA synthesis increased in response to TSA *in vivo*, it is likely that histone acetylase transferase (HATs) or deacetylase complexes (HDACs) play a role in regulating pol III transcription. p300/CBP transcriptional co-activator proteins play many roles in controlling transcription mediated through multiple mechanisms (Chan and La Thangue 2001). As well as acting as protein bridges, connecting transcription factors to the transcriptional apparatus, they can also influence chromatin activity by acetylating nucleosomal histones. Other proteins, including p53, can be acetylated by p300/CBP (Chan and La Thangue 2001). Previously it was demonstrated that the CBP/p300 associated factor PCAF acetylates TAFI68, the second largest subunit of the TATA-box-binding protein (TBP)-containing factor SL1. This modification resulted in stimulation of pol I transcription (Muth et al. 2001). It was investigated whether p300 plays a role in controlling pol III transcription.

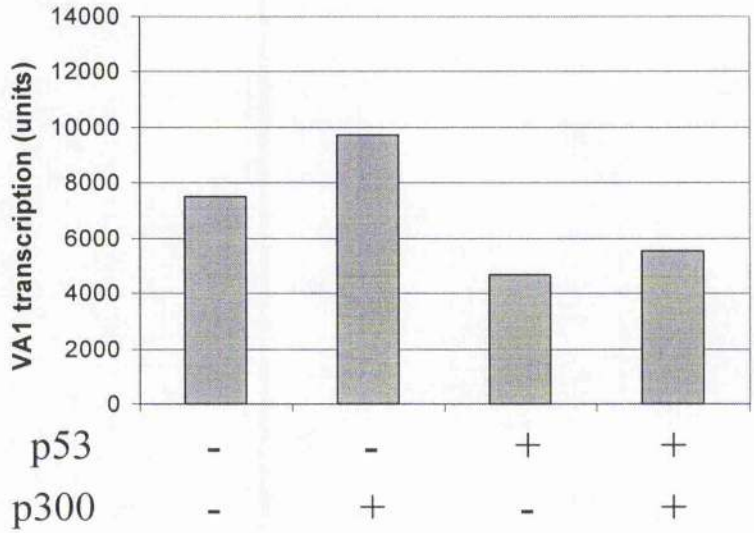
The p53 null osteosarcoma cell line SAOS2 was transfected with the VA1 gene as a reporter for pol III activity and HSV-CAT as an internal control. In addition, vectors encoding p300 and wild-type p53 were co-transfected. Expression of the VA1 and HSV-CAT reporters was then monitored by quantitive primer extension assays. As expected, compared to the vector alone control (Figure 5.15, lane 1), transfection of wild-type p53 resulted in a decrease in VA1 transcription (figure 5.15, lane 3). Cotransfection of p300 slightly relieved this p53-mediated repression (Figure 5.15, lane 4); however, the increase in VA1 expression was comparable to that obtained by

Figure 5.15. p300 stimulates pol III transcription through a p53-independent mechanism. (A) SAOS2 cells were cotransfected with pVA1 (3μg), HSV-CAT (3μg) and 2μg of empty vector (lanes 1 and 2) or vector encoding wild-type p53 (lanes 3 and 4) in the presence (lanes 2 and 4) or absence (lanes 1 and 3) of 2μg p300. RNA and protein were harvested 48hrs after transfection. The upper two panels show primer extension analyses of extracted RNA using primers specific for VA1 (upper panel) and CAT (second panel). (B) Numbers represent the relative levels of VA1 transcription.

A


p53 - - + +
p300 - + - +
VA1
CAT
1 2 3 4

B


VA1 transcription (units)
14000
12000
10000
8000
6000
4000
2000
0
p53 - - + +
p300 - + - +

transfection of p300 alone (Figure 5.15, lane 2). Although the increase in VA1 transcription mediated by p300 is marginal, it may suggest that p300 could play a role in stimulating pol III transcription. This result, however, suggests that the increase in pol III activity by p300 is not mediated through p53. This result is preliminary and requires verification.

5.2.9 p300 but not p53 associates with tRNA genes *in vivo*

One possible mechanism that p300 may utilize to modulate pol III transcriptional activity could be through the direct acetylation of chromatin. Nucleosomes are associated with the coding regions of many pol III-transcribed genes (Paule and White 2000). In addition histone acetylation has been shown to facilitate the access of transcription factors to chromatinised 5S rRNA promoter sequences (Lee et al. 1993). To investigate whether p300 is associated with pol III genes, an antibody against p300 was used in ChIP assays. It was also tested whether p53 or the retinoblastoma protein RB could be found associated with tRNA templates. The results presented here suggest that p53 represses pol III transcription through targeting of TFIIIB. This p53-TFIIIB interaction prevents TFIIIB associating with the other pol III transcription complex components. To elicit this effect, it is unlikely that p53 would be required to associate with the pol III gene itself. Rb also represses pol III transcription. This is achieved through a very similar mechanism, and again it is unlikely that Rb is required to associate with the pol III promoter.

As expected, TBP clearly coprecipitated with $tRNA^{Leu}$ and $tRNA^{Tyr}$ (Figure 5.16, lane 5, upper and middle panels). In contrast, under the conditions of this experiment, neither p53 nor Rb were found to associate with the tRNA genes (Figure 5.16 lanes 6

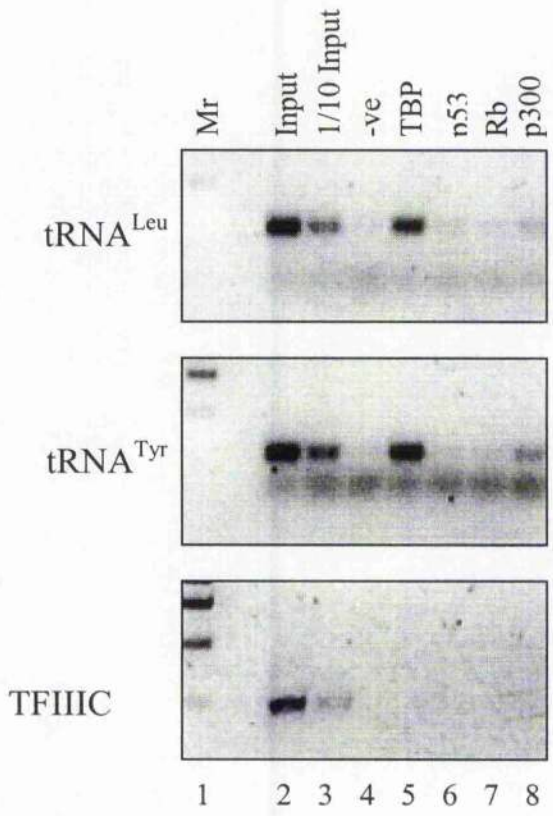


Figure 5.16. p53 is not present at the tRNA promoter in vivo. ChIP was carried out with antibodies against TBP (lane 5), p53 (lane 6), RB (lane 7), p300 (lane 8) or beads alone as control (lane 4). Occupancy at $tRNA^{Leu}$ (upper panel), $tRNA^{Tyr}$ (second panel) and TFIIIC220 genes (lower panel) was analysed in HeLa cells Input DNA is shown in lanes 1 and 2.

and 7) A weak association, however, is observed reproducibly between p300 and the tRNA promoters (Figure 5.16, lane 8). This effect was specific since no association was observed between p300 and the TFIIIC220 control gene fragment (Figure 5.16, lower panel); in addition no DNA was found to co-precipitate with the negative control (Figure 5.16,lane 4). This result suggests that p53 and Rb are not found associated with tRNA genes *in vivo*. These findings are in agreement with the proposed mechanisms by which these factors elicit their effects on pol III transcription, since they both bind TFIIIB and prevent its recruitment to promoters. This assay also suggests that p300 may associate with pol III templates. To confirm this idea further analyses are required.

5.3 Discussion

In order for a functional pol III transcription initiation complex to form, multiple protein-protein, and protein-DNA interactions are required. TFIIIC2 binds to internal promoter elements, which allows recruitment of TFIIIC1 and TFIIIB. This TFIIIB-TFIIIC-DNA complex then facilitates the recruitment of pol III to the start site of transcription (Paule and White 2000; Geiduschek and Kassavetis 2001). p53 has been shown previously (Chesnokov et al. 1996; Cairns and White 1998), and in this study, to target TFIIIB. Such an interaction appears to result in occlusion of TFIIIB from the promoter both *in vitro* and *in vivo*. In addition, functional p53 prevents TFIIIB from forming interactions with either TFIIIC2 or pol III.

Binding studies have revealed that wild-type p53 can interact with two TFIIIB components, TBP and Brf1. However, titration of TBP *in vitro* proved to be sufficient to at least partly restore p53-repressed Alu transcription. This finding is in agreement with unpublished results showing that p53-mediated inhibition of tRNA transcription can be reversed by transient transfection of TBP (D. Johnson, unpublished results). In addition, a TBP-E184R mutant, which is specifically defective for pol II transcription, was also able to rescue p53 repressed pol III transcription (D. Johnson, unpublished results). In contrast, overexpression of Brf1 did not result in a reversal of p53-mediated repression (D. Johnson, unpublished results). These results suggest that although p53 may form an interaction with Brf1, it is its interaction with TBP that is functionally significant for repressing pol III transcription. Although binding of TBP to p53 has been shown extensively, the functional significance of this interaction has remained elusive (Seto et al. 1992; Liu et al. 1993; Martin et al. 1993; Farmer et al. 1996b; Tansey and Herr 1997).

Overexpression of TBP in transient transfections failed to restore p53-repressed pol II transcription (Farmer et al. 1996b), and in this study TAFs were proposed to be mediating repression. The results presented here suggest that the TBP-p53 interaction plays an important role in controlling pol III transcription and may well establish a reason for this well-documented interaction.

Both the immobilised template assays and the ChIP assays demonstrated that TFIIIB occupancy on tRNA promoters is reduced in the presence of p53. As exemplified by ChIP, the decrease in TFIIIB binding in wild-type MEFs compared to p53-knockout cells results in less pol III being recruited and consequently a decrease in pol III transcription. Levels of TFIIIC2 found at the tRNA genes, however, remain unchanged both *in vitro* and *in vivo*. These results are also consistent with the finding that p53 prevents association of TFIIIB with TFIIIC2 or pol III in coimmunoprecipitation assays. EMSA revealed that TFIIIC2 binding activity was unchanged in NARF2 cells upon induction of $p14^{ARF}$. Eichhorn and Jackson previously reported that Brf1 is specifically degraded following induction of p53 in the TR9-7 fibroblast cell line (Eichhorn and Jackson 2001). In contrast to this, no difference in the cellular levels of Brf1 or indeed in any of the tested pol III transcription components was observed between the wild-type and p53-knockout MEFs.

Although interactions between pol III and the transcription factors TFIIIB and TFIIIC2 were diminished in the presence of p53, the interaction between TBP and Brf1 appeared to remain intact. These results also suggest that the interaction between Brf1 and wild-type p53 in Figure 5.1 could be indirect, mediated through

TBP. This may also help explain why TBP is sufficient to relieve p53-mediated repression of pol III transcription. In this study it was not possible to ascertain whether p53 affects the interaction of Bdp1 with TBP and Brf1 because Bdp1 associates very loosely with TFIIIB and has proved to be extremely difficult to coimmunoprecipitate from mammalian cells (Schramm et al. 2000) and data not shown.

Previously it was suggested that the functional and mechanistic parameters for p53 to repress pol II and pol III transcribed genes might vary (Stein et al. 2002a). In transfected SAOS2 cells, the R181L p53 mutant remains capable of repressing the pol III template VA1 (Stein et al. 2002a), whereas this mutant was found to have lost the ability to repress various pol II transcribed genes (Crook et al. 1994). Consistent with this, it is shown here that there are several differences in how p53 represses these two classes of gene. The TBP-p53 interaction plays a fundamental role in regulating pol III transcription, demonstrated by the finding that titration of TBP but not Brf1 can relieve p53-mediated repression (Figure 5.2 and D. Johnson, unpublished observations). Although the TBP-p53 interaction is well established, there has been no direct evidence implicating this interaction as relevant to regulation of pol II transcription (Tansey and Herr 1995; Thut et al. 1995a; Farmer et al. 1996a). Indeed, Tansey and Herr showed that TBP binding is not required for p53 to activate a pol II reporter (Tansey and Herr 1995).

p53-dependant HDAC recruitment has been shown to mediate repression of certain pol II transcribed genes (Murphy et al. 1999). Although HDAC activity modulates pol III transcription, this activity is irrespective of p53 status. Transfection of p300

was found to stimulate VA1 transcription very slightly. This effect was also found to be independent of p53. p53 itself can be acetylated by p300 (Gu and Roeder 1997). Such modifications are thought to modulate the activity of the protein. p300 may however play a more direct role in controlling pol III transcription. ChIP analysis revealed that there may be a weak association between p300 and tRNA genes. The CBP/p300 associated factor PCAF plays a role in controlling pol I transcription (Muth et al. 2001). In this case, acetylation of the basal pol I transcription factor SL1 resulted in increased association with the rDNA promoter and an increase in pol I transcription. It is equally possible that components of the basal pol III transcriptional machinery are subject to acetylation. Transcription can be regulated through acetylation and deacetylation of chromatin directly (Chan and La Thangue 2001). In many cases, hyper-acetylation of core histone tails is associated with increased transcriptional activity, whereas hypo-acetylation correlates with transcriptional repression. Pol III transcription may be regulated by such chromatin remodelling effects. TFIIIC was previously demonstrated to harbour intrinsic HAT activity and partial inhibition of this activity correlates with a partial reduction in transcription from chromatin templates (Kundu et al. 1999).

ChIP analysis indicated that RB is not present on tRNA genes. This finding is consistent with the model in which RB represses pol III transcription through its interaction with TFIIIB, which prevents TFIIIB interacting with TFIIIC2 and being recruited to a promoter (Sutcliffe et al. 2000). Collectively, the data investigating the mechanism by which p53 represses pol III transcription supports a similar model. In the absence of p53, efficient pol III transcription complexes can form (Figure 5.16a).

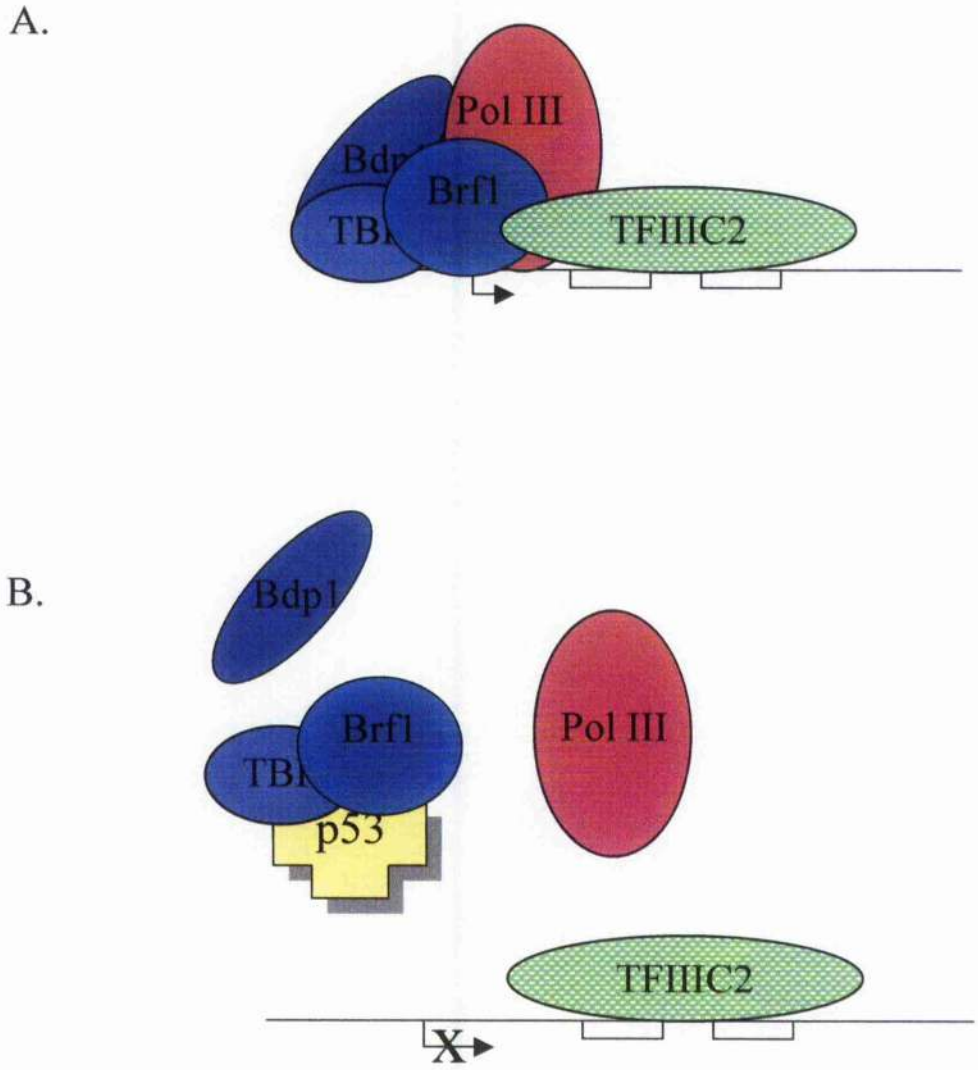


Figure 5.17. Model of p53 mediated pol III transcriptional repression. (A) Specific initiation at class III genes is dependent on the interaction between TFIIIB (blue) and pol III (pink); in most cases TFIIIB must be recruited to promoters through binding to TFIIIC2 (green). (B) p53 associates with TFIIIB and prevents it from interacting with pol III and TFIIIC2. In this way p53 is able to disrupt preinitiation complex formation and inhibit transcription.

In the presence of functional p53, TFIIIB is targeted. p53-TFIIIB interactions prevent TFIIIC2-dependent recruitment of TFIIIB to pol III promoter regions. This ultimately leads to a decrease in pol III transcription, mediated by a reduction in recruitment of pol III to the start sites of genes (Figure 5.16b).

Chapter 6

The Physiological role of p53 in controlling pol III transcription in response to genotoxic stress

6.1 Introduction

Although not required for normal growth and development, p53 is a key player in the prevention of tumour development (Ko and Prives 1996; Levine 1997). Under a range of physiological stress conditions p53, can induce cell cycle arrest or apoptosis (Ko and Prives 1996; Levine 1997). These functions serve to control DNA damage and protect cellular progeny from accumulating genetic mutations (Ko and Prives 1996). Most tumours evade these defences by inactivating p53 (Hollstein et al. 1991). In tumours that do not contain mutated p53, perturbations in the p53 activation or response pathways often occur (Sherr and Weber 2000).

Activation of p53 is observed in response to a variety of cellular stresses. These include DNA damage, hypoxia and ribonucleotide depletion (Larkin and Jackson 1999). Under normal physiological conditions, p53 is maintained at low levels in an inactive state. A variety of mechanisms exist to control p53 activity, with one of the most central regulators being Mdm2 (Momand et al. 1992; Oliner et al. 1993). As well as inhibiting the transcriptional activation function of p53, Mdm2 can also target p53 for proteolytic degradation (Oliner et al. 1993; Haupt et al. 1997; Kubbutat et al. 1997b).

Within minutes of a cell sustaining DNA damage, p53 levels can rise dramatically (Larkin and Jackson 1999). This is mostly achieved through stabilization of p53. Stress can induce multiple post-translational events that have been found to modulate the function of p53 (Giaccia and Kastan 1998; Woods and Vousden 2000). These phosphorylation, dephosphorylation and acetylation events are believed to play major roles in controlling the activity of p53. The nature of the response pathway can vary dramatically between different cell types and different stresses (Giaccia and Kastan 1998; Oren 1999; Zhao et al. 2000). Serine-15 of p53 is phosphorylated in response to DNA damage, but not in response to aberrant oncogene signals (de Stanchina et al. 1998). It was also shown that p53 is phosphorylated on serine-15 in response to IR or UV, whereas phosphorylation of the CK2 site situated at serine-392 is phosphorylated following UV but not IR (Blaydes and Hupp 1998; Kapoor and Lozano 1998; Lu et al. 1998). Although it is established that p53 can regulate pol III transcription, it is yet to be determined under which physiological circumstances this control becomes important. It was therefore investigated whether the DNA damaging agent methyl methane sulfonate (MMS) could induce a p53 response that results in the down regulation of pol III activity.

6.2 Results

6.2.1 p53 induction in response to DNA-damage results in down regulation of pol III transcription.

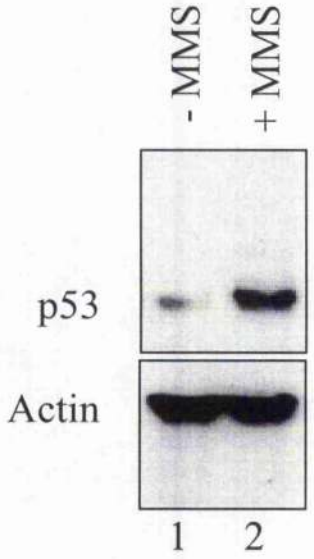
Several forms of DNA damage have been shown to activate p53 (Larkin and Jackson 1999). Such damaging agents include ionising radiation, radio-mimetic drugs, ultraviolet light and a variety of chemicals (Larkin and Jackson 1999). It was tested

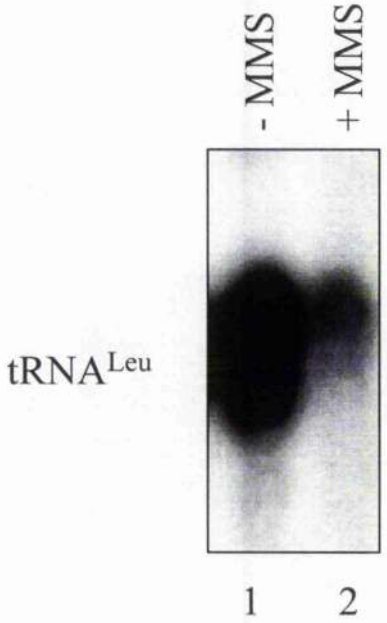
whether changes in pol III activity occur in response to the DNA damage-inducing agent methyl methane sulfonate (MMS). MMS is a DNA alkylating agent that has previously been demonstrated to provoke a p53 response. HeLa cells were grown to 80% confluency then treated for two hours with 0.04% MMS. Cell extracts were then analysed by Western blotting for p53 expression. HeLa cells express low levels of p53, which can be induced upon treatment with MMS (Figure 6.1A, upper panel). This induction was specific, since the levels of the actin control remained unchanged (Figure 6.1A, lower panel). As exemplified by *in vitro* transcription assays, this induction correlated with a decrease in $tRNA^{Leu}$ transcription (Figure 6.1B). In addition RT-PCR analysis revealed that MMS treatment decreased synthesis of $tRNA^{Tyr}$ (Figure 6.1C, top panel). It is well established that p53 can induce synthesis of p21. As shown in Figure 6.1C, middle panel, RT-PCR analysis revealed that MMS treatment of HeLa cells also resulted in an induction of p21 mRNA (Figure 6.1C, middle panel). These changes were specific, since the levels of ARPP P0 mRNA were unresponsive to MMS. This result demonstrates that MMS treatment of HeLa cells results in induction of p53. This increase in p53 correlates specifically with an induction of p21 gene expression, and a repression of pol III transcription.

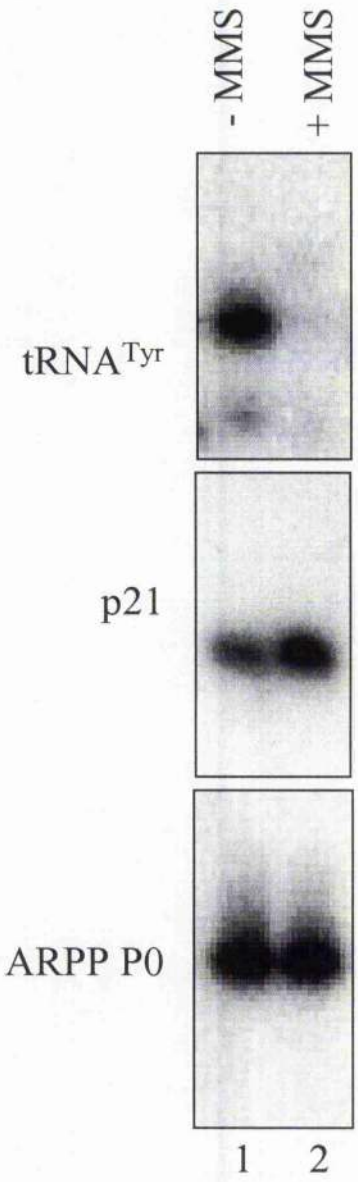
6.2.2 Induction of p53 by MMS specifically reduces TBP occupancy at tRNA genes.

Having established that MMS induces a p53 response that leads to a down regulation of pol III transcription, it was investigated whether changes in TFIIIB occupancy at pol III promoters could be detected in response to MMS. ChIP analysis revealed that significantly less TBP is present at $tRNA^{Leu}$ (upper panel) and $tRNA^{Tyr}$ (middle panel) promoters in MMS treated HeLa cells when compared to untreated cells

Figure 6.1. Induction of endogenous p53 by genotoxic stress represses tRNA expression. (A) p53 levels increase following MMS treatment. Immunoblot analysis of untreated HeLa (lane 1) or HeLa cells treated for 2 hours with 0.04% MMS (lane 2). 50μg protein was probed with DO-1 against p53 (upper panel) or C11 against actin (lower panel). (B) $tRNA^{Leu}$ transcription is down regulated following MMS treatment. pleu (250ng) was transcribed with HeLa cell extract (15μg) that had been untreated (lane 1) or treated with 0.04% MMS for 2 hours (lane 2). (C) p21 is induced following MMS treatment. RT-PCR analysis of cDNA prepared from RNAs extracted from untreated HeLa cells (lane 1) or HeLa cells treated with 0.04% MMS (lane 2) using primers for $tRNA^{Tyr}$ (top panel) p21 (middle panel) or ARPP P0 (bottom panel).


- MMS
+ MMS
p53
Actin
1
2


- MMS
+ MMS
tRNA^{Leu}
1
2

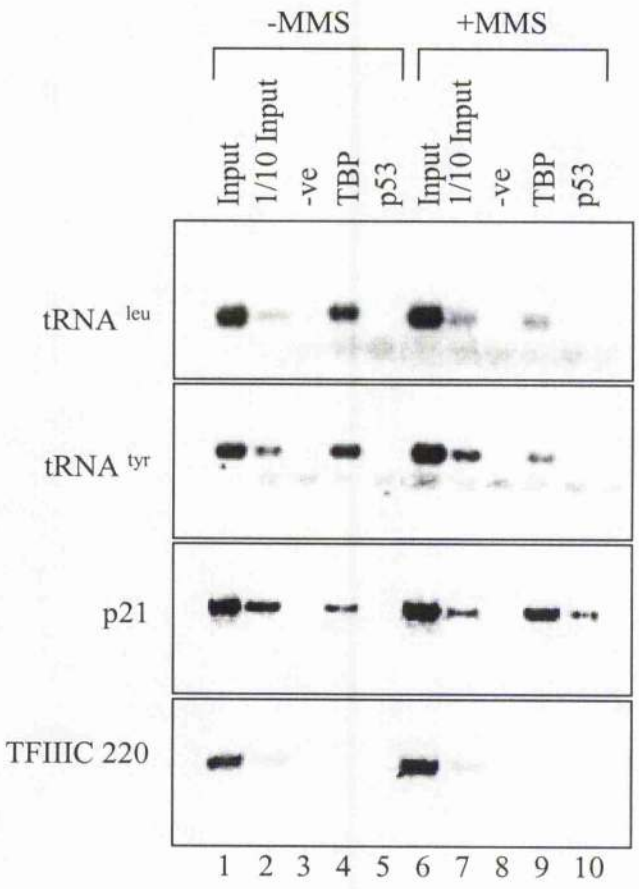

- MMS
+ MMS
tRNA^{Tyr}
p21
ARPP P0
1
2

(Figure 6.2, lanes 4 and 9). In contrast, in response to MMS, TBP occupancy on the p21 promoter increased (Figure 6.2, third panel, lanes 4 and 9). p21 is a well-established target gene of p53. Upon p53 induction, p21 gene expression is induced (El-Deiry et al. 1993). Under these circumstances increased TBP binding would be expected. ChIP with an antibody against p53 revealed that p53 was not detectable on tRNA genes, either before or after MMS treatment. This result further suggests that p53 does not associate with tRNA genes *in vivo*. In contrast, however, p53 is found associated with the p21 locus after MMS treatment (Figure 6.2, third panel, lane 10). This is consistent with previous observations that p53 can bind to the p21 promoter and induce its expression (El-Deiry et al. 1993). These effects were specific, since none of the factors tested were found to be associated with an internal fragment of the TFIIIC220 gene (Figure 6.2, bottom panel). The immunoprecipitation control, in which no antibody was used, revealed no background binding of DNA (Figure 6.2, lane 3 and 8). These results are in agreement with the mechanism by which the p53-TFIIIB complex is excluded from tRNA gene promoters. Firstly these results demonstrate that TBP occupancy on tRNA genes is reduced following MMS treatment. This is a specific effect, since MMS treatment, results in increased association of TBP with the p21 gene locus. It is again suggested that p53 does not associate with pol III genes, even after induction with MMS.

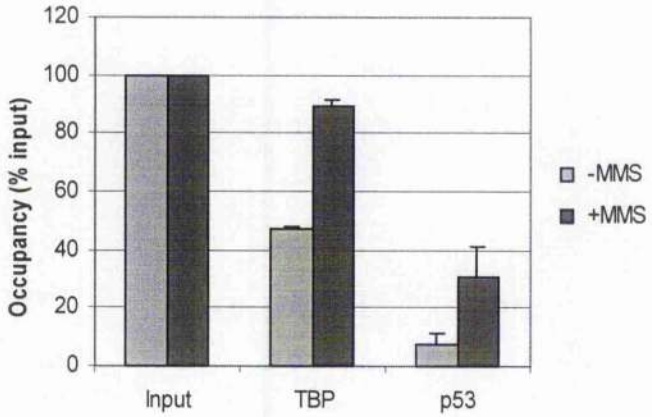
6.2.3 Induction of p53 by MMS results in a decrease in TFIIIC2-tRNA association.

To further investigate the mechanism p53 utilizes to mediate repression of pol III transcription in response to DNA damage, ChIP assays were carried out using antibodies against several components of the basal pol III transcriptional machinery.

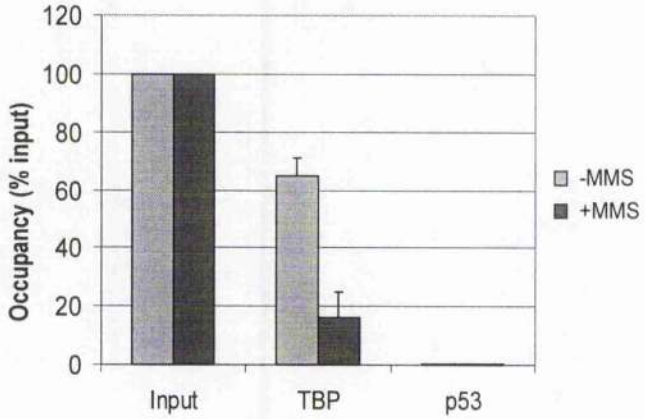
Figure 6.2. Occupancy of TBP at tRNA genes decreases following DNA-damage. ChIP was carried out with antibodies against TBP (lanes 4 and 9) and p53 (lanes 5 and 10) or beads alone as control (lane 3 and 8). Occupancy at $tRNA^{Leu}$ (upper panel), $tRNA^{Tyr}$ (second panel), p21 (third panel) and TFIIIC220 genes (lower panel) was analysed in untreated HeLa cells (lanes 1-5) and HeLa cells treated with 0.04% MMS for 2 hours (lane 6-10). Input DNA is shown in lanes 1, 2, 6 and 7. (B). Values shown represent the % of factor co-precipitating with tRNA in vivo compared with the input DNA which is set at 100% in each case. Values represent the mean and standard deviation of two experiments.


-MMS
+MMS
Input
1/10 Input
-ve
TBP
p53
Input
1/10 Input
-ve
TBP
p53
tRNA leu
tRNA tyr
p21
TFIIIC 220
1 2 3 4 5 6 7 8 9 10

Occupancy on the p21 promoter



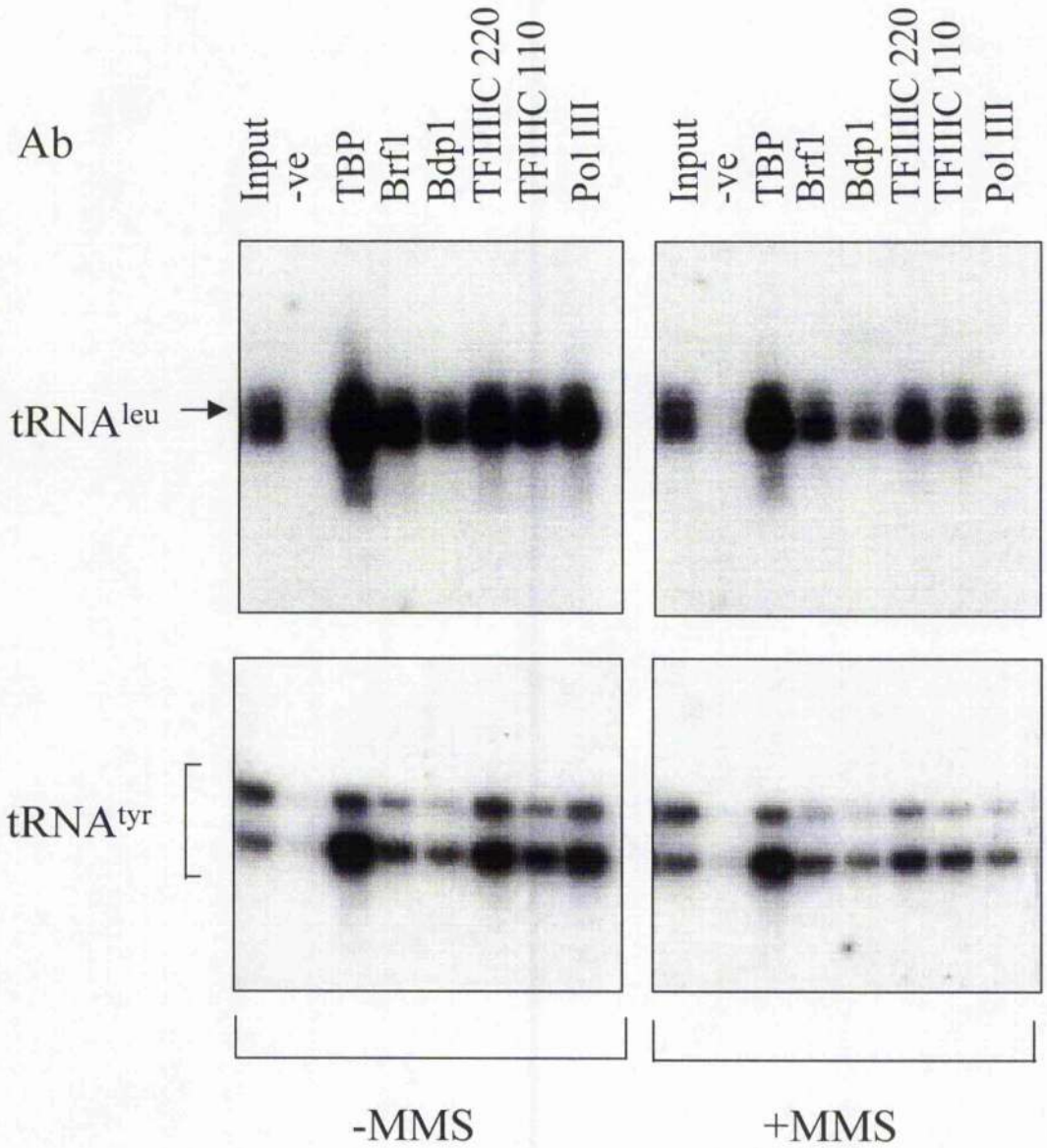
Occupancy on the $tRNA^{Leu}$ promoter

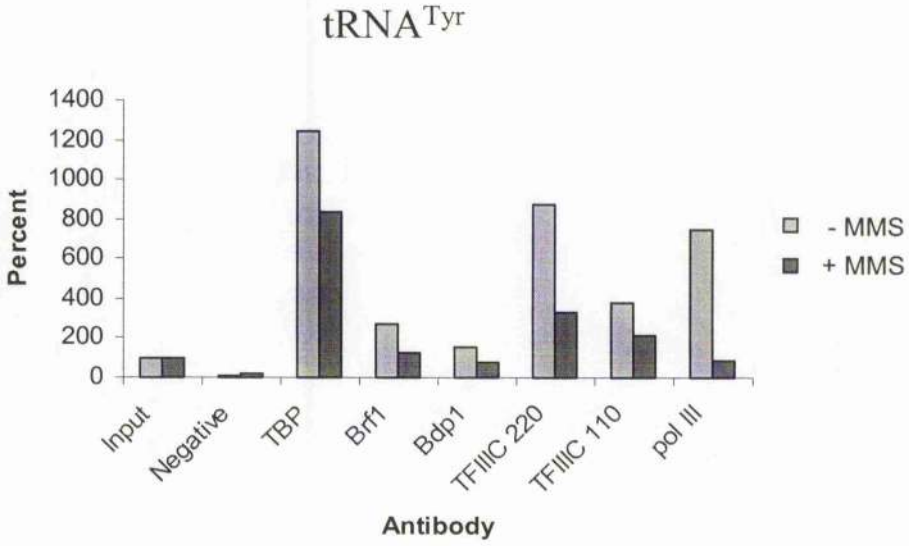


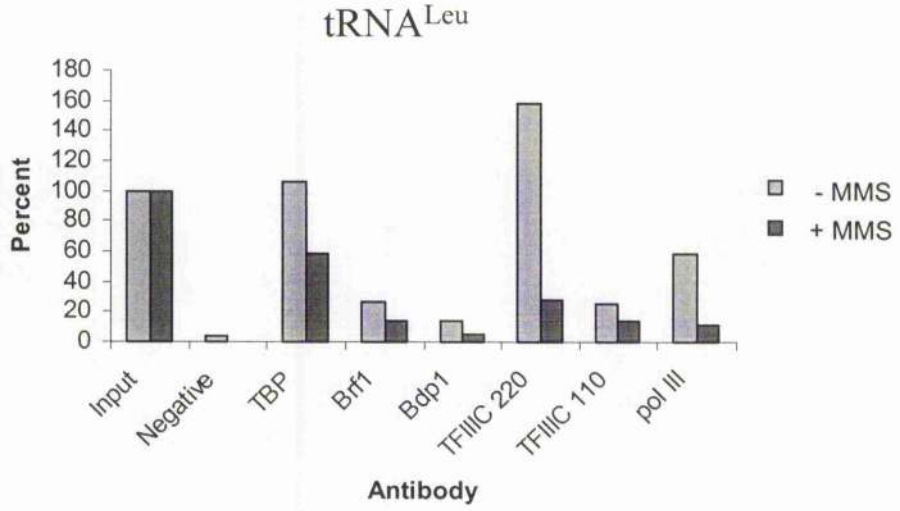
Upon MMS treatment and induction of p53, there is significantly less TBP, Brf1 and Bdp1 present at $tRNA^{Leu}$ (Figure 6.3, upper panel) and $tRNA^{Tyr}$ (Figure 6.3, lower panel) promoters (Figure 6.3, lanes 3, 4 and 5). Promoter occupancy of pol III is also decreased in response to MMS (Figure 6.3, lane 8). These findings are consistent with the previously determined mechanism showing that TFIIIB occlusion from pol III templates occurs in the presence of p53. However, TFIIIC220 and TFIIIC110 levels also decrease in response to MMS (Figure 6.3, lanes 6 and 7). This is in contrast to the finding that TFIIIC2 occupancy on tRNA genes remains comparable in p53 knockout and wild-type fibroblasts (Figure 5.11). The immunoprecipitation control, in which no antibody was used, demonstrated that little background association of DNA was observed in either case (Figure 6.3, lane 2).

To further investigate the activity of TFIIIC2 following MMS treatment, EMSAs were performed. A promoter fragment containing a B-block consensus sequence capable of binding TFIIIC2 was used. After incubation of this B-block oligo with untreated or MMS treated HeLa cell extracts, a major TFIIIC2-DNA binding complex was observed (Figure 6.4 lane 2-5). Treatment of HeLa cells with MMS resulted in a dramatic decrease in the binding activity of this major complex. This result suggests that TFIIIC2 DNA-binding activity is reduced in response to MMS. These results imply that DNA damage induced by MMS can affect both TFIIIB and TFIIIC2 in order to down regulate pol III activity. However, there is no evidence demonstrating that these effects are mediated directly by p53.

Figure 6.3. Occupancy of TFIIIC2 at tRNA genes decreases following DNA-damage. ChIP was carried out with antibodies against TBP (lane 3), Brf1 (lane 4), Bdp1 (lane 5), TFIIIC220 (lane 6), TFIIIC110 (lane 7) and RNA pol III (lanes 8) or beads alone as control (lane 2). Occupancy at $tRNA^{Leu}$ (upper panel) and $tRNA^{Tyr}$ (lower panel) was analysed in untreated HeLa cells (left panel) and HeLa cells treated with 0.04% MMS for 2 hours (right panel). Input DNA is shown in lane 1. (B). Values shown represent the % of factor co-precipitating with tRNA in vivo compared with the input DNA which is set at 100% in each case.


Ab
Input
-ve
TBP
Brf1
Bdp1
TFIIIIC 220
TFIIIC 110
Pol III
Input
-ve
TBP
Brf1
Bdp1
TFIIIIC 220
TFIIIC 110
Pol III
tRNA^leu
tRNA^tyr
-MMS
+MMS


tRNA Tyr
1400
1200
1000
800
600
400
200
0
Percent
Input
Negative
TBP
Brf1
Bdp1
TFIIIC 220
TFIIIC 110
pol III
Antibody
- MMS
+ MMS


tRNA Leu
180
160
140
120
100
80
60
40
20
0
Percent
Input
Negative
TBP
Brf1
Bdp1
TFIIIC 220
TFIIIC 110
pol III
Antibody
- MMS
+ MMS

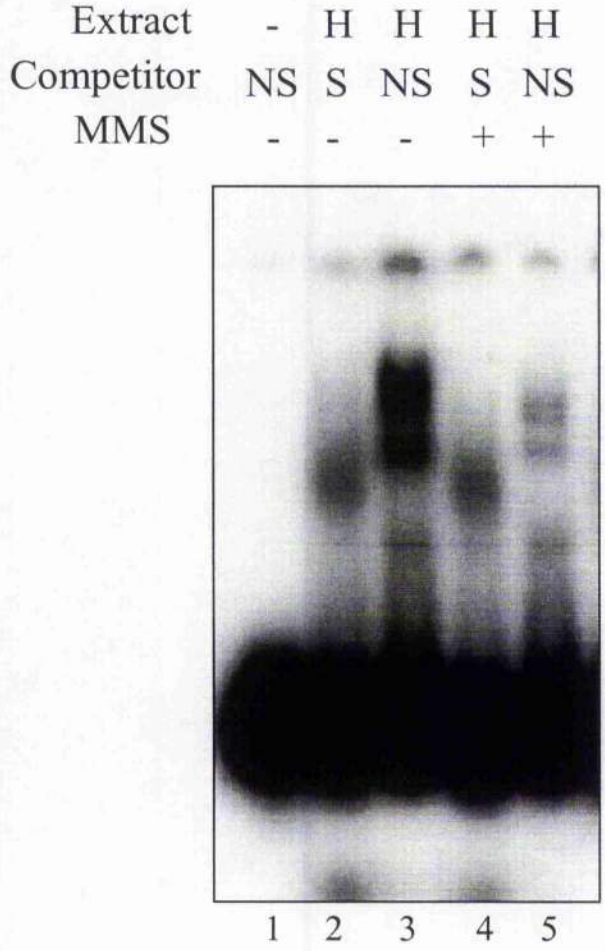


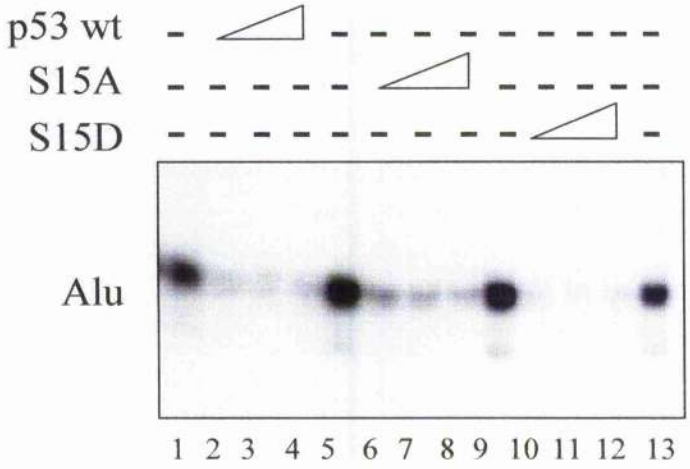
Figure 6.4. DNA-binding activity of TFIIIC2 is reduced upon induction of DNA-damage. EMSA using 1ng of B-block oligo, 1μg polydI-dC, and no protein (lane 1) or 20μg of cellular extract from untreated HeLa cells (lanes 2 and 3) or HeLa cells treated with 0.04% MMS for 2 hours. Lanes 2 and 4 also contained 50ng of unlabelled RJW1 oligo whereas lanes 1, 3 and 5 contained 20ng of non-specific oligo of a similar length.

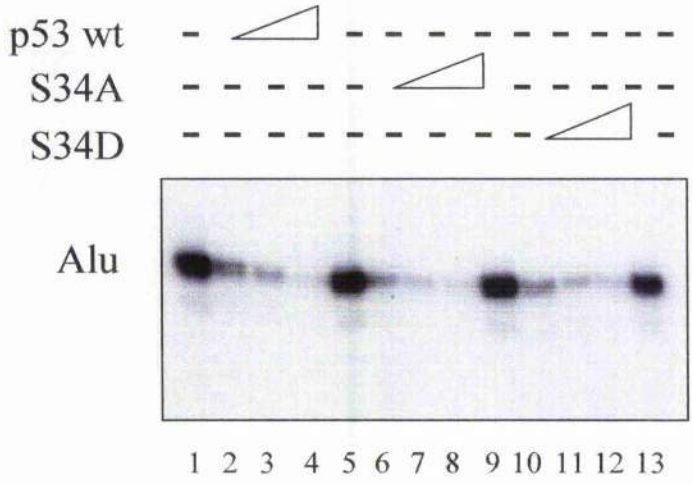
6.2.4 Single amino acid substitutions at residues 15 and 34 do not prevent p53-mediated pol III transcriptional repression.

Phosphorylation of p53, in response to DNA damage, is one mechanism through which the activity of p53 may be regulated. Several lines of evidence have shown that serine-15 and serine-37 (equivalent to serine-34 of murine p53 by sequence homology) of p53 are phosphorylated in response to DNA damage (Milne et al. 1995; Shieh et al. 1997; Siliciano et al. 1997). Having established that DNA damage, induced by MMS, results in down regulation of pol III activity it was tested whether substitution of serine-15 or serine-34 could modulate the activity of p53 to regulate pol III transcription *in vitro*.

Recombinant baculovirus encoding wild-type or mutant His-p53 was expressed in Sf9 cells as described (Okorokov and Milner 1999), and purified to near homogeneity using the polyhistidine tag. When titrated into nuclear extract, wild-type p53 repressed Alu transcription (Figure 6.5A and B, lanes 2, 3 and 4). Substitution of serine-15 to alanine produced no clear difference in transcriptional repression by p53 (Figure 6.5A, lanes 6, 7 and 8). Titration of p53 S15D, where serine-15 of p53 was substituted to glutamic acid resulted in a marginal increase in transcriptional repression by p53 (figure 6.5A, lanes 10, 11 and 12). Substitution of serine-15 to alanine resulted in a slight decrease in transcriptional repression by p53, when compared to that obtained by wild-type His-p53. In contrast substitution of serine-34 of p53 to either alanine (Figure 6.5B, lanes 6, 7 and 8) or glutamic acid (Figure 6.5B, lanes 10, 11 and 12) resulted in no change in repression of Alu transcription by p53. Substitution of a serine to alanine would prevent

Figure 6.5. Mutation at residue 15 or 34 permits efficient p53-mediated pol III transcriptional repression. (A) pRH5.7 (250ng) (top panel) was transcribed using HeLa nuclear extract (15μg) that had been preincubated for 10 min at 30°C in the absence (lanes 1, 5, 9 and 13) or in the presence of 0.4μg, 0.8μg or 1.2μg of wild-type p53 (lanes 2, 3 and 4 respectively), 0.4μg, 0.8μg or 1.2μg of p53(S15A) (lanes 6, 7 and 8) or 0.4μg, 0.8μg or 1.2μg of p53(S15D) (lanes 10, 11 and 12). (B) pRH5.7 (250ng) was transcribed using HeLa nuclear extract (15μg) that had been preincubated for 10 min at 30°C in the absence (lanes 1, 5, 9 and 13) or in the presence of 0.4μg, 0.8μg or 1.2μg of wild-type p53 (lanes 2, 3 and 4 respectively), 0.4μg, 0.8μg or 1.2μg of p53(S34A) (lanes 6, 7 and 8) or 0.4μg, 0.8μg or 1.2μg of p53(S34D) (lanes 10, 11 and 12).


p53 wt
S15A
S15D
Alu
1 2 3 4 5 6 7 8 9 10 11 12 13


p53 wt
S34A
S34D
Alu
1 2 3 4 5 6 7 8 9 10 11 12 13

phosphorylation of this residue. In both cases this substitution at residues 15 or 34 permitted pol III repression, suggesting that phosphorylation of serine-15 or serine-34 is not essential for p53 to repress pol III transcription. A marginal increase in p53-mediated pol III repression is observed when serine-15 was substituted to glutamic acid. This substitution has been shown sometimes to mimic serine phosphorylation. Phosphorylation of serine-15 has previously been reported to result in stabilization of p53 (Sheih et al. 1997), and this may account for this slight increase in repression. These results are preliminary and require further investigation. The analyses would particularly benefit from *in vivo* investigation where one could be certain that p53 was being modified correctly.

6.3 Discussion

Although it is clear that p53 can directly repress pol III transcription, physiological significance of this regulatory mechanism remains unexplored. In this study, the regulation of p53 through the induction of DNA damage is addressed, in the context of its function in controlling pol III transcription. Here it is shown that DNA damage, induced by the chemical alkylating agent MMS, provoked an increase in p53 levels that correlated specifically with a decrease in pol III activity. This finding is in agreement with previous observations in yeast showing that MMS treatment of cells resulted in a down regulation of 5S rRNA and tRNA (Ghavidel and Schultz 2001). A substantial repression of these genes was also observed following UV exposure (Ghavidel and Schultz 2001). Importantly, the authors also showed that repression of pol III transcription by MMS is not an indirect effect of cell cycle arrest, since MMS treatment fully down regulated pol III transcription in arrested cdc mutants (Ghavidel and Schultz 2001)

ChIP analysis revealed that in response to MMS, TBP occupancy at tRNA promoters is decreased. This finding is in concordance with the demonstration that wild-type MEFs have less TBP associated with tRNA promoters when compared with matched p53-knockout cells. In contrast, TBP occupancy on the p21 promoter region increased in response to MMS. RT-PCR analysis demonstrated that p21 gene expression is increased in response to MMS. Following DNA damage, p53-dependant G1 arrest is mediated, at least in part, through p53's induction of p21 (El-Deiry et al. 1994). The findings here are therefore in agreement with this well-established observation. ChIP assays also revealed that whilst p53 is associated with the p21 promoter following MMS treatment, no detectable levels of p53 were found

binding to tRNA genes either before or following MMS treatment. This result suggests that association of p53 with pol III-transcribed genes is not essential for p53 to elicit repression pol III transcriptional repression. p53 binds DNA in a sequence-specific manner. Such sequences are routinely found in p53 target genes, such as p21. p53 consensus motifs are, however, not usually located in pol III transcribed genes. This finding further suggests that p53 does not associate directly with pol III templates. Collectively the results are in agreement with the model in which p53 represses pol III transcription through targeting and subsequent occlusion of TFIIIB from pol III transcribed genes.

Further ChIP analysis of untreated and MMS treated HeLa cells revealed that the occupancy of RNA polymerase III and the TFIIIB subunits TBP, Brf1 and Bdp1 was reduced at the tRNA gene promoters. Furthermore, association of the TFIIIC2 subunits 110 and 220 was also decreased in response to MMS. EMSA also indicated a reduction in TFIIIC2 DNA-binding activity following MMS treatment. These preliminary investigations suggest subtle differences in the mechanistic response to p53 and DNA damage. Ghavidel and Schultz recently demonstrated that partially purified TFIIIB fractions from untreated cells could fully restore pol III transcription in MMS-treated extracts (Ghavidel and Schultz 2001), indicating that TFIIIB was the sole limiting factor in these assays. In contrast fractions containing partially purified pol III and TFIIIC2 but not TFIIIB were inactive (Ghavidel and Schultz 2001). These studies were carried out in yeast, which lack p53, and therefore provides and explanation for the difference in results. The findings in this study are preliminary and certainly require further investigation. It is important to establish that the changes in transcription factor occupancy are promoter specific.

Protein kinase CK2 has been implicated in mediating a cellular response to stress in which pol III activity is reduced. (Ghavidel and Schultz 2001). CK2 was found to physically associate with and inactivate TFIIIB (Ghavidel and Schultz 2001). CK2 is responsible for phosphorylating serine-386, located in the C-terminus of p53 (Hall et al. 1996). Blocking phosphorylation of this residue, through an alanine substitution, was shown to reduce the ability of p53 to repress expression from the c-fos promoter. This effect was specific since mutation of serine-386 did not affect the ability of p53 to activate p21 gene expression (Hall et al. 1996). It would be interesting to ascertain whether this mutation could affect the ability of p53 to repress pol III transcription.

Post-translational modifications are believed to rapidly and effectively regulate p53 in response to DNA damage. Treatment of cell lines expressing p53 with the phosphatase inhibitor okadaic acid was found to result in an increase in p53 phosphorylation and increased levels of p53 protein (Zhang et al. 1994). Multiple lines of evidence have demonstrated that p53 is phosphorylated *in vitro* both in the N- and C-terminal regions by a number of different kinases (Larkin and Jackson 1999). The DNA-dependant protein kinase (DNA-PK) has been demonstrated to phosphorylate p53 *in vitro* at serine-15 and 37 (equivalent to serine-34 of murine p53 by sequence homology) (Lees-Miller et al. 1990; Shieh et al. 1997). ATM kinase was also shown to phosphorylate serine 15, whereas ATR kinase was capable of phosphorylating both serine-15 and 37 (Banin et al. 1998; Canman et al. 1998; Lakin and Jackson 1999; Tibbetts et al. 1999). Whether these modifications have direct relevance *in vivo* remains controversial, but the effects of DNA damage on such modifications are becoming apparent. Phospho-peptide mapping revealed that phosphorylation of mouse p53 at serine-34 occurred in response to UV (Milne et al.

1995). Serine-15 was also identified as a site on p53 that was phosphorylated in response to DNA-damage induced by UV or IR (Shieh et al. 1997; Siliciano et al. 1997). It is unclear at present whether covalent modification of p53 contributes to its control of pol III transcription. Mutation of serine-15 or serine-34 of murine p53 had little effect in modulating p53-mediated pol III transcription *in vitro*. *In vitro* phosphorylation of serine-15 of p53 has been shown to inhibit the interaction of p53 with Mdm2 (Shieh et al. 1997), and it has been proposed that phosphorylation of serine-15 of p53 may be important in controlling the half-life and transcriptional activity of p53 in response to DNA-damage (Larkin and Jackson 1999). In this model, p53 is targeted for ubiquitin-mediated degradation in the undamaged cell by interacting with Mdm2. Upon DNA damage, p53 becomes phosphorylated on serine-15 and no longer interacts with Mdm2 (Shieh et al. 1997; Siliciano et al. 1997). This therefore results in a decrease in Mdm2 dependant degradation. Consistent with this model, substitution of serine-15 with glutamic acid (a residue that sometimes mimics serine phosphorylation) resulted in slight stabilisation of p53 *in vivo* (Ashcroft et al. 1999). The precise role of serine-15 is however unclear (Dumaz et al. 2001). Co-transfection of Mdm2 with p53 was shown to abrogate p53-mediated pol III transcriptional repression (Stein et al. 2002a). Therefore it is likely that destabilisation of the Mdm2-p53 interaction, through phosphorylation of Serine-15, may result in a p53-mediated repression of pol III activity.

Multiple residues in p53 have been reported to undergo phosphorylation. As well as aforementioned N-terminal residues, multiple C-terminal residues are believed to undergo modification. For example, CK2, PKC and Cdks have been demonstrated to phosphorylate the C-terminal regulatory domain of p53. Such phosphorylation events

have been shown to activate p53 sequence-specific DNA binding *in vitro* (Hupp et al. 1992; Hupp and Lane 1994; Takenaka et al. 1995; Wang and Prives 1995). Both N and C-terminal domains of p53 are important in eliciting p53-mediated repression of pol III transcription (Stein et al. 2002b). It is therefore possible that modifications within these regions modulate the activity of p53 with respect to its control of pol III gene expression.

p53-mediated pol III repression may serve a critical role in cells with DNA damage. It could serve to divert cellular resources from transcription to DNA repair and other processes required to maintain viability while the damaged cell is repaired (Ghavidel and Schultz 2001). In yeast, it was demonstrated that DNA repair is inhibited by pol III transcription (Aboussekhra and Thoma 1998). In this situation repression of pol III activity, by p53, may allow adequate access of the repair machinery to damaged DNA. In damaged cells, it is also possible that p53-independent effects contribute to the down-regulation of pol III activity. It is also very likely that multiple mechanisms may exist to repress pol III transcription, depending on which form of cellular stress is elicited.

Chapter 7

Conclusions, discussion and future work

The formation of productive mammalian transcription initiation complexes on tRNA gene promoters, involves the binding of TFIIIC2 to the intragenic promoters, followed by the recruitment of TFIIIB and TFIIIC1 to the complex via interactions of these factors with TFIIIC2. This TFIIIB-TFIIIC-DNA complex then serves to recruit Pol III to the start site of transcription via contacts between TFIIIB and pol III (Paule and White 2000; Geiduschek and Kassavetis 2001). The mechanism utilised by p53 to repress pol III transcription involves binding and inactivation of TFIIIB (Chesnokov et al. 1996; Cairns and White 1998). p53 can interact directly with the TFIIIB subunit TBP. Association between Brf1 and p53 has been observed, although at present it cannot be concluded whether this is a direct interaction or mediated through another molecule such as TBP. The consequences of such interactions are clear, however. Association of p53 with TFIIIB results in diminished interactions between TFIIIB, TFIIIC2 and pol III. It is unlikely that p53 can disrupt preformed interactions between these factors, since assembly of TFIIIB into a preinitiation complex, was shown to confer substantial protection against the inhibitory effects of p53 (Cairns and White 1998). p53 evidently prevents formation of functional preinitiation transcription complexes. In the presence of wild-type p53, less TFIIIB and consequently pol III subunits were found to be associated with $tRNA^{Leu}$ genes *in vitro*. To ascertain the effect of the p53-TFIIIB associations on transcription factor occupancy of tRNA genes *in vivo* ChIP assays were employed. Transcription factor occupancy in non-stressed $p53^{+/+}$ cells was compared to that of $p53^{-/-}$ cells.

Reproducibly, it was found that in the presence of wild-type p53, less TFIIIB and pol III subunits were associated with the tRNA genes. The decrease in TFIIIB and pol III recruitment was not due to a decrease in the cellular concentrations of these transcription components. Eichorn and Jackson previously reported that Brf1 is specifically degraded following p53 induction in the TR9-7 fibroblast cell line. In contrast there were no discernable differences in the levels of Brf1 in the $p53^{+/+}$ and $p53^{-/-}$ cells. Unlike the changes observed for TFIIIB and pol III, the occupancy of TFIIIC2 was unaffected by p53. This suggests that TFIIIC2 is able to stably associate with tRNA gene promoters *in vivo* in the absence of TFIIIB. Similar results have been obtained using a p53 inducible cell line (D. Johnson, unpublished results).

The possibility that p53 might disrupt the TBP-Brf1 interaction was investigated. Dr1 represses pol III transcription when overexpressed *in vitro* or in yeast cells via such a mechanism (White et al. 1994b; Kim et al. 1997). In contrast, p53 appears to be unable to disrupt the TBP-Brf1 interaction. It remains a possibility that the p53 may prevent the association of Bdp1 with the TBP-Brf1 complex. Bdp1 associates very loosely with TFIIIB (Schramm et al. 2000) and consequently this has prevented examination of this interaction.

Although direct binding of p53 to TBP has been demonstrated in many studies, the biological relevance of this interaction to p53-mediated transcriptional regulation has been unclear (Seto et al. 1992; Liu et al. 1993; Martin et al. 1993; Tansey and Herr 1995; Farmer et al. 1996a; Farmer et al. 1996b). Tansey and Herr (1995) identified substitutions in TBP that prevent its binding to p53 *in vitro* but do not compromise its ability to support p53-mediated activation of an RNA pol II reporter *in vivo*.

Repression of pol II transcription has also been shown to involve molecular interactions that are distinct from those with TBP (Farmer et al. 1996b). Overexpression of TBP in transient transfections failed to rescue pol II transcriptional repression by p53 (Farmer et al. 1996b). In contrast, increasing amounts of recombinant TBP could reverse p53 mediated transcriptional repression *in vitro*. Furthermore, p53-mediated inhibition of tRNA transcription can be reversed *in vivo* by TBP (D. Johnton, unpublished results). The TBP-E248R mutant is also able to rescue p53 repressed pol III transcription. This mutant renders TBP specifically defective for pol II transcription (Bryant et al. 1996), which suggests that the TBP-mediated increase in pol III transcription does not require changes in pol II activity. In contrast, overexpression of Brf1 could not reverse p53-mediated repression of pol III gene expression *in vivo*. At present this experiment cannot be carried out *in vitro* due to our inability to produce significant quantities of recombinant Brf1. These observations however provide evidence that the p53-TBP interaction is of functional significance for pol III regulation.

A requirement for TBP binding is also consistent with the fact that pol III repression involves both the N- and C-terminal domains of p53. Both these regions contact TBP (Liu et al. 1993; Horikoshi et al. 1995). The data presented in chapter 3 demonstrate that the N-terminal region of p53 is essential for pol III transcriptional repression *in vitro*. This region encompasses the transcriptional activation domain of p53 and has been shown to interact with several proteins, including TBP. Mutation of residues 22 and 23, within the N-terminal region of p53, has been previously demonstrated to severely reduce the ability of p53 to repress pol III transcription in a transient transfection assay (Stein et al. 2002b). Chesnokov *et al.* (1996) also demonstrated the

importance of these residues in mediating pol III repression. Mutation impaired the ability of p53 to bind to TFIIIB and hence down regulate pol III activity both *in vitro* and *in vivo*. These residues are important in binding TFIID, a function that contributes to the regulation of pol II templates (Lin et al. 1994; Chang et al. 1995; Lu and Levine 1995; Thut et al. 1995b; Farmer et al. 1996a; Farmer et al. 1996b). Deletion of residues 13-19 permitted efficient repression of VA1, when compared to wild-type p53 (Stein et al. 2002b). Clearly efficient repression of pol III transcription by p53 requires N-terminal sequence that includes residues 22 and 23 but excludes residues 13-19.

The C-terminal portion of p53 also contributes to pol III transcriptional repression. Although deletion of the 30 C-terminal residues did not affect the ability of p53 to repress VA1 and Alu transcription *in vitro*, preincubation of wild-type p53 with an antibody raised against residues 371-380 clearly blocked transcriptional activity. Further evidence implicating the C-terminus in pol III transcriptional repression was obtained by transient transfection assays. A p53 mutant lacking the C-terminal 102 residues of p53 was unable to repress VA1 expression *in vivo* (Stein et al. 2002b). Additionally, a p53 mutant carrying a quadruple substitution (ALAL) in residues 365, 372, 379 and 387, was unable to repress pol III transcription in a transient transfection assay (Stein et al. 2002b). This mutant has constitutive DNA-binding activity, and can efficiently transactivate pol II transcription and induce a G1 arrest or apoptosis (Marston et al. 1998). This result therefore supports the idea that the C-terminal region is involved in mediating pol III transcription repression. It also provides an important demonstration that repression observed *in vivo* is not an indirect effect of cell cycle arrest. Residues 339-346 were reported to possess

independent transrepression activity with respect to pol II reporters (Hong et al. 2001). It is possible that this region contributes to the pol III response.

It has also been found that the central core domain of p53 is involved in pol III transcriptional repression. Individual deletions of conserved regions II-V have been shown to abolish the ability of p53 to repress VA1 gene expression (Stein et al. 2002b). In agreement with these observations, preincubation of wild-type p53 with pAb1620, which binds the central core domain, abolishes the ability of p53 to repress pol III gene expression *in vitro*. It is perhaps unexpected that the sequence-specific DNA binding domain of p53 is required for pol III repression, since consensus p53 binding sites are not found in pol III transcribed genes. p53 was undetectable at tRNA promoters using ChIP assays. It would be interesting therefore to establish the role played by this domain in controlling pol III activity. An obvious possibility is that deletions or antibody binding may have interfered with the conformation of the p53 molecule. It is also possible that TFIIIB, or an as yet unidentified cofactor, which may contribute to regulation, might interact with this region. Further experiments will be required to distinguish these possibilities.

p53-mediated repression of pol I transcription also involves a direct interaction between p53 and the TBP-containing SL1 complex (Zhai and Comai 2000). In this instance, p53 binding to SL1 prevents its association with the pol I transcription component UBF. It is therefore possible that p53-mediated repression of pol I transcription involves a mechanism similar to that used for pol III, where p53 directly targets TBP within the SL1 complex.

A mechanism distinct from that described here is used by p53 to repress expression of the pol II transcribed genes, *Map4* and *stathmin*. p53 binds to the recognition sequences within these promoters and recruits a corepressor complex containing HDACs that mediate transcriptional silencing (Murphy et al. 1999). TSA, a potent inhibitor of HDAC activity, enhances pol III transcriptional activity; this finding is consistent with previous observations (Sutcliffe et al. 2000). This effect, however, appears to be independent of p53. Consistent with this result, ChIP assays show that p53 is not bound to the tRNA genes *in vivo*. Mechanistic differences between p53-mediated pol II and pol III repression are supported by their distinct responses to a leucine substitution at p53 residue 181 (Stein et al. 2002a). In transfected SAOS2 cells, the R181L p53 mutant remains capable of repressing VA1 gene expression but has lost the capacity to repress pol II templates such as the SV40 early promoter (Crook et al. 1994; Stein et al. 2002a).

Mutations in the p53 tumour suppressor gene occur in about 50% of all human tumours, making it the most frequent target for genetic alterations in cancer (Hollstein et al. 1991). Inherited mutations in p53 can give rise to LFS, a familial cancer predisposition in humans (Varley et al. 1997b). A wide variety of mutations can be associated with this inherited disease (Varley et al. 1997a). It is shown here that the pol III activity of samples prepared form several Li-Fraumeni patients are substantially elevated when compared to samples obtained from healthy volunteers. Further deregulation of pol III activity was also observed upon deletion of the wild-type p53 gene. RT-PCR analysis of primary p53 knock-out fibroblasts has demonstrated that endogenous p53 contributes significantly towards the control of

pol III transcription *in vivo*. The study of LFS patients provides further insights into the function of endogenous p53 at physiological concentrations.

Since each of the patients in the LFS study has a different genetic background, it cannot be concluded that the elevated pol III transcriptional activity results solely from changes in p53. Evidence from transient transfection assays do, however, demonstrate that naturally occurring p53 mutations can compromise its ability to repress pol III transcription (Stein et al. 2002a). The p53 R175P mutant, originally obtained from an anogenital tumour, was severely compromised in its ability to repress VA1 gene expression *in vivo* (Stein et al. 2002a). This mutant retains the ability to suppress proliferation of SAOS2 cells, providing further evidence that the cell cycle arrest function of p53 is insufficient to mediate a pol III transcriptional repression. Substitution R273C, within a highly conserved region of the DNA-binding domain, completely abolishes the ability of p53 to repress VA1 transcription in SAOS2 cells (Stein et al. 2002a). This mutant is also completely inactive towards various pol II promoters (Crook et al. 1994). Another mutant which is also inactive towards various pol II promoters is R181L (Crook et al. 1994). This mutant was nearly as active as wild-type p53 in repressing pol III transcription (Stein et al. 2002a). Although p53 appears to repress pol III transcription through protein-protein interactions with TFIIIB, rather than direct binding to DNA at a class III gene site, it was unclear whether mutations within the sequence specific DNA-binding domain of p53 would perturb its repression function. Clearly however, the data establish that regulation of pol III transcription can be influenced by substitutions within the central domain. In many cases these effects may be explained by conformational changes within the molecule. Arginine 273 is a DNA contact site within p53 (Cho et

al. 1994). Mutation at this site is not thought to affect p53 conformation, suggesting that conformational changes cannot fully account for perturbations in p53 function. This therefore implies that the core domain of p53 may be directly involved in regulating pol III transcriptional activity.

The p53 R175H substitution accounts for ~6% of the missense mutations identified in human cancers (Greenblatt et al. 1994). When compared with other p53 mutants it is found to be the most tumourigenic in a variety of assays (Finlay et al. 1989; Dittmer et al. 1993); it is also associated with an extremely poor prognosis in human cancer victims (Goh et al. 1995). This mutant has not only lost the ability to repress pol III transcription, but actually displays a capacity to stimulate expression of VA1 when transfected into SAOS2 cells (Stein et al. 2002a). The mechanistic basis of this stimulation remains unclear at present, although there are precedents for such behaviour. For example, the promoter of the gene encoding the insulin-like growth factor I receptor is repressed by wild-type p53, but stimulated by certain tumour-derived p53 mutants (Werner et al. 1996).

Apart from mutation, p53 function can be compromised by the action of various oncoproteins. p53 is bound to and inactivated by the E6 product of HPV in many cervical carcinomas (Werness et al. 1990; Vousden 1995). As expected, cotransfection of E6 abrogates p53 mediated repression of pol III transcription in SAOS2 cells. Mdm2 and its human counterpart hdm2 are cellular oncoproteins that can also bind to and inactivate p53 (Momand et al. 1992). Amplification and overexpression of the gene encoding hdm2 is observed in many tumour types, especially osteosarcomas and soft tissue tumours (Momand et al. 1998). As with E6,

cotransfection of hdm2 was found to counteract the specific repression of pol III transcription by p53 (Stein et al. 2002a). In contrast, an hdm2 mutant with a small deletion in its p53-binding domain (residues 59-89) was unable to overcome repression (Stein et al. 2002a). Both E6 and hdm2 promote the degradation of p53 through ubiquitin-mediated proteolysis (Scheffner et al. 1993; Haupt et al. 1997; Kubbutat et al. 1997b), and it is therefore not surprising that these oncoproteins deregulate pol III transcription. Nevertheless it was important to confirm that E6 and hdm2 stimulate pol III transcription, since both are important aetological agents in the development of specific types of human cancer.

Extensive northern analysis of 80 tumour specimens representing 19 types of cancer revealed that 7SL RNA, a pol III product, is abnormally abundant in every tumour analysed relative to healthy tissue from the same patient (Chen et al. 1997a). In addition, *in situ* hybridisation of lung, breast and tongue carcinomas revealed increased levels of pol III transcripts in neoplastic cells relative to the surrounding healthy tissue (Chen et al. 1997a; Chen et al. 1997b). In a significant number of cases, the consistent deregulation of pol III in transformed cells may be provided by deregulation of p53. Currently it is estimated that p53 function is compromised in over 80% of human tumours (Lozano and Elledge 2000). Derepression of pol III can be expected in a high proportion of these cases.

Under normal cellular conditions, p53 does not interfere with cell cycle progression or cell survival; however, in response to cellular stresses such as hypoxia, ribonucleotide depletion and exposure to genotoxins, p53 is rapidly induced (Oren 1999). Under such circumstances, reducing cellular biosynthesis through a reduction

in pol III transcription may be important for maintaining cell viability. Reducing the production of rRNA and tRNA might be particularly beneficial when ribonucleotide pools are depleted. Indeed, such conditions have been shown to trigger a p53-dependent repression in pol III activity (A. McLees and R. J. White, unpublished). Furthermore a specific block in rRNA processing and hence ribosome production has been shown to trigger a p53-mediated reversible arrest (Pestov et al. 2001).

The DNA-damaging agent MMS was shown to induce a p53 response that correlates with a reduction in pol III transcription. The mechanistic basis of this repression remains unclear. The data presented here demonstrate that less TBP is found associated with tRNA promoters in response to MMS. This is a promoter specific decrease, since more TBP was detected at the p21 locus following treatment. These findings are in agreement with the situation in the p53 knockout and wild-type cells. In contrast, levels of TFIIIC2 were also found to be diminished in response to MMS. These studies are, however, preliminary and require vital controls. It is necessary to show that the changes in factor occupancy are promoter specific and not the result of a cell cycle arrest.

It is entirely possible that different mechanisms are employed by p53 to down regulate pol III activity in response to different stresses. Stress causes p53 to accumulate rapidly, primarily due to its stabilization. It also triggers a complex series of phosphorylation and acetylation events (Hupp et al. 1992; Giaccia and Kastan 1998; Sakaguchi et al. 1998; Oren 1999). The nature of the response pathways and the modifications they induce can vary dramatically between different cell types and distinct kinds of stress (Giaccia and Kastan 1998; Oren 1999; Zhao et al. 2000). For

example, serine 15 of p53 is phosphorylated in response to DNA damage, but not in response to aberrant oncogenic signals (de Stanchina et al. 1998). Substitution of serine 15 to glutamic acid, a residue that can mimic a serine phosphorylation, resulted in a marginal increase in pol III transcriptional repression *in vitro*. Although this modification may enhance the activity of p53 with respect to controlling pol III transcription, it was not essential for its function since mutation to alanine also permitted efficient pol III repression.

It remains to be determined whether acetylation of p53 modulates its function with respect to pol III transcriptional control. Inhibition of HDAC activity with TSA permitted pol III repression *in vitro,* suggesting that in this system acetylation or deacetylation events do not play a major role in controlling pol III activity by p53. Transfection of p300 appeared to slightly increase pol III activity, although this effect seemed to be independent of p53. This result requires conformation, although, if substantiated, it may suggest that the pol III apparatus is subject to acetylation that may enhance its activity. Indeed, a weak association between p300 and tRNA promoters was observed in ChIP analysis. It is entirely possible that p300 may play a role reminiscent to that in the pol I system, where acetylation of the basal pol I transcription factor SL1 results in increased association with the rDNA promoter, and an increase in pol I transcription (Muth et al. 2001).

A variety of mechanisms may contribute to the deregulation of pol III activity found in a wide range of transformed cell lines and tumour samples. The pol III specific transcription factor TFIIIC2 is frequently overexpressed in ovarian carcinomas (Winter et al. 2000). Furthermore, clinically important tumour viruses, such as

hepatitis B virus and human T-cell leukaemia virus type I can increase pol III activity specifically (Aufiero and Schneider 1990; Wang et al. 1995; Gottesfeld et al. 1996; Wang et al. 1997). Like p53, the tumour suppressor retinoblastoma protein, RB, has been shown to downregulate pol III activity through its interaction with TFIIIB. This interaction prevents TFIIIB associating with TFIIIC2 and pol III, thereby inhibiting pol III transcription. This function is compromised by a number of mutations that arose naturally in human carcinomas (White et al. 1996; Brown et al. 2000; Sutcliffe et al. 2000). In addition, three viral oncoproteins, E7, E1A and SV40 large T antigen, can bind to RB releasing pol III from repression (Larminie et al. 1999; Sutcliffe et al. 1999) (White et al. 1996). In a high proportion of cancers, the function of RB is compromised due to its hyperphosphorylation; this can be caused by overexpression of cyclin D or loss of p16, a specific inhibitor of cyclin D-dependent kinases (Hunter and Pines 1994; Bates and Peters 1995). Such hyperphosphorylation has been shown to prevent RB from interacting with TFIIIB *in vivo* (Scott 2001). Clearly, loss of RB function through a variety of mechanisms may contribute to pol III deregulation in a range of transformed cell types (Brown et al. 2000). It is highly likely that, like the loss of RB function, deregulation of p53 may contribute to elevated pol III activity found in many tumour types.

At present there is no direct evidence that p53 can achieve growth restraint by regulating pol III transcription. It is however a possibility, given the clear data showing that p53 directly targets pol III transcription. It is unquestionable that growth can be prevented when the synthesis of rRNA or tRNA becomes limiting. It is however necessary to establish under what physiological conditions this becomes

important, and whether loss of such functions during tumour development may contribute an important step towards neoplastic growth.

Chapter 8

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