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p53 Inhibits RNA Polymerase III-Directed Transcription in a Promoter-Dependent Manner

IGOR CHESNOKOV,[1] WEN-MING CHU,[1] MICHAEL R. BOTCHAN,[2] AND CARL W. SCHMID[3]*

Section of Molecular and Cellular Biology[1] and Department of Chemistry,[3] University of California, Davis, Davis, California 95616, and Department of Molecular and Cellular Biology, University of California, Berkeley, Berkeley, California 94720[2]

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Wild-type p53 represses *Alu* template activity in vitro and in vivo. However, upstream activating sequence elements from both the 7SL RNA gene and an *Alu* source gene relieve p53-mediated repression. p53 also represses the template activity of the U6 RNA gene both in vitro and in vivo but has no effect on in vitro transcription of genes encoding 5S RNA, 7SL RNA, adenovirus VAI RNA, and tRNA. The N-terminal activation domain of p53, which binds TATA-binding protein (TBP), is sufficient for repressing *Alu* transcription in vitro, and mutation of positions 22 and 23 in this region impairs p53-mediated repression of an *Alu* template both in vitro and in vivo. p53's N-terminal domain binds TFIIIB, presumably through its known interaction with TBP, and mutation of positions 22 and 23 interferes with TFIIIB binding. These results extend p53's transcriptional role to RNA polymerase III-directed templates and identify an additional level of *Alu* transcriptional regulation.

Alu transcripts are barely detectable in cultured cells despite the transcriptional potential of one million templates in the human genome having internal A-box and B-box RNA polymerase III (Pol III) promoter elements (24). Because *Alu* transcripts are retropositional intermediates and may also serve a normal physiological role, their regulated expression is important (24, 30). The extremely low level of Pol III-directed *Alu* expression is attributable to a hierarchy of controls, including chromatin structure, repression due to methylation, an inherently weak internal promoter, and posttranscriptional events (5, 7, 17, 24). However, adenovirus infection greatly increases the abundance of *Alu* RNA (21, 23). Adenovirus activates TFIIIC and may also unmask *Alu* chromatin, providing two explanations for its effect on *Alu* RNA (23, 26). However, adenovirus E1A protein also displaces the tumor suppressor gene product p53 from binding with TATA-binding protein (TBP) (9). TBP is an integral component of TFIIIB (reference 22 and references therein), suggesting another possible target for adenovirus's effects on *Alu* transcription.

The tumor suppressor gene product p53 can both activate and repress transcription by directly interacting with the basal transcriptional machinery. p53 stimulates Pol II transcription of genes flanked by p53 response elements (8, 10). To cause this stimulation, one domain of p53 binds the response element, while the interaction of its N-terminal transactivation domain with TFIID-associated TBP-associated factors (TAFs) activates the initiation complex (27). p53 also represses expression of many Pol II-transcribed genes that lack p53 response elements (18, 19, 25, 28) by interaction with TBP. Possibly p53 activates transcription when it is bound to DNA and contacts TFIID but represses transcription when it associates with TFIID without contacting the DNA template.

We report here that, depending on the structure of the promoter, p53 represses Pol III-directed transcription both in vitro and in vivo. Further, in the case of an *Alu* template, p53's N-terminal transactivation domain is necessary for this repression in vivo and is sufficient for this activity in vitro. Additional results indicate that this region of p53 binds TFIIIB.

* Corresponding author. Mailing address: Section of Molecular and Cellular Biology, University of California, Davis, Davis, CA 95616. Phone: (916) 752-9029. Fax: (916) 752-3085. Electronic mail address: cwschmid@ucdavis.edu.

MATERIALS AND METHODS

In vitro transcription assays. Plasmids tested for Pol III template activity in vitro include *Alu* source gene constructs with and without 5′-flanking sequences (4, 13), chimeric 7SL-*Alu* and *Alu*-T constructs (5), and plasmids containing the 7SL RNA, U6 RNA, 5S RNA, adenovirus VAI RNA, and *Xenopus* $tRNA^{Met}$ genes (3, 11, 12, 16). The 7SL-*Alu* chimera consists of 180 bp located 5′ to the 7SL RNA gene fused to an *Alu* repeat (5). The same *Alu* element devoid of any 5′-flanking sequences was fused to a defined terminator for Pol III to construct *Alu*-T (5). Five hundred nanograms of DNA template was used in reactions. Previous control experiments using nuclear extracts show that the resulting transcripts in these assays are directed by Pol III, and the present experiments were performed in the presence of 1 μg of α-amanitin per ml (reference 5 and references therein).

As indicated below, transcription assays included either purified, wild-type human p53 (gift from B. Vogelstein) or various p53–glutathione *S*-transferase (GST) fusion proteins. In these cases, added protein was preincubated with nuclear extract at 30°C for 10 min before the addition of the templates. The vector GST-p53(1-73) contains the first 73 amino acids of p53 fused to GST, and the vector GST-p53(22,23) contains the first 73 amino acids of p53 doubly mutated at positions 22 and 23 (14). GST fusion proteins were overexpressed in *Escherichia coli* XA90 cells and purified by affinity chromatography with glutathione-Sepharose 4B (Pharmacia) according to the manufacturer's recommendations.

Transcripts resulting in transiently transfected cells. Primer extension with reverse transcriptase was used to assay *Alu* transcripts in transiently transfected 293 cells or COS cells (5). A marked U6 RNA gene, su^+C, having four substituted bases was used as a template for transient-transfection assays (6). Three terminal bases on the oligonucleotide AATATGGAAGAA complement the substituted positions in this marked template, thereby specifically targeting its transcript in primer extension assays. To compensate for the shorter length of this oligonucleotide in comparison with others employed in this study, an annealing temperature of 37°C was used, a modification of our standard methods (5).

As a control for the quality and amount of RNA, 7SL RNA was also assayed by primer extension (5). Cotransient transfections were performed with either a plasmid expressing wild-type p53 (pCMVp53wt) (1) or a construct [pCMVp53 (22,23)] expressing p53 doubly mutated at positions 22 and 23 (15). As an additional control, chloramphenicol acetyltransferase (CAT) activity resulting from a CAT reporter gene driven by a p53 response element, pG13CAT, was assayed by standard methods (1).

GST pull-down assay. The GST fusion proteins described above were incubated with nuclear extract at 4°C for 1 h. This incubation was next continued for

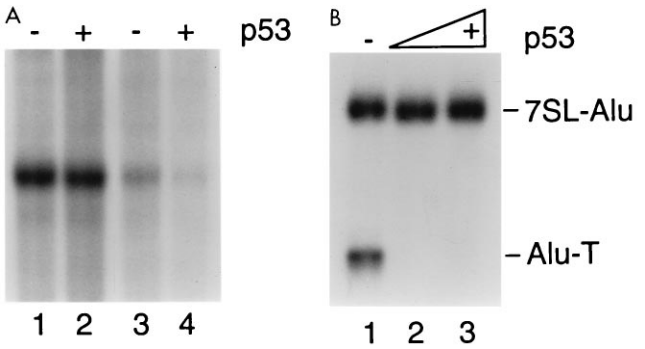

FIG. 1. Effect of p53 on *Alu* transcription in vitro. (A) In vitro transcription of an *Alu* source gene with (lanes 1 and 2) and without (lanes 3 and 4) an endogenous 5′-flanking sequence in the absence (lanes 1 and 3) and presence (lanes 2 and 4) of 100 ng of wild-type p53. (B) In vivo transcription of *Alu*-T and 7SL-*Alu* constructs (lane 1) in the presence of 100 ng (lane 2) or 200 ng (lane 3) of wild-type p53. Transcripts from the *Alu*-T construct are ~25 bp shorter than those of 7SL-*Alu* (5).

3 h with glutathione-Sepharose 4B beads (Pharmacia) which had been preincubated with bovine serum albumin. The beads were washed six times with a solution of 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.5), 100 mM NaCl, 5 mM $MgCl_2$, 5% glycerol, and 0.1% Triton X-100. Subsequently, the beads were eluted by boiling with sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer and analyzed by Western blotting (immunoblotting).

RESULTS AND DISCUSSION

Effect of p53 on *Alu* transcription in vitro. Purified p53 inhibits in vitro transcription of a basal *Alu* template containing just the internal Pol III type 2 promoter (Fig. 1A, lanes 3 and 4). The 5′ end of the *Alu* employed in this study directly abuts plasmid vector sequences so that it is entirely devoid of endogenous 5′-flanking sequences (4). Internal A-box and B-box elements such as those found in this particular *Alu* are sufficient to direct Pol III transcription in vitro (20). In the context of results for the U6 RNA gene which are discussed below, we note that the vector sequence immediately flanking this *Alu* template does not provide any obvious TATA motifs.

This particular *Alu* template is derived from a source gene for a successful *Alu* subfamily, and endogenous human sequences located within a 315-nucleotide upstream region stimulate its transcription in vitro (4). Transcription of a construct with this 315-nucleotide flanking region is not inhibited by p53 (Fig. 1A, lanes 1 and 2). These results suggest that the internal *Alu* promoter is subject to p53-mediated repression but that upstream activating sequences can relieve this repression.

To further test this possibility, we examined the effects that strong Pol III promoter elements located in the 5′-flanking sequences of the 7SL RNA gene might have on p53-mediated repression of *Alu*s (3, 5). *Alu* elements are ~80% similar in sequence to homologous regions within 7SL RNA, which is thought to be an ancestor of *Alu* sequences (24, 30). As a further control for any extraneous effects due to the addition of recombinant p53, transcription was performed on a mixture of two *Alu* templates: *Alu*-T, a basal template having only the internal promoter, and 7SL-*Alu*, the chimera containing the extragenic promoter elements of the 7SL RNA gene (5). Transcripts resulting from these two templates differ in length (Fig. 1B). Under these competitive conditions, p53 completely represses in vitro transcription from the *Alu*-T template but has no effect on the transcription of 7SL-*Alu* chimeric templates (Fig. 1B). In the absence of competition, the effect of p53 on the template activity of *Alu*-T is similar to its effect on the basal template described above (data not shown).

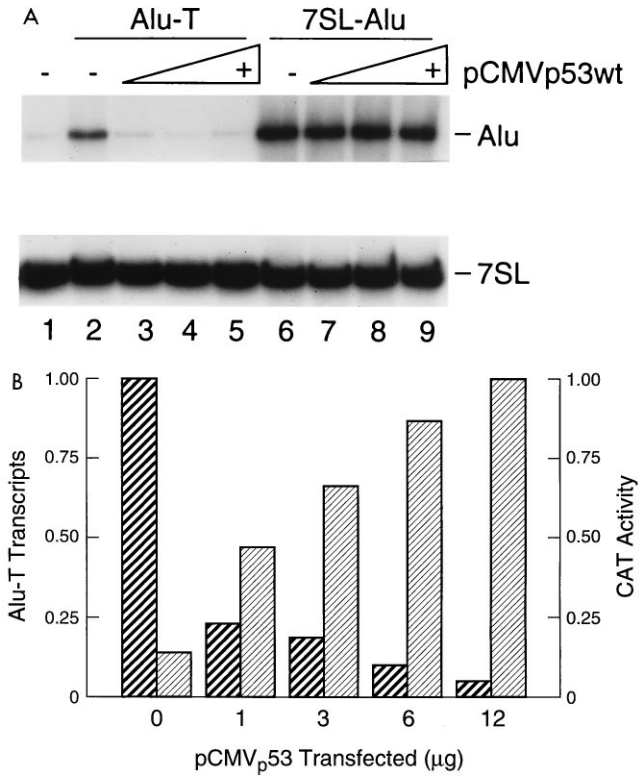

FIG. 2. p53 inhibits *Alu* transcription in vivo. (A) Primer extension analysis of *Alu* transcripts. COS cells were transfected with 10 μg of *Alu*-T (lanes 2 to 5)- or 7SL-*Alu* (lanes 6 to 9)-containing plasmids together with increasing amounts of an expression vector encoding the wild-type human p53 protein: 2 μg (lanes 3 and 7), 4 μg (lanes 4 and 8), or 8 μg (lanes 5 and 9). As a control, RNA from untransfected COS cells was assayed by primer extension (lane 1). Similar results were obtained with 293 cells (data not shown). The *Alu* and endogenous 7SL primer extension products are indicated. (B) Comparison of CAT activation and *Alu* repression by p53. 293 cells were cotransiently transfected with 10 μg of either *Alu*-T or pG13CAT and the indicated amounts of pCMVp53. The abundances of *Alu* transcripts (bars with thick stripes), quantified by phosphorimager analysis of the primer extension products, and CAT activity (bars with thin stripes) are compared by normalization to the highest value of each.

Together, the results of Fig. 1 indicate that p53 represses *Alu* transcription in a promoter-dependent manner. The 7SL RNA gene and corresponding 7SL-*Alu* chimera have upstream ATF sites, and additionally, the *Alu* source gene has an upstream AP1 site located at about the same position (3–5). *Alu*'s type 2 Pol III promoter and the promoter from the 7SL RNA gene form stable Pol III complexes by different pathways presumably involving TFIIIB (2). These differences in TFIIIB recruitment may account for the different effects of p53 on these *Alu* templates (see below).

Effect of p53 on *Alu* transcription in vivo. Extending these observations, cotransient-transfection assays with *Alu* templates and pCMVp53, a p53-overproducing clone, show that p53 has similar effects on *Alu* expression in vivo (Fig. 2A). p53 represses transcription of the basal *Alu*-T template but has virtually no effect on transcription of the 7SL-*Alu* chimera (Fig. 2A).

The following control indicates that the effects of p53 on *Alu* transcription are not an artifact either of saturating the transcriptional apparatus in cotransiently transfected cells or of nonspecific effects resulting from gross overexpression of p53. Cotransient transfection of the p53 expression vector stimu-

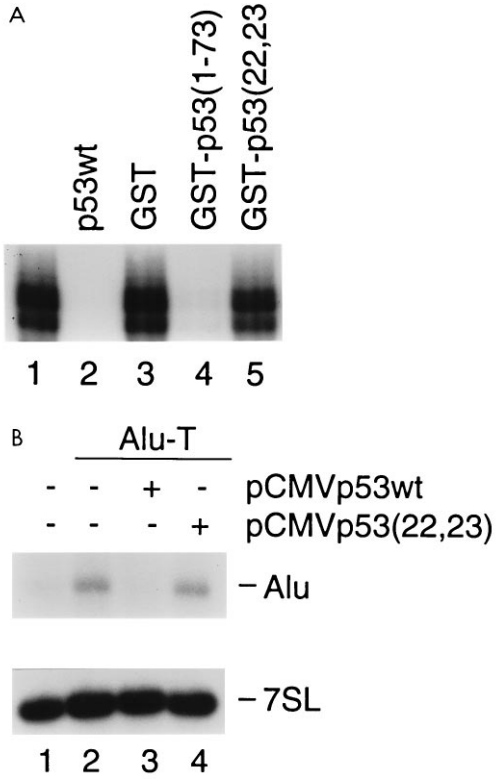

FIG. 3. Mutation in the N-terminal transactivation domain abolishes p53-mediated repression of *Alu* transcription. (A) In vitro transcription of *Alu* sequence. One hundred nanograms of wild-type (wt) p53 (lane 2), GST (lane 3), GST-p53(1-73) (lane 4), or GST-p53(22,23) (lane 5) was preincubated with nuclear extract before addition of the plasmid with the *Alu*-T insertion. Transcription of this template in the absence of recombinant protein is shown in lane 1. (B) Primer extension analysis of *Alu*-T activity (lane 2) in the presence of vectors expressing either wild-type (lane 3) or mutant (lane 4) p53. Ten micrograms of *Alu*-T plasmid was cotransfected into COS cells with 5 μg of a vector expressing wild-type or mutant p53. As a control, RNA from untransfected COS cells was assayed by primer extension (lane 1). Similar results were obtained with 293 cells (data not shown).

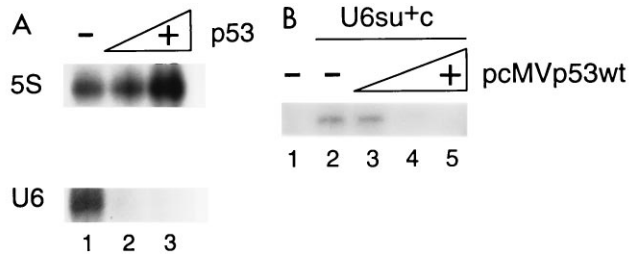

FIG. 4. Effect of p53 on transcription of the 5S RNA gene and the U6 RNA gene. (A) In vitro transcription of the 5S RNA gene and the U6 RNA gene without added p53 (lane 1) and with 100 ng (lane 2) or 200 ng (lane 3) of wild-type p53. (B) Primer extension analysis of U6 transcripts resulting from the plasmid U6 su^+C (lane 2) and from this plasmid cotransfected with 2 μg (lane 3), 4 μg (lane 4), and 8 μg (lane 5) of the vector expressing wild-type p53. Lane 1 contains RNA from untransfected 293 cells, providing a negative control for the primer extension product of the marked U6 gene.

lates activity from a CAT reporter gene driven by p53 response elements (10) (Fig. 2B). In particular, p53-dependent *Alu* repression and CAT activation each occur at approximately the same level of p53 (Fig. 2B).

p53's N-terminal domain is necessary and sufficient for *Alu* repression. To determine whether the N-terminal activation domain of p53 is also responsible for repressing *Alu* transcription, we tested the effects of a fusion protein, GST-p53(1-73), consisting of GST and residues 1 to 73 from human p53. Preincubation of nuclear extract with either the purified GST-p53(1-73) fusion protein or wild-type p53 represses *Alu* transcription in vitro (Fig. 3A). As controls, purified GST and a purified fusion protein consisting of GST and the N-terminal domain mutated at both positions 22 and 23 have no effect on *Alu* transcription (Fig. 3A). The N-terminal domain of p53 contains one TBP binding domain (18, 28), and mutation of residues 22 and 23 abolishes transactivation by p53 (15).

Cotransient-transfection assays with plasmids expressing wild-type or doubly mutated (positions 22 and 23) p53 also show the importance of the N-terminal region (Fig. 3B). Wild-type p53 represses *Alu* transcription in vivo, but the double mutant has no effect. p53 is produced in similar amounts from these two transfected clones (15), implying that this difference in *Alu* repression is attributable to the effect of the double mutation in the N-terminal domain.

Effect of p53 on other Pol III-directed templates. In addition to *Alu* templates, we surveyed the effects of p53 on other Pol III-transcribed genes. We observe that p53 represses in vitro transcription of the U6 RNA gene but has either no effect or a slight stimulatory effect on transcription of the 5S RNA gene (Fig. 4A). Whereas the N-terminal domain of p53 is sufficient for *Alu* repression, GST-p53(1-73) does not repress transcription of the U6 RNA gene (data not shown). Also, we do not observe any effect of p53 on the in vitro transcription of 7SL RNA, adenovirus VAI RNA, and tRNA genes (data not shown).

Primer extension using the oligonucleotide targeted toward base substitutions in the marked U6 RNA gene su^+C distinguishes between endogenous U6 RNA and transcripts resulting from the transfected plasmid (6) (compare lanes 1 and 2 in Fig. 4B). We have used this assay to test the effects of p53 on the in vivo expression of the U6 RNA gene. Cotransient transfection of the marked U6 RNA gene and the vector producing p53 shows that p53 also represses U6 RNA transcription in vivo (Fig. 4B).

p53 binds TFIIIB. Because p53, especially a region within the N-terminal activation domain, binds TBP, TFIIIB is a likely target for the effect of p53 on Pol III transcription (reference 9 and references therein). This was tested by a GST pull-down experiment (Fig. 5). Nuclear extract was incubated with either wild-type or mutated p53-GST fusion protein at the salt concentrations indicated in the legend to Fig. 5. The sample was absorbed onto glutathione beads, and bound protein was tested for TFIIIB90 (Fig. 5A), TBP (Fig. 5B), and TAF130 (a component of TFIID [Fig. 5C]) by Western analysis. TFIIIB binds to residues 1 to 73 of p53, but mutation of positions 22 and 23 largely abolishes this interaction (Fig. 5A). GST alone shows no interaction with TFIIIB90 (data not shown), so the very weak interaction with the double mutant probably reflects a residual binding activity. As expected from previous studies (9, 27), TBP and TFIID are also pulled down by p53 residues 1 to 73, and again the double mutation abolishes these interactions (Fig. 5B and C). The effect of increasing salt concentration is similar in each case: binding is highest at 0.1 M, decreases at 0.2 M, and is almost eliminated at 0.4 M (Fig. 5). This result suggests that p53's N-terminal domain has similar affinities toward TFIIIB containing TFIIIB90 and toward TFIID associated with TAF130.

In summary, p53 represses the in vitro and in vivo transcription of certain Pol III-directed templates, i.e., a basal *Alu*

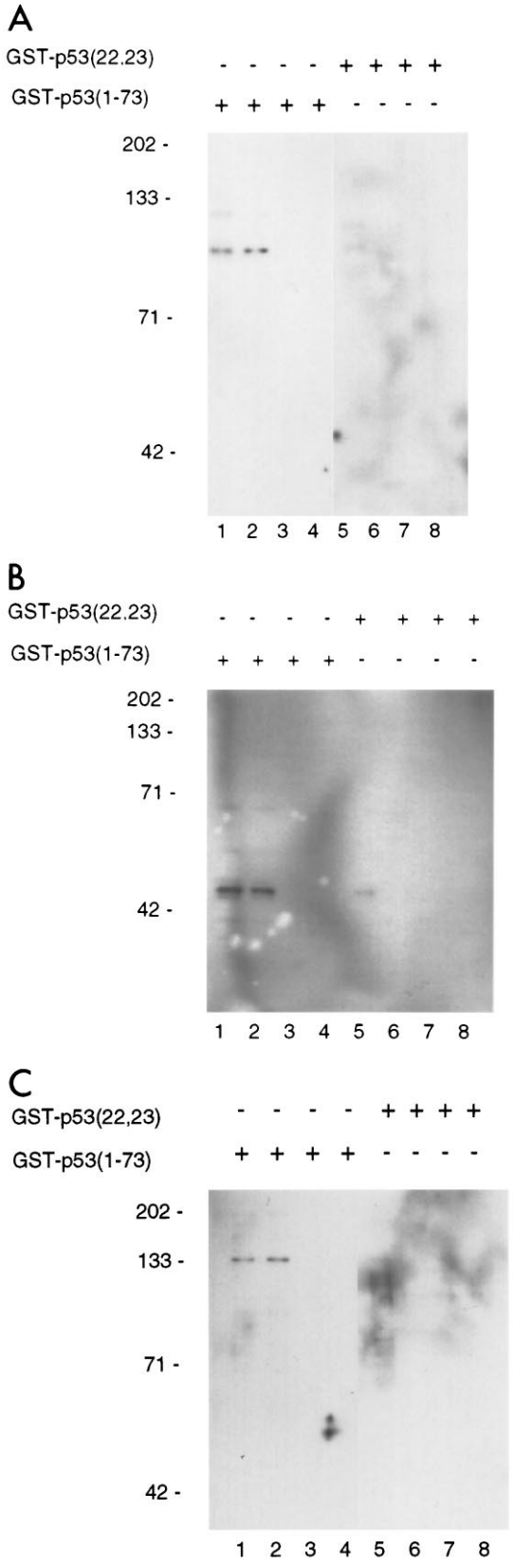

FIG. 5. p53's N-terminal domain binds TFIIIB. Western blotting with antibodies against either TFIIIB90 (A), TBP (B), or TAF130 contained within TFIID (C) is used to detect binding to p53. Nuclear extract was incubated with either GST-p53(1-73) (lanes 1 to 4) or GST-p53(22,23) (lanes 5 to 8) in either 0.1 M (lanes 1 and 5), 0.2 M (lanes 2 and 6), 0.4 M (lanes 3 and 7), or 0.6 M (lanes 4 and 8) NaCl. Incubated extracts were bound to glutathione beads for isolation and analysis. Positions of molecular weight markers (in thousands) are indicated.

template and the U6 RNA gene, but not others, i.e., genes for 5S RNA, 7SL RNA, tRNA, and adenovirus VAI RNA, an intact *Alu* source gene, and an *Alu* chimera closely resembling the 7SL RNA gene. This group of templates represents all known promoter classes employed by Pol III. However, the simplest distinction between templates which are repressed by p53 and those which are not is that a basal *Alu* sequence and the U6 RNA gene are weaker templates than the others tested. Compared with *Alu*s and the U6 RNA gene, these stronger promoters probably use other pathways to assemble the transcription complexes that overcome p53-mediated repression.

For a basal *Alu* template, the N-terminal activation domain is sufficient to cause repression. This region binds both TBP and TAFs, identifying two possible targets for the effect of p53 on *Alu* transcription (9, 27). TBP and TFIIIB90 are sufficient to reconstitute TFIIIB activity (29), so that the observed binding of the N-terminal activation domain to TFIIIB suggests TBP as the likely target. In analogy to p53's effects on Pol II-transcribed genes, these results show that p53 represses Pol III transcription units in a promoter-dependent manner and that the mechanism for *Alu* repression probably involves an interaction between TBP contained within TFIIIB and p53's N-terminal activation domain.

p53 activates Pol II-directed transcription by interaction with TAFs which are associated with TFIID (27). Binding experiments indicate that p53's N-terminal activation domain has similar binding affinities toward TFIIIB and TFIID (Fig. 5). In agreement with this result, repression of Pol III-directed transcription and stimulation of a p53 response element for Pol II-directed transcription occur at similar p53 concentrations in vivo (Fig. 2B). Together, these results indicate that mechanistically and functionally, p53 can act simultaneously on both Pol II- and Pol III-directed transcription.

We have not identified which regions of p53 are required for repression of the U6 RNA gene. However, in contrast to *Alu*s, an upstream TATA motif is an essential element in this gene's promoter (12), indicating again that the pathway by which TBP is recruited determines p53's effects on Pol III transcription. Possibly the second TBP binding domain located near the C terminus of p53 is also required for repression of the U6 RNA gene (reference 9 and references therein).

Activation of p53 has pleiotropic effects on cell physiology, including changes in transcription of Pol II-directed genes, some of which are up regulated and others of which are down regulated (8, 10, 18, 19, 25, 27, 28). Since small RNAs are involved in various stages of the maturation and expression of mRNAs, a corresponding role for p53 in the regulated transcription of certain small RNAs, some of which are Pol III transcribed, might have been anticipated. In agreement with this suggestion, another tumor suppressor protein, Rb, also represses Pol III-directed transcription (31). However, unlike p53, which represses Pol III transcription in a promoter-dependent manner, Rb generally represses all Pol III-directed templates (31), including those investigated in the present study (unpublished data).

Significantly, the *Alu* source gene escapes p53 suppression by virtue of its upstream elements, suggesting a basis for its retropositional success (4, 13). As previously mentioned, the transcriptional potential of one million *Alu*s is repressed at multiple levels so that only a very small number may be poised

for efficient expression (24). By its interaction with TFIIIB, even low levels of p53 activity may be sufficient to deflect limiting factors away from the small number of accessible but weak *Alu* promoters without affecting Pol III-directed transcription of essential genes.

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