p53 Transcriptional Activation Mediated by Coactivators $TAF_{II}40$ and $TAF_{II}60$

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The tumor suppressor protein p53 is a transcriptional regulator that enhances the expression of proteins that control cellular proliferation. The multisubunit transcription factor IID (TFIID) is thought to be a primary target for site-specific activators of transcription. Here, a direct interaction between the activation domain of p53 and two subunits of the TFIID complex, $TAF_{II}40$ and $TAF_{II}60$, is reported. A double point mutation in the activation domain of p53 impaired the ability of this domain to activate transcription and, simultaneously, its ability to interact with both $TAF_{II}40$ and $TAF_{II}60$. Furthermore, a partial TFIID complex containing *Drosophila* TATA binding protein (dTBP), human $TAF_{II}250$, $dTAF_{II}60$, and $dTAF_{II}40$ supported activation by a Gal4-p53 fusion protein in vitro, whereas TBP or a subcomplex lacking $TAF_{II}40$ and $TAF_{II}60$ did not. Together, these results suggest that $TAF_{II}40$ and $TAF_{II}60$ are important targets for transmitting activation signals between p53 and the initiation complex.

One mechanism by which the p53 protein appears to function as a tumor suppressor is by inducing the expression of gene products that are responsible for inhibiting or arresting cell growth and proliferation (*1*). The ability of p53 to regulate transcription of its target genes is critically dependent on both its DNA binding and transcriptional activation domains (*2–5*). The DNA binding domain appears to be sensitive to disruption by mutation, and most lesions associated with human cancers occur within this domain (6). Functional inhibition of the p53 activation domain also appears to play a role in human tumorigenesis. The product of the *MDM2* oncogene, which binds and is believed to mask the activation domain of p53, is overexpressed in certain tumors, resulting in the abrogation of p53 function (*7*). Although the transcriptional activation domain of p53 appears to be critical for its function as a regulator of cell growth, little is known about how this domain functions to mediate transcriptional activation. The activation domain of p53 lies within the first 73 amino acids of the protein, and some research places it within the first 42 amino acids (*3, 5, 8*). On the basis of the large number of acidic amino acid residues in this domain, p53 has been classified as a member of the "acidic" group of transcriptional activators (*2, 5*). Recent studies have provided circumstantial evidence that p53 communicates with the transcriptional machinery by means of a direct interaction with the TATA binding protein (TBP) (9), which is a subunit of the basal transcription factor TFIID (*10*). In contrast, most well-characterized transactivators have been shown to require the presence of the TFIID complex, containing its full complement of TBP-associated factors (TAFs), to stimulate transcription (*11*). To begin to decipher how p53 transmits its activation signal to the basal machinery, we identified functional targets of p53 within the transcription initiation complex.

First, to see whether p53 could in fact activate transcription with TBP substituted for the complete TFIID complex, we performed in vitro transcription reactions using a fractionated HeLa cell transcription system. Both full-length p53 and a Gal4 DNA binding domain fusion to the first 42 amino acids of the protein stimulate transcription in vitro using similar systems (*3, 12*). We produced a recombinant protein containing the Gal4 DNA binding domain fused to two tandem copies of p53 residues 1 to 42 (G4-p53) (Fig. 1A), reasoning that multimerization might make the activation domain more potent. In fractionated HeLa cell extracts, partially purified TFIID but not recombinant human TBP was able to support activation by G4-p53 from a template containing Gal4 sites (Fig. 1B). These results suggest that TBP is not sufficient to mediate activation by this NH_2-terminal portion of the p53 activation domain and that at least one of the eight TAFs in the TFIID complex may be essential for transcriptional activation. The TAFs have been proposed to serve as "coactivators" that mediate signals from transcriptional activators to the basal machinery (*10, 13*). Recent evidence strongly suggests that different classes of activators (for example, acidic, glutamine-rich, and isoleucine-rich) interact with distinct sets of TAFs to transmit their signals (*14–16*). For example, in vitro interaction and antibody inhibition experiments have suggested that $TAF_{II}40$ may mediate the activation signal between VP16, a prototypic member of the acidic class of transactivators, and the basal machinery (*14*). Given the acidic nature of the activation domain of p53, we asked whether $TAF_{II}40$ might also serve as a coactivator of p53.

To determine whether the activation domain of p53 interacts with $TAF_{II}40$, we coupled a protein containing glutathione-S-transferase (GST) fused to amino acids 1 to 73 of human p53 [GST-p53(1–73)] to glutathione-agarose beads. The GST-p53(1–73) beads were incubated with crude *Escherichia coli* lysates containing either *Drosophila* $TAF_{II}40$ ($dTAF_{II}40$) (*14*) or hemagglutinin (HA) epitope–tagged human $TAF_{II}32$ protein ($hTAF_{II}32$), the human homolog of $dTAF_{II}40$ (*17*). After extensive washing of the beads, the bound proteins were eluted with sample buffer, separated by SDS–polyacrylamide gel electrophoresis, transferred to nitrocellulose, and probed with antisera to either $dTAF_{II}40$ or the HA epitope. GST-p53(1–73) beads, but not control GST beads, efficiently retained both $dTAF_{II}40$ and $hTAF_{II}32$ (Fig. 2A). In the reciprocal experiment, the G4-p53(1–42) fusion protein was also able to interact with both GST-$dTAF_{II}40$ and GST-$hTAF_{II}32$ (Fig. 3, A and B). These results indicate that the activation domain of p53 can bind selectively to $dTAF_{II}40$ and to its human counterpart $hTAF_{II}32$ and that a domain sufficient for $TAF_{II}40$ interaction resides within the first 42 amino acids of p53.

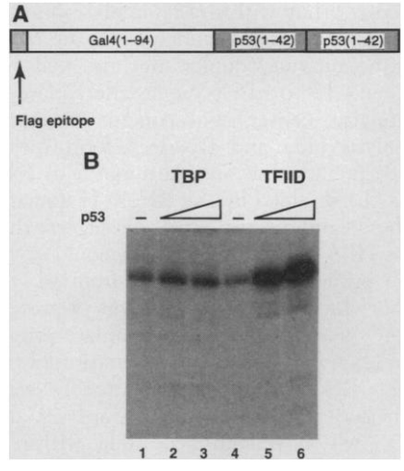

Fig. 1. TBP cannot mediate transcriptional activation by the NH_2-terminal activation domain of p53. (**A**) Schematic representation of the p53 activation domain fusion protein. The protein contains the Flag epitope fused to amino acids 1 to 94 of the Gal4 DNA binding domain and two tandem copies of amino acids 1 to 42 of the activation domain of p53 (*25*). (**B**) TBP is not sufficient to mediate transcriptional activation by a Gal4-p53 fusion construct. The G4-p53 fusion construct was assayed for its ability to activate transcription in the presence of approximately 0.5 ng of purified hTBP expressed in *E. coli* (lanes 1 to 3) or partially purified TFIID from HeLa extracts (lanes 4 to 6) by primer extension with the use of a transcription system with fractionated HeLa cells and the G5BCAT template (*16, 25*).

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To test the specificity of the p53-$TAF_{II}40$ interaction, we also determined the ability of other subunits of TFIID to bind p53. Our results revealed that $TAF_{II}30\beta$, $TAF_{II}80$, $TAF_{II}110$, and $TAF_{II}150$ failed to interact with p53 (*18*). Interestingly, the *Drosophila* and human homolog of an additional TAF, $dTAF_{II}60$ and $hTAF_{II}70$, were found to bind the activation domain of p53 in vitro. In experiments analogous to those previously described with recombinant $TAF_{II}40$, glutathione beads linked to GST-p53 but not to GST alone were capable of retaining both $dTAF_{II}60$ and $hTAF_{II}70$ (Fig. 2B) from crude lysates of Sf9 cells infected with recombinant baculovirus (*19*). Furthermore, by immobilizing the G4-p53(1–42) fusion protein on agarose beads by means of its NH_2-terminal Flag epitope tag, we were able to show that the first 42 amino acids of the activation domain of p53 are also sufficient for binding $hTAF_{II}70$ (Fig. 3C). Thus, the activation domain of p53 interacts in vitro with at least two components of the TFIID complex. The functional significance of these interactions, however, remained to be established.

Recently, it was shown that a double point mutation in the p53 activation domain that changes a Leu at position 22 to Glu and a Trp at position 23 to Ser impairs the ability of full-length p53 to activate transcription in vivo (*20*). One explanation for this defect in transcriptional activation is that the mutant may no longer be able to interact with one or more components of the basal transcription machinery. However, the binding of TBP was not affected by the mutations at residues 22 and 23 (*20*). Having found that this domain of p53 also interacted with $TAF_{II}40$ and $TAF_{II}60$, we wanted to determine whether similar point mutations in the activation domain of p53 would affect binding to these TAFs. To this end, an additional vector was engineered that directed expression of a mutant fusion protein (G4-p53mut) in which both copies of the first 42 amino acids of the activation domain had alanines substituted for the Leu and Trp at positions 22 and 23, respectively (Fig. 4A). To ensure that our mutant fusion protein recapitulated the transcriptional defect observed with full-length, mutant p53 in vivo, we assessed the ability of the mutant fusion protein to stimulate transcription in vitro in a fractionated HeLa cell transcription system using a template containing Gal4 binding sites. Under these in vitro transcription conditions, Gal4 alone (G4) activated transcription weakly (less than twofold), whereas G4-p53 activated transcription at least eightfold (Fig. 4B). In contrast, G4-p53mut was substantially impaired in its ability to enhance transcription and was about as active as the control G4 protein (Fig. 4B). These findings are consistent with previously reported in vivo results (*20*) and confirm that our mutant fusion protein is deficient in its ability to activate transcription.

The mutant G4-p53 protein was next tested for its ability to bind $TAF_{II}40$ and $TAF_{II}60$. As previously described, G4-p53

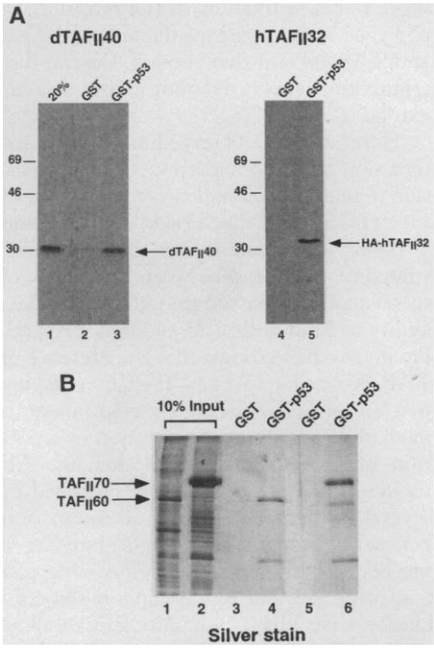

Fig. 2. The activation domain of p53 interacts with the *Drosophila* and human homologs of TFIID components $TAF_{II}40$ and $TAF_{II}60$. (**A**) The activation domain of p53 interacts with $dTAF_{II}40$ and its human homolog $TAF_{II}32$. Fusion proteins containing GST and the first 73 amino acids of p53 or GST alone were expressed in *E. coli* and bound to glutathione agarose beads (*26*). The beads were incubated with crude lysates from *E. coli* expressing either amino acids 1 to 222 of $dTAF_{II}40$ (*14*) (lanes 2 and 3) or recombinant $hTAF_{II}32$ protein fused to an NH_2-terminal HA epitope tag (*17*) (lanes 4 and 5). After washing, the beads were boiled in sample buffer, separated on a 12% SDS-polyacrylamide gel, transferred to nitrocellulose, and probed with rabbit polyclonal serum to $dTAF_{II}40$ (*14*) (lanes 1 through 3) or mouse monoclonal antibodies to the HA epitope (*27*) (lanes 4 and 5). Lane 1 corresponds to 20% of the amount of $dTAF_{II}40$ used in each binding assay. (**B**) The activation domain of p53 interacts with $dTAF_{II}60$ and its human homolog $hTAF_{II}70$. Binding assays using GST-p53 fusion proteins were performed as in (A) with the use of crude Sf9 cell lysates containing $dTAF_{II}60$ (lanes 3 and 4) or $hTAF_{II}70$ (lanes 5 and 6). After separation on a 10% SDS-polyacrylamide gel, proteins were visualized by silver staining.

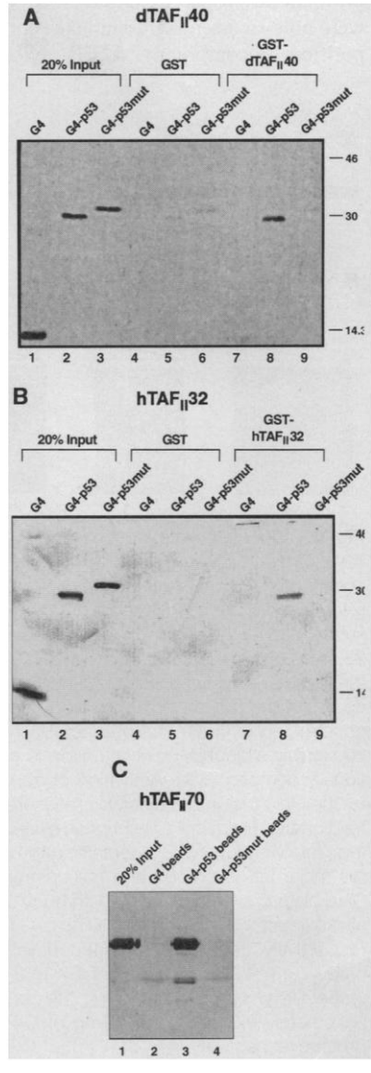

Fig. 3. The transcriptionally compromised p53 mutant fails to bind $dTAF_{II}40$, $hTAF_{II}32$, and $hTAF_{II}70$. (**A**) The mutant G4-p53 fusion protein does not interact with $dTAF_{II}40$. Proteins containing GST fused to amino acids 1 to 222 of $dTAF_{II}40$ (*14*) were expressed in *E. coli* and bound to glutathione agarose beads (*26*). Crude lysates from *E. coli* expressing either the Gal4 DNA binding domain (G4) (lanes 4 and 7), the wild-type p53 activation domain fusion (G4-p53) (lanes 5 and 8), or the mutant fusion protein (G4-p53mut) (lanes 6 and 9) were incubated with the GST and GST fusion protein beads (*26, 28*). After washing, the beads were boiled in sample buffer, separated on a 15% polyacrylamide-SDS gel, transferred to nitrocellulose, and probed with rabbit polyclonal serum raised against the Gal4 DNA binding domain. Lanes 1, 2, and 3 represent 20% of the amount of each protein used in the bead binding assay. Molecular size markers are shown on the right in kilodaltons. (**B**) The mutant G4-p53 fusion protein does not interact with $hTAF_{II}32$. Proteins containing GST fused to the complete coding region of $hTAF_{II}32$ (*17*) were expressed in *E. coli* and bound to glutathione agarose beads. The binding of the Gal4 fusion proteins to the GST beads was assayed as in (A). (**C**) The mutant G4-p53 fusion protein does not interact with $hTAF_{II}70$. Gal4 fusion proteins were immobilized by means of their NH_2-terminal Flag epitope tag on M2 Flag antibody beads and incubated with crude Sf9 cell lysates containing $hTAF_{II}70$. After the proteins retained by the fusion beads were treated as in (A), proteins bound to nitrocellulose were probed with rabbit polyclonal serum raised against $hTAF_{II}70$ (*19*). Lane 1 represents 20% of the amount of $hTAF_{II}70$ protein used in the bead binding assay.

was retained by the GST-dTAF$_{II}$40 and GST-hTAF$_{II}$32 fusion proteins but not by GST alone (Fig. 3, A and B). By contrast, when crude *E. coli* lysates containing G4-p53mut were incubated with control GST beads, GST-dTAF$_{II}$40 beads, or GST-hTAF$_{II}$32 beads, the transcriptionally deficient mutant protein was not retained (Fig. 3, A and B). Likewise, immobilized G4-p53 but neither G4-p53mut nor G4 alone was able to retain hTAF$_{II}$70 from crude Sf9 cell lysates (Fig. 3C). The correlation between the ability to activate transcription and the ability to bind TAF$_{II}$40 and hTAF$_{II}$70 suggested that transcriptional activation by the NH_2-terminal portion of the p53 activation domain might require a direct interaction between p53 and these components of TFIID.

To obtain more decisive evidence that the binding of p53 to TAF$_{II}$40 and TAF$_{II}$60 is involved in transcriptional activation, we assembled partial TFIID complexes in the presence and absence of TAF$_{II}$40 and TAF$_{II}$60 and tested their ability to activate transcription by G4-p53 in vitro. Using a protocol developed by Chen *et. al.* (*16*), we were able to assemble complexes containing purified, recombinant dTBP, hTAF$_{II}$250, dTAF$_{II}$60, and dTAF$_{II}$40. Human TAF$_{II}$250 serves as a core subunit of TFIID that allows the recruitment of many of the remaining TAFs, including dTAF$_{II}$60, which in turn helps recruit TAF$_{II}$40 to the complex (*14, 16, 19*). We therefore generated three sets of complexes, one containing TBP and TAF$_{II}$-250 (TBP-250), one containing TBP, TAF$_{II}$250, and TAF$_{II}$60 (TBP-250-60), and one containing TBP, TAF$_{II}$250, TAF$_{II}$60, and TAF$_{II}$40 (TBP-250-60-40). A silver-stained SDS-polyacrylamide gel of the assembled complexes confirmed the presence of the expected subunits (Fig. 5A).

Next, a fractionated HeLa cell transcription system was used to test the ability of the in vitro–assembled complexes to direct p53-mediated transcriptional activation. In the presence of purified G4-p53 and the TBP-250 complex, no activation of transcription over the basal level was observed (Fig. 5B, lanes 1 and 2). By contrast, the TBP-250-60-40 complex was capable of supporting a significant amount of p53-dependent activation (three- to fivefold) (Fig. 5B, lanes 3 and 4, and lanes 11 and 12). Importantly, the complex containing only TBP, TAF$_{II}$250, and TAF$_{II}$60 also supported transcriptional stimulation by G4-p53 (three- to fivefold) (Fig. 5B, lanes 8 and 9). These results suggest that at least TAF$_{II}$60 is required for p53-dependent activation in this minimal complex. However, the necessary presence of TAF$_{II}$60 in TAF$_{II}$40-containing complexes precludes a direct test of whether TAF$_{II}$40 alone can also mediate p53-dependent activation. Under the conditions of our reconstituted transcription reaction, we find that Gal4(1–94) protein lacking the p53 activation domain displayed no detectable enhancement of transcription with any of the partial complexes (Fig. 5B, lanes 5, 10, and 13). The complete TFIID complex supported G4-p53–dependent activation to a level only twofold greater than that for TBP-250-60-40 (Fig. 5B, lanes 6, 7, 14, and 15) or for TBP-250-60 (lanes 8 and 9). For an additional control, we also tested the glutamine-rich activator Sp1 for its ability to stimulate transcription with the TBP-250-60-40 complex (*16*). As expected, this complex failed to support Sp1-dependent transcription, which suggests that TAF$_{II}$40 and TAF$_{II}$60 do not act as general coactivators of transcription (*18*). These results, in conjunction with the mutant binding data, strongly suggest that activation of transcription by p53 is at least in part mediated by TAF$_{II}$40 and TAF$_{II}$60 and that these TAFs can discriminate between different classes of activators.

Here, we have observed an in vitro interaction between a portion of the activation domain of p53 and two components of the transcription machinery, TAF$_{II}$40 and TAF$_{II}$60. Furthermore, we have demonstrated a correlation between the ability of this domain to activate transcription and its ability to bind both TAF$_{II}$40 and TAF$_{II}$60. Finally, we have shown that the presence of TAF$_{II}$60 or of a TAF$_{II}$40-TAF$_{II}$60 complex in a partial TFIID assemblage is sufficient to mediate transcriptional activation by a portion of the p53 activation domain. Although it had been previously suggested by several groups that an observed interaction between TBP and p53 might indicate a mechanism in which TBP mediates p53 activation (9), our results support the conclusion that TBP is not sufficient to allow the stimulation of transcription by at least

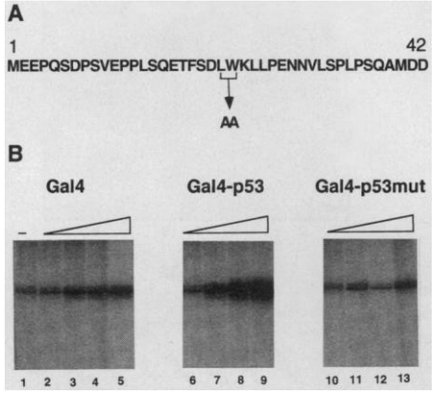

Fig. 4. A double point mutation in the p53 activation domain decreases its ability to activate transcription in vitro. (**A**) Amino acids 1 to 42 of p53 (*29*). The double point mutant has two alanines substituted for the Leu and Trp at positions 22 and 23. A fusion protein consisting of the Flag epitope, the Gal4 DNA binding domain, and two tandem copies of the mutant p53 activation domain was constructed (*25*). (**B**) The p53 activation domain containing a double point mutation is transcriptionally compromised. Activation of transcription by the Gal4 fusion proteins was measured with a fractionated HeLa cell transcription system from a template without any G's containing the adenovirus major late promoter and five upstream Gal4 DNA binding sites (*28*). Lanes 1 through 5 contain approximately 0, 0.3, 1, 3, or 10 ng of G4 protein, respectively. Lanes 6 through 9 contain approximately 0.3, 1, 3, or 10 ng of G4-p53 protein, respectively. Lanes 10 through 13 contain approximately 0.3, 3, 10, or 30 ng of G4-p53mut protein, respectively.

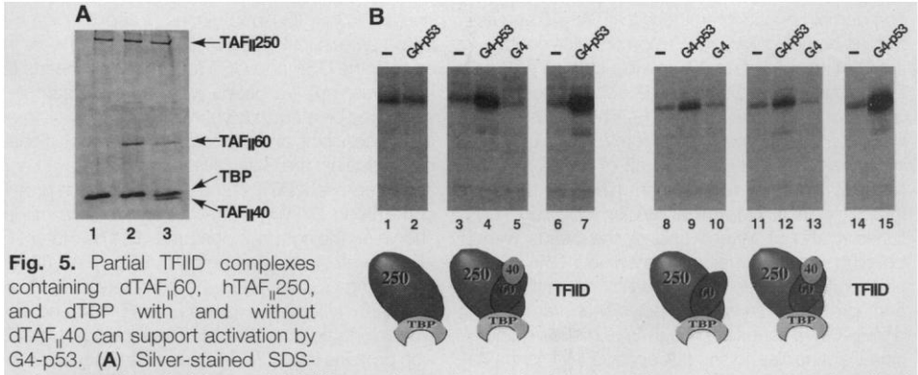

Fig. 5. Partial TFIID complexes containing dTAF$_{II}$60, hTAF$_{II}$250, and dTBP with and without dTAF$_{II}$40 can support activation by G4-p53. (**A**) Silver-stained SDS-polyacrylamide gel of the partial TFIID complexes. Assembly of the partial complexes was as described (*30*). Ten microliters (each) of the dTBP-hTAF$_{II}$250 complex (lane 1), dTBP-hTAF$_{II}$250-dTAF$_{II}$60 complex (lane 2), and the dTBP-hTAF$_{II}$250-dTAF$_{II}$60-dTAF$_{II}$40 complex (lane 3) were electrophoresed on an 8% polyacrylamide-SDS gel and silver-stained. (**B**) In vitro transcription in a fractionated HeLa cell transcription system with the use of partial TFIID complexes. Activated transcription was assayed by primer extension (*25*) with activators added as follows: 3 ng of G4-p53 was added to lanes 2, 4, 7, 9, 12, and 15 and 3 ng of G4 was added to lanes 5, 10, and 13. The partial complexes were added as follows: 1 μl of dTBP-hTAF$_{II}$250 (lanes 1 and 2), 1 μl of dTBP-hTAF$_{II}$250-dTAF$_{II}$60 (lanes 8, 9, and 10), 1 μl of dTBP-hTAF$_{II}$250-dTAF$_{II}$60-dTAF$_{II}$40 (lanes 3, 4, and 5), 0.5 μl of dTBP-hTAF$_{II}$250-dTAF$_{II}$60-dTAF$_{II}$40 (lanes 11, 12, and 13), and 1 μl of TFIID (lanes 6, 7, 14, and 15).

the NH_2-terminal portion of this activation domain. It is possible that full-length p53 contains multiple activation domains that can contact distinct targets in the TFIID complex. We interpret the correlation between the transcriptional defect of the G4-p53 fusion protein containing mutations at residues 22 and 23 and its inability to bind $TAF_{II}40$ and $TAF_{II}60$ as evidence that both of these TAFs may serve as target coactivators to transmit the activation signal from p53 to the basal transcription machinery. Our data suggest that the binding sites for $TAF_{II}40$ and $TAF_{II}60$ reside within the first 42 amino acids of p53, though it is not clear whether these sites are identical or simply overlapping. An alternative explanation for the TAF binding defects of the mutant G4-p53 fusion protein is that residues 22 and 23 are critical to the overall structure of the activation domain. However, TBP, which binds to a similar portion of the p53 activation domain, has been reported to be unaffected by mutations in these residues in the context of the entire p53 protein (*20*).

How p53 and its target TAFs interact with one another during the process of assembly of the transcriptional preinitiation complex is still unknown. If the interaction domains in p53 for $TAF_{II}40$ and $TAF_{II}60$ are nonoverlapping, p53 may simultaneously bind both proteins. Such a three-way interaction model is plausible as $TAF_{II}40$ and $TAF_{II}60$ appear to directly contact one another in the TFIID complex. It is also possible that p53 binds to only a single TAF and that the specific TAF bound is determined by the presence of other activators competing for binding to either $TAF_{II}40$ or $TAF_{II}60$. $TAF_{II}40$ has been implicated in transcriptional activation by the viral acidic activator VP16. Antibodies directed against $TAF_{II}40$ selectively inhibit the ability of VP16 to activate transcription in vitro (*14*). In addition, $TAF_{II}40$ has also been shown to interact with other acidic activators, including EBNA-2, an Epstein-Barr virus transactivator (*21*). A correlation between the ability of wild-type and mutant EBNA-2 proteins to activate transcription and to bind $TAF_{II}40$ suggests that this interaction may also have functional significance. Recently, we obtained evidence that $TAF_{II}60$ also interacts with the acidic activation domain of VP16 and that transcriptionally impaired mutants of the VP16 activation domain are also impaired in their ability to bind both $TAF_{II}40$ and $TAF_{II}60$ (*18*). Taken together, these results are consistent with a model in which both $TAF_{II}40$ and $TAF_{II}60$ serve as coactivators for at least a subset of acidic transactivators (*14*). Whether $TAF_{II}40$ and $TAF_{II}60$ can simultaneously interact with a single acidic activator or whether the activator can choose the most accessible of the two TAFs is presently unknown.

Several proteins that interact with p53 and inhibit the ability of its activation domain to contact the transcription initiation complex have been reported. SV40 T antigen and the hepatitis B virus X protein both appear to block the ability of p53 to bind its DNA site, which decreases the ability of p53 to target normally responsive promoters (*22, 23*). Alternatively, the cellular oncogene *MDM2* appears to directly bind the activation domain of p53 and thus may disrupt the ability of p53 to interact with the basal machinery (*7*). Through their inhibitory effects on p53 function, these proteins appear to contribute to the transformation of cells (*23, 24*). Our finding that $TAF_{II}40$ and $TAF_{II}60$ cannot bind efficiently to activation domains of p53 containing mutations at residues 22 and 23, taken in combination with the previous observation that mutations in these residues also inhibit the binding of MDM2 to p53 (*20*), suggests that these proteins vie for binding to a similar region of the activation domain of p53. Thus, an attractive model for the inhibition of p53 function by the *MDM2* oncogene may be that MDM2 binding precludes the interaction of p53 with $TAF_{II}40$ or $TAF_{II}60$. By dissecting these and other important interactions between p53 and specific functional targets in the transcription complex, we hope to contribute to the eventual understanding of the mechanisms governing p53 activity that will allow the development of therapies to control the cellular effects of p53.

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25. G4(1–94), G4-p53, and G4-p53mut constructs were engineered in a pET 3a vector derivative containing the nucleotide sequence encoding the Flag epitope followed by the first 94 amino acids of the Gal4 DNA binding domain under control of a T7 polymerase promoter and expressed in the *E. coli* BL21 strain. T7 polymerase expression was induced in these cells with 0.4 mM isopropyl-β-D-thiogalactopyranoside for 2 hours. After sonication and centrifugation, fusion proteins used for transcription were purified from the cell lysates by incubation with Flag M2 antibody resin (Eastman Kodak, New Haven, CT) for 4 hours. The resin was washed with binding buffer and binding buffer + 1 M NaCl. We released fusion proteins from the resin by incubating them with Flag peptide (0.2 mg/ml) for 1 hour at 4°C. We standardized relative amounts of the three fusion proteins by assaying their ability to retard the migration of a Gal4 site containing a DNA fragment in nondenaturing gels. In vitro transcription was assayed by primer extension from the G5BCAT template [J. W. Lillie and M. R. Green, *Nature* **338**, 39 (1989)]. Fractionated extracts from HeLa cells were prepared as described [J. Dignam, P. Martin, B. S. Shastry, R. G. Roeder, *Methods Enzymol.* **101**, 582 (1983)] (*16*). Recombinant dTFIIA protein was used in place of the P.1 fraction [K. Yokomori *et al.*, *Genes Dev.* **8**, 2313 (1994)]. All additions of activators and basal factors were carried out at 4°C in the following order: DNA template, activator, TBP or TFIID complex, P.5, DE.1, and dTFIIA. The mixtures were incubated at 30°C for 30 min, and transcription was initiated by adding nucleoside triphosphates to a final concentration of 500 μM and incubating them an additional 30 min. The reaction products were detected by primer extension, visualized by autoradiography, and quantified by phosphoimager analysis (Molecular Dynamics, Sunnyvale, CA).
26. *Escherichia coli* expression of GST fusion proteins, $dTAF_{II}40$, and $hTAF_{II}32$ and the protein-protein interaction assays were performed essentially as described (*14*). Expression of the $dTAF_{II}60$ and $hTAF_{II}70$ proteins in Sf9 cells was as described (*19*). G4 fusion proteins were immobilized on Flag M2 antibody resin (Eastman Kodak) as described (*25*) but without eluting with Flag peptide. The binding and washing buffers contained 20 mM tris (pH 7.9), 5 mM $MgCl_2$, 1 mM EDTA, 20% glycerol, 0.1 M NaCl, and 0.1% NP-40. The vector containing the first 73 amino acids of p53 fused to GST was as described [R. Li and M. Botchan, *Cell* **73**, 1207 (1993)]. We produced a vector containing GST fused to $hTAF_{II}32$ by engineering an Nde I site at the first ATG of the

hTAF$_{II}$32 coding sequence and then inserting the sequence into the pGEX2TK vector.
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28. Transcriptional activation was assayed with fractionated HeLa extracts and a template without G's containing the adenovirus major late promoter with five upstream Gal4 sites (*25*) [M. Sawadoga and R. G. Roeder, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 4394 (1985)]. We incubated 100 ng of template with purified activator for 5 min on ice. HeLa cell fractions P.1/D.3(IIA) and P.5 were added and incubated at 30°C for 30 min. Transcription was initiated by addition of nucleotides [500 mM adenosine triphosphate and cytidine triphosphate and 25 mM [α-^{32}P]uridine triphosphate (UTP); 5 mCi per reaction] and allowed to proceed for 15 min. After separation on a denaturing polyacrylamide gel, [α-^{32}P]UTP incorporation was quantitated by phosphoimager (Molecular Dynamics, Sunnyvale, CA).
29. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
30. Partial complex assembly and in vitro transcription were performed essentially as described (*16*). Briefly, HA-hTAF$_{II}$250 bound to HA antibody beads served as the basis of the complex assembly. Purified, recombinant proteins were added in the order of dTBP, dTAF$_{II}$60, and dTAF$_{II}$40, with extensive washing between each addition. The complexes were eluted from the beads in 60 μl of buffer containing 1 mg/ml of HA peptide at room temperature for 30 min. In vitro transcription was performed with the G5BCAT template (*25*). Reaction products were detected by primer extension (*25*).
31. We thank R. Li of M. Botchan's laboratory for providing the GST-p53(1–73) plasmid; S. Ruppert for providing recombinant hTAF$_{II}$70; K. Yokomori for providing recombinant dTFIIA; M. Maxon, E. Wang, H. Rye, D. Rio, G. Gill, and J. Oliner for critical comments on the manuscript; J. Lin for helpful discussions; S. Albright and G. Cutler for excellent graphics advice; and J. Goodrich for providing HeLa nuclear extracts, a pET 3a vector derivative, helpful discussions, and encouragement. Supported by an NSF graduate fellowship (C.J.T.) and in part by a grant from NIH (R.T.).

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Functional Isolation and Characterization of Human Hematopoietic Stem Cells

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Hematopoietic cells differentiate in steps marked by the acquisition or loss of specific phenotypic characteristics. Human bone marrow cells that were responsive to the early-acting cytokines Kit ligand and interleukin-3 were forced to a metabolic death. The subfraction remaining represented 1 in 10^5 bone marrow mononuclear cells, was determined to be quiescent by cell cycle analysis, and had a stem cell immunophenotype. The cells were highly enriched for long-term culture-initiating cells, were capable of secondary colony formation, and produced both myeloid and lymphoid progeny. Thus, this technically simple strategy led to the efficient purification of cells with characteristics of hematopoietic stem cells.

The efficient isolation of hematopoietic stem cells would enhance both the investigation of the processes of lineage commitment and self-renewal and the clinical strategies of bone marrow transplantation and gene therapy with hematopoietic cells. The fraction of human bone marrow that is CD34$^+$, CD33$^-$, CD38$^-$, human lymphocyte antigen (HLA) DR$^-$, Thy-1^{lo}, negative for lineage-specific antigens, and stains lightly with the dye rhodamine-123 is enriched for repopulating stem cells (*1*, *2*). However, methods that depend on cell sorting require considerable mechanical manipulation of the cells, and antibody binding to cell surface structures may induce perturbations of the cells' physiology. Other enrichment methods have been based on functional characteristics of cells, including soybean agglutinin binding (*3*) and resistance to either the alkylating agent 4-hydroxycyclophosphamide (4-HC) (*4*) or the antimetabolite 5-fluorouracil (5-FU) (*5*). However, these methods result in persistent heterogeneity within the selected cell population.

We reasoned that by combining cytokine stimulation with antimetabolite treatment, we could induce cell death in responding cells and therefore select for cells resistant to the proliferative effects of the cytokines. The earliest identifiably committed progenitor cells respond to Kit ligand (KL) and interleukin-3 (IL-3) and have been identified as primitive cells capable of forming high proliferative capacity colonies type 1 (HPP-CFC-1), an in vitro marker of a very primitive myeloid cell (*6*). Because the IL-3 receptor is not thought to be present on the hematopoietic stem cell (*7*), we incubated these cells with KL and IL-3 in the presence of serum plus 5-FU for a prolonged period to functionally select for primitive stem cells.

We assessed the selection technique by flow cytometric analysis with DNA staining dyes (Fig. 1). When either bulk bone marrow mononuclear preparations or bone marrow cells selected with CD34$^+$ immunobeads were used, the selection strategy efficiently eliminated cells in the S or G_2-M phase. Remaining cells were shown to be in the G_0 phase by staining with the ribosomal RNA dye Pyronin Y (Polysciences, Warrington, Pennsylvania). Testing a number of different selection strategies, we determined that the maximal efficiency was attained when high concentrations of 5-FU were used (>200 μg/ml) and when selection was continued for at least 7 days. At high concentrations, 5-FU inhibits RNA splicing as well as DNA synthesis and may therefore act to more effectively eliminate metabolically activated cells (*8*).

We evaluated whether our selection had isolated cells resistant to the proliferative effects of KL and IL-3 by use of the fluorescent membrane dye PKH26, which is stable

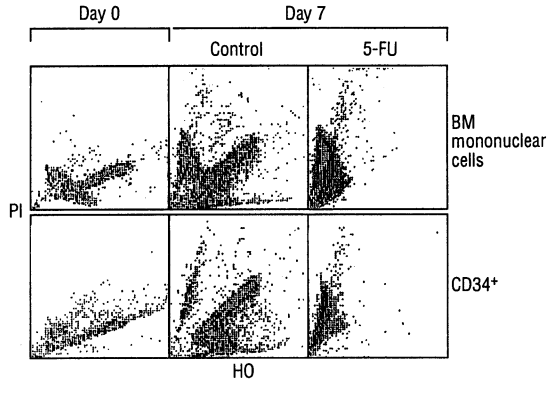

Fig. 1. Cell cycle status of CD34$^+$-selected or unselected bone marrow (BM) mononuclear cells cultured under control or 5-FU–selected conditions (*23*). Cells were stained with propidium iodide (PI) and Hoechst 33342 (HO) (Sigma) (*24*) and then analyzed by flow cytometry on days 0 and 7. HO staining intensity varies with DNA content and increases as cells transit from G_0-G_1 into the S and G_2-M phases of the cell cycle. PI staining increases with either loss of viability ($PI_{mod,hi}$, HO_{lo}) or as cells undergo the S, G_2, and M phases ($PI_{mod,hi}$, $HO_{mod,hi}$). Under control conditions, the full spectrum of the cell cycle was represented as indicated by variable HO staining, with more nonviable cells ($PI_{mod,hi}$, HO_{lo}) present after the 7-day incubation period, which results from terminally differentiated cells in liquid culture. However, the 5-FU–treated cells are restricted to HO_{lo} staining and had an increased proportion of nonviable cells. A remaining small subpopulation of viable cells (PI_{lo}, HO_{lo}) in the 5-FU–treated cultures was confirmed by trypan blue exclusion analysis. Similar results were obtained with either CD34$^+$-selected (bottom) or bulk preparations of bone marrow mononuclear cells (top).