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Regulation of human small nuclear RNA gene transcription by the tumor suppressor protein p53

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REGULATION OF HUMAN SMALL NUCLEAR RNA GENE TRANSCRIPTION BY THE TUMOR SUPPRESSOR PROTEIN P53

By

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ABSTRACT

REGULATION OF HUMAN SMALL NUCLEAR RNA GENE TRANSCRIPTION BY THE TUMOR SUPPRESSOR PROTEIN P53

By

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Activation of the tumor suppressor protein p53 or loss of the Cockayne syndrome complementation group B (CSB) protein induces fragile site formation at RNA polymerase II-transcribed U1 and U2 snRNA gene loci and at RNA polymerase III-transcribed 5S rRNA gene loci. Yu et al. (2000) hypothesized that p53 interferes with transcription elongation functions of CSB, resulting in accumulation of stalled RNA polymerase and impaired chromatin condensation at these gene loci. However, a role for p53 and CSB in transcription of these genes has not been investigated.

In this study I show that both p53 and CSB are involved in human U1 snRNA and 5S rRNA gene transcription. I found that p53 represses U1 snRNA and 5S rRNA gene transcription by RNA polymerases II and III, respectively. p53 also represses U6 snRNA gene transcription by RNA polymerase III. Both DNA binding competent and defective forms of p53 revealed similar levels of snRNA promoter occupancy during transcription repression, suggesting that sequence-specific DNA binding by p53 is not essential for repression of snRNA gene transcription. I further demonstrated that CSB plays a positive role in snRNA gene transcription by both polymerases II and III and a negative role in transcription of those other classes of RNA polymerase III-transcribed genes that contain an intragenic arrangement of promoter elements.

The functional interplay between p53 and CSB in snRNA gene transcription was also investigated. Firstly, removing CSB from cell extracts modulates p53 transcription

activity in vitro. CSB immunodepletion potentiates the inhibitory effect of p53 on U1 snRNA gene transcription, but does not affect p53-mediated repression of U6 snRNA gene transcription. Interestingly, at low amounts p53 activates and at higher amounts represses 5S rRNA gene transcription when transcription is performed with CSB depleted extracts. Secondly, CSB association with snRNA gene promoters was diminished after UV light treatment concomitant with increased p53 promoter association. As CSB was described as an elongation factor for RNA polymerase II, p53 may affect elongation by interfering with CSB promoter association. Thirdly, transient transfection of p53 results in snRNA gene transcription repression concomitant with accumulation of covalently modified forms of RNA polymerase III. These forms of RNA polymerase III are more enriched in CSB cells, suggesting that p53 and CSB have opposing roles in post-translational modifications of RNA polymerase III. I speculate that p53 represses elongation by RNA polymerase III by facilitating post-translational modifications of the polymerase. Together, these results suggest that p53 may modulate CSB transcriptional activity and support the hypothesis that fragile sites at U1 and U2 snRNA and 5S rRNA gene loci may be caused by the inhibitory effect of p53 on CSB-mediated elongation by RNA polymerases.

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KEY TO SYMBOLS AND ABBREVIATIONS

ASSP	apoptotic-stimulating proteins of p53
ATM	ataxia telangiectasia mutated kinase
Brf1-TFIIIB	TFIIIB complex composed of TBP, Brf1 and Bdp1
Brf2-TFIIIB	TFIIIB complex composed of TBP, Brf2 and Bdp1
CDK	cyclin-dependent kinase
CHH	cartilage-hair hypoplasia
ChIPs	chromatin immunoprecipitations
CKII	casein kinase II
CO-IP	co-immunoprecipitation
CS	Cockayne syndrome
CSB	the Cockayne syndrome complementation group B protein
CTD	carboxy terminal domain of RNA polymerase II largest subunit
C-terminal	carboxy-terminal
Da	Dalton
DBD	DNA binding domain
ds	double stranded
DSE	distal sequence element
ELL	the Eleven Lysine-rich Leukemia protein
EMSA	electrophoretic mobility shift assay
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HAT	histone acetyl transferase

HDAC	histone deacetylase
Hdm2	the human double minute protein
IE	intermediate element
IP	immunoprecipitation
JMY	junction mediating and regulatory protein
Mdm2	the murine double minute 2 protein
mRNA	messenger RNA
NER	nucleotide excision repair
NES	nuclear export signal
NLS	nuclear localization signal
NTB	nucleotide binding domain
N-terminal	amino-terminal
PSE	proximal sequence element
P-TEFb	positive transcription elongation factor-b
PTF	proximal sequence element transcription factor
RB	the retinoblastoma tumor suppressor protein
RD	the regulatory domain
RNAP	RNA polymerase
RPA	the replication protein A
rRNA	ribosomal RNA
RT-PCR	reverse transcriptase polymerase chain reaction
$SNAP_C$	the snRNA activating protein complex
snRNAs	small nuclear RNAs

snRNPs	small nuclear ribonucleoprotein particles
ss	single stranded
SUMO	the small ubiquitin-like modifier
SV40	Simian virus 40 large T antigen
TAD	the transactivation domain
TAF	TBP-associated factor
TBP	TATA binding protein
TCR	transcription coupled repair
TD	the tetramerization domain
tRNA	transfer RNA
UV	ultraviolet light

CHAPTER 1

INTRODUCTION

1. Human small nuclear RNA

1.1. Diverse functions in cells

Small nuclear (sn) RNAs are short, stable, nontranslated RNAs that are evolutionarily well conserved and found in the nuclei of all eukaryotic cells. These RNAs are not polyadenylated and contain unusual 5' cap structures. They exist in the cell packaged with proteins as small nuclear ribonucleoprotein particles (snRNPs). Newly synthesized snRNAs are immediately exported to the cytoplasm where they undergo maturation and assembly into snRNPs prior to their reentry into the nucleus (55).

SnRNA perform essential functions in cells. As part of snRNPs, uridine rich snRNAs are involved in messenger (m) RNA splicing (e.g. U1, U2, U4, U5 and U6) and ribosomal (r) RNA processing (e.g. U3) (55). It is estimated that up to 15% of all point mutations causing human genetic disease result in an mRNA splicing defect (70, 138).

In addition to their roles in RNA metabolism, some snRNAs also participate in regulation of transcription initiation (e.g. U1 snRNA and B2 RNA) or transcription elongation (e.g. 7SK and components of the splicing apparatus) through association with transcription factors (36, 39, 76, 108, 153). U1 snRNA associates with TFIIH and stimulates transcription initiation in vitro (76). In contrast, B2 RNA represses transcription initiation when it binds to RNA polymerase II upon heat shock of mouse cells (36). Repression of transcription elongation by 7SK RNA occurs through the

inhibition of the kinase activity of the positive transcription elongation factor (P-TEFb) (108, 153).

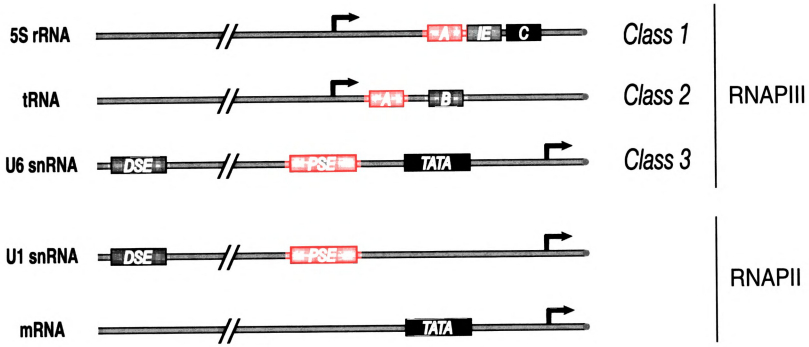
Recent evidence suggests additional unexpected snRNA cellular functions in innate immunity (58), human hereditary diseases (e.g. cartilage-hair hypoplasia (CHH) disease) (116) and cancer (70, 138).

1.2. Promoter organization of human snRNA genes

Human snRNA genes are transcribed by either RNA polymerase II (e.g. U1 snRNA gene) or by RNA polymerase III (e.g. U6 snRNA gene) and yet have very similar promoter architecture (Figure 1-1). Both RNA polymerase II- and III- transcribed snRNA genes contain the proximal sequence element (PSE) within the core promoter region. This essential element is located at approximately –45 bp upstream of the start site of transcription. In addition, the RNA polymerase III-transcribed snRNA genes contain a TATA box located in the core promoter adjacent to the PSE. In humans, the TATA box acts as dominant element for determining the specificity of RNA polymerase III-transcribed snRNA gene promoters (56, 75, 88). Both the RNA polymerase II and III snRNA gene promoters contain the distal sequence element (DSE), located in the regulatory region around –220 (105). The DSE functions as an enhancer and is required for maximum promoter activity.

As shown in Figure 1-1, human U6 snRNA genes are distinct from other RNA polymerase III-transcribed genes. U6 snRNA genes belong to class 3 promoters, which are defined by their extragenic RNA polymerase III promoters. In contrast, the class 1 and 2 RNA polymerase III-specific genes contain intragenic promoter elements and are

Figure 1-1. Schematic representation of RNA polymerase II- and III- transcribed promoters. Class 1 and 2 RNA polymerase III-transcribed promoters are intragenic and are exemplified by the 5S rRNA and tRNA promoters, respectively. Class 1 genes contain A and C boxes that are separated by an intermediate element (IE). Class 2 promoters consist of A and B boxes. Class 3 promoters are defined as extragenic RNA polymerase III promoters and are exemplified by the human U6 snRNA promoter. The architecture of the class 3 RNA polymerase III-transcribed snRNA gene promoters is similar to other snRNA gene promoters that are transcribed by RNA polymerase II (e.g. U1 snRNA gene). Both RNA polymerase II- and III- transcribed snRNA gene promoters contain the proximal sequence element (PSE) and the distal sequence element (DSE). In addition, class 3 RNA polymerase III-transcribed promoters have a TATA box.


5S rRNA
A
IE
C
Class 1
tRNA
A
B
Class 2
RNAPIII
U6 snRNA
DSE
PSE
TATA
Class 3
U1 snRNA
DSE
PSE
RNAPII
mRNA
TATA

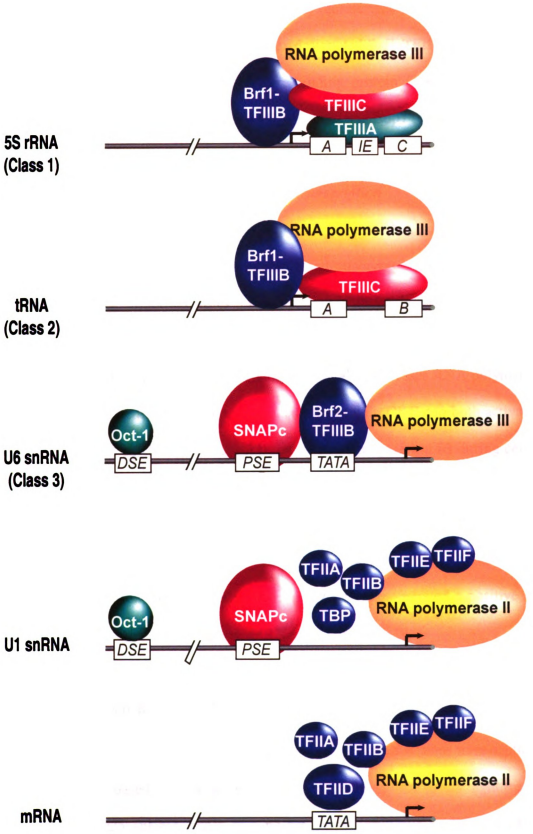
exemplified by the 5S rRNA and tRNA promoters, respectively. Class 1 genes contain A and C boxes that are separated by an intermediate element (IE). These sequence elements constitute the internal control region that is required for transcription (55, 113). Class 2 promoters consist of an A and B box (40, 55).

1.3. The General Transcriptional Machinery

As illustrated in Figure 1-2, the PSE of class 3 RNA polymerase III-transcribed and RNA polymerase II-transcribed snRNA genes is recognized by the snRNA activating protein complex ($SNAP_C$), also referred to as the PSE-binding transcription factor (PTF) (119, 154). It is a multi-protein complex, composed of at least five proteins SNAP190, SNAP50, SNAP45, SNAP43 and SNAP19 (53). $SNAP_C$ is essential for the activity of both RNA polymerase II- and III-transcribed snRNA promoters (54). The DSE for both types of snRNA promoters is recognized by the POU domain Oct-1 protein (105, 136). Other snRNA transcription factors are more promoter-specific. The TATA box serves as a binding site for a TFIIIB-like complex (119) designated Brf2-TFIIIB, which is composed of the TBP and TBP-associated factors Brf2 and Bdp1 (126). TBP is also a required factor for transcription of snRNA genes transcribed by RNA polymerase II (74). Additionally, basal transcription from RNA polymerase II-transcribed snRNA gene promoters requires TFIIA, TFIIB, TFIIF, and TFIIE (74).

RNA polymerase III-transcribed class 1 promoters recruit the transcription factor TFIIIA that belongs to the zinc finger family of DNA binding proteins (98). The binding of TFIIIA then allows the recruitment of TFIIIC (55). In contrast to class 1 promoters, class 2 promoters recruit TFIIIC directly, without prior binding of TFIIIA (55). In both

Figure 1-2. Schematic representation of the general transcription machinery of the RNA polymerase II- and III- transcribed genes. RNA polymerase III-transcribed class 1 promoters require the TFIIIC complex and TFIIIB complex. Class 1 genes have an additional requirement for TFIIIA. The PSE and the DSE for both class 3 RNA polymerase III-transcribed and RNA polymerase II-transcribed snRNA genes are recognized by the multisubunit protein complex SNAPc and Oct-1 proteins, respectively. Class 3 genes have an alternative TFIIIB complex, which binds to the TATA element.


5S rRNA
(Class 1)
RNA polymerase III
Brf1-
TFIIIB
TFIIIC
TFIIIA
A
IE
C
tRNA
(Class 2)
RNA polymerase III
Brf1-
TFIIIB
TFIIIC
A
B
U6 snRNA
(Class 3)
Oct-1
SNAPc
Brf2-
TFIIIB
RNA polymerase III
DSE
PSE
TATA
U1 snRNA
Oct-1
SNAPc
TFIIA
TFIIB
TFIIE
TFIIF
TBP
RNA polymerase II
DSE
PSE
mRNA
TFIIA
TFIIB
TFIIE
TFIIF
TFIID
RNA polymerase II
TATA

class 1 and 2 RNA polymerase III promoters, the recruitment of TFIIIC is followed by the recruitment of the TBP-containing complex designated Brf1-TFIIIB (99).
Brf1-TFIIIB is composed of the TATA box binding protein (TBP) and at least two additional TBP-associated factors called Brf1 (99, 100) and Bdp1 (126).

1.4. Regulation of human snRNA gene transcription

In addition to the general transcription machinery snRNA gene transcription was found to be regulated by the tumor suppressors and oncoproteins, suggesting that these snRNAs may play important roles in controlling cellular homeostasis. The tumor suppressor proteins RB and p53 were shown to repress snRNA gene transcription by RNA polymerase III (14, 17, 43, 57). In addition p53 also represses RNA polymerase II-transcribed snRNA genes (43). Interestingly, an oncoprotein CKII can both activate (61) and repress (60) U6 snRNA gene transcription by RNA polymerase III during cell cycle progression. CKII-dependent phosphorylation of RNA polymerase III stimulates U6 snRNA gene transcription (61). In contrast, CKII-dependent phosphorylation of the Bdp1 subunit of the Brf2-TFIIIB complex during mitosis results in U6 snRNA gene transcription repression (60). Recently, Gu et al. (2005) demonstrated a positive role of CKII for U1 snRNA gene transcription by RNA polymerase II (45).

2. Tumor suppressor protein p53

The role of the tumor suppressor protein p53 in snRNA gene transcription was initially suggested based on their unusual transcriptional response to UV light treatment. The U1 through U5 snRNA genes showed prolonged inhibition of RNA synthesis in

response to UV light treatment (34, 103), as well as to other DNA damaging agents (158), also known to activate the tumor suppressor protein p53; thus, suggesting a role of p53 in snRNA gene transcription. Indeed, my studies revealed that p53 is directly involved in snRNA gene transcription repression by both RNA polymerases II and III (see chapter 2 and references 14, 17, 43).

2.1. Discovery of p53

The p53 protein was discovered in 1979 as a 53-kDa protein that is bound by the large T-antigen of the sarcoma-associated virus SV40 (86). It was first described as an oncoprotein, because overexpression of p53 appeared to cause oncogenic transformation of cells (110). Later studies revealed that uncontrolled cellular proliferation results from p53 inactivation and demonstrated that wild type p53 is a tumor suppressor protein (79).

The gene encoding p53 is lost or mutated in more than 50% of human cancers. The other half of human tumors are thought to contain alterations in other components of the p53 pathway (59). Furthermore, germline mutations in p53 are responsible for the majority of cases of the inherited cancer family syndrome known as Li-Fraumeni syndrome (92). Thus, p53 inactivation is considered to be an important step in carcinogenesis.

2.2. Cellular functions of the p53 tumor suppressor protein

Once p53 is activated by a stress signal, p53 can induce any of several different cell fates, including cell cycle arrest, apoptosis and repair of damaged DNA. How this decision is determined is not well understood, however, multiple factors contribute to the

cellular fate choice, including cell type and the specific stress activation. The choice of response is determined by the subset of genes activated or inhibited by p53 as described below (69).

2.2.1. Cell cycle control

p53 arrests the cell cycle in G1 and G2 phases to potentially give cells time to repair damaged DNA that could otherwise lead to mutations and genomic instability. In the DNA damage response, the most important transcriptional target for p53-induced G1 arrest is $p21^{WAF1/CIP1}$, which is a potent inhibitor of several cyclin-dependent kinase (CDK) complexes. p53 activates p21 gene expression by binding to two response elements located in the p21 promoter (33).

p53 regulates the G2/M transition at the level of the cyclin-dependent kinase Cdc2, which is essential for entry into mitosis (114). Several of the transcriptional targets of p53 can inhibit Cdc2 levels and activity, including p21, 14-3-3δ and GADD45. p21 inhibits Cdc2 directly, 14-3-3δ anchors Cdc2 in the cytoplasm where it cannot induce mitosis and Gadd45 prevents the association of Cdc2 with Cyclin B1. p53 also represses transcription of *cyclin B1* and *cdc2* genes, which further enforces G2 cell cycle arrest. In addition, p53 can bind to and inhibit CAK activity, which may cause a drop in the extent of phosphorylation and activity of Cdc2 (140).

2.2.2. Apoptosis

Apoptosis is an evolutionarily conserved process by which an organism removes unwanted or damaged cells. Two major apoptotic pathways are defined in mammalian

cells: (i) the extrinsic and (ii) the intrinsic pathways (109). p53 is implicated in the induction of both apoptotic signaling pathways (51). p53 can activate the extrinsic apoptotic pathway through the induction of genes encoding death receptors (Fas/APO1, KILLER/DR5, and PERP) (5, 106, 141, 150). p53 was also shown to activate transcription of pro-apoptotic genes (Apaf-1, Bax, NOXA, p53AIP1, and PUMA), which are involved in the intrinsic apoptotic pathway (66, 156).

In addition, there is a transcription-independent p53 function in apoptosis. In this case, p53 binds anti-apoptotic proteins Bcl-2 and Bcl-xL in the mitochondria. These two proteins are otherwise known to inhibit pro-apoptotic proteins Bax and Bak, which are involved in the release of cytochrome c from mitochondria. It has been proposed that p53 binds directly to Bcl-2 and Bcl-xL and hence liberates Bax and Bak, allowing the caspase cascade to occur (18).

2.2.3. DNA repair

p53 is required for efficient nucleotide excision repair (NER) pathway by the direct interaction with components of the repair machinery. p53 binds and modulates the activities of the NER-associated helicases XPB and XPD (49, 147). Also, p53 associates with and inhibits the replication protein A (RPA), which is needed for DNA replication, homologous recombination, and NER. The inhibitory interaction is disrupted upon UV radiation allowing RPA to participate in DNA repair (1). In addition, p53 has been shown to interact with the Cockayne syndrome complementation group B (CSB) protein (147, 155). CSB is required for the transcription-coupled repair (TCR) pathway and may be a DNA repair protein (142, 145). It has been proposed that CSB recruits the nucleotide

excision repair (NER) machinery to sites of stalled RNA polymerase to permit rapid repair of the transcribed strand (11). In addition, p53 can recruit histone acetyl transferases (HAT) complexes to chromatin and promote chromatin relaxation, which results in increased chromatin accessibility for NER factors (118).

2.2.4. Cellular response to p53 and p53 protein partners

Whether a cell undergoes cell cycle arrest or apoptosis in response to p53 depends on several factors. Firstly, the p53 activity itself can contribute to the choice of response. The type and the magnitude of the cellular stress may control p53 functions by affecting the level or activity of the p53 protein that is induced. Activation of apoptosis has been associated with higher levels of p53 than those required for cell cycle arrest (16). It was also suggested that promoters regulating expression of apoptotic genes bind p53 with a lower affinity then the cell cycle arrest targets (16). Affinity of p53 to target promoters might be regulated by covalent modifications of p53. For example, phosphorylation of p53 on Ser46 is required for the induction of the apoptotic target gene p53AIP1 (p53-regulated apoptosis inducing protein 1) (111). Secondly, the strength of p53 interaction with a particular promoter might also be regulated by interactions with other cellular factors. The ASPP (Apoptotic-stimulating proteins of p53) proteins bind to the evolutionarily conserved DNA binding domain (DBD) of p53 and specifically increase the transactivation of pro-apoptotic p53-responsive genes such as Bax and PIG3, but have little effect on other p53 target genes that are involved in other functions such as cell cycle arrest (122). Junction mediating and regulatory (JMY) protein and p53 family members, p63 and p73, were also shown to be required for p53-induced apoptosis (38,

131). The new studies reveal an extra layer of complexity by showing that even when both cell cycle arrest and apoptotic target genes are induced by p53, the resultant response may depend on factors that cause selective inhibition of p53 target genes that encode a survival function (129).

2.3. p53 as a transcriptional factor

2.3.1. Role of p53 in regulation of the RNA polymerase I, II and III transcription

p53 has been shown to either activate or repress a variety of cellular promoters transcribed by RNA polymerase II. p53 activates RNA polymerase II-transcribed genes by binding to its cognate DNA-binding sites in target gene promoters (33). In addition, p53 represses certain RNA polymerase II-transcribed promoters, many of which lack a p53 response element (130). A novel/alternative p53 DNA-binding site was identified at the promoters of some p53-repressed genes (e.g. *MDR1, Cyclin A, Cyclin B1*). This novel p53 DNA-binding site has defined consensus quarter-sites arranged in head-to-tail orientation (64). p53 can also inhibit transcription from a variety of TATA-containing RNA polymerase II-transcribed promoters. Direct interactions between p53 and TBP (and/or TAFs) are thought to play a role in mediating p53 repression of certain RNA polymerase II-dependent genes (37).

In addition to a role in regulating transcription from RNA polymerase II-dependent promoters, p53 has been also shown to repress transcription of genes transcribed by RNA polymerase I (157) and RNA polymerase III (14, 17). The major product of these genes, rRNA, tRNA, and snRNA are essential for the translational capacity of cells, and regulation of their synthesis is closely linked to cellular growth

rates. Thus, p53-mediated down-regulation of RNA polymerase I- and III-dependent transcription may help restrict cell growth and, perhaps, tumor formation (27). Consistently, synthesis of tRNA and 5S rRNA is elevated significantly in fibroblasts derived from p53 knockout mice (14) and inherited mutations in the p53 protein are often associated with aberrant RNA polymerase III activity in primary fibroblasts from patients with Li-Fraumeni syndrome (134).

2.3.2. Possible mechanisms for transcriptional control by p53

2.3.2.1. p53 as a transcriptional activator

Recent studies revealed that p53-mediated transcription activation is tightly associated with chromatin modifying activities of p53 (3, 118). During transcription activation, promoter-bound p53 can recruit general transcription factors (e.g. TBP, TAFs) or co-activators such as histone acetyl transferases p300/CBP or arginine methyltransferases PRMT1 and CARM1 to chromatin (3, 47). Other co-activator complexes have been shown to function in p53-dependent transcription regulation, including SAGA (Spt-Ada-Gcn5-acetylase) and NuA4/Tip60 complexes, whose common subunit, the ATM-related protein TRRAP, can bind directly to p53 (4).

2.3.2.2. p53 as a transcriptional repressor

It has been shown that p53 can repress RNA polymerase II transcription by a variety of mechanisms that regulate pre-initiation complex formation. Transcriptional repression by p53 can be mediated by p53 binding to consensus DNA elements or in the apparent absence of p53 binding to promoter DNA. For example, p53 can bind directly to

its consensus DNA sequences and either prevent binding of transcriptional activators or the basal transcriptional machinery to promoters (14, 27, 157). Alternatively, p53 can interact with a promoter-bound transcriptional activator and prevent the subsequent recruitment of co-activators such as histone acetyl transferases (p300/CBP) (73). p53 may also directly recruit co-repressors such as histone deacetylases (HDACs) to repress transcription (65). p53-mediated transcriptional repression may also be achieved through the physical interaction of p53 with the general machinery or activators rendering proteins inactive for binding to the promoter and for transcriptional activation.

In addition to affecting pre-initiation complex assembly, p53 can regulate transcription elongation (9). p53 interacts with and inhibits the activity of two transcription elongation factors – the Eleven Lysine-rich Leukemia (ELL) protein and the CSB protein (132, 155). ELL increases the overall rate of transcription elongation by RNA polymerase II via suppression of transient pausing by the polymerase at many sites along DNA, and it is thought that the CSB protein functions as an elongation factor to promote transcription of genes encoding RNA products with significant secondary structure, such as human U1 and U2 snRNA genes (132, 155). Additionally, p53 represses transcription elongation by interacting with and inhibiting the activity of the CAK kinase – a component of the general transcription factor TFIIH (124). CAK plays a role in RNA polymerase II promoter escape through phosphorylation of the C-terminal domain (CTD) on RNA polymerase II (2).

Wild-type p53 can repress not only RNA polymerase II promoters but also RNA polymerase I- and III-transcribed genes (14, 17, 27, 43, 135, 157). RNA polymerase I transcription repression by p53 is probably achieved by inhibiting interactions between

general transcription factors SL1 and UBF and preventing the formation of a productive initiation complex at rRNA gene promoters (157).

The mechanism for p53 regulation of RNA polymerase III transcription is controversial. Several in vivo and in vitro studies demonstrated a direct role of p53 in RNA polymerase III transcription repression. It was proposed that p53 represses RNA polymerase III transcription by interacting with the TBP component of TFIIIB and preventing recruitment of TFIIIB to the promoters of repressed genes (14, 17, 27, 43, 135). An indirect role of p53 in RNA polymerase III transcription repression has also been suggested. A kinetic analysis of RNA polymerase III transcription repression, using p53 expressed from a stably integrated inducible p53 gene, revealed that RNA polymerase III repression can be mediated indirectly through p53-dependent degradation of TFIIIB (31).

2.4. p53 stability, cellular stress and p53 post-translational modifications

Under normal conditions p53 undergoes rapid turnover, and is thus maintained at low steady state levels that restrict its impact on cell fate. Rapid p53 turnover in normal cells is largely due to the murine double minute 2 (Mdm2) (or the human double minute 2 (Hdm2)) oncoprotein. Mdm2 binds to the N-terminal TAD region of p53 and represses p53 activity via two mechanisms: by promoting p53 degradation and by blocking p53 transcriptional activation (52, 71, 102). Mdm2 is an E3 ubiquitin ligase that promotes the covalent conjugation of ubiquitin residues to p53. Mdm2 can only mono-ubiquitylate p53 (78). The mono-ubiquitylation exposes a nuclear export signal (NES), which allows p53 to be exported from the nucleus into the cytoplasm. The poly-ubiquitylation of p53 is

achieved by histone acetyl transferase (HAT) p300, which has intrinsic ubiquitin ligase activity (44). In addition, Pirh2 and Cop1 have recently been identified as other RING finger proteins that bind p53 and mediate its poly-ubiquitylation (81). HAUSP has been shown to deubiquitylate and stabilize p53 by removing its ubiquitin modifications (82). The poly-ubiquitylated p53 is then subjected to proteasomal degradation by the 26S proteasome. The binding of Mdm2 to p53 is inhibited by phosphorylation of p53 by ATM-family and Chk-family kinases and phosphorylation of Mdm2 by ATM-family kinases in response to DNA damage (15).

In unstressed cells, in addition to the low levels of the p53 protein, p53 exists in a latent form, inactive for transcription. p53 activation is accomplished by post-translational modifications in response to a variety of intrinsic and extrinsic cellular stress signals. Different cellular stresses result in different patterns of post-translational modifications of p53. The extensive covalent modifications affect p53 stability, oligomerization, sub-cellular localization and association of p53 with cellular factors (12, 22, 47, 120, 151).

The multiple lysine residues at the extreme C-terminal RD of p53 can be post-translationally modified by multiple mechanisms. The five lysine residues of p53 (Lys370, -372, -373, -381, and 382) can be acetylated by CBP/p300 and Lys320 by P/CAF (46, 120). The role of p53 acetylation has been studied extensively. Gu et al. (1997) suggested that CBP/p300 mediated acetylation of p53 can increase p53 sequence-specific DNA-binding activity in vitro (46). However, subsequent studies showed that acetylation does not increase the p53 DNA-binding activity when the protein is assayed for binding to artificially reconstituted chromatin, but instead, p53 acetylation is

important for the recruitment of co-activators (35). Also, the same lysines can be ubiquitylated by E3 ligases Mdm2, p300, Pihr2 and Copl (29, 52, 81). Several studies suggested that acetylation and ubiquitylation of p53 at the C-terminus can regulate p53 stability. In contrast to ubiquitylation, p53 acetylation can stabilize p53 (63, 72). In addition to acetylation and ubiquitylation, the C-terminal lysine residue Lys386 of p53 can be modified by conjugation to small ubiquitin-like protein SUMO-1, however the functional consequence of p53 sumoylation remains controversial (117). Several studies demonstrated both positive and negative roles of sumoylation for p53-mediated transactivation of target genes (42, 117, 123). However, studies by Kwek et al. (2001) did not confirm these observations (77). Interestingly, using a yeast-two-hybrid approach, Dr. Min-Hao Kuo (Michigan State University) found that some Sumo-modifying E1 and E2 enzymes preferentially interact with p53 acetylated at K320. My subsequent studies revealed that sumoylated p53 is enriched in the acetylated p53 population, suggesting that p53 acetylation may be a prerequisite for subsequent p53 sumoylation. Recent studies also showed that the ubiquitin-like protein Nedd8 can be covalently linked to p53 at Lys370, -372, and –373. Neddylation of p53 negatively regulates its transcriptional activity (151). Additionally, methylation of p53 at Lys372 by Set9 methyltransferase has been identified and suggested to stabilize p53 and restrict it to the nucleus (22).

Phosphorylation has also been shown to regulate p53 transcriptional activity. For example, the rapid phosphorylation on the C-terminus on Ser392 in response to UV light (67) may stimulate sequence-specific DNA binding activity of p53 (62, 67). Also, an ATM-dependent dephosphorylation of p53 at Ser376 creates a binding site for 14-3-3 proteins, which can activate sequence-specific DNA binding of p53 (148).

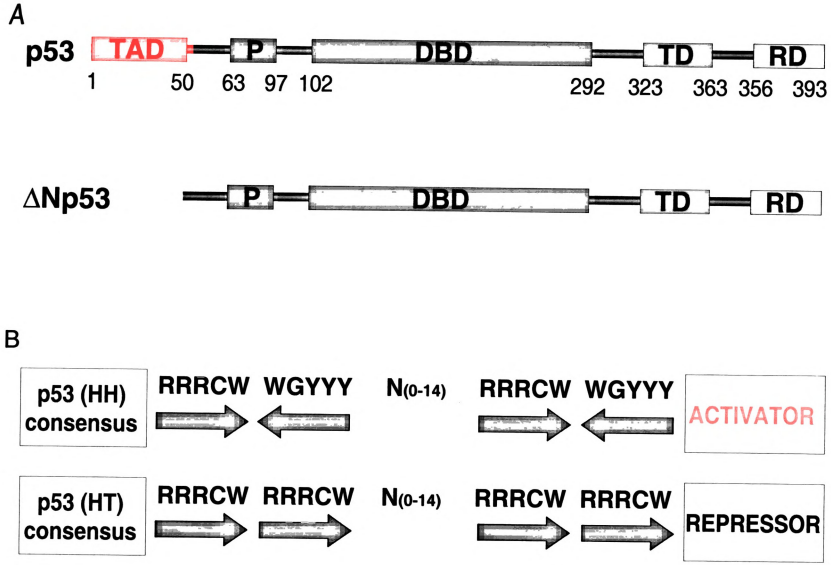
2.5. Structure of the p53 protein

2.5.1. Crystal structure of the p53 domains

The human p53 protein contains 393 amino acids and has been divided structurally and functionally into five domains (Figure 1-3A): (i) the transactivation domain (TAD), (ii) the proline-rich domain, (iii) the DNA binding domain (DBD), (iv) the tetramerization domain (TD) and (v) the regulatory domain (RD) (69). The TAD (amino acids 1-42) is located in the N-terminal part of the protein. Next to a TAD is a proline-rich area (63-97). The proline-rich SH3-target subdomain contains five copies of the amino acid sequence PXXP, which contributes to the apoptotic functions of p53 (121, 146). Sequence-specific DNA binding is mediated through the DBD of p53 (amino acids 102-292). The C-terminal part of p53 is subdivided in the TD (amino acids 326-353) and the RD (amino acids 363-393) (69).

Studies on the structural organization of p53 domains revealed that both TAD and RD of p53 are natively unfolded (8, 28). The structure of the DBD and TD were determined both by X-ray crystallography and NMR (19, 25, 68, 149). The DBD of p53 comprises nine anti-parallel β-strands, which form a β-barrel. β-strands are held close together by three loop regions (L1, L2 and L3). Loops L2 and L3 are stabilized by a zinc atom. In addition to three loops, the DBD of p53 also has a strand-loop-helix region called the SLH motif. It connects the β-barrel with the α-helix at the C-terminus of the DBD of p53. The C-terminal α-helix and loop L1 of p53 bind to the major groove of the DNA. In addition, loop L3 contacts DNA in the minor groove (19). The majority of p53 mutations found in human tumors occur within DBD, including six hot spot mutations most commonly found in human cancers. Two of these hot spot mutations (R248 within

Figure 1-3. Schematic representation of the structure of p53 and ΔNp53 proteins and the consensus DNA binding sites. (A) Schematic representation of the tumor suppressor protein p53 and ΔNp53: TAD = Transactivation domain, P = Proline-rich region, DBD = DNA binding domain, TD = tetramerization domain, RD = regulatory domain. (B) Schematic of p53 consensus DNA binding sites. The consensus p53 DNA binding site consists of 2 half sites, each comprised of two copies of the sequence 5'-Pu-Pu-Pu-C-A/T-3' (Pu is purine), arranged head-to-head (HH) (for p53 acting as a transcriptional activator) or head-to-tail (HT) (for p53 acting as a transcriptional repressor), and separated by 0 to 14 nucleotides.


A
p53
TAD
P
DBD
TD
RD
1
50
63
97
102
292
323
363
356
393
ΔNp53
P
DBD
TD
RD
B
p53 (HH) consensus
RRRCW
WGYYY
N(0-14)
RRRCW
WGYYY
ACTIVATOR
p53 (HT) consensus
RRRCW
RRRCW
N(0-14)
RRRCW
RRRCW
REPRESSOR

L3 and R273 within the C-terminal α-helix) contact DNA directly. The other four (R175, G245, R249 and R282) stabilize the surrounding protein structure (19, 149). The TD is represented by a β-strand linked to an α-helix. A TD monomer has a V-shape. Two monomers form a dimer through interactions between the β-strands and the α-helices arranged in an anti-parallel fashion. The interactions that hold two monomers together as dimers are mainly provided through hydrogen bonds in the β-strands and hydrophobic interactions from both the β-strands and the α-helices. Two dimers are held together as a tetramer by a large hydrophobic surface of each α-helix (25).

2.5.2. DNA binding activity

As shown in figure 1-3B, the consensus p53 DNA binding site consists of 2 half sites, each comprised of two copies of the sequence 5'-Pu-Pu-Pu-C-A/T-3' (Pu is purine), arranged head-to-head (for p53 acting as a transcriptional activator) or head-to-tail (for p53 acting as a transcriptional repressor), and separated by 0 to 14 nucleotides (33, 64). Some variations within consensus PuPuPuC(A/T) sequence are also permissible (115). These specific cognate sites can bind tightly to the DBD of p53. It was suggested that one p53 DBD dimer binds first to one half of the consensus DNA-binding site, increasing the probability for the binding of the second p53 dimer to the adjacent half of the site (95, 96).

In addition to binding specifically to DNA at p53 consensus sites, p53 also binds non-specifically to ssDNA, nicked DNA, damaged DNA with ds breaks, and DNA with Holliday junctions (87). These DNA structures represent the intermediates of DNA damage and repair. Binding to non-specific DNA was primarily mapped to the C-terminal domain of p53 (112, 159). It was generally accepted that the C-terminus of p53

is a negative regulator of p53 sequence-specific DNA binding. Several groups have demonstrated that various alterations of the C-terminal domain (deletion, post-translational modifications and interaction with antibodies directed at a C-terminal epitope) result in an increase of p53 DNA binding (46, 62, 120). However, more recent studies have shown that p53 binds its target sites in vitro and in vivo in the absence of DNA damage or extensive modifications of the C-terminus (6, 35) and the C-terminal-deleted p53 is substantially less efficient at binding and transactivation of its targets in vivo (94). It suggests that the C-terminus does not maintain p53 in a state that is inactive for DNA binding, but rather that it is required for efficient binding of p53 to its target promoters.

2.6. The p53 family

2.6.1. p53/p63/p73

Since the discovery of p53 in 1979, two more members have been added to the p53 superfamily, p63 and p73. The p53 family members share very significant homology both at the genomic and at the protein levels (152). Each contains a TAD, a DBD, and TD. In addition, p63 and p73 contain long C-termini. As a result of the alternative splicing of their C-termini, three p63 isoforms (α to γ) and seven p73 isoforms (α to η) were identified. Additional complexity is also achieved because these isoforms (called the TA and ΔN isoforms, respectively) are transcribed from the upstream promoter as well as from a cryptic promoter within intron 3 (152). The highest level of homology between the p53 family of proteins is reached in the DBD, which suggests that the three proteins can bind to the same DNA sequence and regulate the same genes. Indeed, p63

and p73 bind to p53 consensus DNA elements and trans-activate certain p53 target genes (38). p63 and p73 may also inhibit the transcriptional activity of p53 by competing for the same binding sites on DNA (38). High conservation of the TD sequence between p53 family members results in formation of hetero-oligomers as well as homo-oligomers. ΔNp63 and ΔNp73 forms lack TAD and may inhibit p53 in a dominant-negative fashion through forming hetero-complexes with p53 (101).

Unlike p53, the genes encoding p63 and p73 are rarely mutated in human cancer. The phenotype of the p63- and p73- deficient mice suggests that the primary biological function of these proteins is to regulate development, which is in contrast to p53-null mice, which are highly tumor prone but lack a developmental phenotype (101).

2.6.2. Alternative forms of p53

The human ΔNp53 and its murine counterpart p44 are naturally occurring isoforms of p53 (26, 91, 127). ΔNp53 is encoded by the *p53* locus, but uses an alternative translation start site located in exon 4 at codon 40 in human RNA. The resultant 44 kDa protein lacks the corresponding N-terminal amino acids (Figure 1-3A). Choice of start site depends on an interaction between p53 and its cognate RNA, which requires the N-terminal domain of p53 and its DNA binding domain. The complex of the p53 protein with newly synthesized RNA prevents the ribosome at ATG (codon 1) from being activated and translating full-length p53. Instead, translation of ΔNp53 by the ribosome at ATG (codon 41) occurs. The RNA – p53 protein complex is destabilized by Mdm2, which competes with RNA for binding to N-terminus of p53 (127). Since the ΔNp53

lacks the Mdm2 binding site, it is not a subject to Mdm2-mediated degradation. This results in a prolonged half-life of ΔNp53.

ΔNp53 is severely compromised in ability to activate target genes due to complete absence of N-terminal TAD that binds to the basal transcription machinery. Since ΔNp53 is able to hetero-tetramerize with full length p53, the ratio of full-length to ΔNp53 can determine cellular functions of p53. At low levels, ΔNp53 would be exclusively found in tetramers with full-length. At high levels, however, excess short form would also be present as homo-tetramers or even as non-tetrameric forms, such as monomers or dimer, which could have several different effects (127). Homo- and hetero- tetramers of ΔNp53 would be severely compromised in their ability to activate target genes due to complete absence of N-terminal TAD in ΔNp53 (97). Also, non-tetrameric forms of ΔNp53 could replace p53 in transcription-independent activities, such as mitochondrial cytochrome c release, altering the ability of p53 to mediate apoptosis (97).

3. Cockayne Syndrome factor B protein

3.1. Functional interplay with p53

Several studies demonstrated that infection of human cells by Adenovirus type 12 induces specifically four fragile sites including efficiently induced fragility of the U1 and U2 snRNA gene loci and U1 snRNA pseudogenes in several different cell lines and weakly induced fragility of the 5S rRNA gene in primary human embryonic kidney cells (30, 41, 84, 125). Formation of the same fragile sites were also observed after treatment of cells with actinomycin D or cytosine arabinoside C (83, 90). All these factors are known to activate p53 and do not induce fragility in cells lacking functional p53 (83). In

addition, these fragile sites can be induced by over-expression of the full length wild type p53 or just the C-terminal domain of p53 alone (155). Together, these observations suggest that p53 plays a direct role in fragile site formation at U1 and U2 snRNA and 5S rRNA genes, and the C-terminus of p53 is required for p53-mediated induction of fragile sites. Interestingly, loss of functional CSB in cells also results in fragile site formation at the U1 snRNA, U2 snRNA and 5S rRNA gene loci (155). The requirement of p53 activation or loss of CSB activity for fragile site formation at these loci suggests a possible role for these factors in controlling transcription of these genes, even though U1 and U2 snRNA genes are transcribed by RNA polymerase II and 5S rRNA genes are transcribed by RNA polymerase III. Studies by Selby and Sancar (1997) suggested a role of CSB for elongation of gene, encoding highly structured RNAs (such as U1 and U2 snRNAs) (128). Interestingly, CSB has been shown to interact with p53 within the C-terminal region (147, 155). It was suggested that p53 binding to CSB may interfere with CSB activity; thus, mimicking loss of CSB (155). Yu et al. (2000) proposed that p53 may repress RNA polymerase II transcribed snRNA genes by interfering with Cockayne syndrome group B (CSB)-mediated elongation, causing RNA polymerase II stalling at U1 and U2 snRNA genes. Stalled RNA polymerase protein complexes may interfere with local chromatin condensation, causing locus-specific chromosome fragility (155).

3.2. Functions of CSB

3.2.1. Role in DNA repair

The CSB protein was originally identified as a DNA repair protein and it was cloned on the basis of its ability to complement the transcription coupled repair (TCR)

defect in CS cells (142, 145). It has been proposed that CSB recruits the NER apparatus to sites of stalled RNA polymerase II to permit rapid repair (145). CSB is also critical for the repair of nucleotide base damage induced by reactive oxygen species when such lesions are located on the transcribed strand of active genes (80). CSB chromatin remodeling activity may be required to open chromatin around lesions, thereby stimulating repair. In addition, the CSB protein may also play a role in clearing the stalled RNA polymerase II molecule from the lesion site so that repair can occur and transcription resume (49). The observed interaction of CSB with RNA polymerases I and II both in vivo and in vitro might specifically target CSB to sites of blocked transcription (11).

3.2.2. Role in transcription regulation

Various in vitro and in vivo experiments point to a possible role for CSB in transcription. CSB was shown to associate with RNA polymerase I and II protein complexes (11, 144). Gel mobility shift assays revealed that CSB interacts with a ternary complex of DNA, RNA polymerase II, and nascent RNA (139) and in vitro transcription experiments showed that CSB stimulates elongation by RNA polymerase II (128). It is suggested that CSB functions as an elongation factor to promote transcription of genes encoding RNA products with significant secondary structure, such as human U1 and U2 snRNA genes (155).

Additionally, CSB is also implicated in chromatin remodeling during transcription. It was demonstrated that CSB remodels nucleosomes at the expense of ATP hydrolysis and interacts with core histones in vitro (24). Also, CSB can alter DNA double helix

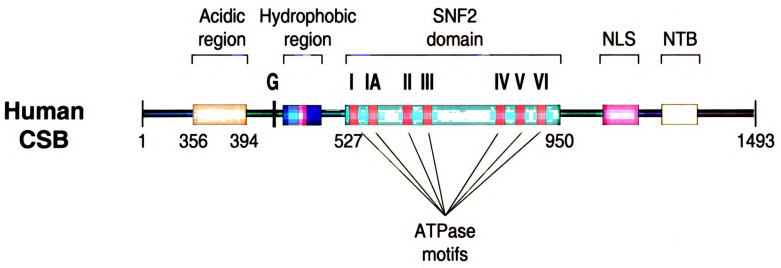
conformation upon binding by wrapping DNA using energy from ATP hydrolysis (7). One possibility is that by changing DNA conformation CSB may disrupt the histone-DNA interactions, as well as the interaction of stalled RNA polymerase with damaged DNA.

3.3. Structure of CSB

The CSB gene encodes a 168 kDa protein, containing several conserved motifs (142). As illustrated in figure 1-4, the central part of CSB is represented by the seven consecutive ATPase motifs I, Ia, II, III, IV, V, and VI from amino acids 527-950. These motifs form a nucleotide-binding fold and are conserved among three superfamilies of RNA and DNA helicases. The region of CSB encompassing these motifs is highly homologous to proteins of the SNF2-like family (32). The N-terminus of CSB has an acidic (A) domain (amino acids 356-394) (142). Between the acidic and SNF2 domains is a glycine-rich (G) stretch and a highly hydrophilic region (H). C-terminal to the ATPase motifs is a putative nucleotide-binding (NTB) domain. The protein also contains a bipartite nuclear-localization signal (NLS). Studies by Christiansen et al. (2005) demonstrated that CSB protein functions as a dimer. The homodimerization occurs via the central ATPase domain of the CSB protein and is essential for ATP hydrolysis by CSB (21).

Of the seven ATPase motifs, motifs I and II contain consensus NTP-binding sequences and are involved in ATP binding by CSB (48). Mutant CSB protein with point mutations in ATPase motifs I and II of CSB cannot complement UV sensitivity,

Figure 1-4. Predicted motifs of CSB: A = Acidic domain, G = glycine-rich stretch, NLS = Nuclear localization sequence, NTB = Nucleotide binding motif.


Acidic region
Hydrophobic region
SNF2 domain
NLS
NTB
G
I IA II III IV V VI
Human CSB
1
356
394
527
950
1493
ATPase motifs

suggesting that ATP hydrolysis by CSB protein is required for TCR of DNA damage (20, 23). Studies on CSB ATPase motif Ia and III mutants revealed only partial complementation of the sensitivity of CS cells towards UV and recovery of RNA synthesis after UV irradiation (104). Studies of homologous proteins suggest the involvement of motifs Ia and III in energy transduction between the ATPase site and the nucleic acid binding site (48). The ATPase motif V and VI CSB mutants exhibit a similar inhibition of ATPase activity and reduced ATP binding in vitro (20). These mutants also have comparable defects in recovery of RNA synthesis after UV irradiation (143). So far, there have been no reported structure-function experiments involving the putative NLS, the glycine-rich stretch, the hydrophobic segment, or the ATPase motif IV. Also, no functions have thus far been assigned to the acidic domain and putative NTP box (13, 137).

3.4. Mutant CSB protein and Cockayne syndrome

Transcription-coupled repair (TCR) is a cellular pathway for the removal of the many DNA lesions that block and arrest transcription. TCR is responsible for the rapid and preferential repair of damage in the transcribed strand of active genes (10). It occurs in both prokaryotic and eukaryotic organisms. In humans, the absence of TCR is associated with Cockayne Syndrome (CS). The majority of CS cases are caused by defects in the Cockayne Syndrome complementation group B (CSB) protein (85).

CS is a premature aging syndrome with complex symptoms, including developmental abnormalities, neurologic disfunction and a short average life span. Cellular characteristics include hypersensitivity to UV light, and failure of RNA synthesis

to recover to normal rates following UV irradiation (93, 107, 145). The photosensitivity of CS patients can be attributed to the TCR defect. It is assumed that accumulation of the DNA damage causes growth arrest and apoptosis, which may explain the progressive course of the disease. Interestingly, CS patients, despite their DNA repair deficiency, do not have a predisposition to cancer. However, CSB deficiency itself has antineoplastic effect in cancer predisposed mice (89). It has been suggested that precancerous cells in CS patients are more efficiently eliminated by apoptosis than they are in healthy person (50, 89). In contrast, neurological and developmental features of this disease can be a result of the problems with transcription (50).

Could misregulation of U1 snRNA gene transcription contribute to development of the CS phenotypes? It was shown that in addition to mutations in *CSB* and *CSA* genes the CS phenotype can result from mutations in *XPB*, *XPD*, and *XPG* genes (133). The products of these genes were shown to be a part or directly associate with the TFIIH protein complex involved in both basal and activated transcription and NER (85), suggesting that misfunctioning of TFIIH may be a common step in developing the CS phenotypes. Interestingly, U1 snRNA was also shown to associate with TFIIH and stimulate transcription initiation by TFIIH (76). Thus, misregulation of U1 snRNA in CSB deficient cells may result in misfunctioning of TFIIH similarly to the *XBP*, *XBD* and *XPG* gene mutations and may contribute to development of the CS disease. Another interesting possibility is that CS patients may additionally experience misregulation of other non-coding RNAs, which may contribute to the pleotropic phenotype of the CS disease.

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CHAPTER TWO

THE P53 TUMOR SUPPRESSOR PROTEIN REPRESSES HUMAN SNRNA GENE TRANSCRIPTION BY RNA POLYMERASES II AND III INDEPENDENTLY OF SEQUENCE-SPECIFIC DNA BINDING[1]

Abstract

Human U1 and U6 snRNA genes are transcribed by RNA polymerases II and III, respectively. While the p53 tumor suppressor protein is a general repressor of RNA polymerase III transcription, whether p53 regulates snRNA gene transcription by RNA polymerase II is uncertain. The data presented herein indicate that p53 is an effective repressor of snRNA gene transcription by both polymerases. Both U1 and U6 transcription in vitro is repressed by recombinant p53, and endogenous p53 occupancy at these promoters is stimulated by UV light. In response to UV light, U1 and U6 transcription is strongly repressed. Human U1 genes, but not U6 genes, contain a high-affinity p53 response element located within the core promoter region. Nonetheless, this element is not required for p53 repression and mutant p53 molecules that do not bind DNA can maintain repression, suggesting a reliance on protein interactions for p53 promoter recruitment. Recruitment may be mediated by the general transcription factors TATA-box binding protein and snRNA-activating protein complex, which interact well with p53 and function for both RNA polymerase II and III transcription.

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Introduction

The p53 tumor suppressor protein plays a critical role in preventing unwarranted cellular proliferation by activating transcription of key target genes that influence cell growth and apoptosis (reviewed in references 28, 31, 35, and 64). Though p53 can enable both pathways, the switch controlling which cellular outcome is enacted is uncertain (reviewed in references 65 and 66), but both the p53 level and the nature of the DNA damage can influence apoptotic response (8). Altogether, p53 activity serves to prevent passage of mutations to daughter cells after DNA damage.

Recent evidence suggests that p53 regulates transcription of genes that are not obviously involved in controlling cell cycle arrest or apoptosis. Indeed, p53 can repress RNA polymerase I (3, 72) and III (5, 9) transcription of genes encoding a variety of nontranslated RNAs that play critical roles at numerous points during global gene expression. RNA polymerase III activity is elevated in p53-/- knockout fibroblasts (5) and in a variety of cancer-derived cell lines that lack p53 function (57). However, the mechanism for p53 regulation of RNA polymerase III transcription is controversial. A kinetic analysis of RNA polymerase III repression using p53 expressed from a stably integrated inducible p53 gene suggested that RNA polymerase III repression is mediated indirectly through p53-dependent degradation of TFIIIB (11). In contrast, recombinant p53 can repress in vitro transcription from a variety of RNA polymerase III-specific promoters and can interact with components of the general transcription machinery required for RNA polymerase III transcription (5, 9, 10, 58), indicating that p53 might directly repress transcription by RNA polymerase III.

Within the group of genes transcribed by RNA polymerase III, the human snRNA gene family is intriguing because these genes contain similar sets of promoter elements, and yet only some genes are transcribed by RNA polymerase III while others are transcribed by RNA polymerase II (see references 19, 23, 24, and 42 for review). Regardless of polymerase specificity, human snRNA genes contain a distal sequence element in the upstream promoter region that serves as the recognition element for activator proteins, including Oct-1, STAF, and Sp1 (33, 54). These factors activate transcription from the core promoters that commonly contain a proximal sequence element (PSE). The PSE is directly recognized by a general transcription factor called the snRNA activating protein complex ($SNAP_C$) (52), which is also known as the PSE transcription factor (49). $SNAP_C$ is involved in human snRNA gene transcription by both RNA polymerases II and III (20–22, 51, 69). RNA polymerase III-transcribed snRNA genes also contain a TATA box that serves to recruit the TATA-box binding protein (TBP) as part of an snRNA-specific TFIIIB complex (45, 55, 60).

The conservation of important promoter elements among human snRNA genes suggests that transcription of these genes by RNA polymerases II and III may be coordinately regulated. However, it is not known whether p53 can regulate human snRNA gene transcription by RNA polymerase II. A role for p53 in this process is suggested from two sources. Firstly, in response to UV light treatment, human U1 and U2 snRNA genes exhibit a delayed and prolonged reduction in transcription by RNA polymerase II (14, 47, 48). In part, this reduction may be attributable to increased hyperphosphorylation of the carboxy-terminal domain of the RNA polymerase II largest subunit in response to UV light (27). However, in normal human diploid fibroblasts, the

balance of hyper- and hypophosphorylated RNA polymerase II is restored by 6 h after UV light treatment (46), suggesting additional cellular mechanisms that enable snRNA gene repression after UV light exposure. Potentially, p53 activation by DNA damage might play a direct role in the prolonged repression of these genes.

Secondly, infection of human cells by adenovirus serotype 12 causes metaphase fragility at four chromosomal sites, including the U1 snRNA (RNU1) and U2 snRNA (RNU2) loci (1, 36), in a process that requires p53 (38, 39). It was postulated that fragile site formation occurs during viral infection, because RNA polymerase II stalls at these genes and interferes with chromosome condensation during metaphase (37). Interestingly, p53 that harbors mutations in the DNA binding domain supports fragile site formation (39), and overexpression of the C-terminal domain of p53 alone, which lacks the DNA binding domain, induces fragility during transient transfection (71). Together, these data indicate that p53 is important for generation of fragile sites at the U1 and U2 snRNA gene loci and may play a role in regulation of these genes in a fashion that does not require sequence-specific binding of p53 to DNA.

In this study, the role of p53 in governing human snRNA gene transcription by RNA polymerase II and III was examined. We show that recombinant p53 represses both U1 and U6 transcription by RNA polymerase II and III, respectively. Repression is supported by the C-terminal region of p53 alone, indicating that sequence-specific DNA binding by p53 is not critical for repression. Both the full-length and C terminus of p53 alone can associate with the U1 and U6 promoters during repression, and promoter recruitment may be assisted through interactions with the general transcription factors $SNAP_C$ and TBP, which are commonly required for transcription of both U1 and U6

snRNA genes. In vivo, p53 can bind to both U1 and U6 snRNA genes in untreated human MCF-7 cells, and promoter occupancy is stimulated after UV light treatment. These results further indicate that p53 contributes to snRNA gene regulation in response to DNA damage.

Materials and Methods

Cell culture and UV irradiation

Human MCF-7 and HeLa cells were maintained in Dulbecco's modified Eagle's medium supplemented with penicillinstreptomycin and 10% (MCF-7) or 5% (HeLa) fetal bovine serum. Cells grown to 70 to 80% confluence were washed with phosphate-buffered saline and irradiated with 50 J of UV light (254-nm peak)/m^2 by using a UV Stratalinker (Stratagene). After irradiation, growth medium was added and cells were incubated at 37°C under 5% CO_2 for the indicated times. Additionally, HeLa cells were grown to 50% confluence in 150-mm plates and were then transiently transfected with 2.5 μg of the pRc/RSV or pRc/RSV-p53-Flag.wt plasmids by using Lipofectin reagent (Invitrogen) for 6 h. Subsequently, the medium was replaced and cells were incubated for 48 h for further analysis in nuclear run-on assays.

Nuclear run-on assays

Nuclear run-on assays were performed as described elsewhere (6) in the presence of [α-^{32}P]UTP using approximately 10^7 nuclei that were isolated from MCF-7 or HeLa cells before or 8 h after exposure to UV light. Additional assays were performed using HeLa cells transiently transfected with pRc/RSV or pRc/RSV-p53-Flag.wt as described.

Labeled RNA was recovered and hybridized to a nitrocellulose membrane containing approximately 7 μg of U1, U6, and 5S rRNA target gene DNAs or 10 μg of pUC119 plasmid, as a negative control. Target gene DNAs corresponding to the coding regions of the indicated genes were generated by PCR and were immobilized on a nylon membrane at levels calculated to be in excess relative to the corresponding snRNA population in the nuclei. Hybridizations were performed for 16 h at 42°C in hybridization buffer containing 50% formamide. Membranes were then washed extensively in 2X SSC (1X SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.1% sodium dodecyl sulfate (SDS) at 50°C. Hybridized RNA transcripts were visualized by autoradiography for 7 days. Similar results were observed when signals were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression by varying the exposure time (data not shown).

RNA isolation and RT-PCR

RNA was isolated using the TRIzol reagent as recommended by the manufacturer (Gibco-BRL). RNA preparations were quantified by UV spectrometry and examined for integrity by agarose formaldehyde morpholinepropanesulfonic acid gel electrophoresis. Reverse transcription-PCR (RT-PCR) was performed by a two-step procedure using U1-, U6-, and GAPDH-specific primers. The primers used for amplification of each gene were the following: U1 forward, 5'-ATACTTACCTGGCAGGGGAG-3'; U1 reverse, 5'-CAGGGGGAAAGCGCGAACGCA-3'; U6 forward, 5'-GGAATCTAGAACATATACT AAAATTGGAAC-3'; U6 reverse, 5'-GGAACTCGAGTTTGCGTGTCATCCTTGCGC-3'; GAPDH forward, 5'-AGGTCATCCCTGAGCTGAAC-3'; and GAPDH reverse, 5'-GCAATGCCAGCCCCAGCGTC-3'.

Expression and purification of recombinant proteins

Glutathione *S*-transferase (GST), GST-tagged full-length human p53, and a GST-tagged C terminus of human p53 (amino acids 301 to 393) [p53 (301-393)] were expressed in *Escherichia coli* BL21(DE3) codon+ cells (Stratagene) and were affinity purified by binding to glutathione agarose beads (Sigma). GST and GST-p53 were then eluted from beads in HEMGT-150 buffer containing 50 mM glutathione for 4 h at 4°C or, alternatively, untagged p53 was obtained by digestion with thrombin. Proteins were further purified by chromatography using a Mono-Q (HR5/5) column (Pharmacia) and were concentrated by centrifugation using a Centricon YM-30 spin column (Millipore) in HEMGT-80 buffer (20 mM HEPES [pH 7.9], 0.1 mM EDTA, 10 mM MgCl2, 10% glycerol [vol/vol], 0.1% Tween 20, 80 mM KCl) containing protease inhibitors and 1 mM dithiothreitol.

In vitro transcription assays

In vitro transcription assays were performed as described previously (21, 43) using 18, 2, 2, and 10 μL of HeLa cell nuclear extract for the U1 snRNA, U6 snRNA, 5S rRNA, and adenovirus major late promoter (AdML) transcription reactions, respectively. The pU1-4.0 (1 μg), pU6/Hae/RA.2 (250 ng), pH5Ssa (250 ng), and M13-AdML (250 ng) templates were used for the U1 snRNA, U6 snRNA, 5S rRNA, and AdML transcription reactions, respectively. Purified p53 or GST-tagged p53 proteins were added in the amounts indicated in the figure legends. Transcription reactions were performed for 1 h at 30°C. Transcripts were separated by denaturing 6% polyacrylamide gel electrophoresis (PAGE) and visualized by autoradiography.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation assays were performed as described previously (25). Human MCF-7 cells were grown to 60 to 80% confluence and were then cross-linked with 1% formaldehyde for 30 min at room temperature. After cell lysis and sonication, immunoprecipitation reactions were performed overnight at 4°C using chromatin from approximately 10^7 cells per reaction mixture and 1 μg of each antibody. The anti-p53 antibodies used were the following: anti-p53 (21-25) (Ab-6; Oncogene), anti-p53 (371-380) (Ab-1; Oncogene), anti-p53 (213-217) (Ab240; Pharmingen), anti-acetyl-p53 (373-382) (Upstate), and anti-acetyl-p53 (320) (Upstate). Recovered chromatin was suspended in 50 μL of H_2O, and PCR analysis was performed using 5 μL of immunoprecipitated chromatin or input chromatin. The primers used for amplification of each gene were the following: U1 forward, 5'-CACGAAGGAGTTCCCGTG-3'; U1 reverse, 5'-CCCTGCCAGGTAAGTATG-3'; U2 forward, 5'-AGGGCGTCAATAGCGC TGTGG-3'; U2 reverse, 5'-TGCGCTCGCCTTCGCGCCCGCCG-3'; U6 forward, 5'-GTACAAAATACGTGACGTAGAAAG-3'; U6 reverse, 5'-GGTGTTTCGTCCTTTCC AC-3'; GAPDH forward, 5'AGGTCATCCCTGAGCTGAAC-3'; GAPDH reverse, 5'-GCAATGCCAGCCCCAGCGTC-3'; U1 upstream forward, 5'-GAACTTACTGGGATC TGG-3'; U1 upstream reverse, 5'-GAGACAACTGAGCCACTTG-3'; p21 upstream forward, 5'-CCGCTCGAGCCCTGTCGCAAGGATCC-3'; p21 upstream reverse, 5'-GGGAGGAAGGGGATGGTAG-3'. PCR products were separated by 2% agarose electrophoresis in Tris-borate-EDTA buffer and were stained with ethidium bromide.

Immunoprecipitations from in vitro transcription reactions

In vitro transcription assay mixtures containing U1 or U6 promoter plasmids and equal molar amounts of pUC119 were performed as described previously (25) in the absence or presence of full-length GST-p53 (wild-type or R175H), GST-p53 (301-393), or GST. Five microliters of each transcription reaction mixture was diluted to 500 μL and was cross-linked in 1% formaldehyde for 10 min at room temperature, quenched with 125 mM glycine for 10 min at room temperature, and immunoprecipitated with immunoglobulin G (IgG), anti-SNAP43 (CS48), or anti-p53 (Ab-1; Oncogene) antibodies. Recovered plasmid DNA was analyzed by PCR using primers specific to the U1 and U6 promoter regions or to pUC119 as a negative control.

DNase I footprinting

Footprinting assays were generally performed as described elsewhere (4). Linear DNA encompassing the human U1 promoter from -151 to +13 was generated by PCR using primers that were end labeled with [γ-^{32}P]ATP by using T4 polynucleotide kinase (New England BioLabs). U1 promoter probes were incubated with increasing amounts of recombinant GST-p53 for 40 min at room temperature and were then digested with 0.04 U of DNase I (Roche) for 2 min at room temperature. The resultant fragments were purified and separated by 8% denaturing PAGE. Footprints were visualized by autoradiography and were mapped relative to sequencing ladders generated from the same labeled primers used to generate the U1 promoter probes.

EMSA

Electrophoretic mobility shift assays (EMSA) were performed as described elsewhere (7). The amounts of p53 used are indicated in the figure legends. Reaction mixtures were incubated at room temperature for 20 min prior to addition of radiolabeled probes. Unless otherwise noted, the U1 probe encompasses -312 to +13 and the U6 probe encompasses -267 to +1. DNA binding reactions were carried out at room temperature for 20 min, and resulting DNA-protein complexes were separated on a 4% polyacrylamide gel in 0.5X Tris-borate-EDTA running buffer at 150 V. Complexes were visualized by autoradiography.

Coimmunoprecipitation and GST pull-down experiments

GST pull-down assays were performed as previously described (25). Coimmunoprecipitation assays were performed using 5 mg of total protein contained in MCF-7 nuclear extracts from untreated or UV-treated cells and 2 μg of rabbit anti-SNAP43 antibodies (CS48) (22). Western blot analyses of recovered proteins were performed using anti-SNAP43 (CS48), anti-p53 (Ab-6; Oncogene), and anti-galectin-3 (Mac2) antibodies. The reciprocal immunoprecipitation reactions were performed using 2 μg of anti-p53 antibody (Ab-6) with approximately 1.6 or 5 mg of MCF-7 extract, followed by anti-SNAP43 Western blot analysis.

Results

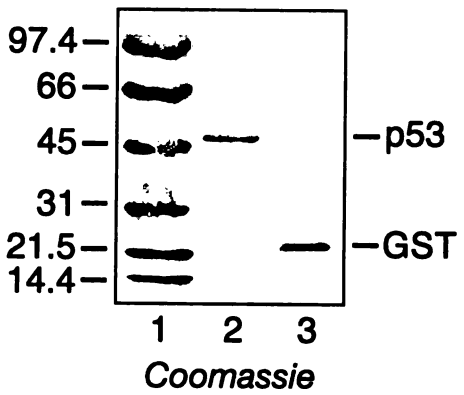
p53 represses human snRNA gene transcription by both RNA polymerases II and III.

To determine whether p53 can repress U1 transcription by RNA polymerase II, as has been previously shown for U6 transcription by RNA polymerase III (5, 9), the effect of recombinant p53 on human U1 in vitro transcription was tested. The recombinant full-length wild-type p53 and the GST proteins used for these experiments are shown in Fig. 1A. As shown in Fig. 1B, p53 effectively repressed correctly initiated U1 transcription (labeled U1 5') by RNA polymerase II, and this repressive effect was specific, because concomitant RNA polymerase II transcription of an mRNA read-through transcript derived from the same plasmid was unaffected in these reactions. As a positive control for p53 activity, human U6 snRNA gene transcription was tested. Indeed, the same amounts of p53 effectively repressed U6 snRNA transcription by RNA polymerase III, while RNA polymerase II transcription from the AdML was unaffected. Therefore, p53 can repress human snRNA gene transcription by both RNA polymerases II and III.

As a first step towards understanding the mechanism for p53 repression of U1 and U6 transcription, a time course for p53 repression was performed (Fig. 1C). As a positive control, p53 repression of 5S rRNA gene transcription was also examined. To ensure maximal repression, an excess of p53 was used, because 5S rRNA gene transcription appears less sensitive to p53 repression (9) (data not shown). As was demonstrated previously (5), p53 can repress 5S rRNA transcription when added to reactions concomitantly with nuclear extract (lane 2) or nuclear extract plus template DNA (lane 3) prior to initiation of transcription by nucleotide addition. However, p53 did not repress

Figure 2-1. p53 represses human snRNA gene transcription by both RNA polymerases II and III in vitro. (A) Recombinant full-length wild-type p53 and GST proteins were separated by SDS–12.5% PAGE and were stained with Coomassie blue. (B) In vitro transcription from U1, U6, and AdML promoter constructs was tested using HeLa nuclear extracts containing 0, 50, 200, and 800 ng of p53 (lanes 1 to 4) or 800 ng of GST (lane 5). Fifty nanograms of p53 represents an approximate 2:1 molar ratio of monomeric p53 to U1 promoter template DNA and an approximate 8:1 molar ratio to the U6 and AdML promoter plasmids. p53 effectively repressed correctly initiated U1 transcription (U1 5) and U6 transcription (U6 5), but it didn't affect read-through (RT) transcription from the U1 reporter plasmid or transcription from the AdML promoter. (C) U1, U6, and 5S rRNA in vitro transcription reaction mixtures were supplemented with 800 ng of active or heat-inactivated p53 (lanes 2 to 4 and lanes 5 to 7, respectively) at different times, as indicated. Transcription was allowed to proceed for an additional 60 min. Recombinant p53 repressed U6 gene transcription both prior to and after preinitiation complex assembly but did not repress U1 and 5S rRNA gene transcription after the formation of a preinitiation complex.

A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GST
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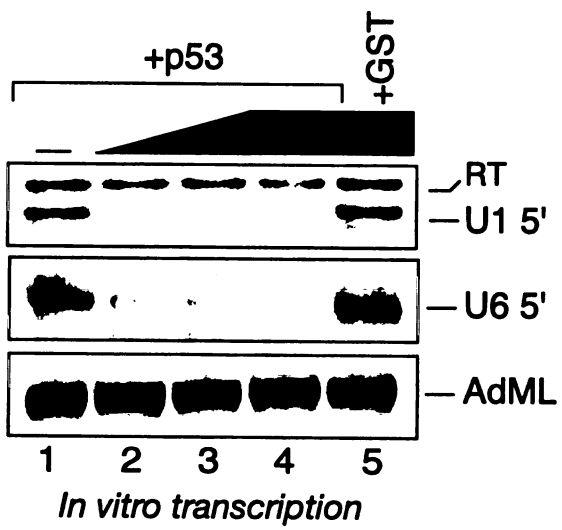
Coomassie

B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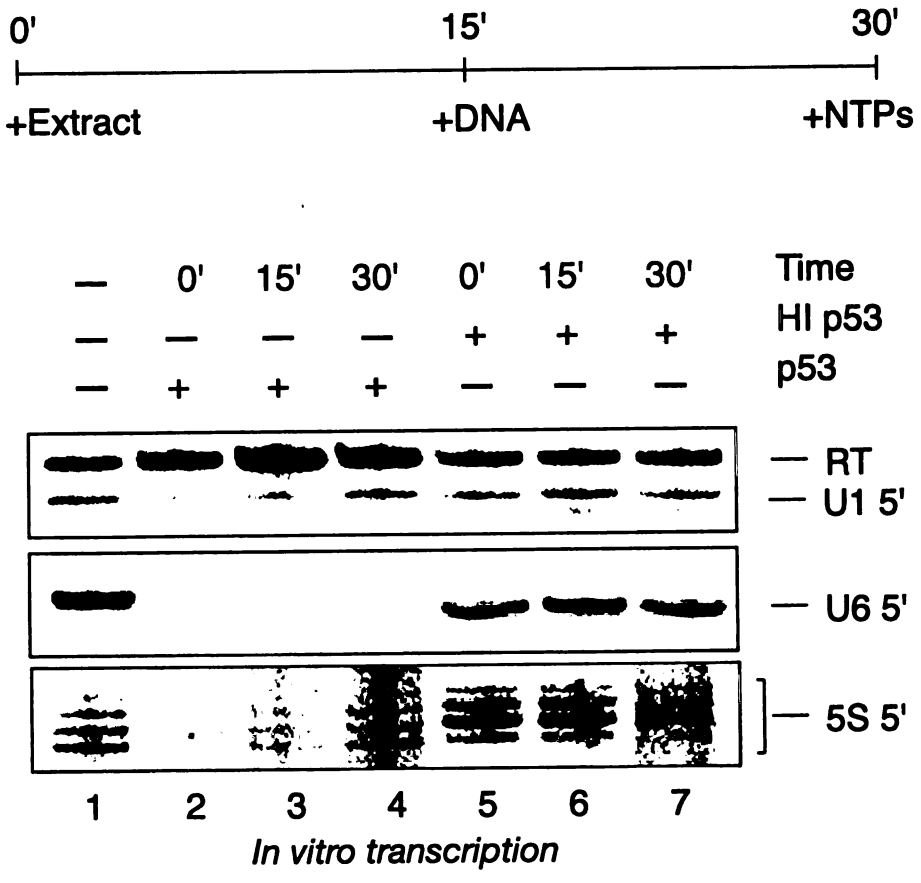
+GST
RT
U1 5'
U6 5'
AdML
1 2 3 4 5
In vitro transcription

C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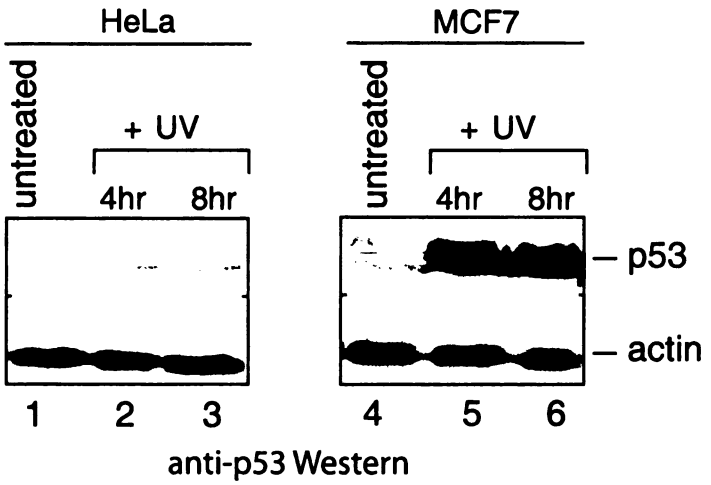
15'
30'
+Extract
+DNA
+NTPs
— 0' 15' 30' 0' 15' 30' Time
— — — — + + + HI p53
— + + + — — — p53
— RT
— U1 5'
— U6 5'
— 5S 5'
1 2 3 4 5 6 7
In vitro transcription

5S rRNA transcription when the nuclear extract was preincubated with template DNA prior to p53 addition (lane 4). Presumably, p53 cannot repress 5S rRNA gene transcription after the formation of a preinitiation complex. For all time points, repression was specific for functional p53, because repression was disabled by heat inactivation of p53 (lanes 5 to 7). In contrast, p53 could effectively repress U6 transcription by RNA polymerase III even after the nuclear extract had been preincubated with the template DNA. This result suggests that the U6 preinitiation complex is not recalcitrant to p53 repression. Surprisingly, the pattern for p53 repression of U1 transcription by RNA polymerase II was similar to that of 5S rRNA rather than the U6 repression pattern. This observation suggests that formation of a preinitiation complex could render U1 snRNA genes refractory to p53 repression. This result also suggests that p53 represses U1 and U6 snRNA gene transcription by different mechanisms.

As UV light exposure activates p53, this treatment was used here to determine whether p53 is involved in human snRNA transcriptional regulation in vivo. The majority of in vivo studies presented herein were performed with human MCF-7 breast adenocarcinoma cells, because these cells exhibit a robust increase in p53 levels in response to UV light treatment (Fig. 2A, lanes 4 to 6) compared to human HeLa cervical carcinoma cells, wherein p53 levels are low and remain unchanged after UV light exposure (lanes 1 to 3).

To determine the effect of UV light on snRNA gene transcription, nuclear run-on experiments were performed using nuclei harvested from HeLa and MCF-7 cells before and 8 h after UV light exposure. As shown in Fig. 2B, UV light treatment of HeLa cells did not substantially affect U1 transcription by RNA polymerase II compared to cells that

A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untreated
+ UV
4hr
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+ UV
4hr
8hr
p53
actin
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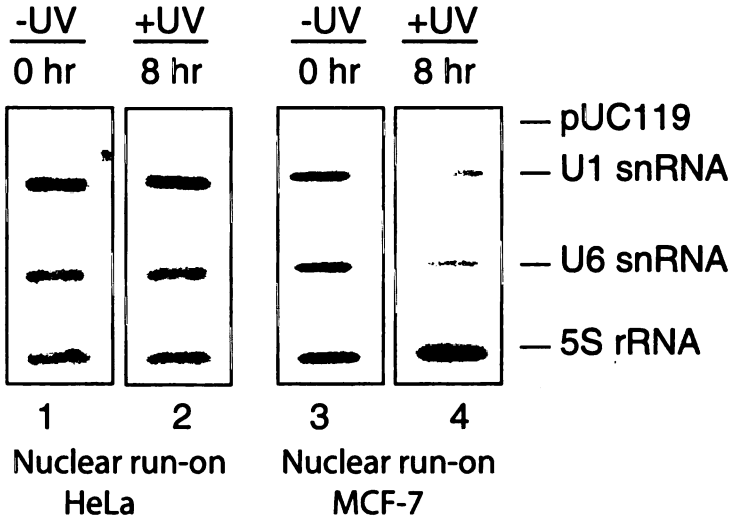
2
3
4
5
6
anti-p53 Western

B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8 hr
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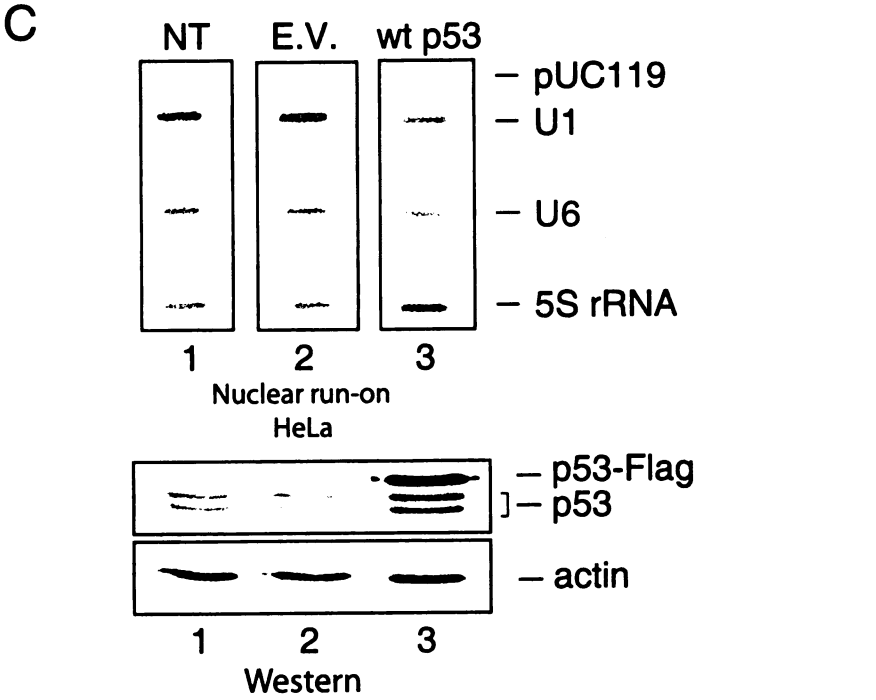
+UV
0 hr
8 hr
pUC119
U1 snRNA
U6 snRNA
5S rRNA
1
2
3
4
Nuclear run-on
HeLa
Nuclear run-on
MCF-7

Figure 2-2. UV light inhibits snRNA gene transcription and stimulates p53 binding to human snRNA gene promoters. (A) Whole-cell extracts from untreated and UV light-treated HeLa (lanes 1 to 3) and MCF-7 (lanes 4 to 6) cells were analyzed by SDS–12.5% PAGE and Western blot analysis of endogenous p53 and actin. MCF-7 cells exhibited robust accumulation of endogenous p53 in response to UV light treatment, whereas no change was observed in HeLa cells. (B) UV light represses transcription of endogenous human U1 and U6 snRNA genes in MCF-7 cells, but not in HeLa cells. Nuclear run-on assays measuring polymerase density at U1 snRNA, U6 snRNA, and 5S rRNA genes in nuclei from untreated HeLa cells (lane 1) or MCF-7 cells (lane 3) were compared to results with nuclei harvested 8 h after UV light treatment (lanes 2 and 4). After hybridization, membranes were exposed to film for 7 days. Similar trends were also obtained when exposure times were varied to normalize to GAPDH gene transcription, which was unaffected by UV light treatment in these assays (data not shown). (C) Transiently transfected p53 represses U1 snRNA gene transcription in HeLa cells. Nuclear run-on assays were performed on HeLa cells (lane 1) or HeLa cells transiently transfected with either the empty vector pRC/RSV (lane 2) or pRC-RSV expressing wild-type full-length Flag-tagged p53 (pRc/RSV-p53-Flag) (lane 3). Levels of p53 expression were determined by Western blotting (bottom panel). (D) Endogenous p53 associates with human snRNA gene promoters. Chromatin immunoprecipitation experiments were performed using chromatin harvested from MCF-7 cells prior to or 8 h after UV light treatment and using antibodies directed against SNAP43 (lane 3), various epitopes within p53 (lanes 4 to 7), and nonspecific IgG (lane 8) as a negative control. Enrichment of U1 and U6 promoter regions was measured by PCR and was compared to the p21 promoter (-1.4 kb site), as a positive control, and the U1 upstream region and GAPDH exon 2, as negative controls. (E) Endogenous p53 was not detected at human snRNA gene promoters in untreated or UV light-treated HeLa cells. Chromatin immunoprecipitation experiments were performed using HeLa cell chromatin with the indicated antibodies. (F) UV light causes a decrease in steady-state U1 snRNA levels. (Top panel) Total RNA was isolated from untreated MCF-7 cells and was titrated (0, 0.1, 0.3, 1, 3, and 10 ng [lanes 2 through 7, respectively]) into RT-PCRs performed using U1 gene-specific primers. The amount of U1 cDNA amplification is proportional to the amount of total RNA used for RT-PCR. (Bottom panel) Steady-state levels of U1, U6, and GAPDH RNA were measured by RT-PCR using 2 ng, 10 ng, and 1 μg of total RNA, respectively, harvested before (lane 1) or after (lanes 2 to 7) UV light treatment.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– pUC119
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3
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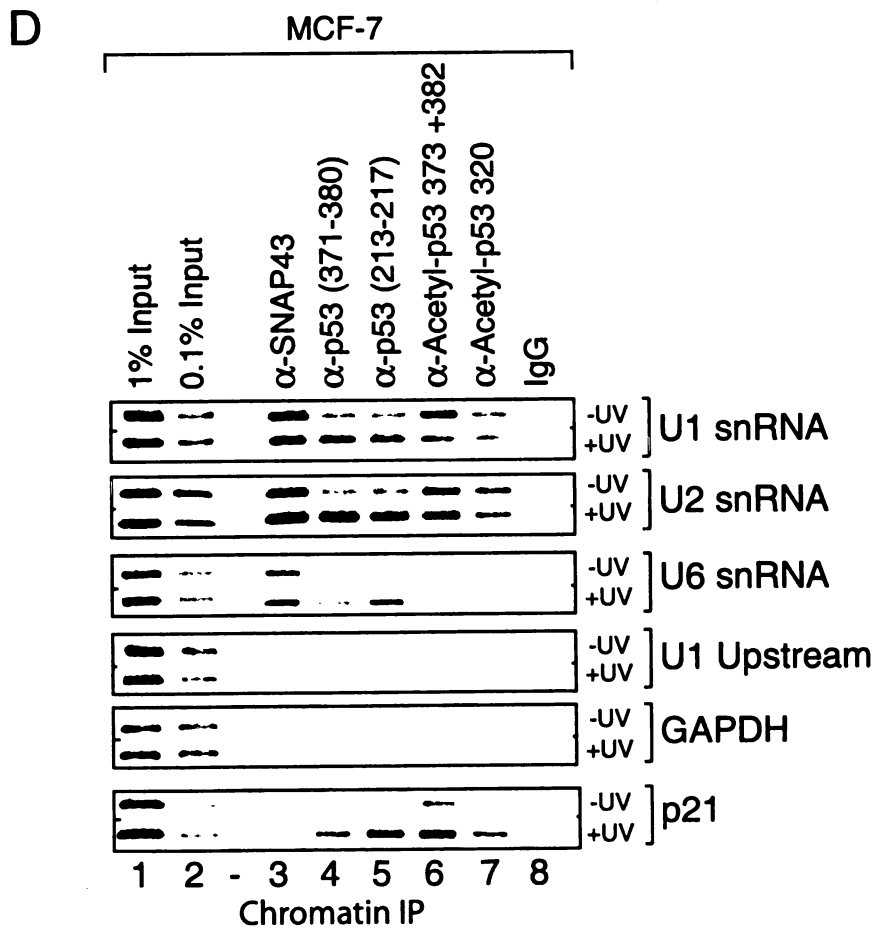
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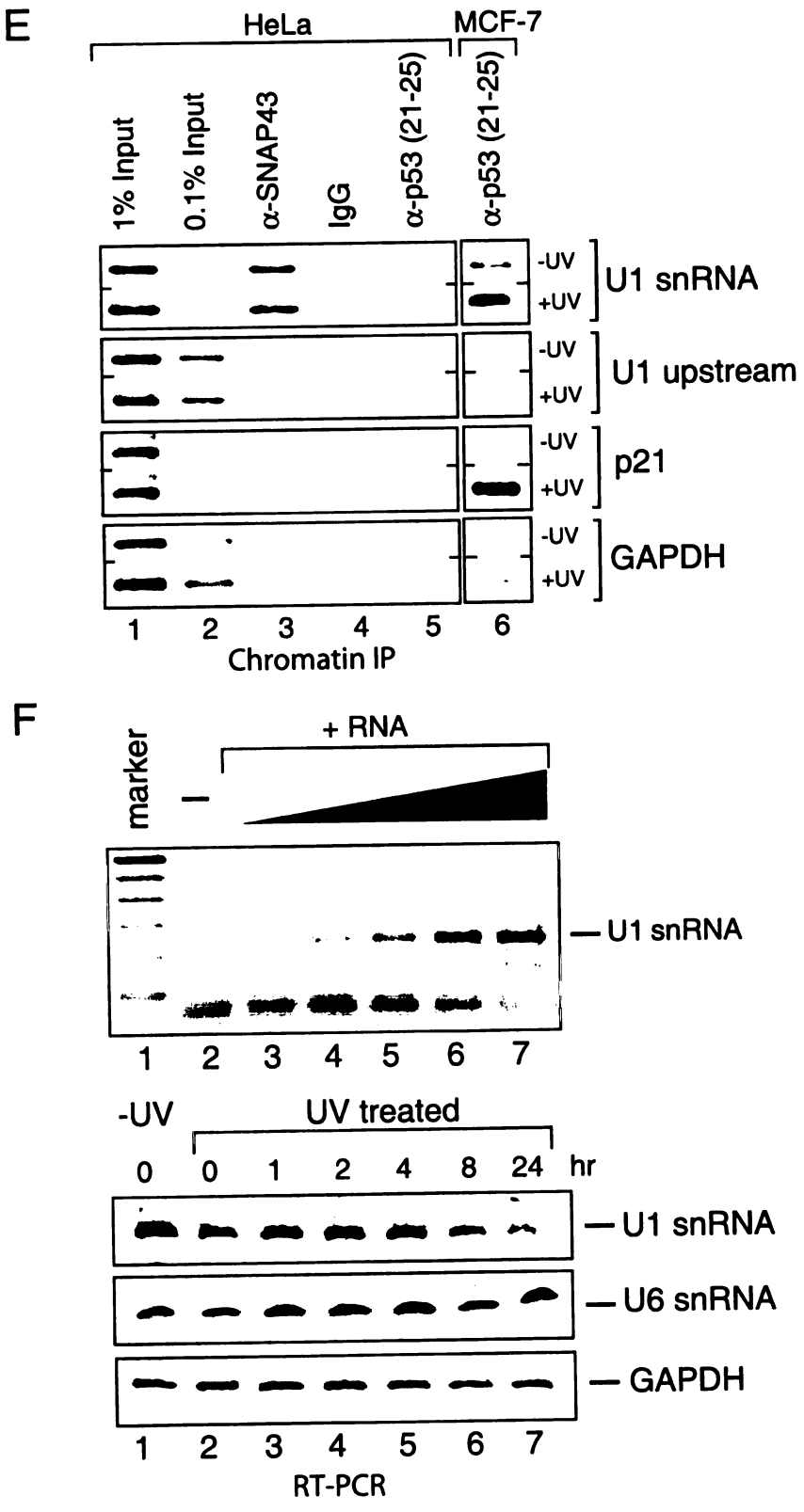
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-UV
+UV
U1 snRNA
U1 upstream
p21
GAPDH
1 2 3 4 5 6
Chromatin IP
F
marker
+ RNA
U1 snRNA
1 2 3 4 5 6 7
-UV
UV treated
0 0 1 2 4 8 24 hr
U1 snRNA
U6 snRNA
GAPDH
1 2 3 4 5 6 7
RT-PCR

did not receive UV light exposure. Similarly, U6 snRNA and 5S rRNA transcription by RNA polymerase III was unaffected, suggesting that the transcription of these genes is insensitive to UV light. These results for U1 transcription are in contrast with that previously described wherein U1 transcription in nuclear run-on assays was markedly reduced 2 h after UV light treatment of HeLa cells (48). In the present study, the 8-h posttreatment time point was selected because RNA polymerase II is ubiquitylated and degraded in response to UV light but normal levels are restored by 6 h after UV light treatment (46). Additionally, a longer recovery period after UV light treatment was desirable to allow sufficient time for DNA damage repair.

In contrast with HeLa cells, MCF-7 cells exhibited a marked reduction in U1 transcription 8 h after UV light treatment. Additional studies revealed that repression was already established by 4 h posttreatment (data not shown). Interestingly, UV light elicited different effects on RNA polymerase III-transcribed genes, causing reduced U6 transcription while stimulating 5S rRNA transcription. In all cases, the signals detected for these transcripts are specific, because no hybridization to pUC119 was detected in any of these experiments. Therefore, U1 and U6 transcription exhibits cell type-specific responses to UV light treatment, with UV light invoking a prolonged repressive effect in MCF-7 cells but not in HeLa cells. In two independent replicates of this experiment, U1 and U6 transcription levels were reduced to 42 and 39%, respectively, of the untreated sample levels, whereas 5S RNA transcription rates were increased to 170%. Stimulated 5S rRNA gene transcription under these conditions was unexpected but does indicate that the 5S rRNA transcriptional response to DNA damage depends upon cellular p53 status.

To determine whether p53 contributes to regulation of endogenous snRNA genes, p53 was overexpressed in HeLa cells and the effect on endogenous U1, U6, and 5S rRNA gene transcription was again measured by nuclear run-on assays. As shown in Fig. 2C, increased p53 expression was correlated with diminished U1 transcription. In contrast, U6 transcription was unaffected, whereas 5S rRNA transcription was stimulated. The reason for the unresponsiveness of U6 transcription to p53 expression is unknown, but p53 may require additional UV light-stimulated modification for activity at this gene. Interestingly, the expression pattern for U1 snRNA and 5S rRNA transcription in response to p53 expression is similar to that observed with UV light treatment of MCF-7 cells, consistent with the idea that p53 regulates these genes in response to DNA damage.

Endogenous p53 associates with human snRNA gene promoters.

Chromatin immunoprecipitation experiments were then performed to determine whether p53 is directly involved in the regulation of endogenous snRNA genes in response to UV light. As shown in Fig. 2D, substantial enrichment of U1 and U2 snRNA promoter DNA was observed in anti-p53-immunoprecipitated samples (lanes 4 and 5) with chromatin harvested from MCF-7 cells prior to UV light treatment, and these levels were markedly enriched by using chromatin harvested from cells 8 h after UV light treatment. In contrast, only low levels of U6 promoter DNA were enriched in the anti-p53-immunoprecipitated samples prior to UV light treatment, but promoter recovery was noticeably enhanced after treatment.

Previously, it was shown that p53 is acetylated within its C terminus in response to DNA damage, which may stimulate DNA binding by p53 (18, 44) and increase

recruitment of coregulatory proteins (2). Furthermore, acetylation but not phosphorylation of p53 within the C-terminal domain contributes to fragile site formation at the U2 snRNA gene loci, indicating that p53 acetylation may be important for p53 function at snRNA genes (71). Therefore, immunoprecipitation reactions were also performed with antibodies that specifically recognize p53 acetylated at K320 or at K372 and K382 (Fig. 2D). Interestingly, significant levels of U1 and U2 promoter enrichment were observed with antibodies that recognize acetylated p53 (lanes 5 and 6), but UV light treatment either did not affect promoter enrichment or caused a modest reduction. In contrast, no significant recovery of U6 promoter DNA was obtained with antibodies that recognize acetylated p53. Enrichment of the p21 promoter (-1.4 kb site) in the anti-p53-immunoprecipitated samples was low prior to UV light treatment, but recovery increased significantly after treatment, as has been previously demonstrated (30). UV light treatment also resulted in increased p21 promoter enrichment for those reactions performed using anti-acetylated p53 antibodies. Together, these data indicate that p53 associates with the endogenous U1 and U2 snRNA gene promoters prior to genotypic stress and UV light stimulates p53 association with these promoters, although the apparent proportion of p53 that is acetylated decreases. Second, low levels of p53 associate with the U6 promoter prior to stress and UV light stimulates p53 promoter association, but this p53 is not acetylated to a significant degree. These observations are in contrast to those seen with the p21 promoter, where p53 association is low but the total p53 level and proportion that is acetylated increase in response to UV light treatment. The data in Fig. 2E show that neither U1 nor p21 promoter association by p53 was observed in HeLa cells (lane 5) using an anti-p53 antibody that efficiently recovered

these DNA segments from MCF-7 cells (lane 6). As previously observed for MCF-7 cells, UV light also did not affect $SNAP_C$ occupancy at the U1 promoter in HeLa cells (lane 3).

Human snRNA molecules are abundant and very stable (13, 17, 68) and, thus, it is not clear what effect diminished U1 transcription would have on overall U1 snRNA levels. Therefore, RT-PCR assays were employed to determine the effect of UV light on steady-state U1 and U6 snRNA levels. As shown in Fig. 2F (top panel), addition of increasing amounts of total cellular RNA harvested from untreated MCF-7 cells resulted in a linear amplification of U1 sequences (lanes 3 to 7), thus demonstrating that this assay is suitable for measuring changes in steady-state U1 snRNA levels. Similar preliminary experiments were performed to determine the range for linear amplification of both U6 snRNA and GAPDH mRNA (data not shown). Under these conditions, U1 steady-state levels were noticeably reduced 8 h after UV light treatment, whereas U6 snRNA and GAPDH mRNA levels remained relatively stable before and after treatment. In three independent replicates of this experiment, the steady-state level of U1 snRNA at 8 h posttreatment was 43% of levels in untreated cells (data not shown). The reduction in U1 steady-state levels in response to UV light could be attributable to increased degradation of this RNA, decreased transcription from U1 snRNA genes, or a combination of both factors. U1 snRNA is traditionally viewed as extremely stable, with a half-life greater than 24 h and, thus, little change was expected in steady-state U1 levels by 8 h, even if transcription were completely repressed. The kinetics of the decrease in U1 steady-state levels and the results shown in Fig. 2B suggest that both a reduction in U1 transcription and an increase in U1 snRNA degradation contribute to reduced U1 snRNA levels after UV light treatment. Together, these results indicate that UV light initiates a complicated

network of control governing expression of human snRNA genes.

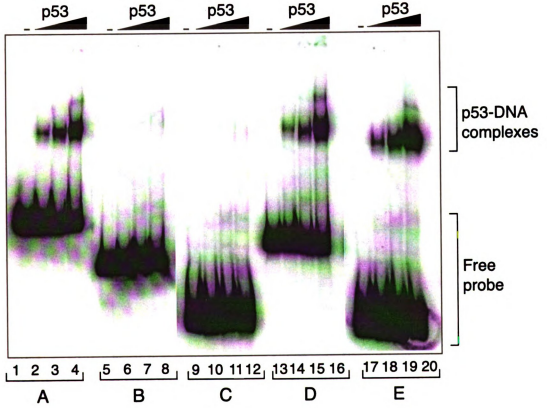
Human U1 snRNA gene core promoters contain a high affinity p53 binding site.

As a first step towards understanding the mechanism for p53 repression, EMSA were performed to determine whether p53 could bind directly to human U1 snRNA gene promoters. Indeed, recombinant p53 bound extremely well to a U1 probe encompassing the region from -422 to +13 of the promoter (Fig. 3A, lanes 2 to 4). Competition experiments suggest that p53 affinity for the U1 promoter is comparable to the p53 binding element contained within the GADD45 promoter (data not shown). Interestingly, two different complexes formed on the U1 promoter probe, suggesting that the U1 promoter may contain two p53 binding elements. The protein-DNA complexes formed on these probes are due to p53, because inclusion of anti-p53 antibodies in the DNA binding reactions retarded the migration of these complexes (data not shown). To determine the location of the high-affinity p53 binding element, EMSA was also performed with probes containing different regions of the promoter. Strong binding of p53 to DNA was observed in reactions with equivalently labeled probes containing the -312 to +13 and -150 to +13 regions of the promoter. Consistently, low-affinity binding was observed in probes containing the -422 to +152 and -312 to +152 promoter regions. Together, these data indicate that the high-affinity p53 binding element is contained between -150 and +13 of the U1 core promoter.

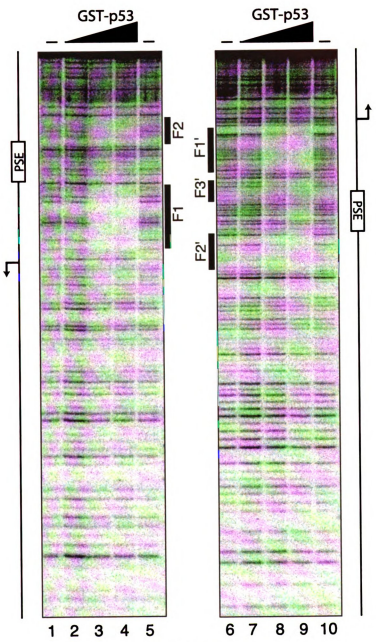
DNase I footprinting experiments were then performed to further map the location of the high-affinity p53 binding element. As shown in Fig. 3B, those reactions in which

Figure 2-3. Human U1 snRNA gene core promoters contain a high-affinity p53 binding site. (A) EMSA with increasing amounts of recombinant p53 (0, 80, 160, and 320 ng) were performed with double-stranded DNA probes encompassing various regions of the core U1 promoter, as indicated. At higher p53 concentrations, two p53-dependent complexes were formed on those probes that exhibited high-affinity p53 binding. (B) DNase I footprinting reactions were performed using labeled template stand (lanes 1 to 5) or nontemplate stand (lanes 6 to 10) U1 promoter probes encompassing -150 to +13 with increasing amounts of GST-p53 (0, 100, 200, and 400 ng). Digestion of the probe DNA without added p53 is shown in lanes 1, 5, 6, and 10. The relative positions of the PSE and transcription start site are indicated. Two protected regions within the template strand (labeled F1 and F2) overlap with the three protected regions (labeled F1', F2', and F3') from the nontemplate strand.

A


p53
p53
p53
p53
p53
p53-DNA complexes
Free probe
1 2 3 4
5 6 7 8
9 10 11 12
13 14 15 16
17 18 19 20
A
B
C
D
E
EMSA
-422
+13
A
-422
-152
B
-312
-152
C
-312
+13
D
-150
+13
E

B



DNase I footprinting

the template strand was end labeled exhibited two sites of protection (labeled F1 and F2) in the presence of GST-p53 (left panel), whereas three regions (F1', F2', and F3') were protected in reactions with the labeled nontemplate strand (right panel). The F1' and F3' regions correspond to the same region as the F1 footprint (hereafter referred to as p53 footprint 1), whereas the F2 and F2' regions map to the same region (hereafter referred to as p53 footprint 2). Interestingly, the regions protected by p53 flank the PSE, which is required for DNA binding by $SNAP_C$ and is an essential promoter element for high level expression of human snRNA genes. The juxtaposition of p53 footprints1 and 2 with the PSE raises the possibility that p53 represses U1 transcription by occluding $SNAP_C$ promoter binding. However, to date we have not observed any effect of p53 on promoter recognition by $SNAP_C$ (data not shown).

p53 repression and sequence-specific DNA binding are separable activities.

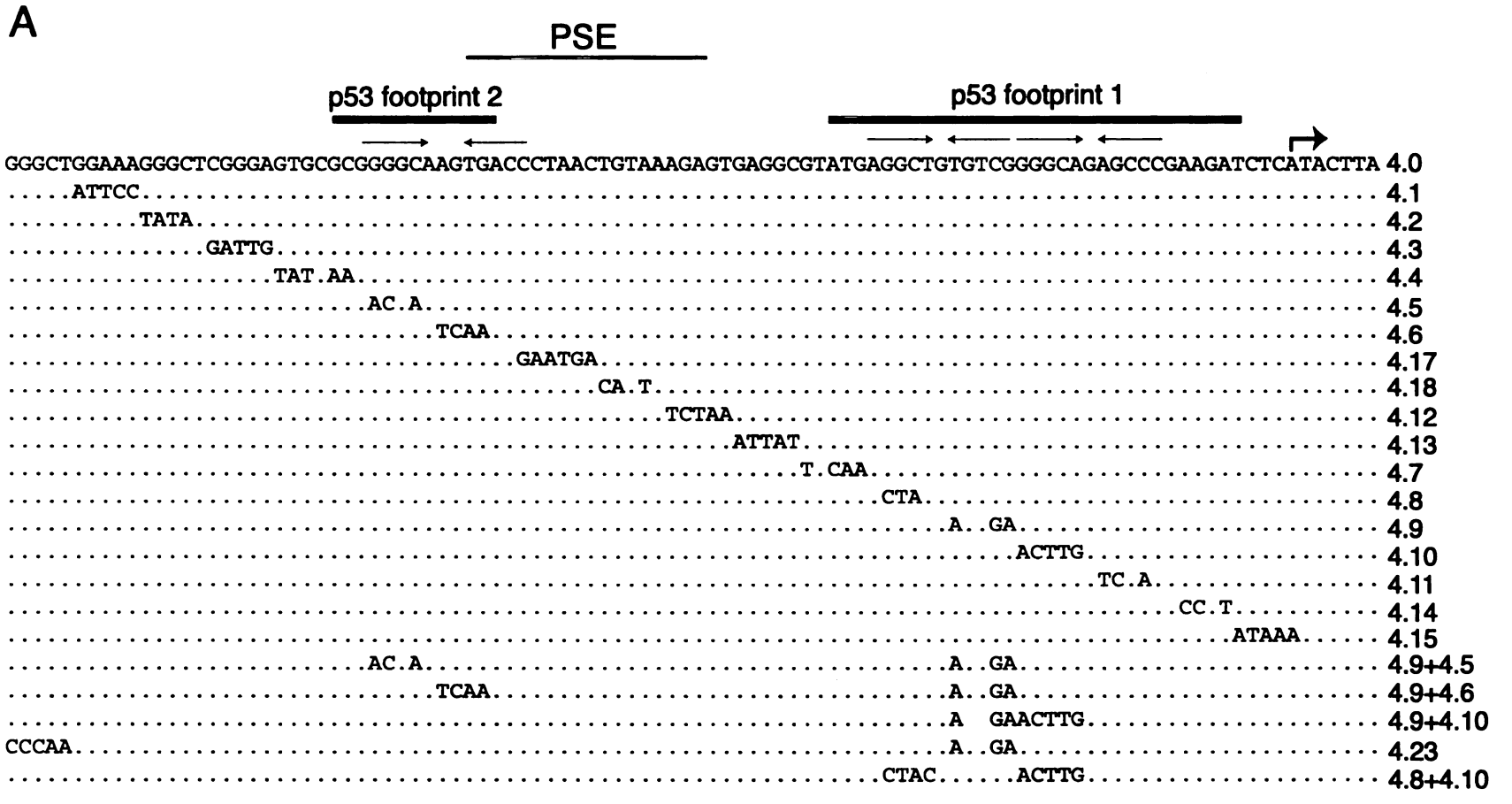
As a transcription factor, p53 can either activate or repress transcription, depending upon the structure of the target gene promoter. Notably, p53 can activate transcription from gene promoters that contain consensus p53 binding elements (12). A few examples have been described where p53 represses target genes that contain consensus p53 binding elements (reviewed in reference 26), but it is generally believed that p53 can repress transcription of other target genes whose promoters lack specific p53 recognition elements or contain a noncanonical p53 binding element (29). A comparison of p53 binding elements from target genes that are activated by p53 revealed that a consensus p53 binding site contains two half sites separated by 0 to 13 bp (50). Each half site contains two quarter sites containing the sequence PuPuPuC(A/T) arranged in a head-

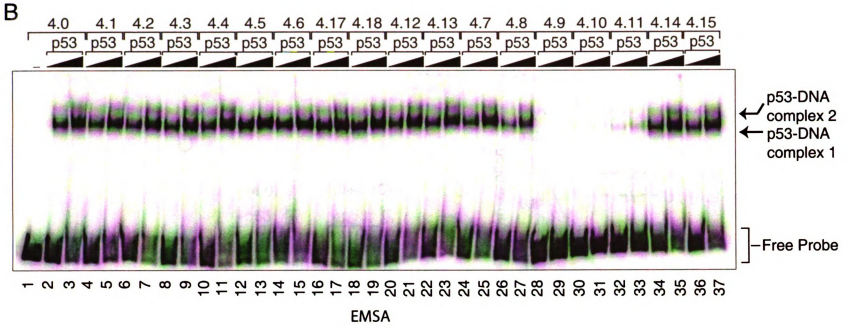
to-head orientation. Interestingly, the p53 footprint 1 region contains a sequence that is similar to the consensus p53 binding element usually associated with transcriptional activation by p53 (Fig. 4A).

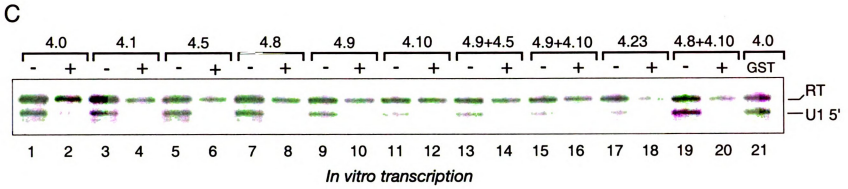
To investigate the contribution of the high-affinity p53 binding element for U1 promoter recognition and transcriptional repression, a scanning mutagenesis of the U1 core promoter was performed. The sequence of the U1 core promoter region and the location of the p53 footprints are shown in Fig. 4A. This figure also shows the location and identity of the mutations introduced into the U1 core promoter. The ability of recombinant p53 to bind DNA probes harboring these mutations was tested by EMSA (Fig. 4B). The majority of mutations throughout the U1 core promoter, including mutations within the region corresponding to p53 footprint 2 (U1-4.5 and U1-4.6 probes), had no significant effect on DNA binding by p53. A slight reduction for p53-DNA complex 2 formation for the U1-4.5 and U1-4.6 probes seen in this figure was not observed in replicates of this experiment. In contrast, mutations within the p53 footprint 1 region caused a marked reduction in p53 binding to the U1 promoter. The strongest effect was seen with the U1-4.9, U1-4.10, and U1-4.11 probes, whereas the U1-4.8 probe exhibited only a modest reduction in p53 binding. Formation of both p53-DNA complex 1 and p53-DNA complex 2 was affected by these mutations. These data indicate that the region adjacent to the U1 transcriptional start site contains the high-affinity p53 binding element.

Next, the requirement of the high-affinity p53 binding element for p53 repression was tested. As shown in Fig. 4C, recombinant p53 effectively repressed transcription from all U1 reporter constructs tested. Neither single nor double sets of mutations within

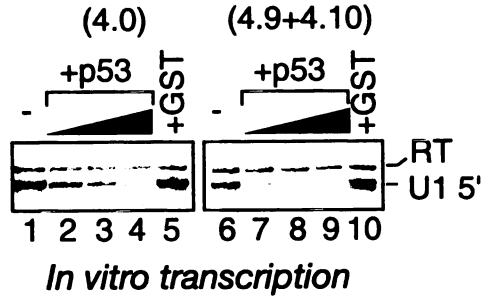
Figure 2-4. The high-affinity p53 element in the U1 promoter is not essential for p53 repression in vitro. (A) Primary sequence of the U1 core promoter region and the location of the p53 footprints. The plasmid pU1-4.0 contains a wild-type promoter sequence from -151 to +13. Scanning mutagenesis across this region was performed, and the introduced mutations and plasmid identity are indicated. Dots represent positions of identity to this wild-type sequence. (B) Mutations in the p53 footprint 1 region disrupt p53 binding. EMSA was performed using the various U1 promoter probes and either 200 or 400 ng of p53, as indicated (lanes 2 to 37). Lane 1 contains the wild-type U1 promoter probe and no added p53. Mutations within the p53 footprint 1 region caused a marked reduction in p53 binding to the U1 promoter (probes 4.8, 4.9, 4.10, and 4.11). (C) Mutations within the p53 footprint 1 region do not affect p53 repression in vitro. Selected mutations were incorporated into a U1 G-less reporter plasmid for in vitro U1 transcription assays (lanes 1 to 20) in the absence or presence of p53 (800 ng). Lane 21 shows transcription from the wild-type U1 reporter in the presence of GST (800 ng). The read-through (RT) and correctly initiated transcripts (U1 5) are indicated. (D) p53 represses transcription of the wild-type and mutant U1 reporter constructs to similar extents. (Left panel) An extensive titration of p53 (0, 20, 40, 80, and 160 ng) into U1 transcription assay mixtures was performed using the wild-type (U1 4.0; lanes 1 to 4) and mutant (U1 4.9 4.10; lanes 6 to 9) U1 reporter. Lanes 5 and 10 show transcription from the wild-type U1 plasmid in the presence of 160 ng of GST, as a negative control. (Right panel) Transcription levels for two independent experiments were normalized to the signals from transcription reactions containing no added p53, and the average dose-response curves are shown.

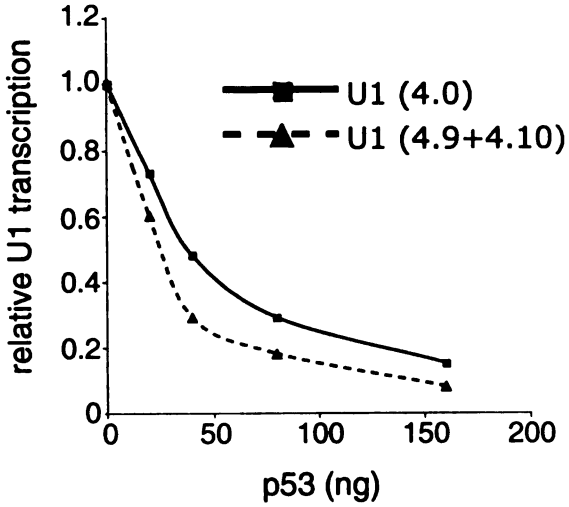

A
PSE
p53 footprint 2
p53 footprint 1
GGGCTGGAAAGGGCTCGGGAGTGCGCGGGGCAAGTGACCCTAACTGTAAAGAGTGAGGCGTATGAGGCTGTGTCGGGGCAGAGCCCGAAGATCTCATACTTA 4.0
.....ATTCC..... 4.1
.....TATA..... 4.2
.....GATTG..... 4.3
.....TAT.AA..... 4.4
.....AC.A..... 4.5
.....TCAA..... 4.6
.....GAATGA..... 4.17
.....CA.T..... 4.18
.....TCTAA..... 4.12
.....ATTAT..... 4.13
.....T.CAA..... 4.7
.....CTA..... 4.8
.....A..GA..... 4.9
.....ACTTG..... 4.10
.....TC.A..... 4.11
.....CC.T..... 4.14
.....ATAAA..... 4.15
.....AC.A.....A..GA..... 4.9+4.5
.....TCAA.....A..GA..... 4.9+4.6
.....A GAACTTG..... 4.9+4.10
CCCAA.....A..GA..... 4.23
.....CTAC.....ACTTG..... 4.8+4.10


B
4.0 4.1 4.2 4.3 4.4 4.5 4.6 4.17 4.18 4.12 4.13 4.7 4.8 4.9 4.10 4.11 4.14 4.15
p53
p53-DNA complex 2
p53-DNA complex 1
Free Probe
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37
EMSA


C
4.0 4.1 4.5 4.8 4.9 4.10 4.9+4.5 4.9+4.10 4.23 4.8+4.10 4.0
- + - + - + - + - + - + - + - + - + - + GST
RT
U1 5'
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
In vitro transcription

D


(4.0)
(4.9+4.10)
+p53
+GST
-
RT
U1 5'
1 2 3 4 5 6 7 8 9 10
In vitro transcription


relative U1 transcription
1.2
1.0
0.8
0.6
0.4
0.2
0
0
50
100
150
200
p53 (ng)
U1 (4.0)
U1 (4.9+4.10)

the p53 footprint 1 region or within both p53 footprint 1 and 2 regions had a major effect on p53 repression ability. In some cases, mutation of the U1 core promoter caused a slight reduction in transcription in the absence of p53, which suggests that factors binding to these regions may have a positive role in U1 transcription in the absence of added p53. To more carefully examine the contribution of the high-affinity p53 binding element to p53 transcriptional repression, a titration of p53 was performed on the wild-type U1 reporter (U1-4.0) and mutant U1 reporter (U1-4.9 + 4.10), which harbors two sets of mutations within the high-affinity p53 binding element (Fig. 4D, left panel). Transcription initiated from the U1 promoter was quantified, and levels were normalized to levels in transcription reactions containing no added p53 for each U1 reporter construct. The average U1-specific response from two replicates of this experiment is shown in Fig. 4D (right panel). This analysis revealed that both U1 reporter constructs exhibited similar repression responses to increasing amounts of p53. Together these data indicate that the high-affinity p53 binding element contained within the human U1 core promoter region is not required for in vitro transcriptional repression by p53.

The p53 C terminus contributes to both RNA polymerase II and III repression.

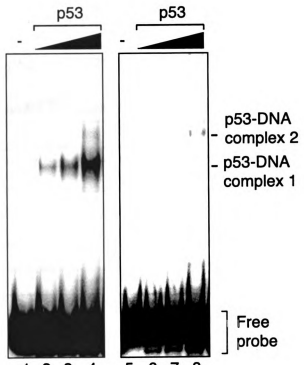
Our previous data suggested that p53 is an effective repressor of U6 transcription by RNA polymerase III. Therefore, p53 binding to the U6 promoter was next investigated to determine the contribution of sequence-specific DNA binding by p53 to the repression of RNA polymerase III transcription. As shown in Fig. 5A, p53 binds well to the U1 promoter probe in EMSA, as expected, but approximately 10- to 20-fold less well to the U6 promoter probes (lanes 6 to 8). The weak binding by p53 to the U6 promoter probe

suggests that p53 may not rely on direct promoter recognition to mediate RNA polymerase III repression. However, it is possible that p53 binds DNA specifically elsewhere in the reporter constructs during repression.

To determine whether DNA binding by p53 is important for repression of U1 and U6 transcription, a point mutation in the p53 DNA binding domain was introduced to eliminate sequence-specific DNA binding. In particular, the arginine at position 175 was changed to a histidine (R175H) to mimic a hot spot mutation commonly found in human cancers. Additionally, the C-terminal region of p53 (301-393) was tested for activity, because this region is sufficient for chromosome fragile site formation at the multicopy U1 and U2 snRNA gene loci during adenovirus infection (38, 71). First, the wild-type and mutant GST-p53 proteins were tested for DNA binding activity in EMSA using probes encompassing the U1 core promoter region. As shown in Fig. 5B, only the wild-type recombinant GST-p53 bound effectively to the U1 promoter DNA while the GST-p53 (R175H) and GST-p53 (301-393) proteins were completely inactive in this assay. Next, the ability of these proteins to regulate in vitro U1 and U6 transcription by RNA polymerases II and III, respectively, were tested (Fig. 5C). Interestingly, GST-p53 (R175H) repressed U1 transcription as effectively as wild-type GST-p53, whereas GST-p53 (301-393) was approximately twofold less effective, but still capable of repression. Both mutant GST-p53 proteins repressed U6 transcription, but GST-p53 (R175H) was less effective than either wild-type GST-p53 or GST-p53 (301-393), which were equivalently active in these assays. In these assays, the repression of RNA polymerase III transcription by p53 (R175H) is in contrast with previous experiments, wherein the introduction of the R-to-H mutation switched p53 from a repressor to an activator of

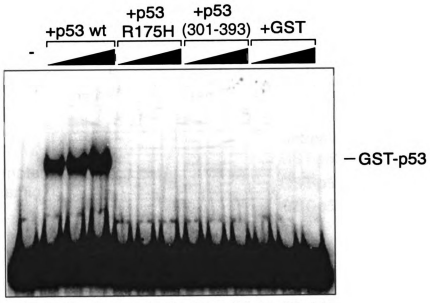
Figure 2-5. The p53 C terminus is sufficient for transcriptional repression and promoter association. (A) The U6 core promoter does not contain a high-affinity p53 binding element. EMSA was performed with increasing amounts of recombinant p53 (0, 80, 160, and 320 ng) and equivalently labeled double-stranded DNA probes encompassing the U1 core promoter (lanes 1 to 4) and U6 core promoter (lanes 5 to 8). (B) Direct U1 promoter recognition requires the p53 DNA binding domain. EMSA were performed with a wild-type U1 promoter probe (151 to 13) using 250, 500, and 1,000 ng of full-length wild-type or mutant (R175H) GST-p53 (lanes 2 to 4 and 5 to 7, respectively). Reactions containing truncated GST-p53 (301-393) or GST alone are shown in lanes 8 to 10 and 11 to 13, respectively. Lane 1 contains no added protein. (C) Wild-type and mutant GST-p53 repress transcription similarly. Approximately 100, 200, and 400 ng of wild-type GST-p53 (lanes 2 to 4), GST-p53 (R175H) (lanes 5 to 7), GST-p53 (301-393) (lanes 8 to 10), or 400 ng of GST (lane 11) were titrated into U1, U6, and AdML in vitro transcription reaction mixtures. Both wild-type and mutant p53 molecules repressed U1 and U6 transcription, whereas AdML transcription was unaffected. (D) The p53 DNA binding domain is not required for U1 and U6 promoter association during repression. A portion of untreated or p53 repressed U1 and U6 in vitro transcription reaction mixtures (lanes 1, 4, 7, 10, and 11 in panel C) was cross-linked with formaldehyde and subjected to immunoprecipitation with anti-SNAP43 (lanes 3 and 8), IgG (lanes 4 and 9), or anti-p53 (lanes 5 and 10) antibody. Enrichment of the U1 and U6 reporter plasmids or negative control pUC119 DNA was compared by PCR using promoter-specific or pUC-specific primers. Lanes 1, 2, 6, and 7 show the amplification directly from the input DNA (10 and 1% in lanes 1 and 2 for U1 and lanes 6 and 7 for U6, respectively) contained in the transcription assay mixtures prior to immunoprecipitation. The results of PCRs using the pUC-specific primers under all conditions were indistinguishable and are shown only for the reactions containing added wild-type GST-p53.

A


p53
p53
-
-
p53-DNA
complex 2
p53-DNA
complex 1
Free
probe
1 2 3 4
5 6 7 8
U1
U6

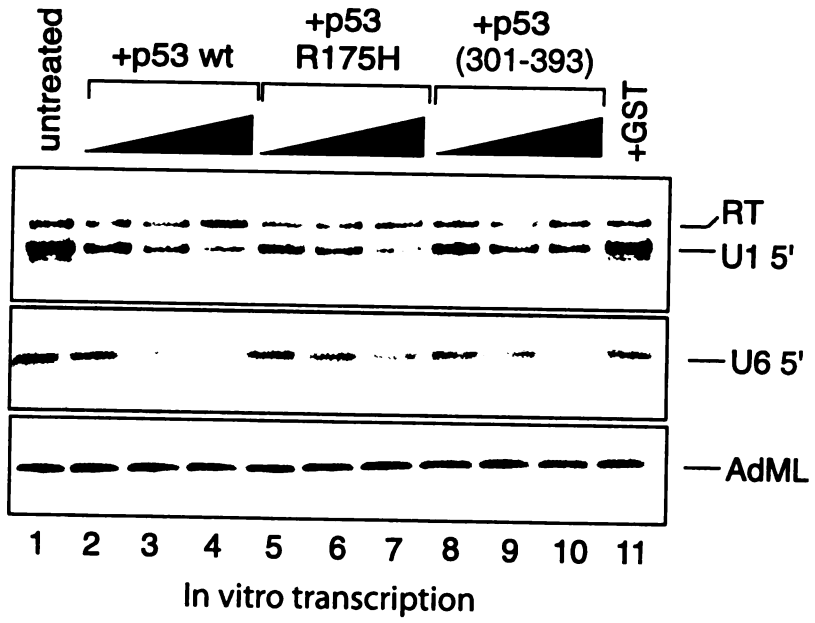
EMSA

B

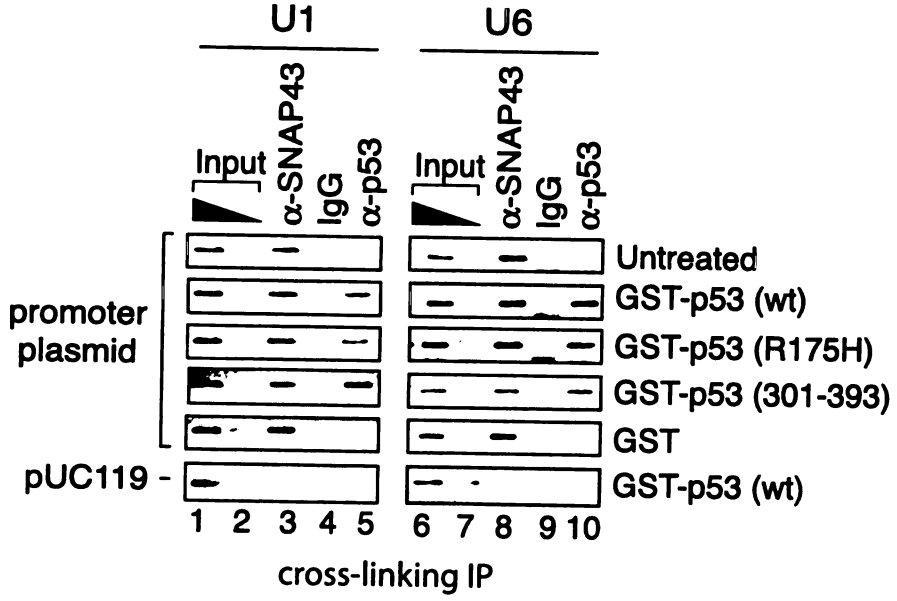

+p53 wt
+p53
R175H
+p53
(301-393)
+GST
-
GST-p53
1 2 3 4 5 6 7 8 9 10 11 12 13

EMSA

C


untreated
+p53 wt
+p53 R175H
+p53 (301-393)
+GST
RT
U1 5'
U6 5'
AdML
1 2 3 4 5 6 7 8 9 10 11
In vitro transcription

D


U1
U6
Input
α-SNAP43
IgG
α-p53
promoter plasmid
pUC119 -
Untreated
GST-p53 (wt)
GST-p53 (R175H)
GST-p53 (301-393)
GST
GST-p53 (wt)
1 2 3 4 5 6 7 8 9 10
cross-linking IP

general RNA polymerase III transcription (57). These data herein indicate that DNA binding by p53 is not essential for repression of U1 and U6 snRNA gene transcription by RNA polymerases II and III, respectively, and the C-terminal region encompassing amino acids 301 to 393 is sufficient for p53 repression activity.

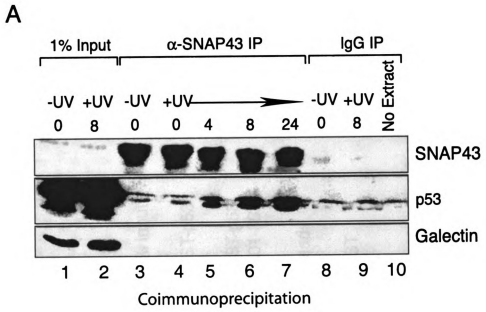
Next, recruitment of the mutant p53 proteins to the U1 and U6 promoters during repression was examined. Portions of in vitro transcription reaction mixtures containing wild-type or mutant GST-p53 proteins were cross-linked with formaldehyde prior to immunoprecipitation by using antibodies directed against SNAP43 or p53. Transcription reaction mixtures containing GST or no added proteins were also tested. The specific recoveries of the U1 and U6 promoter-containing plasmids were compared to recovery of an irrelevant plasmid (pUC119) included in the original transcription assay mixtures. As shown in Fig. 5D, significant U1 and U6 reporter plasmid recovery by anti-SNAP43 immunoprecipitation was obtained from the untreated transcription reaction mixture (lanes 3 and 8). Neither wild-type nor mutant p53 affected reporter enrichment by anti-SNAP43 immunoprecipitation, suggesting that p53 may not disrupt $SNAP_C$ promoter binding during repression. However, it is difficult to eliminate the possibility that $SNAP_C$ can bind to both actively transcribed and inactive templates, whereas p53 may be specifically bound to only those active templates containing a complete preinitiation complex. When the untreated U1 and U6 transcription assays were used for anti-p53 immunoprecipitation, only background levels of reporter plasmid enrichment were observed (lanes 5 and 10). This result was expected, because these assays were performed using HeLa cell nuclear extracts, which contain very low levels of endogenous p53. However, significant recovery of the U1 and U6 promoter plasmids was observed in

reactions containing additional wild-type p53. Interestingly, U1 and U6 reporter DNA enrichment was also observed in reactions containing the mutant p53 (R175H) and p53 (301-393) proteins. In all cases, recovery of the reporter-containing plasmids was substantially greater than that observed for the irrelevant pUC119 plasmid (Fig. 5D, bottom panel, and data not shown). These results indicate that p53 can associate with the U1 and U6 promoters during repression. The preferential association of the mutant p53 proteins with the promoter-containing plasmids suggests that during repression p53 is actively recruited to U1 and U6 promoters independently of sequence-specific DNA binding by p53. Furthermore, the C-terminal domain of p53 is likely critical for promoter recruitment.

p53 associates with general transcription factor $SNAP_C$.

Promoter association by p53 in the absence of direct DNA binding suggests that protein-protein interactions are critical for p53 recruitment. Furthermore, p53 was capable of repressing both U1 and U6 transcription by RNA polymerases II and III, raising the possibility that a factor commonly used for these genes could be targeted by p53. Both the general transcription factors $SNAP_C$ and TBP are utilized during transcription of these genes and are thus candidate p53 targets. Indeed, TBP has been shown to interact strongly with p53 within the N-terminal activation domain and the C-terminal oligomerization domain (16, 41, 61). To determine whether p53 could potentially target $SNAP_C$, coimmunoprecipitation assays were performed using antibodies directed against the SNAP43 subunit of $SNAP_C$. The association of endogenous p53 with SNAP43 was measured by Western analysis before and after UV

Figure 2-6. Endogenous p53 associates with the general transcription factor $SNAP_C$. (A) Immunoprecipitation with anti-SNAP43 antibodies was performed from whole-cell extracts prepared from untreated and UV-treated MCF-7 cells at different time points after UV light treatment. Western analysis revealed significant differences in recovered p53 between the negative control IgG immunoprecipitations (lanes 8 to 10) and anti-SNAP43 immunoprecipitation by the 4-h time point (lane 5). The amount of coimmunoprecipitated p53 continued to increase up to 24 h after UV light treatment (lanes 6 and 7), whereas the amount of precipitated SNAP43 did not change from 0 to 24 h after UV exposure. No galectin-3 was observed in any of the immunoprecipitations. Lanes 1 and 2 contain 1% of the extract used for immunoprecipitation reactions. (B) Immunoprecipitation with anti-p53 (Ab-6) antibodies was performed from whole-cell extracts (5 mg, lanes 3, 4, and 6; 1.7 mg, lanes 5 and 7) prepared from untreated and UV-treated MCF-7 cells 8 h after UV light treatment, as indicated. Lanes 1 and 2 contain 150 g of extract that was used as input for immunoprecipitation reactions. Western analysis with anti-SNAP43 antibodies revealed increased SNAP43 association with p53 by 8 h after UV light exposure. (C) p53 interacts with SNAP43, SNAP190, and TBP. GST pull-down experiments were performed with 1 μg of recombinant full-length GST-p53 (lane 2), GST-p53 (301-393) (lane 3), GST (lane 5), or beads alone (lane 4) with individual $SNAP_C$ subunits and TBP that were translated in vitro and labeled with [^{35}S]methionine. Lane 1 contains 5% of radiolabeled proteins added to each pull-down reaction mixture.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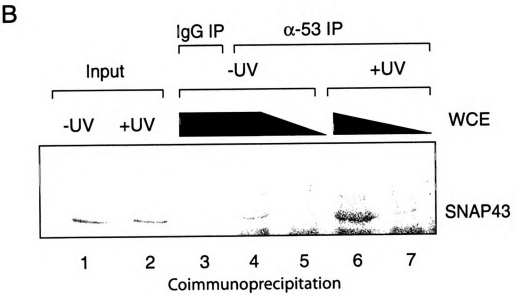
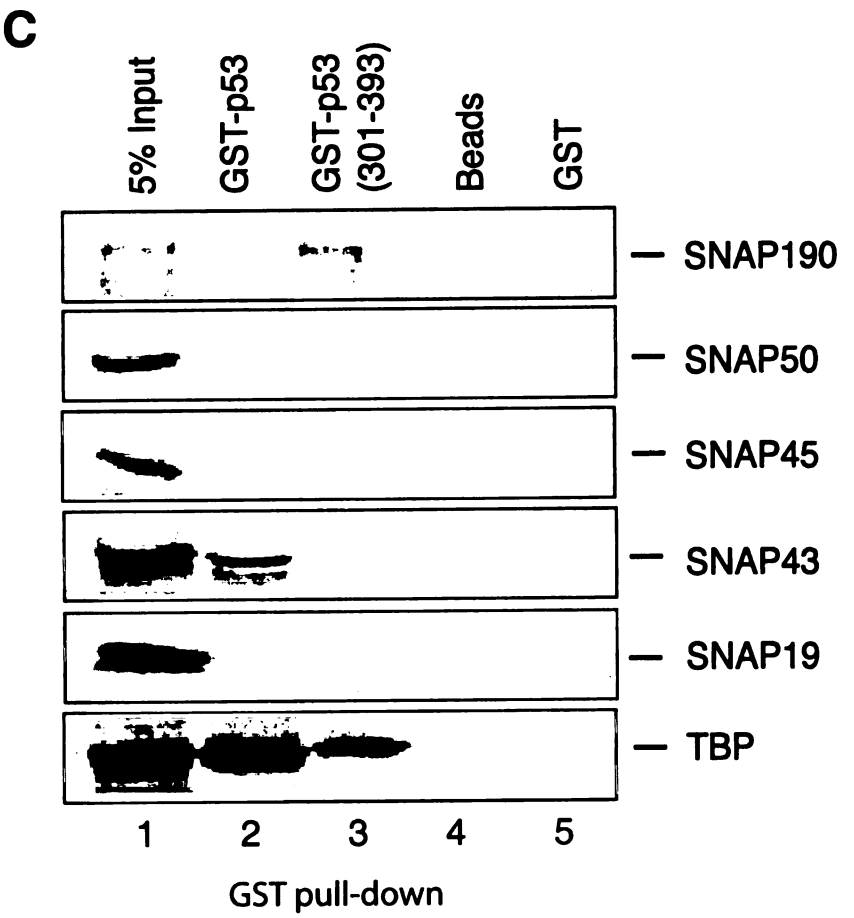
1% Input
α-SNAP43 IP
IgG IP
No Extract
-UV +UV -UV +UV -UV +UV
0 8 0 0 4 8 24 0 8
SNAP43
p53
Galectin
1 2 3 4 5 6 7 8 9 10
Coimmunoprecipitation


B
IgG IP
α-53 IP
Input
-UV
+UV
-UV +UV
WCE
SNAP43
1 2 3 4 5 6 7
Coimmunoprecipitation


C
5% Input
GST-p53
GST-p53 (301-393)
Beads
GST
SNAP190
SNAP50
SNAP45
SNAP43
SNAP19
TBP
1
2
3
4
5
GST pull-down

light treatment. As shown in Fig. 6A, p53 was not detected in anti-SNAP43 immunoprecipitation reactions prior to UV treatment, but p53 recovery increased by 4 h and continued to increase by 24 h after UV light treatment. No recovery of the splicing factor galectin-3 was observed in these reactions. Reciprocal anti-p53 immunoprecipitation assays were performed using extracts prepared from MCF-7 cells that were untreated or UV light treated (8 h posttreatment). As shown in Fig. 6B, UV light treatment had little effect on the levels of SNAP43 present in the extract (lanes 1 and 2) but caused increased SNAP43 association with p53 (compare lanes 4 and 5 to 6 and 7). Together, these data indicate that UV light modulates the association between endogenous p53 and $SNAP_C$.

GST pull-down experiments were then performed to determine whether p53 could interact with TBP and individual components of $SNAP_C$ (Fig. 6C). In these assays, full-length GST-p53 interacted well with TBP and SNAP43 while SNAP190 interacted only weakly in this assay. No significant interactions were observed for GST-p53 with SNAP50, SNAP45, and SNAP19. Interestingly, truncating p53 to include only the C-terminal domain abolished interactions with SNAP43 but stimulated interactions with SNAP190. These observations suggest that p53 may interact with TBP and $SNAP_C$ to correctly target human snRNA genes for repression.

Discussion

The p53 tumor suppressor protein acts as a transcriptional activator in response to cellular stress, but it is also competent for concomitant repression of other sets of target genes. The data presented herein demonstrate that endogenous p53 associates with human snRNA gene promoters and that UV light exposure stimulates p53 association with both U1 and U6 snRNA genes. These in vivo data indicate that p53 is directly involved in the regulation of human snRNA gene transcription by both RNA polymerases II and III. That recombinant p53 effectively repressed both U1 and U6 snRNA gene transcription in vitro suggests that these genes similarly could be repressed by p53 in response to DNA damage in vivo. Indeed, cellular U1 and U6 snRNA gene transcription is down regulated in p53-positive MCF-7 cells after UV light exposure. In contrast, transcriptional repression was not observed in p53- deficient HeLa cells after UV light exposure.

The data presented herein also suggest that p53 uses different mechanisms to repress U1 and U6 transcription. First, U1 snRNA genes contain a high-affinity p53 binding element located with the core promoter region adjacent to the PSE. Second, U6 snRNA genes remain sensitive to p53 repression after preinitiation complex assembly, whereas U1 snRNA genes become refractory to p53 influence, suggesting that the potential targets required for RNA polymerase II repression may be unavailable for p53 interaction after preinitiation complex assembly. Thus, it is tempting to speculate that p53 interferes with $SNAP_C$ or TBP promoter occupancy during repression, which for U1 genes could involve direct competition between p53 and $SNAP_C$ for adjacent promoter elements. However, the high-affinity p53 binding element is clearly not required for U1 repression in vitro, and the levels of $SNAP_C$ detected on U1 and U6 snRNA gene

promoters by chromatin immunoprecipitation do not substantively change in response to UV light, even though there is a substantial increase in p53 association. Consistently, we have not observed any effect of full-length p53 on PSE binding by $SNAP_C$ in EMSA (data not shown). We currently favor the hypothesis that p53 interferes with snRNA gene transcription at steps occurring after promoter association by $SNAP_C$.

As U1 and U6 snRNA gene transcription relies on different assemblies of transcription factors, it is likely that to enact repression p53 targets different factors specifically required for RNA polymerase II or III transcription. For RNA polymerase II transcription, one candidate is TFIIH, which can interact with p53 (67, 70). TFIIH may be utilized during transcription of human U1 snRNA genes, as removal of TFIIH from extracts impairs U1 snRNA gene transcription (32). However, U1 transcription was not reconstituted in the depleted extracts by addition of purified TFIIH, suggesting that additional factors may complement the purified TFIIH for activity (32). A second candidate coregulator is the Cockayne syndrome group B (CSB) protein, which also can interact with p53 (67). CSB was originally suggested to play a role in DNA repair and, more recently, was shown to interact with RNA polymerase II (63). CSB may be an elongation factor (34, 56, 59, 62) which could mediate transcription of genes encoding RNA products with significant secondary structure, such as human U1 and U2 snRNA genes. As previously described for p53, mutations in CSB also cause chromosomal fragile site formation at U1 and U2 snRNA genes (71), suggesting that CSB may play a role in transcription of these genes by RNA polymerase II. It was hypothesized that activated p53 interferes with CSB-mediated elongation to cause RNA polymerase II stalling at U1 and U2 snRNA genes (71). The data presented herein support a role for p53

in mediating repression of U1 snRNA gene transcription by RNA polymerase II, but whether CSB is involved in this process is not yet known.

During genomic threats, p53 is stabilized from destruction and activated by a cascade of posttranslational modifications, including phosphorylation and acetylation. Acetylation of p53 has been suggested to increase sequence-specific DNA binding by p53 (18, 44) and to stimulate cofactor recruitment, such as the p300 histone acetyltransferase, during activated transcription of p53 target genes (2, 15). Thus, it is interesting that while acetylated p53 was detected at human U1 genes prior to UV light exposure, the level of acetylated p53 at these genes was not stimulated by UV light even though a substantial increase in the total p53 levels at these genes was observed. Furthermore, little to no acetylated p53 was observed at a U6 snRNA gene promoter after UV light treatment, indicating that most of the p53 associated with this gene is not acetylated. One possibility is that p53 recruits a histone deacetylase to enact repression, and this enzyme deacetylates p53. Another possibility is that p53 acetylation is not compatible with transcriptional repression. For example, the sites of p53 acetylation are all located in the C-terminal region (18, 40, 53), and this region is sufficient for transcriptional repression of human U1 and U6 snRNA gene transcription. Presumably, the p53 C terminus makes key protein-protein contacts to enact repression and, as we have shown, this region interacts with SNAP190 and TBP. Acetylation of p53 may disrupt these contacts and prevent p53 from being recruited to these snRNA promoters. However, it is difficult to exclude the possibility that p53 acetylation is important for p53 repression where the sites of acetylation are intimately involved in protein-protein contacts and are not available for antibody recognition during chromatin

immunoprecipitation. Nonetheless, p53 acetylation is unlikely to be essential for repression, because the recombinant wild-type and mutant p53 used for the U1 and U6 repression assays were not substantially acetylated. The presence of acetylated p53 at the U1 snRNA gene promoters in untreated MCF-7 cells suggests that activated p53 can bind to these genes, possibly through direct recognition of the high-affinity p53 binding element contained within the U1 snRNA gene core promoter even though this element is not required for U1 repression in vitro. An intriguing possibility is that p53 may have dual roles at human U1 snRNA genes, with direct promoter recognition and activation functions prior to substantial DNA damage but repressive activity mediated via a different promoter-targeting mechanism after DNA damage, such as that caused by UV light.

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CHAPTER THREE

THE COCKAYNE SYNDROME COMPLEMENTATION GROUP B PROTEIN MODULATES TRANSCRIPTION REGULATORY FUNCTIONS OF THE TUMOR SUPPRESSOR PROTEIN P53

Abstract

Activation of the tumor suppressor protein p53 or loss of the Cockayne syndrome complementation group B (CSB) protein induces chromosomal fragile site formation at the U1 snRNA, U2 snRNA, and 5S rRNA gene loci, suggesting a possible role for these factors in controlling transcription of these genes even though U1 and U2 snRNA genes are transcribed by RNA polymerase II and 5S rRNA genes are transcribed by RNA polymerase III. p53 acts as a transcriptional repressor of both 5S rRNA gene and U1 snRNA gene transcription. However, the relationship between CSB and these transcription programs has not been established.

In this study I show that CSB associates with human 5S rRNA and U1 snRNA promoters and has opposing roles in transcription of these genes. CSB represses 5S rRNA gene transcription but activates U1 snRNA gene transcription. Also, CSB was found to be involved in general RNA polymerase III transcription, including U6 snRNA gene transcription. Human U6 snRNA genes, even though they are transcribed by RNA *pol*ymerase III, have promoter structures that are more similar to the RNA polymerase II-transcribed U1 snRNA genes. Surprisingly, the role of CSB in U6 snRNA gene transcription is more similar to U1 snRNA, rather than 5S rRNA gene transcription.

Interestingly, CSB association with U1 and U6 snRNA gene promoters was diminished after UV light treatment concomitant with increased p53 promoter association. Also, removal of CSB from cell extracts enhances the inhibitory effect of p53 on U1 snRNA gene transcription by RNA polymerase II, but does not affect p53-mediated repression of U6 snRNA gene transcription by RNA polymerase III. At low amounts p53 activates 5S rRNA transcription, but at higher amounts p53 represses 5S rRNA gene transcription better when CSB is depleted from in vitro transcription reactions. Together, these results suggest that p53 modulates CSB transcription regulatory functions at U1 and U2 snRNA genes and 5S rRNA genes, which are susceptible for fragile site formation during metaphase.

Introduction

Chromosomal fragile sites are non-randomly located non-staining gaps in metaphase chromosomes caused by incompletely condensed chromatin or, more rarely, by chromosome breaks. Fragile sites have been proposed to be not only susceptible to DNA instability in cancer cells, but also associated with genes that contribute to the neoplastic process (27). Infection of human cells at low multiplicity by adenovirus serotype 12 (Ad12), but not adenovirus 2 or 5, or transient expression of Ad12 E1B 55 kDa protein induces four sites of metaphase chromosome fragility that were found to co-localize with four tandemly repeated multigene families: *RNU1* locus (contains U1 snRNA genes), *RNU2* locus (contains U2 snRNA genes), *RN5S* locus (contains 5S rRNA genes), and *PSU1* locus (contains U1 pseudogenes) (9, 14, 24, 29, 37). It was subsequently found that p53 was required for Ad12-induced chromosome fragility (24,

37). Surprisingly, the same chromosomal loci – *RNU1*, *RNU2*, and *RN5S* – are constitutively fragile in Cockayne syndrome group B (CSB) cells that fail to express functional CSB protein (37).

The involvement of p53 and CSB in fragile site formation suggests that both factors are involved in the transcription of these genes, even though U1 and U2 snRNA genes are transcribed by RNA polymerase II and 5S rRNA genes are transcribed by RNA polymerase III. Indeed, a role for p53 in transcription of these genes has been demonstrated. p53 acts as a transcriptional repressor of 5S rRNA gene transcription by RNA polymerase III (4, 6) and U1 snRNA gene transcription by RNA polymerase II (15). However, any role for CSB in transcription of these genes has not been investigated. CSB belongs to the SWI2/SNF2 family of proteins and has many of the activities expected for a SWI2/SNF2 protein (10). It has DNA-stimulated ATPase activity and can remodel nucleosomes at the expense of ATP hydrolysis (8). CSB was originally suggested to function in transcription-coupled repair of RNA polymerase II-transcribed genes after DNA damage (23, 33, 35). Recent studies also revealed a role of CSB in transcription. It was demonstrated that CSB associates with RNA polymerase I and II protein complexes, and in vitro transcription experiments showed that CSB stimulates elongation by RNA polymerase II (2, 30, 34). The observed interaction of CSB with RNA polymerase I and II in vivo and in vitro suggested that CSB might be specifically targeted to sites of blocked transcription (2). In addition, the CSB protein may also play a role in clearing the stalled RNA polymerase II molecule from the lesion site so that repair can occur and transcription resume (16). To-date, the relationship between CSB and RNA polymerase III transcription has not been established.

RNA polymerase III-transcribed genes are divided into three classes based on the structure of promoters (18). Class 1 and 2 genes are represented by the 5S rRNA and tRNA genes respectively and contain internal promoter elements. In contrast, class 3 genes, represented by the U6 snRNA, have external promoters. Even though class 3 genes are transcribed by RNA polymerase III, they share similar promoter architectures as well as some general transcription machinery requirements with RNA polymerase II-transcribed snRNA genes.

In this current study I examined the role of CSB in RNA polymerase II and III transcription and its functional interplay with p53. I found that CSB associates with U1 snRNA gene promoters and with a variety of RNA polymerase III-transcribed gene promoters but CSB has different roles for RNA polymerase II and III transcription. CSB plays a positive role in U1 snRNA gene transcription by RNA polymerase II and in U6 snRNA (class 3) gene transcription by RNA polymerase III, but plays a negative role in transcription of other classes of RNA polymerase III-transcribed genes. In addition, I present several pieces of data that suggest a modulatory role of p53 on CSB transcription functions.

Materials and Methods

Cell culture and UV irradiation

Human MCF-7, HeLa and H1299 (p53-/-) cells were maintained in Dulbecco's modified Eagle's medium supplemented with penicillin-streptomycin and 10% fetal bovine serum. Cells grown to 70 to 80% confluence were washed with phosphate-buffered saline and irradiated with 50 J of UV light (254-nm peak)/m^2 by using a UV Stratalinker (Stratagene). After irradiation, growth medium was added and cells were incubated at 37°C under 5% CO_2 for the indicated times. Additionally, H1299 cells were grown to 50% confluence in 150-mm plates and were then transiently transfected with 2 μg of the pRc/RSV or pRc/RSV-p53.wt or pRc/RSV-KR5A-p53 plasmids by using Lipofectin reagent (Invitrogen) for 6 h. Subsequently, the medium was replaced and cells were incubated for 48 h for further analysis.

Nuclear run-on assays

Nuclear run-on assays were performed as described elsewhere (5) in the presence of [α-^{32}P] UTP using approximately 10^7 nuclei that were isolated from MCF-7 cells. Labeled RNA was recovered and hybridized to a nitrocellulose membrane containing approximately 7 μg of U1, U6, and 5S rRNA target gene DNAs or 10 μg of pUC119 plasmid DNA, as a negative control. Target gene DNAs corresponding to the coding regions of the indicated genes were generated by PCR and were immobilized on a nylon membrane at levels calculated to be in excess relative to the corresponding snRNA population in the nuclei. Hybridizations were performed for 16 h at 42°C in hybridization buffer containing 50% formamide. Membranes were then washed extensively in 2X SSC

(1X SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.1% sodium dodecyl sulfate (SDS) at 50°C. Hybridized RNA transcripts were visualized by autoradiography.

Expression and purification of recombinant proteins

Glutathione *S*-transferase (GST), GST-tagged full-length human p53 and GST-tagged human CSB (528-1222) were expressed in *Escherichia coli* BL21 (DE3) codon+ cells (Stratagene) and were affinity purified by binding to glutathione agarose beads (Sigma). GST and GST-CSB (528-1222) were then eluted from beads in HEMGT-150 buffer containing 50 mM glutathione for 4 h at 4°C. Untagged p53 was obtained by digestion with thrombin. p53 was further purified by chromatography using a Mono-Q (HR5/5) column (Pharmacia) and were concentrated by centrifugation using a Centricon YM-30 spin column (Millipore) in HEMGT-80 buffer (20 mM HEPES [pH 7.9], 0.1 mM EDTA, 10 mM MgCl2, 10% glycerol [vol/vol], 0.1% Tween 20, 80 mM KCl) containing protease inhibitors and 1 mM dithiothreitol.

In vitro transcription assays

In vitro transcription assays were performed as described previously (17) using 18, 2, 2, and 10 μL of HeLa cell nuclear extract for the U1 snRNA, U6 snRNA, 5S rRNA, and adenovirus major late promoter (AdML) transcription reactions, respectively. The pU1-4.0 (1 μg), pU6/Hae/RA.2 (250 ng), pH5Ssa (250 ng), and M13-AdML (250 ng) templates were used for the U1 snRNA, U6 snRNA, 5S rRNA, and AdML transcription reactions, respectively. Purified proteins were added in the amounts indicated in the figure legends. Transcription was performed for 1 h at 30°C. Transcripts were separated

by denaturing 6% polyacrylamide gel electrophoresis and visualized by autoradiography.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation assays were performed as described previously (19). Human MCF-7 cells were grown to 60 to 80% confluence and were then cross-linked with 1% formaldehyde for 30 min at room temperature. After cell lysis and sonication, immunoprecipitation reactions were performed overnight at 4°C using chromatin from approximately 10^7 cells per reaction mixture and 1 μg of each antibody: anti-TBP (SL2), pre-immune IgG (Sigma), anti-p53 (21-25) (Ab-6; Oncogene), anti-CSB (N-17, Santa Cruz), anti-RNA polymerase II (8WG16, Covance Research Products) and anti-RNA polymerase III (TB2). Recovered chromatin was suspended in 50 μL of H_2O, and PCR analysis was performed using 5 μl of immunoprecipitated chromatin or input chromatin. The primers used for amplification of each gene were described previously (15). PCR products were separated by 2% agarose electrophoresis in Tris-borate-EDTA buffer and were stained with ethidium bromide.

Immunoprecipitations from in vitro transcription reactions

In vitro transcription assay mixtures containing U1 or U6 promoter plasmids and equal molar amounts of pUC119 were performed as described previously (19) in the absence or presence of full-length wild type p53 or GST. Five microliters of each transcription reaction mixture was diluted to 500 μL and was cross-linked in 1% formaldehyde for 10 min at room temperature, quenched with 125 mM glycine for 10 min at room temperature, and immunoprecipitated with anti-TBP (SL2), pre-immune

(Sigma), anti-p53 (Ab-6; Oncogene), anti-CSB (N-17, Santa Cruz) and anti-RNA polymerase II (8WG16, Covance Research Products) or anti-RNA polymerase III (TB2) antibodies. Recovered plasmid DNA was analyzed by PCR using primers specific to the U1 and U6 promoter regions or to pUC119 as a negative control.

Immunoprecipitation experiments

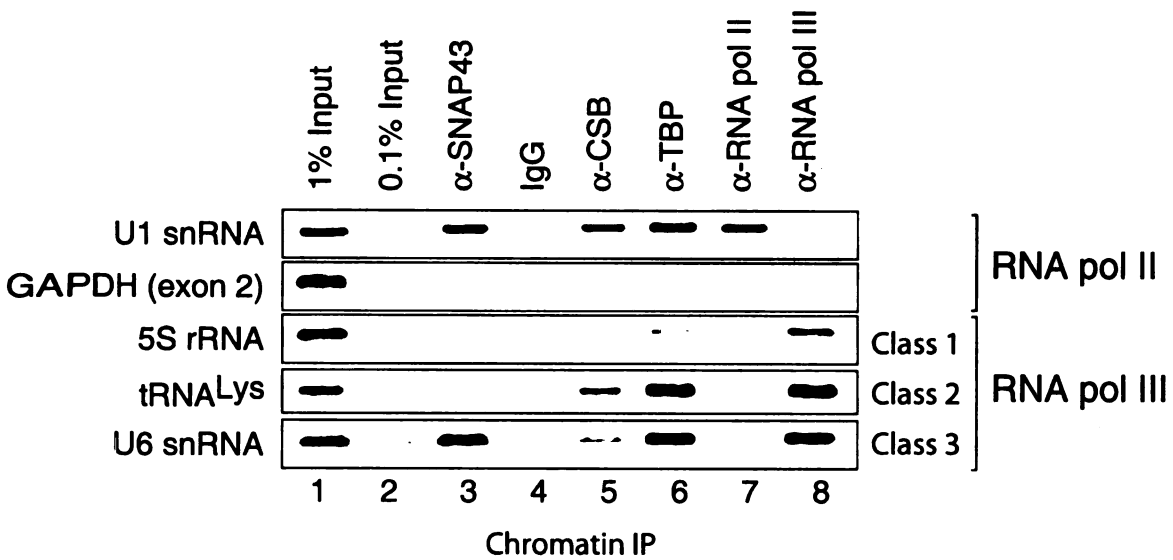
Approximately 5 mg of HeLa cell nuclear extract was incubated with 1 μg of antibodies directed against CSB (N-17, Santa Cruz) or goat IgG (Sigma) 90 minutes at room temperature. Reactions were diluted to 1 mL in HEMGT-100 buffer and stable complexes were affinity purified by incubation with Protein-G Fast Flow sepharose beads (Upstate Biotechnology) for 90 minutes at room temperature. Beads were washed in HEMGT-100 buffer and boiled for 5 minutes in Laemmli Buffer. Bound proteins were separated by 12.5% SDS-PAGE and transferred to nitrocellulose. Western blot analyses of recovered proteins were performed using anti-RNA polymerase III (TB2), anti-RNA polymerase II (8WG16, Covance Research Products) and anti-actin (Sigma) antibodies.

Results

Endogenous CSB occupies human U1 and U6 snRNA gene promoters.

The *RNU1*, *RNU2*, and *RN5S* loci are constitutively fragile in CSB deficient cells that fail to express functional CSB protein (37) and it was postulated that loss of CSB transcription functions is important for fragile site formation (37). To determine whether CSB is involved in transcription of these genes, the association of endogenous CSB with U1 snRNA gene promoter and with three different classes of RNA polymerase III-transcribed promoters was examined using chromatin immunoprecipitation experiments. As shown in figure 3-1, substantial recovery of U1 snRNA promoter DNA was observed in anti-CSB-immunoprecipitated samples (lane 5) as compared to negative control IgG-immunoprecipitated reactions (lane 4). Interestingly, enrichment of all three classes of RNA polymerase III-transcribed promoters was also observed in anti-CSB-immunoprecipitated samples. No CSB was detected on the negative control GAPDH exon 2 region. The presence of CSB on RNA polymerase II-transcribed U1 snRNA gene promoters and RNA polymerase III-transcribed 5S rRNA gene is in agreement with the observation that CSB is required for 'fragile site' formation on these genes. Together, these data suggest a role of CSB in regulation of RNA polymerase II-transcribed U1 snRNA gene expression and RNA polymerase III gene transcription.

Figure 3-1. Endogenous CSB associates with RNA polymerase II-transcribed snRNA gene promoters and RNA polymerase III-transcribed promoters. Chromatin from MCF-7 cells was immunoprecipitated with anti-SNAP43, IgG (negative control), anti-CSB, anti-TBP and anti-RNA polymerase II and III antibodies. CSB enrichment was observed on RNA polymerase II-transcribed U1 snRNA gene and on the promoters of all three classes of RNA polymerase III transcribed genes. No CSB was detected on the GAPDH exon 2 region, which served as a negative control for immunoprecipitations.


1% Input
0.1% Input
α-SNAP43
IgG
α-CSB
α-TBP
α-RNA pol II
α-RNA pol III
U1 snRNA
GAPDH (exon 2)
5S rRNA
tRNALys
U6 snRNA
Class 1
Class 2
Class 3
RNA pol II
RNA pol III
1 2 3 4 5 6 7 8
Chromatin IP

Endogenous CSB associates with RNA polymerase III protein complexes.

The relationship of CSB to the RNA polymerase I and II transcription has been examined previously. CSB was shown to associate with RNA polymerase I and II protein complexes (2, 34); however, to-date a role of CSB in RNA polymerase III transcription has not been investigated. Thus, we examined the relationship of CSB to the RNA polymerase III using confocal microscopy. The antibody staining patterns in these immunofluorescent studies revealed that both CSB and RNA polymerase III are primarily confined to the nucleus. The overlay of the confocal images showed that approximately 30% of RNA polymerase III co-localizes with CSB (Figure 3-2A).

To further test whether CSB associates with RNA polymerase III complex, HeLa nuclear extract was subjected to immunoprecipitation with anti-CSB or negative control IgG antibodies. As shown in figure 3-2B, RNA polymerase III was specifically enriched in anti-CSB-immunoprecipitated samples, but not in negative control IgG-immunoprecipitated samples. Also, no significant recovery of RNA polymerase II or actin was observed in anti-CSB-immunoprecipitations. CSB was shown to be in the complex with RNA polymerase II when immunoprecipitations are performed under less stringent lower salt concentrations (2). Together, the co-localization of CSB and RNA polymerase III in cells and the presence of these two proteins in a complex suggests a role of CSB in RNA polymerase III transcription.

Figure 3-2. Endogenous CSB associates with RNA polymerase III. (A) HeLa cells were immunostained using antibodies against RNA polymerase III and CSB for visualization by confocal microscopy. This experiment was performed by Chen Wang in the laboratory of Dr. Sui Huang, Feinberg School of Medicine, Northwestern University. (B) Co-immunoprecipitations with anti-CSB antibody (lane 3) or negative control IgG antibody (lane 2) were performed from HeLa nuclear extract. Lane 1 contains 1% of the HeLa nuclear extract that was used for each immunoprecipitation reaction. Western analysis revealed significant differences in recovery of RNA polymerase III in the CSB immunoprecipitation reaction but not in the negative control IgG immunoprecipitation. No actin or RNA polymerase II were recovered in any of the tested conditions.

HeLa cells
l CSB for
en Wang in
University.
ontrol IgG
1% of the
n. Western
in the CSB
ecipitation.

CSB has positive and negative regulatory activities.

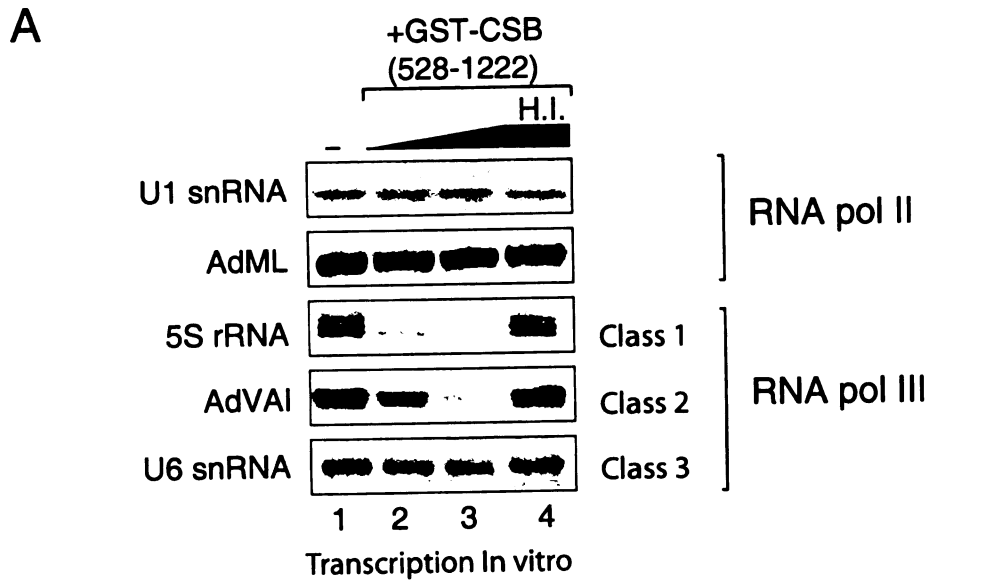
To examine the effect of CSB on RNA polymerase II and III transcription, recombinant GST-CSB (528-1222) protein, containing SNF2-like domains, was titrated into RNA polymerase II-transcribed U1 snRNA and AdML in vitro transcription reactions as well as RNA polymerase III-transcribed 5S rRNA (class 1), AdVAI (class 2) and U6 snRNA (class 3) reactions (Figure 3-3A). In these experiments addition of recombinant GST-CSB (528-1222) repressed RNA polymerase III-transcribed 5S rRNA (class 1) and AdVAI (class 2) gene expression. The repressive effect of CSB on these RNA polymerase III-transcribed genes was specific because the same amounts of the GST-CSB protein did not repress either RNA polymerase III-transcribed U6 snRNA (class 3) gene expression or RNA polymerase II-transcribed U1 snRNA or AdML gene transcription.

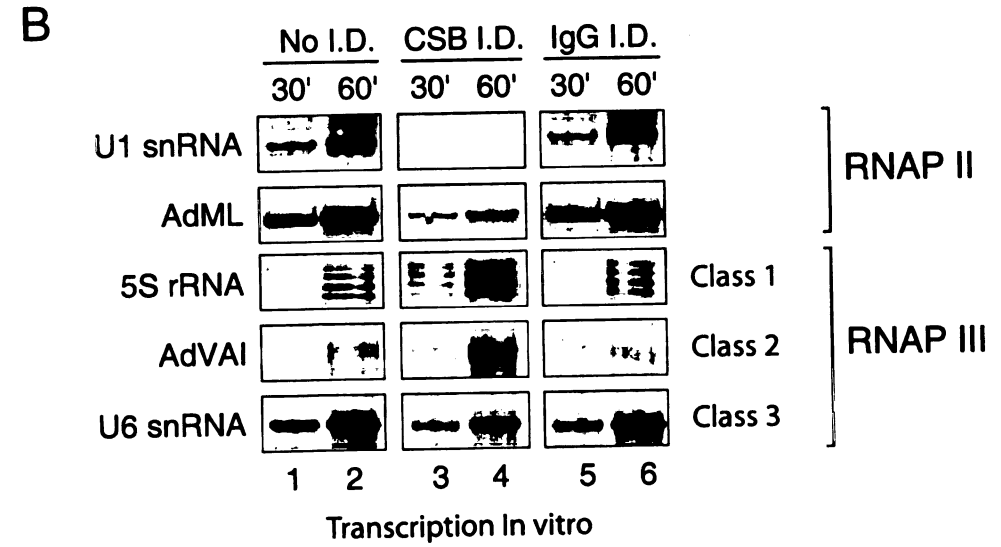
To further examine the role of CSB in transcription in vitro transcription reactions were assembled in the presence of endogenous CSB or in its absence as a result of immunodepletion of CSB from nuclear extracts (Figure 3-3B). The CSB depleted HeLa nuclear extracts did not support expressions of the RNA polymerase II-transcribed U1 snRNA and AdML genes, which is in agreement with suggested positive role of CSB in RNA polymerase II transcription (30). U6 gene expression by RNA polymerase III (class 3) was also modestly decreased in reactions performed with CSB depleted nuclear extracts. In contrast to the effect of CSB immunodepletion on U6 snRNA (class 3) gene expression, RNA polymerase III transcription of 5S rRNA (class 1) and AdVAI (class 2) were stimulated in the absence of CSB. This result is in agreement with the observed repressive functions of exogenous CSB in transcription of class 1 and class 2 RNA

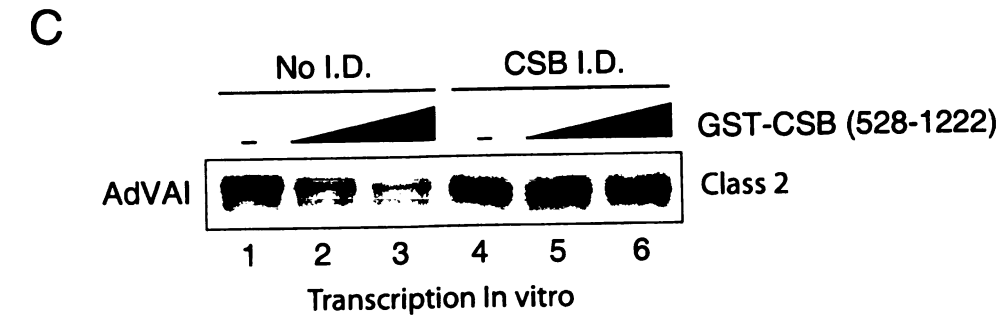
polymerase III-transcribed genes. Interestingly, AdVAI repression was not observed by addition of CSB to nuclear extracts that were immunodepleted for endogenous CSB (Figure 3-3C, compare lanes 5 and 6 to lanes 2 and 3), suggesting that CSB may require a co-repressor for its transcription repression functions.

To determine whether CSB plays a role in RNA polymerase II and III gene transcription in vivo, nuclear run-on experiments were performed using nuclei harvested from human fibroblast cells with normal CSB status (NF) and fibroblast cells with non-functional CSB originally isolated from CS patients (CSB) (Figure 3-3D). Interestingly, U1 snRNA gene transcription was reduced in CSB cells and represented 78% of the U1 snRNA gene transcription in NF cells (p-value = 0.0027, 2-tailed *t*-test). Levels of U6 snRNA gene transcription in CSB cells were also reduced to 90% of that in NF cells (p-value = 0.041, 2-tailed *t*-test). Reduced levels of endogenous U1 and U6 snRNA gene transcription in CSB cells is consistent with the positive role of CSB in snRNA gene transcription regulation. However, 5S rRNA gene transcription was comparable between NF and CSB cells. A possible explanation for the absence of detectable effect of CSB on endogenous 5S rRNA gene transcription is that in contrast to endogenous CSB, the recombinant protein lacks any covalent modifications that may be required for function in certain contexts. Indeed, a study by Christiansen et al., (2003) showed that CSB is phosphorylated in vivo and this can be reversed by UV irradiation (7). The modification status of the CSB protein may determine its ability to interact with its co-factors. For example, endogenous CSB, when phosphorylated, may not be able to interact with its co-repressors. This speculation is in agreement with a conclusion from the experiment 3-3C, which suggests that CSB may require a co-repressor for AdVAI transcription repression.

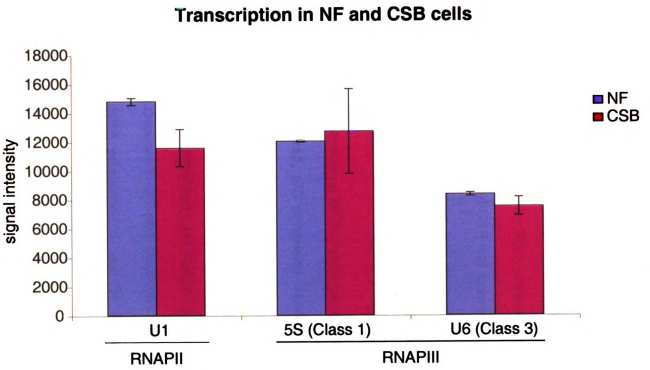
Figure 3-3. CSB regulates snRNA gene transcription. (A) 100 and 400 ng of recombinant GST-CSB (528-1222) were titrated to U1, AdML, 5S, AdVAI and U6 in vitro transcription reactions (lanes 2 and 3). Effect of GST-CSB on transcription was compared to levels of transcription in untreated reactions (lane 1) and reactions containing heat-inactivated (HI) GST-CSB. GST-CSB (528-1222) effectively repressed 5S and AdVAI transcription but it did not affect U1, AdML and U6 transcription. (B) U1, AdML, 5S, AdVAI and U6 in vitro transcription reactions were performed for 30 or 60 minutes with HeLa nuclear extracts containing endogenous CSB (lanes 1 and 2) or immunodepleted for CSB with anti-CSB antibodies (lanes 3 and 4). As a control, immunodepletion of HeLa nuclear extracts was also performed with IgG antibodies (lanes 5 and 6). CSB immunodepleted nuclear extracts supported RNA polymerase II-transcribed U1 snRNA and AdML gene transcription less efficiently as compared to the level of transcription from untreated HeLa nuclear extracts or HeLa nuclear extracts immunodepleted with IgG antibodies. RNA polymerase III-transcribed U6 snRNA gene transcription was also reduced in CSB immunodepleted nuclear extracts. In contrast, transcription of RNA polymerase III-transcribed class 1 (5S rRNA) and class 2 (AdVAI) genes was stimulated in the absence of CSB. (C) 100 and 400 ng of GST-CSB (528-1222) were titrated into AdVAI in vitro transcription reactions performed with HeLa nuclear extracts containing endogenous CSB or immunodepleted for CSB. GST-CSB was not able to repress AdVAI transcription in reactions where endogenous CSB complexes had been removed. (D) Nuclear run-on experiments were performed with nuclei from normal human fibroblasts (NF) and CSB-defective (CSB) human fibroblast cells. Transcription of U1 and U6 snRNA genes was reduced in CSB cells as compared to NF cells. Levels of 5S rRNA gene transcription was comparable between these two cell lines.


A
+GST-CSB
(528-1222)
H.I.
–
U1 snRNA
AdML
5S rRNA
AdVAI
U6 snRNA
RNA pol II
RNA pol III
Class 1
Class 2
Class 3
1 2 3 4
Transcription In vitro


B
No I.D.
CSB I.D.
IgG I.D.
30' 60' 30' 60' 30' 60'
U1 snRNA
AdML
5S rRNA
AdVAI
U6 snRNA
RNAP II
RNAP III
Class 1
Class 2
Class 3
1 2 3 4 5 6
Transcription In vitro


C
No I.D.
CSB I.D.
–
–
GST-CSB (528-1222)
AdVAI
Class 2
1 2 3 4 5 6
Transcription In vitro

D


Transcription in NF and CSB cells
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
signal intensity
NF
CSB
U1
5S (Class 1)
U6 (Class 3)
RNAPII
RNAPIII

p53 antagonizes CSB promoter occupancy.

As activation of p53 or loss of CSB function both cause fragility of the RNU1, RNU2, and 5S loci, the functional interplay between p53 and CSB in U1 snRNA gene transcription was examined because both proteins were shown to associate with U1 snRNA gene promoters. Human U6 snRNA gene promoter occupancy by these proteins was also examined in this study because U6 and U1 snRNA genes have similar promoter structures but U6 snRNA genes are transcribed by RNA polymerase III instead of RNA polymerase II. Promoter occupancy by p53 and CSB was examined in MCF-7 and HeLa cells in response to UV treatment, which causes activation of the endogenous p53 in MCF-7 cells, but not in HeLa cells. As shown in figure 3-4, CSB associates with U1 and U6 snRNA gene promoters in unstressed MCF-7 cells; however, less CSB was detected on the U1 and U6 snRNA gene promoters in MCF-7 cells harvested 4 and 8 hours after UV light exposure (lane 5). In contrast, p53 occupancy increased on the U1 and U6 snRNA gene promoters in MCF-7 cells after UV light treatment (lane 4). In contrast with MCF-7 cells, U1 and U6 snRNA gene promoter occupancy by CSB was unaffected in HeLa cells after UV light treatment. Interestingly, no change was noted in TBP promoter occupancy in both cell types in response to UV light treatment. Thus, UV light exposure decreases CSB occupancy on RNA polymerase II- and III- transcribed snRNA gene promoters in a process that requires functional p53.

The connection between p53 accumulation and CSB loss on snRNA gene promoters was examined in vitro using U6 snRNA gene promoters during p53-mediated transcription repression. Portions of U6 in vitro transcription reaction mixtures (Figure 3-5A) containing wild-type p53 or GST were cross-linked with formaldehyde prior to

Figure 3-4. UV light exposure affects p53 and CSB occupancy on snRNA gene promoters in vivo. Chromatin from untreated or UV light treated MCF7 and HeLa cells was immunoprecipitated with TBP antibodies (lanes 2 and 7), negative control IgG antibodies (lanes 3 and 8), antibodies against the C-terminus of p53 (lanes 4 and 9), and CSB antibodies (lanes 5 and 10). Treatment of MCF7 cells with UV light caused accumulation of p53 on the U1 and U6 snRNA gene promoters concomitant with decreased detectable CSB association. In contrast, CSB association with these snRNA promoters was unchanged in HeLa cells that lack functional p53.

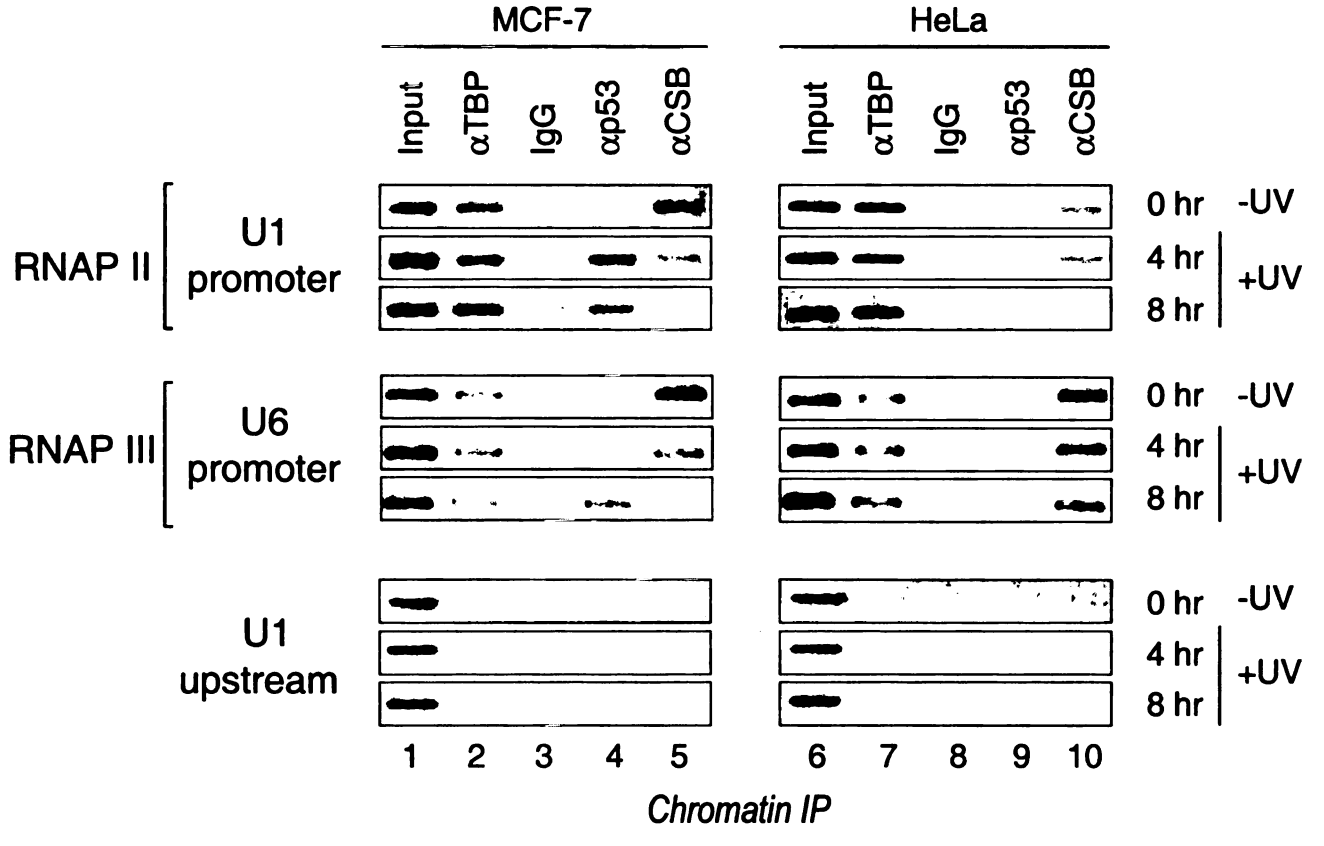
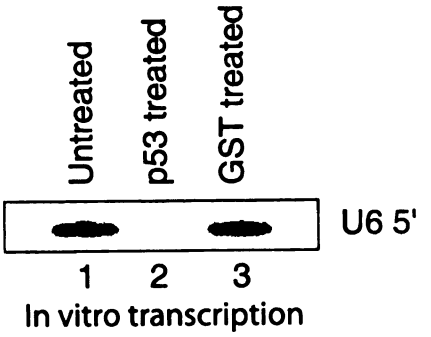
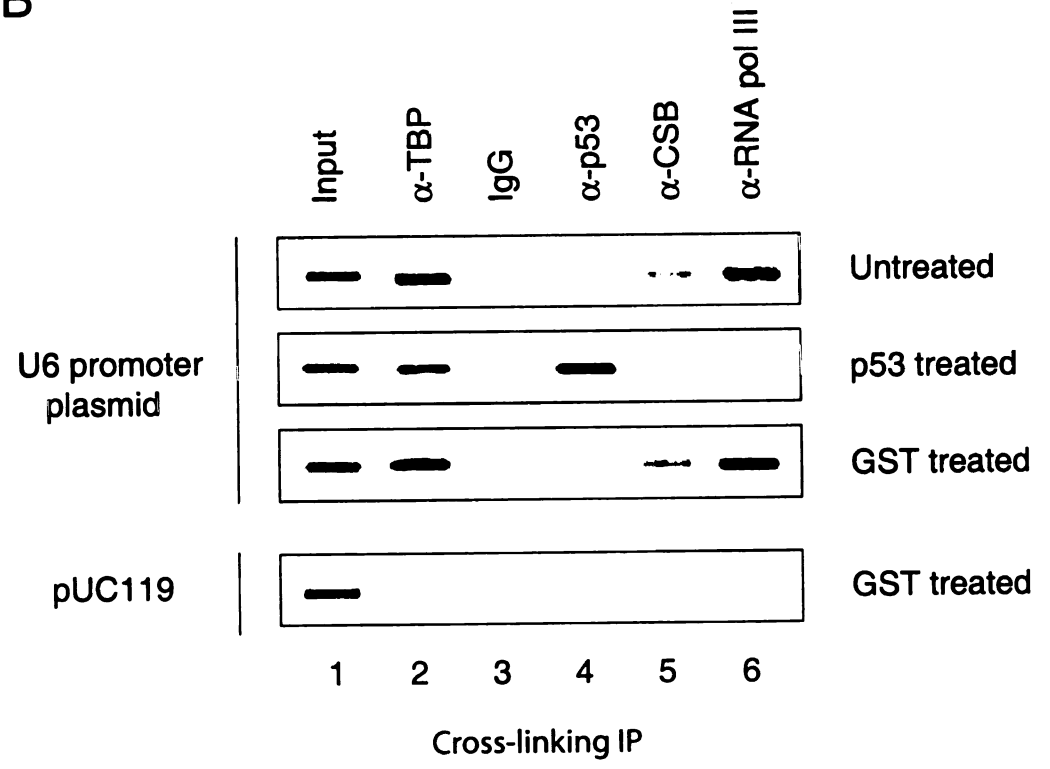

MCF-7
HeLa
Input
αTBP
IgG
αp53
αCSB
Input
αTBP
IgG
αp53
αCSB
RNAP II
U1
promoter
RNAP III
U6
promoter
U1
upstream
0 hr
4 hr
8 hr
-UV
+UV
1 2 3 4 5 6 7 8 9 10
Chromatin IP

Figure 3-5. p53 affects CSB and RNA polymerase III occupancy on U6 snRNA gene promoters in vitro. (A) U6 snRNA in vitro transcription reaction. 400 ng of recombinant p53, but not GST, effectively repressed U6 gene transcription. (B) Portions of the U6 transcription reactions shown in Figure 3-5A were cross-linked with formaldehyde and were subjected to immunoprecipitation with anti-TBP (positive control), IgG (negative control), anti-p53, anti-CSB and anti-RNA polymerase III antibodies. U6 reporter gene DNA was enriched in anti-CSB and anti-RNA polymerase III immunoprecipitation reactions from untreated or GST-treated extracts, whereas p53 addition caused decreased U6 reporter gene recovery by anti-CSB and anti-RNA polymerase III immunoprecipitations. PCR reactions performed directly from the U6 transcription reactions revealed similar amplification of the U6 reporter plasmid (lane 1). The specific recoveries of the U6 promoter-containing plasmid were compared to recovery of an irrelevant pUC119 promoter-less plasmid included in the original transcription assay mixtures.

A


Untreated
p53 treated
GST treated
U6 5'
1 2 3
In vitro transcription

B

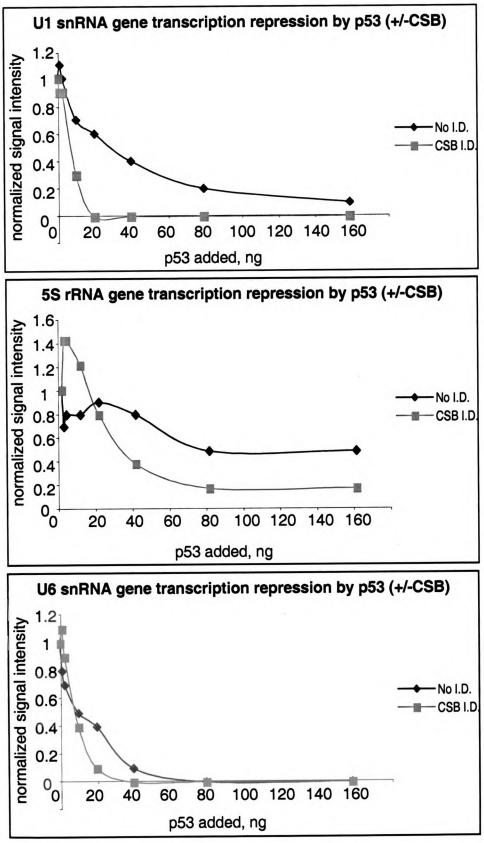

Input
α-TBP
IgG
α-p53
α-CSB
α-RNA pol III
U6 promoter plasmid
Untreated
p53 treated
GST treated
pUC119
GST treated
1 2 3 4 5 6
Cross-linking IP

immunoprecipitations by antibodies directed against TBP, IgG, p53, CSB or RNA polymerase III (Figure 3-5B). A ranscription reaction untreated with any proteins was also tested. The specific recovery of the U6 promoter-containing plasmids were compared to recovery of an irrelevant plasmid (pUC119) included in the transcription assays. As shown in figure 3-5B, significant U6 reporter plasmid recovery by anti-CSB immunoprecipitation was obtained from the untreated or GST treated transcription reaction mixtures, but not from p53 treated reactions, suggesting that p53 may disrupt CSB promoter association. Interestingly, association of RNA polymerase III with U6 promoters was also reduced in p53 treated reactions. In contrast to the effect of p53 on CSB and RNA polymerase promoter occupancy, no change was noted in TBP occupancy. Together, these data suggest that p53 antagonizes CSB promoter occupancy.

CSB modulates transcription regulatory functions of p53.

To test whether CSB is required for p53-mediated U1, 5S rRNA and U6 transcription repression, increasing amounts of recombinant p53 were titrated into in vitro transcription reactions performed using untreated HeLa nuclear extracts or extracts immunodepleted of CSB using anti-CSB antibodies (Figure 3-6). These experiments revealed that p53 repressed U1 snRNA gene transcription better in the absence of CSB. In contrast, the presence or absence of CSB in transcription mixtures did not significantly affect U6 snRNA gene transcription repression by p53. Interestingly, 5S rRNA gene transcription was activated by low amounts of p53 (5 and 10 ng) in the absence of CSB. However, higher amounts of p53 (40, 80 and 160 ng) exhibited stronger repression of 5S

Figure 3-6. Loss of CSB accentuates the RNA polymerase II and III transcriptional response to p53. Approximately 0, 2.5, 5, 10, 20, 40, 80 and 160 ng of p53 were added to U1, 5S and U6 transcription reactions that were performed in vitro using either HeLa nuclear extracts containing CSB or HeLa nuclear extracts immunodepleted for CSB. For comparison, the transcription levels for both types of nuclear extracts with no added p53 were set to one. In these experiments, p53 repressed U1 and U6 transcription better in the absence of CSB. In contrast, 5S rRNA gene transcription was enhanced by low amounts of p53 but was further repressed by higher amounts of p53 in extracts lacking CSB.

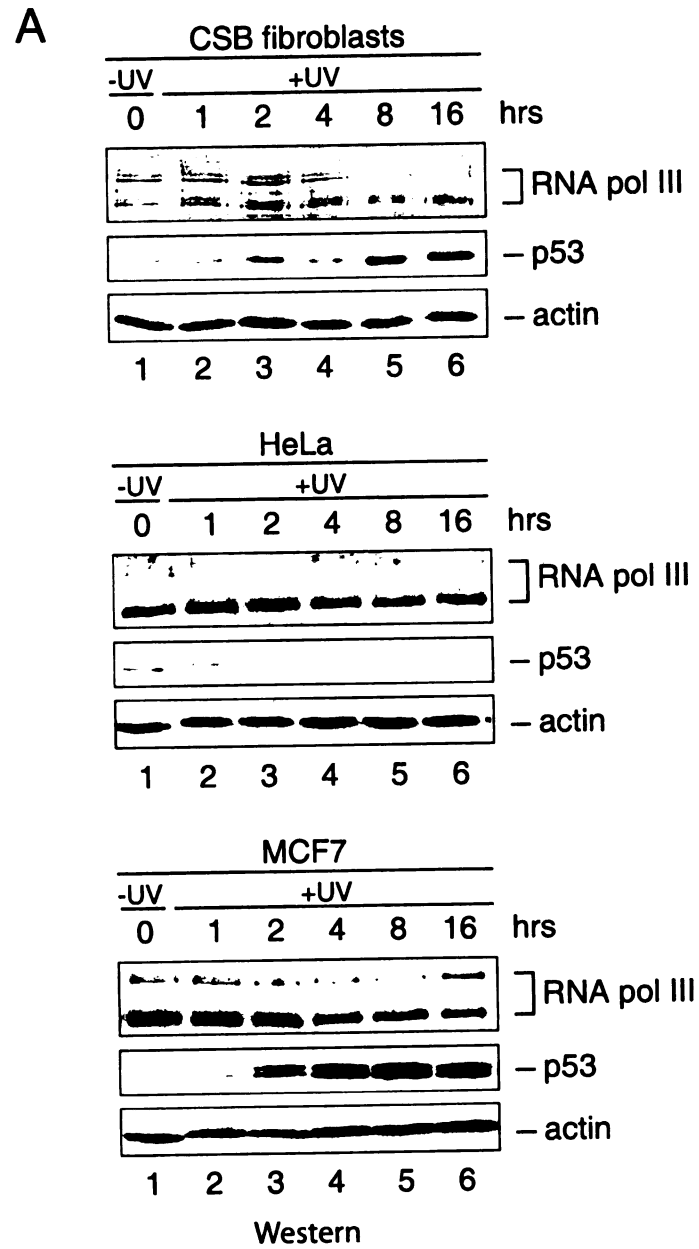

U1 snRNA gene transcription repression by p53 (+/-CSB)
normalized signal intensity
1.2
1
0.8
0.6
0.4
0.2
0
0
20
40
60
80
100
120
140
160
p53 added, ng
No I.D.
CSB I.D.
snRNA (RNAP II)
5S rRNA gene transcription repression by p53 (+/-CSB)
normalized signal intensity
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
20
40
60
80
100
120
140
160
p53 added, ng
No I.D.
CSB I.D.
Class 1 (RNAP III)
U6 snRNA gene transcription repression by p53 (+/-CSB)
normalized signal intensity
1.2
1
0.8
0.6
0.4
0.2
0
0
20
40
60
80
100
120
140
160
p53 added, ng
No I.D.
CSB I.D.
Class 3 (RNAP III)

rRNA gene transcription in the absence of CSB as compared to the extent of p53-mediated 5S rRNA gene transcription repression when CSB was present. Together, these data suggest a modulatory role of CSB on p53 transcription repression functions on these promoters.

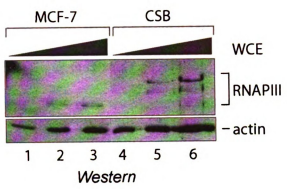
CSB modulates p53-dependent modifications of RNA polymerase III.

It has been reported that RNA polymerase II large subunit (LS) becomes ubiquitylated and proteolytically degraded following UV light treatment in normal but not in CSB deficient fibroblasts. The degradation of elongation incompetent RNA polymerase at sites of DNA damage may be important for the efficient recovery of transcription as cells recover from stress (3). As with RNA polymerase II, CSB may also affect RNA polymerase III stability. To understand the role of p53 - CSB functional interplay for RNA polymerase III stability, I compared RNA polymerase III levels in cells with and without functional CSB or p53 under normal conditions and in response to UV light treatment (Figure 3-7A). Western blot analysis of RNA polymerase III levels in CSB deficient cells revealed multiple slower migrating forms of the polymerase III, which may represent post-translational covalently modified forms of RNA polymerase. These modified forms of RNA polymerase III were less pronounced in MCF-7 cells containing functional CSB and were undetectable in HeLa cells. The proportion of the slower migrating forms of RNA polymerase III to the putative unmodified RNA polymerase III was the highest in CSB deficient cells (Figure 3-7B), suggesting a negative role of CSB in formation of these forms of RNA polymerase III. Interestingly,

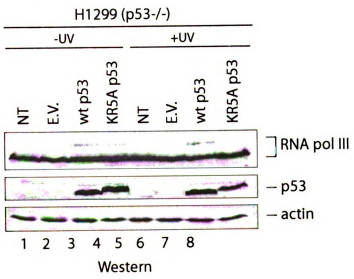
Figure 3-7. CSB modulates p53-dependent modifications of RNA polymerase III. (A) The Cockayne syndrome complementation group B (CSB) protein-defective human fibroblast cells (CSB), HeLa and MCF7 cells were treated with UV light and harvested at indicated times after UV exposure. Western blot analysis revealed slower migrating forms of RNA polymerase III, which were more prominent in untreated CSB cells as compared to MCF-7 cells containing functional CSB. These RNA polymerase III forms were underrepresented in HeLa cells with defective p53. UV light triggered p53 accumulation in CSB and MCF-7 cells and concomitant reduction of modified forms of RNA polymerase III in these cells. (B) Western blot analysis of whole cell extracts from MCF-7 (lanes 1 through 3) and CSB (lanes 4 through 6) cells revealed the enrichment of modified forms of RNA polymerase III in CSB deficient cells as compared to MCF-7 cells. (C) Transient transfection of wild type or acetylation-incompetent KR5A mutant p53 expression plasmids into H1299 (p53-/-) cells causes formation of the higher molecular weight form of RNA polymerase III. After exposure of H1299 cells to UV light, transient transfection of wild type p53, but not acetylation-incompetent KR5A p53, resulted in formation of the slower migrating form of RNA polymerase III.


A
CSB fibroblasts
-UV
+UV
0 1 2 4 8 16 hrs
RNA pol III
– p53
– actin
1 2 3 4 5 6
HeLa
-UV
+UV
0 1 2 4 8 16 hrs
RNA pol III
– p53
– actin
1 2 3 4 5 6
MCF7
-UV
+UV
0 1 2 4 8 16 hrs
RNA pol III
– p53
– actin
1 2 3 4 5 6
Western

B


MCF-7
CSB
WCE
RNAPIII
actin
1 2 3 4 5 6
Western

C


H1299 (p53-/-)
-UV
+UV
NT
E.V.
wt p53
KR5A p53
NT
E.V.
wt p53
KR5A p53
RNA pol III
p53
actin
1 2 3 4 5 6 7 8
Western

HeLa cells with non-functional p53 had the smallest fraction of modified forms of RNA polymerase III, suggesting a positive role of p53 in the formation of modified forms of RNA polymerase III. Intriguingly, UV light treatment of cells resulted in reduction of modified forms of RNA polymerase III in CSB and MCF-7 cells, suggesting that these RNA polymerase III modifications may serve as a 'code' for subsequent modification in response to stress stimuli (e.g. UV light), which may result in intermediate forms of RNA polymerase III, that are more vulnerable for degradation.

To determine whether p53 contributes to RNA polymerase III post-translational modifications, wild type p53 or acetylation-incompetent mutant p53 (KR5A p53) were over-expressed in human osteosarcoma H1299 (p53-/-) cells and levels of RNA polymerase III were evaluated by Western blot analysis (Figure 3-7C). The KR5A p53 was chosen because in contrast to the wild type p53, p53 with mutations that prevent its acetylation was not being able to cause fragile sites induction on U1 and U2 snRNA and 5S rRNA gene loci (37), suggesting that p53 acetylation status may be an important determinant for p53 in this process. Upon transient transfection of wild type or KR5A mutant p53 expression plasmids in H1299 cells a slower migrating form of RNA polymerase III was observed. This form of RNA polymerase III was not present in cells transfected with a negative control empty vector. Interestingly, in UV light treated cells over-expression of wild-type p53, but not acetylation-incompetent KR5A p53, resulted in RNA polymerase III modification, which suggests that the acetylation of p53 may be important for RNA polymerase III modifications in UV light treated cells.

Discussion

A role of CSB in U1 snRNA gene transcription by RNA polymerase II and 5S rRNA gene transcription by RNA polymerase III was suggested based on observation that loss of functional CSB in cells causes metaphase fragility of these genes (37). The same study revealed that fragility in the same genes can be induced by over-expression of p53 (24, 37). The data presented herein directly demonstrate a role of CSB in regulation of RNA polymerase II- and III transcription. In contrast to the positive role of CSB in RNA polymerase II-transcribed snRNA genes, CSB has a negative role in RNA polymerase III-transcribed class 1 and 2 genes. In contrast to other RNA polymerase III-transcribed genes, class 3 (U6 snRNA) was not repressed by CSB, but was modestly activated by CSB similarly to RNA polymerase II-transcribed snRNA genes. Class 3 RNA polymerase III promoters are structurally similar to RNA polymerase II-transcribed snRNA gene promoters (18). Furthermore, class 3 RNA polymerase III genes share some of the general transcription factors with snRNA genes transcribed by RNA polymerase II (18). Thus, CSB may function as a transcriptional activator or repressor depending on promoter architecture and transcription machinery.

A positive role of CSB in RNA polymerase II transcription is suggested from the observation that CSB can modestly stimulate the rate of transcription by RNA polymerase II and thus, may function as an elongation factor (30). The ability of CSB to remodel nucleosomes may also contribute to its functions as a transcriptional activator (8). An interesting feature of the CSB protein is its ability to bind and wrap DNA (1). This could affect the distance between regulatory and core promoter elements and either activate or repress gene transcription. Indeed, snRNA gene transcription activation

requires the cooperative binding of Oct-1 and $SNAP_C$ (13, 38). This cooperativity is mediated by a positioned nucleosome that resides between the DSE and PSE and brings these two snRNA gene promoter elements in proximity (38). The ability of CSB to bind and wrap DNA could also modulate distance between DSE and PSE, thus mimicking the effect of positioned nucleosome on snRNA genes. Also, a negative role of CSB in RNA polymerase III transcription may be the result of CSB-mediated degradation of RNA polymerase III. It has been reported that RNA polymerase II large subunit (LS) becomes ubiquitylated and proteolytically degraded following UV light treatment in normal but not in CSB deficient fibroblasts, suggesting that CSB play role in RNA polymerase II degradation. Following UV-irradiation, the degradation of elongation incompetent RNA polymerase at sites of DNA damage may be important for the efficient recovery of transcription as cells recover from stress (3). As with RNA polymerase II, CSB may also affect RNA polymerase III stability. When levels of RNA polymerase III were analyzed in different human cell with different status of CSB and p53 proteins, we observed that CSB deficient cells have lower levels of unmodified RNA polymerase (Figure 3-7B). We also detected slower migrating and likely covalently modified forms of RNA polymerase III in cells (Figure 3-7A). Interestingly, a larger portion and variety of these modified RNA polymerase III forms was observed in CSB defective fibroblasts (Figure 3-7A and 3-7B), suggesting that presence of functional CSB in HeLa and MCF-7 cells interferes with the accumulation of modified RNA polymerase III in cells. We speculate that these modified RNA polymerase III forms may represent elongation incompetent forms of RNA polymerase III. The absence of CSB-triggered release or degradation of elongation

incompetent RNA polymerase III may result in the accumulation of these forms of RNA polymerase in CSB deficient cells.

Based on the present study I suggest a model for the roles of p53 and CSB in RNA polymerase III modifications and degradation (Figure 3-8). In this model I hypothesize that slower migrating forms of RNA polymerase III represent post-translationally modified elongation incompetent forms of RNA polymerase III. p53 acts as a general repressor of RNA polymerase III transcription by affecting RNA polymerase III transcription elongation or re-initiation, because accumulation of elongation incompetent forms of RNA polymerase III directly correlates with presence of p53 in cells. In contrast to p53, the presence of functional CSB in cells antagonizes accumulation of elongation incompetent forms of RNA polymerase III, suggesting that CSB either helps RNA polymerase III to resume elongation or plays a role in removing elongation incompetent RNA polymerase from the gene, so it can undergo degradation. Cellular stress (e.g. UV light) triggers activation of multiple cellular signaling cascades, which may cause additional modifications of RNA polymerase III, affecting RNA polymerase III gene association and stability. In the future it will be important to determine what types of RNA polymerase III post-translational modifications exist and are induced by p53 in cells.

In summary, I found that in addition to the role of CSB in RNA polymerase I and II transcription, CSB is also involved in RNA polymerase III transcription. Interestingly, CSB can function both as activator or repressor of RNA polymerase III-transcribed genes depending on promoter architecture. I also discovered a novel role of CSB as a modulator of p53 functions. The functional antagonism between p53 and CSB most likely occurs at

Figure 3-8. Model: Roles of p53 and CSB in RNA polymerase III elongation and stability. p53 plays a role in RNA polymerase III modifications, which associates with elongation incompetent RNA polymerase. In contrast, CSB helps RNA polymerase III to resume elongation. In response to UV light exposure elongation incompetent RNA polymerase III could be a substrate for additional modifications triggered by UV light activated signaling cascades. This results in dissociation of RNA polymerase III from gene and its subsequent degradation.

RNA polymerase III
Elongating form
p53 p53
p53 p53
CSB
RNA polymerase III
Covalently modified
elongation incompetent
form
+UV
RNA polymerase III
Covalently modified
elongation incompetent
form
RNA polymerase III
Degradation

the level of RNA polymerase III stability. As p53 triggers RNA polymerase to an elongation incompetent mode, CSB works antagonistically by helping RNA polymerase III to resume elongation or targeting the elongation incompetent RNA polymerase III for destruction.

Cockayne syndrome (CS) is a rare autosomal disease that caused by mutations in CSB. Interestingly, mutations in CSB cause at least four different diseases: Cockayne syndrome (CS), UV-sensitive syndrome ($UV^{S}S$), DeSanctis-Cacchione syndrome (DS-C) and cerebro-oculo-facio-skeletal syndrome (COFS) (25, 31), suggesting that CSB may be involved in different cellular functions possibly via its general role in transcription by all three RNA polymerases. The CSB role in transcription of non-coding RNAs by both RNA polymerase II and III is intriguing. Non-coding RNAs have been shown to be involved in controlling a variety of cellular functions via their role in transcription and RNA metabolisms (11, 12, 22, 26, 36) and misregulations of some non-coding RNAs were recently linked to different human diseases such as human hereditary cartilage-hair hypoplasia disease (28) and cancer (21, 32). Multiple transcription targets for CSB helps to explain multiple phenotypes of CS patients.

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CHAPTER FOUR

SUMMARY

Non-translated snRNAs perform essential cellular functions by controlling RNA synthesis and RNA metabolism (8). The involvement of snRNAs in controlling diverse cellular functions suggests that these genes should be tightly regulated under normal conditions and in response to cellular stress. Indeed, misregulation of some snRNAs have been recently linked to several human diseases, such as cartilage-hair hypoplasia (CHH) disease (15) and cancer (13, 17). In addition, both tumor suppressor proteins (e.g. p53 (3, 4) and RB (6)) and oncoproteins (e.g. Myc (6) and CKII (7, 10, 11)) have been linked to snRNA gene transcription, suggesting that regulated snRNA production plays a role in controlling cellular homeostasis.

Depending on the promoter architecture, snRNA genes are transcribed by either RNA polymerase II or by RNA polymerase III (8). Regardless of RNA polymerase specificity, snRNA genes have similar promoter structure and share some of the general transcription factors (reviewed in 8), suggesting that these genes may be similarly regulated in response to cellular stress stimuli.

The overall goal of my research project was to understand the role of the tumor suppressor protein p53 in human snRNA gene transcription. The tumor suppressor protein p53 is a multifunctional transcription factor known to control cellular proliferation and genomic stability by activating or repressing transcription of target genes by all three classes of RNA polymerases (3-5, 16, 21). It was previously

demonstrated that p53 represses snRNA gene transcription by RNA polymerase III (3, 4); however, the importance of p53 for transcription of RNA polymerase II-transcribed snRNA genes was not investigated.

My studies revealed that in addition to the role of p53 in snRNA gene transcription by RNA polymerase III, p53 also represses snRNA gene transcription by RNA polymerase II. Thus, p53 is a general repressor of snRNA gene transcription by both polymerases. I found that p53 associates with human U1 and U6 snRNA genes during transcription repression and represses endogenous snRNA gene transcription upon transient transfection or in response to UV-light, suggesting a role of p53 in snRNA gene transcription regulation in response to DNA damage.

It has been shown that p53 can repress transcription of its target genes by a variety of mechanisms that regulate pre-initiation complex formation, elongation by RNA polymerase and at the level of chromatin (reviewed in 9). It was proposed that p53-mediated repression of RNA polymerase III transcription occurs via TBP-mediated p53 interaction with TFIIIB, which interferes with TFIIIB binding and subsequent recruitment of RNA polymerase III to RNA polymerase III promoters (3, 5). In contrast, similar levels of TBP at snRNA gene promoters before and during p53-mediated transcription repression were observed, suggesting that p53 interferes with snRNA gene transcription at steps occurring after TBP or TFIIIB recruitment for RNA polymerase II- and III-transcribed genes respectively. Indeed, RNA polymerase III was not detected at U6 snRNA gene promoters during repression by p53, suggesting that p53 interferes with RNA polymerase III recruitment to snRNA gene promoters. Whether p53 also affects the recruitment of RNA polymerase II to snRNA gene promoters remains to be investigated.

Interestingly, upon p53 accumulation on snRNA gene promoters the concomitant enrichment of HDACs was observed, suggesting that p53 repression of snRNA gene transcription may in part occur by recruitment of the chromatin remodeling activities of HDACs. The HDAC-mediated p53 transcription repression has been described for some genes (12, 14) and the role of HDACs in deacetylation of core histones as well as p53 itself was suggested (12). What are the determinants for p53 decision to recruit HDAC for transcription repression? Recently, SUMO modification of several transcription factors has been linked to HDACs recruitment and transcription repression (19). Interestingly, I observed accumulation of sumoylated forms of p53 in cells after UV light treatment. When the proportion of sumoylated protein population was analyzed on snRNA gene promoters, the direct correlation between levels of total p53, sumoylated proteins and accumulation of HDACs on snRNA gene promoters was detected, suggesting that promoter-bound sumoylated p53 may recruit HDACs during transcription repression of snRNA genes.

As part of our investigation of the mechanism of p53-mediated snRNA gene transcription repression, I tested a hypothesis that p53 represses RNA polymerase II-transcribed snRNA genes by interfering with CSB-mediated elongation causing stalling of RNA polymerase II (20). Our studies reveal a direct role of CSB not only in snRNA gene transcription by RNA polymerase II, but also in transcription by RNA polymerase III, including class 3 RNA polymerase III-transcribed snRNA genes. Thus, CSB may be targeted by p53 during snRNA gene transcription repression by both polymerases. Indeed, our data suggests that CSB modulates p53 transcription repression functions and, in the absence of CSB, p53 exhibits stronger repression of U1 snRNA and 5S rRNA gene

transcription. In addition to the role of CSB as an elongation factor for RNA polymerase II-transcribed genes, CSB has also been reported to play a role in ubiquitylation and degradation of RNA polymerase II large subunit (LS) following UV light treatment (2); however, whether CSB also plays a role in RNA polymerase III stability was uncertain. Degradation of elongation incompetent forms of RNA polymerases at sites of DNA damage may be important for the efficient recovery of transcription as cells recover from stress. Interestingly, when we compared RNA polymerase III levels in human cells with different status of CSB, we observed that levels of RNA polymerase III are lower in CSB deficient cells, suggesting that similarly to RNA polymerase II, CSB may also affect RNA polymerase III stability. However, we found that CSB defective fibroblasts have a larger portion of slower migrating and possibly covalently modified forms of RNA polymerase III. As CSB was described as an elongation factor for RNA polymerase II, we speculate that these modified forms of RNA polymerase III in CSB deficient fibroblasts represent elongation incompetent forms of RNA polymerase III. Interestingly, as p53 accumulation in cells correlates with RNA polymerase III transcription repression, it also results in enrichment of slower migrating forms of RNA polymerase III in cells. Thus, I hypothesized that p53 represses elongation by RNA polymerase through its post-translational modifications. At this point it is not clear what kinds of covalent modifications of RNA polymerase III exist in cells and which modifications serve as 'markers' for elongation incompetent RNA polymerase III. As of now, these modifications may represent either phosphorylated, ubiquitylated, sumoylated or neddylated forms of RNA polymerase III. So far, only ubiquitin-modification of proteins are considered to be a marker for protein degradation by the 26S proteasome (18).

Though both mono- and poly-ubiquitylation of proteins were described, poly-ubiquitylated proteins are more likely be targeted to proteasomal degradation (18). The function for protein mono-ubiquitylation has not been yet well characterized. It was proposed that mono-ubiquitylation is a prerequisite for poly-ubiquitylation and several ubiquitin-ligases are only capable to mono-ubiquitylate their substrates (1). We speculate that p53 plays a role in mono-ubiquitylation of RNA polymerase III, which results in elongation incompetent forms of RNA polymerase III. What is the mechanism for p53-mediated RNA polymerase III modification? We do not yet have an answer to this question. It is possible that p53 itself serves as an E3 ubiquitin ligase for RNA polymerase III modification; however, to-date neither p53 interactions with polymerases nor p53 activity as an E3 ubiquitin ligase were reported. An other possibility is that p53 may recruit ubiquitin ligases (e.g. Mdm2 and/or others) during transcription repression.

Together, our data suggests that p53-mediated snRNA gene transcription repression is complex multilevel process, which may be important to assure snRNA gene repression by p53 in response to cellular stress.

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APPENDIX

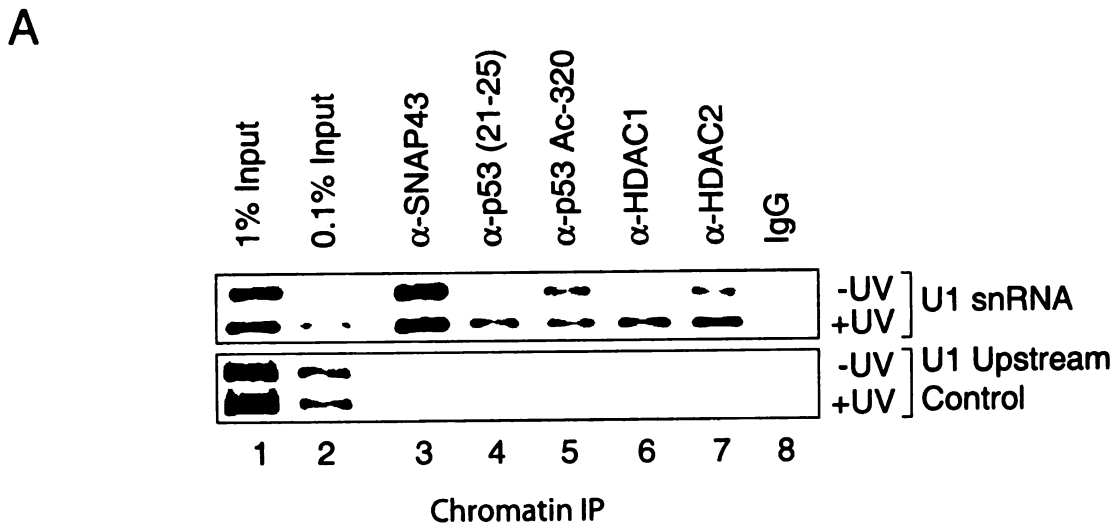
POSSIBLE ROLE OF HDACs IN P53-MEDIATED snRNA GENE TRANSCRIPTION REPRESSION

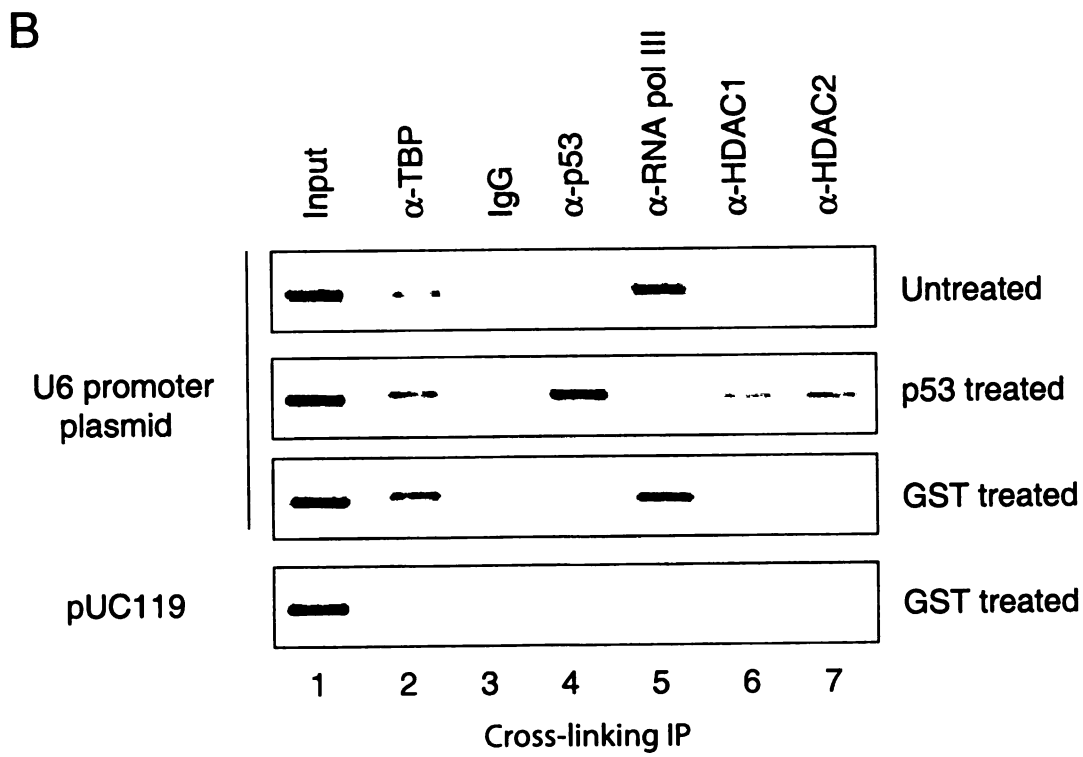
p53 has been shown to repress transcription of its target genes by mechanisms that regulate pre-initiation complex formation and elongation by RNA polymerase. In addition, transcriptional repression by p53 can be also achieved through p53-mediated alteration of chromatin (reviewed in 6).

The ability of p53 to repress transcription at the level of chromatin was linked to recruitment of histone deacetylases (HDACs) to target genes (7, 8). p53 was shown to associate with HDACs via direct interaction of p53 with Sin3a protein of the Sin3/HDAC co-repressor complex (8). Recruitment of HDACs by p53 is believed to result in p53-mediated repression by at least two different mechanisms: core histone deacetylation and p53 deacetylation (7). HDAC-mediated histone deacetylation results in chromatin condensation and reduced promoter accessibility to the transcriptional machinery and/or co-activators (11). Consistent with the positive role of p53 acetylation for transactivation activity of p53 (1), p53 deacetylation by HDACs results in p53-mediated transrepression of target gene transcription (7, 8).

To test whether HDACs are involved in snRNA gene transcription repression, snRNA gene promoter occupancy by HDACs was analyzed using immunoprecipitation experiments (Figure A-1). As shown in figure A-1A, p53 and HDAC1 and 2 occupancy on the endogenous U1 snRNA gene promoter was concomitantly increased in response to

Figure A-1: Endogenous HDAC1 and HDAC2 associate with human snRNA gene promoters. (A) Chromatin from untreated or UV light treated MCF-7 cells was immunoprecipitated with SNAP43 antibodies, antibodies against total p53 (21-25) or K320 acetylated p53, and HDAC1, HDAC2 or negative control IgG antibodies (lanes 3 through 8, respectively). Lanes 1 and 2 represent input titration. p53, HDAC1 and HDAC2 occupancy on U1 snRNA promoter (but not on negative control U1 upstream region) was increased after UV light treatment. No change in U1 snRNA promoter occupancy by acetylated p53 was observed after UV light exposure. (B) A portion of untreated or p53 or GST treated U6 in vitro transcription reaction mixtures was cross-linked with formaldehyde and subjected to immunoprecipitation with anti-TBP, IgG, anti-p53, anti-RNA polymerase III, anti-HDAC1 and anti-HDAC2 antibodies (lanes 2 through 7, respectively). Enrichment of the U6 reporter plasmids or negative control pUC119 DNA was compared by PCR using promoter-specific or pUC-specific primers. Lane 1 represents 10% input DNA.

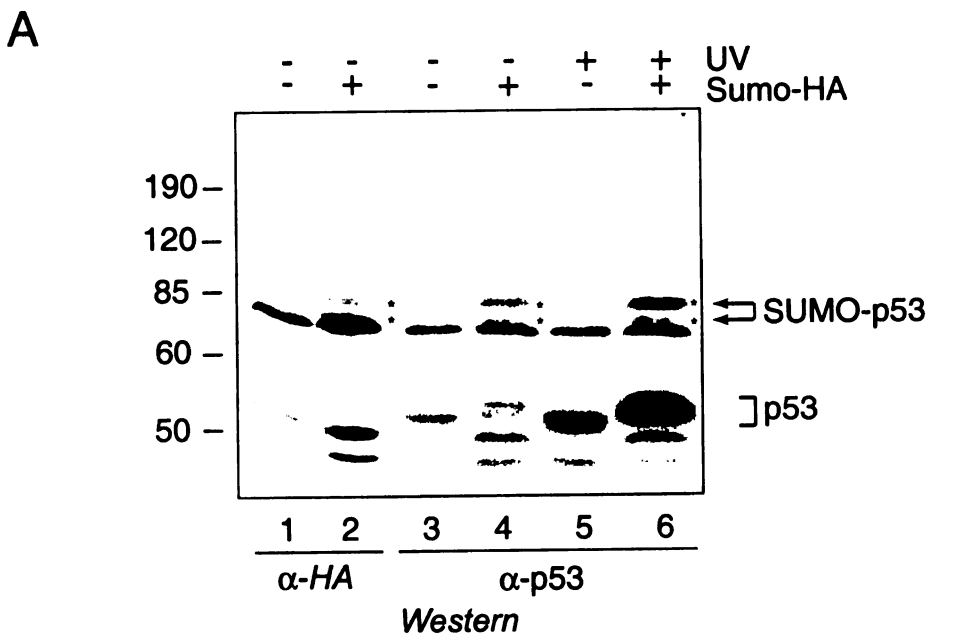

A
1% Input
0.1% Input
α-SNAP43
α-p53 (21-25)
α-p53 Ac-320
α-HDAC1
α-HDAC2
IgG
-UV
+UV
U1 snRNA
-UV
+UV
U1 Upstream
Control
1 2 3 4 5 6 7 8
Chromatin IP

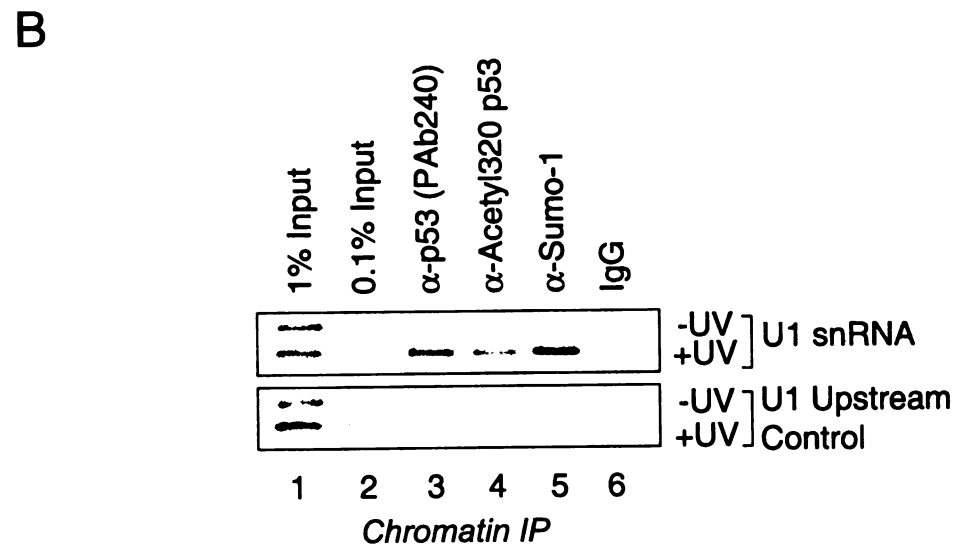

B
Input
α-TBP
IgG
α-p53
α-RNA pol III
α-HDAC1
α-HDAC2
U6 promoter plasmid
Untreated
p53 treated
GST treated
pUC119
GST treated
1 2 3 4 5 6 7
Cross-linking IP

UV light treatment, suggesting that HDACs are involved in snRNA gene transcription in response to UV light treatment and that p53 may recruit HDACs to U1 snRNA gene promoters during transcription repression. Specific enrichment of HDAC1 and 2 was also observed on exogenous U6 snRNA gene promoters during p53-mediated repression using in vitro transcription assays (Figure A-1B, lanes 6 and 7 of the p53 treated reactions); however, no recovery of U6 snRNA gene promoters was observed in anti-HDAC1 and 2 immunoprecipitated samples from untreated or GST treated transcription reactions. Together, these data suggest that p53 may recruit HDAC1 and 2 during snRNA gene transcription repression.

What are the determinants for p53-mediated recruitment of HDACs during transcription repression? Recently, it has been reported that sumoylation of proteins may promote HDAC recruitment and transcription repression (12). It has been shown that p53 can be sumoylated at Lys386 (9) and both positive and negative roles of sumoylation for p53-mediated transactivation of target genes were described (2, 9, 10). Thus, we asked whether p53 sumoylation mediates HDACs recruitment and transcription repression by p53. Interestingly, as UV light exposure results in accumulation of total p53 as well as sumo-modified forms of p53 (Figure A-2A), the concomitant enrichment of p53 and sumoylated protein population was observed on the endogenous U1 snRNA gene promoters in response to UV light treatment in chromatin immunoprecipitation experiments (Figure A-2B). Since there are no commercially available antibodies against sumoylated p53, we can only speculate that U1 snRNA gene promoter occupancy by sumoylated p53 was also enriched in response to UV light exposure and contribute to the signal from the total sumoylated protein population recognized by anti-Sumo-1

Figure A-2: Sumoylated protein population is enriched on U1 snRNA gene promoters after UV exposure. (A) UV light exposure of MCF-7 cells results in accumulation of total p53 as well as sumo-modified forms of p53 as measured by Western blotting. Whole cell extracts from untransfected and pCDNA-HA-Sumo-1, -2, and -3 cotransfected MCF-7 cell harvested prior and 8 hours after UV light treatment were analyzed by 12.5% SDS-PAGE and Western blot analysis of HA-tagged sumoylated proteins (lanes 1 and 2) and p53 (lanes 3 through 6). Co-migrating bands that were detected by both anti-HA and anti-p53 antibodies were labeled as SUMO-p53. (B) Chromatin from untreated or UV light treated MCF-7 cells was immunoprecipitated with antibodies against total and K320 acetylated p53, antibodies against Sumo-1 or negative control IgG antibodies (lanes 3 through 6, respectively). Lanes 1 and 2 represent input titration. p53 and sumoylated proteins were concomitantly enriched at endogenous U1 snRNA gene promoters after UV light treatment. No change in U1 snRNA promoter occupancy by acetylated p53 was observed after UV light exposure.


A
- - - - + + UV
- + - + - + Sumo-HA
190 –
120 –
85 –
60 –
50 –
SUMO-p53
p53
1 2 3 4 5 6
α-HA
α-p53
Western


B
1% Input
0.1% Input
α-p53 (PAb240)
α-Acetyl320 p53
α-Sumo-1
IgG
-UV
+UV
U1 snRNA
-UV
+UV
U1 Upstream Control
1 2 3 4 5 6
Chromatin IP

antibodies (lane 5). Thus, we observed direct correlation between levels of p53, sumoylated proteins and HDAC1 and 2 on U1 snRNA promoters after UV light treatment (Figures A-1 and A-2B), suggesting that sumo-modified p53 may recruit HDACs for repression of snRNA gene transcription.

I further tested whether Sumo-modification of p53 is required for p53-mediated snRNA gene transcription repression by comparing the ability of wild type GST-p53 and GST-p53 (K386R) to repress U1 and U6 snRNA gene transcription (Figure A-3). Both GST-p53 and GST-p53 (K386R) were expressed in *E. coli* and supposedly lack any post-translational modifications. However, during in vitro transcription assays these proteins could undergo covalent modifications by enzymes present in the HeLa nuclear extract. Thus, during in vitro transcription wild type GST-p53 could become sumoylated; in contrast, GST-p53 (K386R) should lack the sumo-modification. These experiments revealed that sumoylation-deficient mutant GST-p53 (K386R) repressed both U1 snRNA gene transcription by RNA polymerase II and U6 snRNA gene transcription by RNA polymerase III approximately two fold less efficiently than wild type GST-p53, suggesting that sumoylation of p53 may contribute to p53-mediated snRNA gene transcription repression. A possible explanation of the modest effect of p53 K386R mutation on snRNA gene transcription repression is that in vitro transcription assays were performed using naked DNA, but not a chromatinized template. If p53 sumoylation is indeed important for HDAC recruitment, HDAC-mediated repression of snRNA genes may require a chromatin context. Indeed, we did not observe any effect of the HDAC inhibitor sodium butyrate on p53-mediated snRNA gene transcription repression using non-chromatinized DNA templates (data not shown). In addition, the proportion of wild

Figure A-3: p53 sumoylation contributes to p53-mediated snRNA gene transcription repression in vitro. Recombinant wild type GST-p53 (lanes 2 through 4) and GST-p53 (K386R) (lanes 6 through 8) were titrated into U1, U6 and AdML in vitro transcription reactions. 0.5, 1 and 2 μg of proteins were used in U1 and AdML in vitro transcription assays and 0.15, 0.3 and 0.6 μg of proteins were added into U6 in vitro transcription mixtures. Heat-inactivated GST-p53, GST-p53 (K386R) and GST were also tested in these transcription reactions (lanes 5, 9 and 10, respectively). Lane 1 shows level of transcription when no proteins were added.

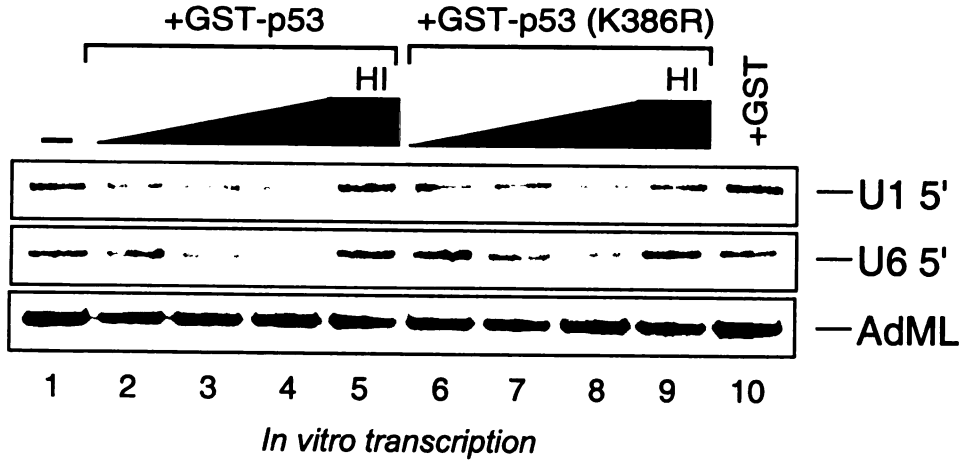
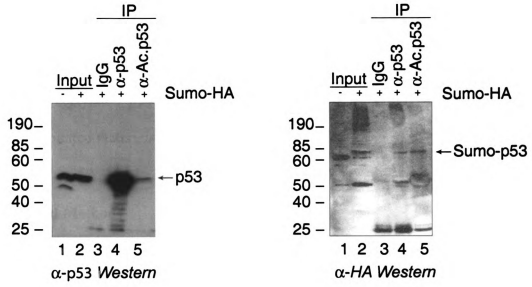

+GST-p53
+GST-p53 (K386R)
HI
HI
+GST
—U1 5'
—U6 5'
—AdML
1 2 3 4 5 6 7 8 9 10
In vitro transcription

Figure A-4: Sumoylated p53 is enriched in acetylated population of p53 in vivo. Whole cells extracts from UV light treated MCF-7 cells expressing HA-Sumo-1, -2 and – 3 proteins were immunoprecipitated with either full length p53 antibodies (lane 4), antibodies against p53 acetylated at K320 (lane 5) or negative control IgG antibodies (lane 3). Presence of p53 and Sumo-p53 was detected by immunoblotting with another p53 (Ab-6) (left panel) and anti-HA (right panel) antibodies.


IP
Input
IgG
α-p53
α-Ac.p53
Sumo-HA
190
85
60
50
40
25
p53
1 2 3 4 5
α-p53 Western
IP
Input
IgG
α-p53
α-Ac.p53
Sumo-HA
190
85
60
50
40
25
Sumo-p53
1 2 3 4 5
α-HA Western

type p53 that gets sumoylated during in vitro transcription is low and majority of the wild type GST-p53 was not sumoylated, but may repress transcription via sumoylation-HDAC-independent mechanism. Thus, it would be useful to know what proportion of the wild type p53 (if any) gets sumoylated during in vitro transcription assays.

Interestingly, I observed that sumoylated forms of p53 are enriched in acetylated p53 population, suggesting that acetylation may be a prerequisite for a subsequent sumoylation of p53. As shown in figure A-4, despite the fact that more p53 was immunoprecipitated with antibodies against total cellular p53 (lane 4, left panel) as compared to acetylated p53 (lane 5, left panel), levels of sumoylated p53 in these immunoprecipitated materials were comparable.

Materials and Methods

Cell culture and UV irradiation

Human MCF-7 cells were maintained in Dulbecco's modified Eagle's medium supplemented with penicillin-streptomycin and 10% fetal bovine serum. Cells grown to 70 to 80% confluence were washed with phosphate-buffered saline and irradiated with 50 J of UV light (254-nm peak)/m^2 by using a UV Stratalinker (Stratagene). After irradiation, growth medium was added and cells were incubated at 37°C under 5% CO_2 for the indicated times. Additionally, MCF-7 cells were grown to 50% confluence in 150-mm plates and were then transiently co-transfected with 5 μg of pCDNA3-HA-Sumo-1, pCDNA3-HA-Sumo-2 and pCDNA3-HA-Sumo-3 plasmids (gift from Dr. Kwok, University of Michigan) using 3 μl Lipofectin reagent (Invitrogen) per transfection. Transfections were performed for 6 h. Subsequently, the medium was replaced and cells

were incubated for 48 h for further analysis.

Expression and purification of recombinant proteins

Glutathione *S*-transferase (GST), GST-tagged full-length human p53 and GST were expressed in *Escherichia coli* BL21 (DE3) codon+ cells (Stratagene) and were affinity purified by binding to glutathione agarose beads (Sigma). GST-p53 and GST proteins were then eluted from beads in HEMGT-150 buffer containing 50 mM glutathione for 4 h at 4°C and concentrated by centrifugation using a Centricon YM-30 spin column (Millipore) in HEMGT-80 buffer (20 mM HEPES [pH 7.9], 0.1 mM EDTA, 10 mM MgCl2, 10% glycerol [vol/vol], 0.1% Tween 20, 80 mM KCl) containing protease inhibitors and 1 mM dithiothreitol.

In vitro transcription assays

In vitro transcription assays were performed as described previously (4) using 18, 2, and 10 μL of HeLa cell nuclear extract for the U1 snRNA, U6 snRNA, and adenovirus major late promoter (AdML) transcription reactions, respectively. The pU1-4.0 (1 μg), pU6/Hae/RA.2 (250 ng), and M13-AdML (250 ng) templates were used for the U1 snRNA, U6 snRNA, and AdML transcription reactions, respectively. Purified proteins were added in the amounts indicated in the figure legends. Transcription was performed for 1 h at 30°C. Transcripts were separated by denaturing 6% polyacrylamide gel electrophoresis and visualized by autoradiography.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation assays were performed as described previously (5). Human MCF-7 cells were grown to 60 to 80% confluence and were then cross-linked with 1% formaldehyde for 30 min at room temperature. After cell lysis and sonication, immunoprecipitation reactions were performed overnight at 4°C using chromatin from approximately 10^7 cells per reaction mixture and 1 μg of each antibody: anti-SNAP43 (CS48), anti-p53 (21-25) (Ab-6; Oncogene), anti-acetyl-p53 (320) (Upstate), anti-HDAC1 (Santa Cruz), anti-HDAC2 (Santa Cruz), or pre-immune IgG (Sigma) antibodies. Anti-Sumo-1 antibodies were purchased from Zymed. Recovered chromatin was suspended in 50 μL of H_2O, and PCR analysis was performed using 5 μL of immunoprecipitated chromatin or input chromatin. The primers used for amplification of each gene were described previously (3). PCR products were separated by 2% agarose electrophoresis in Tris-borate-EDTA buffer and were stained with ethidium bromide.

Immunoprecipitations from in vitro transcription reactions

In vitro transcription assay mixtures containing U1 or U6 promoter plasmids and equal molar amounts of pUC119 were performed as described previously (5) in the absence or presence of full-length wild type p53 or GST. 5 μL of each transcription reaction mixture was diluted to 500 μL and was cross-linked in 1% formaldehyde for 10 min at room temperature, quenched with 125 mM glycine for 10 min at room temperature, and immunoprecipitated with anti-TBP (SL2), pre-immune IgG (Sigma), anti-p53 (Ab-6; Oncogene), anti-RNA polymerase III (TB2) and anti-HDAC1 (Santa Cruz) or anti-HDAC2 (Santa Cruz) antibodies. Recovered plasmid DNA was analyzed by PCR using

primers specific to the U1 and U6 promoter regions or to pUC119 as a negative control.

Immunoprecipitation experiments

Whole cell extracts from pCDNA-HA-Sumo-1, -2, and -3 cotransfected MCF-7 cell harvested 8 hours after UV light treatment were diluted to 1 mL using HEMGT-150 buffer containing protease inhibitors and incubated with 1 μg of antibodies directed against goat IgG (Sigma), full length p53 (Upstate) or anti-acetyl-p53 (320) (Upstate) 90 minutes at room temperature. Stable complexes were then affinity purified by incubation with Protein-G Fast Flow sepharose beads (Upstate Biotechnology) for 90 minutes at room temperature. Beads were washed in HEMGT-150 buffer and boiled for 5 minutes in Laemmli Buffer. Bound proteins were separated by 12.5% SDS-PAGE and transferred to nitrocellulose. Western blot analyses of recovered proteins were performed using anti-p53 (21-25) (Ab-6; Oncogene) and anti-HA antibodies.

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