p53 is a general repressor of RNA polymerase III transcription

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p53 is a major tumour suppressor that is inactivated in a large proportion of human cancers. We show that p53 serves as a general repressor of transcription by RNA polymerase (pol) III. It can inhibit the synthesis of a range of essential small cellular RNAs including tRNA, 5S rRNA and U6 snRNA, as well as viral products such as the adenovirus VA₁ RNA. Fibroblasts derived from p53 knock-out mice display a substantial increase in pol III transcriptional activity. Endogenous cellular p53 is shown to interact with the TATA-binding protein (TBP)-containing general factor TFIIB, thereby compromising its function severely. However, assembly of TFIIB into a pre-initiation complex confers substantial protection against the inhibitory effects of p53. Since TFIIB is an essential determinant of the biosynthetic capacity of cells, its release from repression by p53 may contribute to a loss of growth control during the development of many tumours.

Keywords: p53/RNA polymerase III/TATA-binding protein/TFIIB/transcription

Introduction

The p53 gene is highly conserved amongst vertebrate species and has been shown to encode an important tumour suppressor (reviewed by Cox and Lane, 1995; Haffner and Oren, 1995; Ko and Prives, 1996; Levine, 1997). p53 is lost or mutated in more than half of all human tumours and its inactivation is considered to be an important step in carcinogenesis (Hollstein *et al.*, 1991). Wild-type p53 can arrest cell growth (Mercer *et al.*, 1990; Michalovitz *et al.*, 1990; Martinez *et al.*, 1991; Crook *et al.*, 1994). However, p53 is not an essential cell cycle regulator, since mice that are homozygously null for the p53 gene develop normally (Donehower *et al.*, 1992). These p53^{-/-} mice display a strong predisposition to cancer, such that 74% develop tumours by the age of 6 months (Donehower *et al.*, 1992). This observation provides a strong indication that p53 contributes an important control against aberrant growth and neoplastic transformation.

The p53 protein displays a variety of biochemical activities, including the ability to regulate transcription (Cox and Lane, 1995; Haffner and Oren, 1995; Ko and Prives, 1996; Levine, 1997). It can bind to DNA in a sequence-specific fashion and stimulate expression of proximal genes (Bargonetti *et al.*, 1991; Kern *et al.*, 1991; Farmer *et al.*, 1992; Zambetti *et al.*, 1992). Transcriptional

activation is mediated through an acidic domain at the N-terminus that binds directly to the TATA-binding protein (TBP) (Seto *et al.*, 1992; Liu *et al.*, 1993; Martin *et al.*, 1993; Truant *et al.*, 1993; Horikoshi *et al.*, 1995) and TBP-associated factors (TAFs) in the TFIID complex (Lu and Levine, 1995; Thut *et al.*, 1995; Farmer *et al.*, 1996). In addition to activating genes with p53-binding sites, p53 can also repress promoters that lack its response element (Ginsberg *et al.*, 1991; Mercer *et al.*, 1991; Santhanam *et al.*, 1991; Seto *et al.*, 1992; Subler *et al.*, 1992; Mack *et al.*, 1993; Ragimov *et al.*, 1993; Crook *et al.*, 1994; Horikoshi *et al.*, 1995; Chesnokov *et al.*, 1996). p53 has been shown to specifically inhibit the synthesis of c-Fos (Kley *et al.*, 1992), PCNA (Mercer *et al.*, 1991; Jackson *et al.*, 1994) and cyclin A (Yamamoto *et al.*, 1994). Since these gene products are involved in promoting cell-cycle progression, it has been suggested that the transcriptional repression function of p53 may contribute to its ability to suppress proliferation and/or tumour formation (Cox and Lane, 1995; Ko and Prives, 1996). This possibility is supported by the fact that many tumour-derived p53 mutants have lost the capacity to inhibit transcription (Ginsberg *et al.*, 1991; Mercer *et al.*, 1991; Santhanam *et al.*, 1991; Seto *et al.*, 1992; Subler *et al.*, 1992; Ragimov *et al.*, 1993; Crook *et al.*, 1994). Furthermore, two oncoproteins have been shown to block p53-mediated repression without affecting activation (Crook *et al.*, 1994).

Overexpression of p53 has been shown recently to repress transcription of Alu and U6 snRNA genes by RNA polymerase (pol) III both *in vitro* and in transfected cells (Chesnokov *et al.*, 1996). Point mutations near the N-terminus of p53 abolish its ability to regulate pol III (Chesnokov *et al.*, 1996). Furthermore, a fusion protein containing the N-terminal 73 amino acids of p53 was shown to bind to TFIIB, a TBP-containing factor that is specific for the pol III system (Chesnokov *et al.*, 1996). TFIIB is required for all pol III transcription (reviewed by Willis, 1993; White, 1994). Nevertheless, several pol III templates have been reported to be unresponsive to p53 (Chesnokov *et al.*, 1996).

The current study has extended the characterization of p53 as a regulator of pol III. We have reproduced the previous observation that recombinant p53 can inhibit expression of U6 and Alu genes. However, we find that it can also repress tRNA, 5S rRNA, VA, B2 and EBER genes, and therefore functions as a general regulator of pol III transcription. We also demonstrate that p53 can inactivate TFIIB specifically. Once TFIIB has been assembled into a transcription complex, it becomes far less susceptible to repression by p53. Overexpression of regulatory proteins can often force interactions that would not occur at physiological concentrations. We have therefore investigated whether endogenous p53 associates with the pol III machinery. Co-fractionation and co-immunopre-

ipitation data suggest that a proportion of cellular p53 interacts specifically with TFIIB. Furthermore, fibroblasts derived from p53 knock-out mice transcribe pol III templates at abnormally elevated levels. This increase is due to a specific rise in TFIIB activity. On the basis of these data, we propose that TFIIB is a target for repression by endogenous p53 and that this regulation may contribute to the proper control of pol III transcription in untransfected cells.

Results

p53 is a general repressor of pol III transcription

Chesnokov *et al.* (1996) reported previously that p53 can inhibit expression of U6 and Alu genes *in vitro* and in transfected cells. We have reproduced this result using baculovirus-expressed recombinant p53, which repressed transcription by up to 8-fold when added to a HeLa cell extract (Figure 1A). Full-length p53 expressed in bacteria as a fusion with glutathione *S*-transferase (GST) also represses the U6 promoter, whereas an equal amount of GST alone has little or no effect (Figure 1B, lanes 9 and 10). In contrast to Chesnokov *et al.* (1996), we found that a variety of other pol III templates are also inhibited by GST-p53 (Figure 1B, lanes 1–8). These include the 5S rRNA, tRNA, VA, B2 and EBER2 genes. The ability of GST-p53 to repress pol III transcription is substantially reduced following its pre-incubation with an affinity-purified anti-p53 antibody (Figure 1C). This effect is specific, since efficient repression is still obtained following pre-incubation of GST-p53 with a control antibody against the unrelated factor Oct-1. These observations provide evidence against the possibility that the inhibitory effect of GST-p53 is due to a contaminant. Figure 1D provides an indication of the purity of our GST-p53 preparations, as revealed by Coomassie-staining of an SDS-polyacrylamide gel. To determine the specificity of the pol III response, we tested the effect of GST-p53 on an artificial TATA-less pol II template, pG₆I, which contains an initiator element and six Sp1-binding sites. Sp1-driven initiator activity has been shown to be resistant to p53 overexpression both *in vitro* and *in vivo* (Mack *et al.*, 1993). Consistent with this, we found that GST-p53 is no more effective than GST alone at inhibiting pol II transcription of pG₆I (Figure 1E). This provides evidence against the possibility that recombinant p53 is acting in a non-specific fashion under the conditions of our assays. Instead, the results suggest that p53 is a general repressor of pol III transcription.

Assembly of a pre-initiation complex confers protection against p53

We investigated whether assembly of a class III gene into a stable pre-initiation complex can influence the repressive effects of p53 (Figure 2). GST or GST-p53 were added to an extract either 15 min prior to the addition of a 5S rRNA gene (Figure 2, lanes 1 and 2), simultaneously with the addition of 5S rDNA (Figure 2, lanes 3 and 4), or 15 min after the addition of 5S template (Figure 2, lanes 5 and 6). Nucleotides were then added to allow transcription. Since pol III factors assemble rapidly onto the human 5S gene promoter under the conditions of our assays (data not shown), the procedure used in Figure 2, lanes 5 and

6, tests the ability of p53 to repress a pre-formed initiation complex. Relative to GST alone, GST-p53 was found to inhibit transcription 14-fold when added to the extract prior to the template and 7-fold when added simultaneously with the template. However, virtually no inhibition was observed when GST-p53 was added 15 min after the template. Very similar results were obtained when the VA₁ or a tRNA gene were used instead of the 5S gene (data not shown). These observations suggest that the class III transcription factors are much more susceptible to p53 when free in solution, and receive substantial protection when assembled into a pre-initiation complex.

TFIIB is a specific target for repression by p53

If p53 represses class III genes by inactivating an individual component of the pol III transcription apparatus, it should be possible to overcome this repression by adding more of that factor to the system. Consistent with this, we found that adding Mono Q-purified TFIIB can efficiently relieve VA₁ gene expression from repression by p53 (Figure 3A, lanes 1–3). This effect is specific, since a fraction containing TFIIC and pol III does not overcome repression (Figure 3A, lane 4). The activity of this TFIIC fraction is demonstrated in lanes 5–7, where it is shown to reconstitute transcription when combined with the TFIIB fraction. Further experiments carried out with additional templates have shown that TFIIB can also relieve 5S rRNA and tRNA genes from repression by p53 (data not shown).

The TFIIB fraction used in Figure 3A was gradient-purified on Mono Q, after an initial phosphocellulose step. Nevertheless, the possibility remained that the effect of this fraction reflects a contaminating activity rather than TFIIB itself. To address this concern, we tested the effect of immunodepleting the fraction with an antiserum against the TFIIB-specific TAF TFIIB-related factor (BRF). The Mono Q fraction was no longer able to counteract the effect of p53 after it had been depleted using the anti-BRF antiserum (Figure 3B, lane 5). In contrast, the fraction remained fully competent at overcoming repression following immunodepletion with the corresponding pre-immune serum (Figure 3B, lanes 1–4). These results suggest that TFIIB itself, or a closely associated factor, can overcome repression by p53. This in turn implicates TFIIB as the target for p53-mediated repression of pol III transcription.

Heat-treating fractionated TFIIB at 47°C for 15 min inactivates its TBP subunit whilst the TAF components of the complex retain their function (White *et al.*, 1995a,b). A TFIIB fraction that had been treated in this way was found to have lost its ability to overcome repression by p53 (Figure 3C). This suggests that TBP is at least one of the components of TFIIB that is targeted by p53.

Endogenous cellular p53 associates with TFIIB

The experiments described so far establish that p53 can repress pol III transcription when it is added in excess. However, overexpression of a regulator may force interactions that would not occur at physiological concentrations. It is therefore important to test whether cellular p53 is involved in the regulation of pol III activity. If endogenous p53 plays a significant role in repressing TFIIB, one would expect to find these factors associated in cell extracts. This possibility was investigated by

Fig. 1. Recombinant p53 represses transcription of all class III genes tested. (A) p53 represses U6 and Alu transcription. Transcription of pU6/Hae/RA.2 (500 ng; lanes 1–4) and pRH5.7 (500 ng; lanes 5–8) using HeLa nuclear extract (10 µg) pre-incubated for 15 min at 30°C with 33 (lanes 2 and 6) or 100 ng (lanes 3 and 7) of baculovirus-expressed recombinant p53. (B) p53 is a repressor of pol III transcription. Transcription of pHu5S3.1 (lanes 1 and 2), Mcet1 (lanes 3 and 4), pTB14 (lanes 5 and 6), E2-160 (lanes 7 and 8) and pU6/Hae/RA.2 (lanes 9 and 10) templates (500 ng) using HeLa nuclear extract (10 µg) pre-incubated for 15 min at 30°C with 300 ng of GST (lanes 1, 3, 5, 7 and 9) or GST-p53 (lanes 2, 4, 6, 8 and 10). (C) An antibody that binds p53 blocks repression of 5S gene transcription by GST-p53. Transcription of pHu5S3.1 (500 ng) using HeLa nuclear extract (10 µg) pre-incubated for 15 min at 30°C with 250 ng of GST (lane 1) or GST-p53 (full-length; lanes 2 and 3). Prior to use, the GST-p53 was incubated for 2 h at 4°C with 0.5 µg of DO-1 or C-21 IgG (lanes 2 and 3, respectively). (D) Coomassie-stained gel of purified GST-p53. Molecular weight markers (lane 1) and purified GST-p53 (lane 2; 400 ng) were electrophoresed on a 7.8% SDS-polyacrylamide gel and then visualized by staining with Coomassie Blue. (E) Repression by p53 is template-specific. Transcription of pG6I (100 ng) using HeLa nuclear extract (10 µg) pre-incubated for 30 min at 30°C with 450 ng of GST (lane 1), or 150 ng, 300 ng or 450 ng (lanes 2–4, respectively) of GST-p53.

immunoblotting fractionated pol III factors. Mono Q-purified TFIIB was found to contain readily detectable amounts of p53 (Figure 4A). To assess whether this is due to fortuitous co-fractionation, we followed the elution of p53 and TFIIB on the Mono Q gradient. Functional assays revealed that TFIIB elutes in a sharp peak in

fractions 41 and 42, corresponding to a KCl concentration of 480–560 mM (Figure 4B, upper panel). p53 is readily detectable by immunoblotting in fractions 41 and 42, but there is little or none in the surrounding fractions (Figure 4B, lower panel). Thus, TFIIB and p53 co-elute closely during gradient elution of Mono Q.

Fig. 2. Pre-assembly of a class III transcription complex confers protection against repression by p53. HeLa nuclear extract (10 μ g) was incubated for 15 min at 30°C before the addition of pHu5S3.1 (250 ng); after a further 15 min at 30°C, nucleotides were added and transcription was allowed to proceed. Reactions were supplemented with 400 ng of GST (lanes 1, 3 and 5) or GST-p53 (lanes 2, 4 and 6), which were added at the indicated times.

As a further test of specificity, we examined TFIIB preparations that had been generated using different fractionation protocols (Figure 4C). p53 was detected in TFIIB fractions that had been prepared using phosphocellulose and a heparin gradient (Figure 4C, lane 2), a Q-Sepharose gradient (Figure 4C, lane 3) or a DEAE-Sephadex step (Figure 4C, lane 6). A cross-reacting species that does not co-migrate with p53 is also detected following heparin chromatography (Figure 4C, lane 2); this appears to be a contaminant, as it is not recognized by an alternative p53 antibody. As was the case for Mono Q, when we monitored p53 and TFIIB across a heparin gradient, we found that they co-elute closely (data not shown). In contrast to its consistent co-fractionation with TFIIB, p53 was not found in fractions containing TFIIC or pol III (Figure 4C, lanes 4 and 5, respectively). These data are consistent with a stable and specific interaction between p53 and TFIIB.

We carried out immunoprecipitation assays as a more stringent test of whether or not endogenous cellular p53 associates with TFIIB (Figure 5A). The TFIIB-specific TAF subunit BRF was 35 S-labelled and then mixed with a crude nuclear extract. Immunoprecipitation with an antibody against TBP confirmed that the added BRF is assembled into complexes with factors present in the nuclear extract (Figure 5A, lane 5). BRF was also co-precipitated using antibodies that recognize either the N- or C-terminal domains of p53 (Figure 5A, lanes 7 and 9). None of these antibodies was able to immunoprecipitate BRF in the absence of cellular extract (Figure 5A, even-numbered lanes). These results suggest that BRF can associate with endogenous p53. The specificity of this interaction is demonstrated by the fact that little or no

BRF was immunoprecipitated using antibodies against Oct-1 (Figure 5A, lane 3), the TFIID subunit TAF_{II}250 (Figure 5A, lane 11) or SV40 large T antigen (Figure 5A, lane 13), even in the presence of nuclear extract. We conclude that the TFIIB subunit BRF is assembled into a specific complex that includes cellular p53.

Additional immunoprecipitations were carried out to test whether endogenous BRF associates with p53 when both factors are present at physiological ratios. Crude fractions were immunoprecipitated using various monoclonal antibodies and the precipitates were immunoblotted and probed for the presence of BRF (Figure 5B). As a positive control we used an anti-TBP antibody and as a negative control we used an antibody against SV40 large T antigen, which is not present in the HeLa extracts that were used for these experiments. We also tested a monoclonal antibody that recognizes p53. BRF was co-precipitated strongly by the antibodies against TBP and p53 (Figure 5B, lanes 2 and 3), but was barely detectable in the negative control (Figure 5B, lane 4). Figure 5C demonstrates the specificity of the antiserum, which clearly detects BRF that has been translated in a reticulocyte lysate. An alternative antiserum raised against a distinct epitope also detects BRF in anti-p53 immunoprecipitates (data not shown). A second antibody against p53 was also found to immunoprecipitate endogenous BRF (data not shown). We do not detect co-precipitation of p53 with TFIIC (data not shown). These experiments provide direct evidence for a stable and specific association between endogenous p53 and TFIIB.

TFIIB activity is elevated in p53 knock-out fibroblasts

To examine the importance of p53 in controlling cellular pol III activity, we made use of fibroblast lines derived from p53 knock-out mice or their wild-type equivalent. Northern blot analysis of RNA isolated from these cells revealed that pol III transcripts of the B2 gene family are significantly elevated in two different p53^{-/-} lines relative to an equivalent p53^{+/+} line (Figure 6A, upper panel). This effect is specific, since the wild-type cells produce at least as much tubulin mRNA as the knock-out cells (Figure 6A, lower panel). Glyceraldehyde phosphate dehydrogenase mRNA was also produced at equivalent levels by these three fibroblast lines (data not shown). After correction for the level of tubulin mRNA, the B2 pol III transcripts were found to be 3- to 7-fold more abundant in the p53 knock-out cells.

We carried out run-on assays to compare directly the rates of transcription in intact nuclei of p53^{+/+} and p53^{-/-} fibroblasts. Transcripts initiated *in vivo* were radioactively labelled and resolved on a sequencing gel, as previously (White *et al.*, 1996). The high molecular weight transcripts are synthesized primarily by pol II, as shown by their sensitivity to low doses of α -amanitin (Figure 6B, upper panel). The rate of synthesis of these pol II transcripts is very similar in p53^{+/+} and p53^{-/-} fibroblasts. In contrast, the α -amanitin-resistant synthesis of 5S rRNA and tRNA is significantly increased following disruption of the p53 gene (Figure 6B, lower panel). Thus, transcription of 5S rRNA and tRNA genes is 6- and 4-fold more active, respectively, in the p53^{-/-} lines relative to the wild-type

Fig. 3. TFIIIB is sufficient to relieve repression by p53.

(A) Repression of 5S rRNA synthesis by p53 can be overcome using TFIIIB but not TFIIIC. Transcription of pVA₁ (200 ng) using 10 μ g of HeLa nuclear extract (lanes 1–4), 2.5 μ l of Mono Q-purified TFIIIB (fraction 41; lanes 3, 5 and 7) and 2.5 μ l of CHeP-1.0 fraction containing TFIIIC and pol III (lanes 4, 6 and 7). Extract was pre-incubated (15 min, 30°C) with 400 ng of GST (lane 1) or GST-p53 (lanes 2–4) prior to the addition of template and nucleotides. The reactions shown in lanes 1–7 were all carried out in parallel; however, a shorter exposure is shown of lanes 1–4 than of lanes 5–7, because of the greater activity of the unfractionated extract. (B) Immunodepletion of TFIIIB prevents restoration of 5S gene transcription in the presence of p53. Transcription of pHu5S3.1 (250 ng) using 10 μ g of HeLa nuclear extract pre-incubated (15 min, 30°C) with 400 ng of GST (lane 1) or GST-p53 (lanes 2–5) in the presence of 2 (lane 3) or 5 μ l (lanes 4 and 5) of Mono Q-purified TFIIIB (fraction 41). Prior to use, the TFIIIB fraction was immunodepleted for 3 h on ice with either pre-immune serum (lanes 3 and 4) or anti-BRF antiserum 128 (lane 5) bound to protein A-Sepharose. (C) Heat inactivation of TBP prevents TFIIIB from relieving repression by p53. Transcription of pVA₁ (250 ng) using 10 μ g of HeLa nuclear extract pre-incubated (15 min, 30°C) with 400 ng of GST (lanes 1 and 8) or GST-p53 (lanes 2–7) in the presence of 2 (lanes 3 and 5) or 5 μ l (lanes 4 and 6) of Mono Q-purified TFIIIB (fraction 41). The TFIIIB used in lanes 5 and 6 was heated at 47°C for 15 min prior to use in order to inactivate TBP.

cells. We conclude that loss of the p53 gene can result in a specific increase in the rate of pol III transcription *in vivo*.

Whole-cell extracts were prepared from these fibroblasts and tested for their ability to transcribe the VA₁ gene (Figure 7A). When assayed at a high ratio of protein to template, the p53^{-/-} cell extracts were found to transcribe

VA₁ 3- to 5-fold more actively than an equal amount of p53^{+/+} cell extract (Figure 7A, lanes 1–3). When the protein:DNA ratio was halved, the differential in transcription between null and wild-type increased to 8- to 10-fold (Figure 7A, lanes 4–6). Similar results were obtained using Alu, tRNA and 5S rRNA gene templates (Figure

Fig. 4. Endogenous p53 co-fractionates with TFIIIB. (A) p53 is present in Mono Q-purified TFIIIB. Mono Q-purified TFIIIB (lane 1, fraction 41, 20.6 μ g) and baculovirus-expressed recombinant p53 (lanes 2 and 3, 5 ng and 15 ng, respectively) were resolved on an SDS-7.8% polyacrylamide gel and then analysed by Western immunoblotting with anti-p53 antibody DO-1. (B) A population of endogenous p53 molecules co-fractionates with TFIIIB on a Mono Q gradient. The upper panel shows the TFIIIB activity of individual Mono Q fractions and the lower panel shows p53 content. The upper panel shows transcription reactions carried out using 500 ng of pVA_I, 2 μ l of PC-C and 2 μ l of PC-B starting material (lane 1), flow-through (lane 2), gradient fractions 37-48 (lanes 3-13, respectively) or LDB buffer (lane 14), as indicated. The lower panel shows a Western blot of an SDS-7.8% polyacrylamide gel containing 15 μ l of gradient fractions 38-46 (lanes 1-9, respectively), as indicated. The blot was probed with anti-p53 antibody DO-1. (C) p53 is readily detectable in fractions containing TFIIIB but not in fractions containing pol III or TFIIIC2. Fractionated factors (20 μ l), as indicated, were resolved on an SDS-7.8% polyacrylamide gel and then analysed by Western immunoblotting with anti-p53 antibody DO-1. The TFIIIB fractions in lanes 2, 3 and 6 were BHep-52 (3.9 μ g), QS-PC-B (10.5 μ g) and A25(0.15) (1.6 μ g), respectively. Lane 4 contained affinity-purified TFIIIC2 (3.8 μ g). The pol III fraction in lane 5 was A25(1.0) (2.2 μ g). Lane 1 contains 10 ng of baculovirus-expressed recombinant p53.

7B). These data suggest that disruption of the p53 gene can produce a significant increase in the activity of the general pol III transcription apparatus.

Complementation assays were carried out to measure directly the activity of TFIIIB in extracts prepared from the matched p53^{+/+} and p53^{-/-} cells (Figure 8A). TFIIIB was found to be significantly more active in the p53 knock-out extracts than in the wild-type extract. In contrast to the specific pol III product, synthesis of a template-independent transcript (slightly below the VA_I RNA) was not increased by the loss of p53 (Figure 8A, lanes 1-3). We also examined TFIIIC2 activity in the same extracts. Band-shift assays were carried out using as probe a B-block binding site for TFIIIC2 (Figure 8B). Two distinct TFIIIC-DNA complexes can be resolved by this approach

(Hoeffler *et al.*, 1988; White *et al.*, 1990). The specificity of these complexes is shown by the fact that they are efficiently competed with unlabelled B-block oligonucleotide, but not by a competitor of unrelated sequence (Figure 8B, lanes 2-4). It has been reported that the slowly-migrating complex contains transcriptionally active TFIIIC2, whereas the higher mobility complex contains an inactive form (Hoeffler *et al.*, 1988). We could detect little or no qualitative or quantitative difference in the TFIIIC-DNA complexes formed by p53^{+/+} and p53^{-/-} extracts (Figure 8B, lanes 1 and 2). These results suggest that the DNA-binding activity of TFIIIC2, and perhaps also its transcriptional activity, is not affected significantly by disruption of the p53 gene.

The observed increase in TFIIIB activity that accom-

Fig. 5. Endogenous p53 co-immunoprecipitates with TFIIIB. **(A)** BRF co-precipitates with TBP and p53. Reticulocyte lysate (10 μ l; Promega) that had been used to translate BRF in the presence of [35 S]Met and [35 S]Cys was immunoprecipitated in the presence of 10 μ l LDB buffer (lanes 2, 4, 6, 8, 10 and 12) or 10 μ l of HeLa nuclear extract (lanes 3, 5, 7, 9, 11 and 13) using anti-Oct-1 antibody C-21 (lanes 2 and 3), anti-TBP antibody MTBP-6 (lanes 4 and 5), anti-p53 antibodies pAb421 (lanes 6 and 7) and DO-1 (lanes 8 and 9), anti-TAF $_{250}$ antibody 6B3 (lanes 10 and 11) and T antigen antibody pAb 108 (lanes 12 and 13). Lane 1 shows 2 μ l of input reticulocyte lysate containing translated BRF. Samples were resolved on an SDS-7.8% polyacrylamide gel and then detected by autoradiography. **(B)** Endogenous BRF is detected in complexes with TBP and p53. Proteins from 35 μ g of PC-B that were immunoprecipitated using anti-TBP antibody MTBP-6 (lane 2), anti-p53 antibody DO-1 (lane 3) or anti-T antigen antibody pAb108 (lane 4) were resolved on an SDS-7.8% polyacrylamide gel and then analysed by Western immunoblotting with anti-BRF antibody 128. Lane 1 contains 17.5 μ g of PC-B. **(C)** Control for the specificity of anti-BRF antibody 128. Reticulocyte lysate (5 μ l; Promega) that had been used to translate BRF (lane 1) or TBP (lane 2) mRNA was resolved on an SDS-7.8% polyacrylamide gel and then analysed by Western immunoblotting with anti-BRF antibody 128.

panies inactivation of p53 might not be expected to stimulate transcription unless TFIIIB is limiting in this system. We therefore carried out add-back experiments to determine whether this is the case. Mono Q-purified TFIIIB was found to produce a substantial increase in VA $_1$ expression in p53 $^{+/+}$ extracts (Figure 8C, lanes 1–4). This effect is specific, since a fraction containing TFIIIC and pol III makes little difference to transcription in the wild-type extract (Figure 8C, lane 5). Reconstitution assays confirmed that this fraction is active, since it supports transcription in the presence of Mono Q-purified TFIIIB, whereas neither fraction alone gives detectable expression (Figure 8C, lanes 6–8). These results suggest that TFIIIB activity is indeed limiting in the p53 $^{+/+}$ extracts. When the same fractions were added to knock-out extracts, the TFIIIB fraction caused no stimulation whereas the TFIIIC–pol III fraction caused slight activation (Figure 8C, lanes 9–16; lanes 13–16 show a shorter exposure of lanes 9–12). This indicates that TFIIIB activity is in relative excess in p53 $^{-/-}$ extracts, unlike the case for the p53 $^{+/+}$ extracts. We conclude that TFIIIB activity is limiting in the wild-

type, but disruption of the p53 gene increases TFIIIB activity to such an extent that it is no longer limiting and the overall rate of pol III transcription is significantly increased.

Discussion

The data suggest that p53 can inhibit expression of every class III gene tested, including tRNA, 5S rRNA and U6 snRNA genes. This regulation is achieved by binding and inactivating TFIIIB. Once it has been assembled into a pre-initiation complex, TFIIIB is far less susceptible to the action of p53. Endogenous p53 associates with TFIIIB in a relatively stable complex. Disruption of the p53 gene results in a specific increase in TFIIIB activity and pol III transcription. We conclude that p53 is a general repressor of class III gene expression.

Chesnokov *et al.* (1996) recently reported that transcription of Alu and U6 snRNA genes by pol III can be inhibited by the overexpression of p53 *in vitro* or in transfected cells. In contrast to our findings, they observed

Fig. 6. p53 knock-out fibroblasts display a specific increase in pol III activity *in vivo*. (A) B2 transcript levels are elevated specifically in p53 knock-out fibroblasts. Northern blot analysis of total RNA (10 μ g) from jps-1 (lane 1, p53^{-/-}), j-140 (lane 2, p53^{+/-}) or jps-13 (lane 3, p53^{-/-}) cells. The top panel shows the blot hybridized to a B2 gene probe and the bottom panel shows the same blot reprobed with an α -tubulin gene. (B) Nuclear run-on assays reveal a specific increase in the rates of synthesis of tRNA and 5S rRNA in p53 knock-out fibroblasts. Nuclei (10⁷) from jps-1 (lane 1, p53^{-/-}), j-140 (lane 2, p53^{+/-}) or jps-13 (lanes 3 and 4, p53^{-/-}) cells were used in a transcriptional run-on experiment in the presence (lane 4) or absence (lanes 1–3) of 0.5 μ g/ml α -amanitin. Radiolabelled RNA was resolved on a 7% polyacrylamide gel and detected by autoradiography. The upper panel shows transcripts larger than ~1000 bases, whereas the lower panel shows transcripts in the 70–140 base range.

no effect of recombinant p53 on the *in vitro* transcription of 5S, 7SL, VA₁ and tRNA genes (Chesnokov *et al.*, 1996). As these authors pointed out, the two templates they found to be responsive to p53 have much weaker promoters than the other class III genes that they tested (Chesnokov *et al.*, 1996). This may account for the differential sensitivity that was observed. We found that preparations of recombinant p53 with relatively low specific activity were able to repress U6 and Alu genes, but had little effect when tested in parallel with a much more powerful template such as VA₁. However, more active p53 preparations clearly can inhibit the strong pol III promoters such as VA₁, 5S and tRNA (Figures 1–3). Furthermore, every class III gene tested is transcribed more actively in p53^{-/-} cell extracts when compared with wild-type extracts (Figure 7). Perhaps most importantly,

Fig. 7. pol III transcriptional activity is abnormally elevated in p53 knock-out fibroblasts. (A) The abilities of p53^{-/-} and p53^{+/-} cell extracts to transcribe the VA₁ gene were compared at two different extract concentrations. Transcription of pVA₁ (200 ng) using 16 (lanes 1–3) or 8 μ g (lanes 4–6) of whole-cell extract prepared from jps-1 (lanes 1 and 4), j-140 (lanes 2 and 5) or jps-13 (lanes 3 and 6) cells. (B) p53^{-/-} and p53^{+/-} cell extracts were tested for their ability to transcribe a range of pol III templates. Transcription of pRH5.7 (lanes 1 and 2), Mcet1 (lanes 3 and 4) and pHu5S3.1 (lanes 5, 6 and 7) templates (200 ng) using whole-cell extract (16 μ g) prepared from jps-1 (lanes 1, 3 and 5), j-140 (lanes 2, 4 and 6) or jps-13 (lane 7) cells.

nuclear run-on assays demonstrate that tRNA and 5S rRNA synthesis are significantly elevated *in vivo* following disruption of the p53 gene (Figure 6B). We conclude from these data that endogenous p53 is a general repressor of pol III transcription. Nevertheless, we agree with Chesnokov *et al.* (1996) that U6 and Alu are more sensitive to p53 than the other pol III templates we have tested. This is seen when recombinant p53 is added to extracts and also when wild-type and knock-out extracts are compared. For example, in the experiment shown in Figure 7B, the difference in transcription between p53^{-/-} and p53^{+/-} extracts is 6-fold for the 5S rRNA gene and 25-fold for the Alu gene. Thus, different class III promoters display distinct sensitivities to p53. Induction of p53 *in vivo* may therefore elicit differential regulation, with Alu and U6 becoming inhibited at a lower p53 threshold than some of the other pol III templates. Sensitivity to p53 appears to be inversely correlated with promoter strength, although a more complex explanation cannot be excluded.

The rate of growth of p53^{-/-} fibroblasts is approximately twice that of the corresponding wild-type cells (Harvey *et al.*, 1993). It is possible that this accelerated growth contributes to the high pol III activity of the knock-out cells. However, the difference in tRNA synthesis between serum-stimulated and quiescent fibroblasts is only ~3-fold (Johnson *et al.*, 1974; Mauck and Green, 1974). It is therefore unlikely that a mere doubling in growth rate is sufficient to account for the substantial activation of pol III transcription that is observed following disruption of the p53 gene.

Several independent lines of evidence suggest that p53 is able to repress pol III transcription by specifically inactivating TFIIB. First, Chesnokov *et al.* (1996) have demonstrated that an N-terminal fragment of p53, over-expressed as a GST fusion protein, can bind to TFIIB in a 'pull-down' assay. Our data provide evidence that this interaction can occur at physiological ratios. Our immunoprecipitation experiments demonstrate a stable association between endogenous cellular p53 and TFIIB (Figure 5). The consistent co-fractionation of these factors provides additional support for such an interaction (Figure 4). We have also produced functional evidence that p53 represses class III genes by specifically inactivating TFIIB. Thus, transcription can be restored in the presence of excess p53 simply by adding more TFIIB to the system (Figure 3). Furthermore, TFIIB activity is elevated in p53 knock-out fibroblasts, whereas TFIIC2 activity seems to be unchanged (Figure 8). The TBP-binding capability of p53 is well-documented (Seto *et al.*, 1992; Liu *et al.*, 1993; Martin *et al.*, 1993; Truant *et al.*, 1993; Horikoshi *et al.*, 1995; Tansey and Herr, 1995; Chesnokov *et al.*, 1996; Farmer *et al.*, 1996) and appears to be required for repression of TFIIB. Heating TFIIB at 47°C for 15 min inactivates its TBP subunit without compromising the function of the other components of the complex (White *et al.*, 1995a,b). Such treatment abolishes the ability of TFIIB to restore transcription in the presence of p53 (Figure 3C). This suggests that p53 targets TBP within the TFIIB complex, although it does not rule out additional interactions with other subunits of TFIIB. We also cannot exclude the possibility that other components of the pol III transcription apparatus might be regulated by p53 as well, but we have no evidence that this is the case.

It remains to be determined how the association between p53 and TFIIB results in decreased transcription. Analogy may be drawn with the TBP-binding repressor protein Dr1, which inactivates TFIIB and thereby represses pol III transcription both *in vitro* and *in vivo* (White *et al.*, 1994; Kim *et al.*, 1997). Dr1 achieves this by displacing the essential pol III TAF BRF from its interaction with TBP (White *et al.*, 1994). Our immunoprecipitation data and previous pull-down experiments (Chesnokov *et al.*,

Fig. 8. TFIIB activity is elevated specifically in p53 knock-out fibroblasts. (A) TFIIB is more active in p53^{-/-} extracts than in p53^{+/+} extracts. pVA₁ (500 ng) was transcribed using 4 µl of PC-C, 25 ng of recombinant TBP plus either 4 µl of Mono Q-purified TFIIB (lane 5), 2.5 µg of whole extract prepared from jps-1 (lane 1), j-140 (lane 2) or jps-13 (lane 3) cells, or no additional factors (lane 4). The whole-cell extracts and TFIIB were heated at 47°C for 15 min prior to use in order to inactivate endogenous TBP and TFIIC. The band that runs beneath the VA₁ RNA is a template-independent transcript. (B) The B-block-binding activity of TFIIC is similar in p53^{-/-} and p53^{+/+} extracts. Band-shift assay using 1 ng of radiolabelled B-block oligonucleotide probe, 500 ng of polydI-dC competitor, and 14.6 µg of j-140 (lane 1) or jps-13 (lanes 2-4) cell extract, or no extract (lane 5). Lanes 3 and 4 also contained 50 ng of unlabelled B-block or MSV control oligonucleotide, as indicated. The complex labelled NS is due to non-specific binding. (C) TFIIB is limiting for VA₁ transcription in extracts from wild-type fibroblasts but not in extracts from p53 knock-out equivalents. pVA₁ (500 ng) was transcribed using extract (20 µg) prepared from jps-13 (lanes 1 and 9-12) or j-140 (lanes 2-5) cells. Mono Q-purified TFIIB (fraction 41) was included as follows: reactions 3 and 11, 2.5 µl; reactions 4 and 12, 5 µl; reactions 6 and 8, 2 µl. Fraction CHep-1.0 (containing TFIIC and pol III) was included as follows: reactions 5 and 10, 5 µl; reactions 7 and 8, 2 µl. Lanes 13-16 show a shorter exposure of the reactions in lanes 9-12.

1996) demonstrate that BRF is not displaced from TFIIB by p53. Nevertheless, mammalian TFIIB contains at least one additional TAF for which molecular probes are not yet available (Lobo *et al.*, 1992; Taggart *et al.*, 1992; Mital *et al.*, 1996), and it is possible that this subunit is released from the complex by p53 binding. Alternatively, p53 might interfere with the ability of TFIIB to interact with TFIIC and/or pol III.

A number of studies have linked p53 to the control of protein synthesis. It has been shown to influence the translation of specific mRNAs (Ewen *et al.*, 1995; Mosner *et al.*, 1995). Furthermore, a proportion of cytoplasmic p53 is covalently bound to 5.8S rRNA and associated with ribosomes (Fontoura *et al.*, 1992, 1997). p53 has also been found in a complex containing the ribosomal L5 protein and 5S rRNA, leading to the suggestion that it may play a role in controlling ribosome biogenesis (Marechal *et al.*, 1994). Strong support for this idea is provided by our demonstration that p53 can control the synthesis of 5S rRNA.

TFIIB is a target for repression by the retinoblastoma protein RB (White *et al.*, 1996; Chu *et al.*, 1997; Larminie *et al.*, 1997). It is therefore particularly striking that this same factor should also be regulated by another unrelated tumour suppressor, p53. TFIIB is a major determinant of the biosynthetic capacity of cells, controlling the production of a variety of small RNAs including tRNA and 5S rRNA. It has been suggested that the repression of pol III transcription might contribute to growth control by limiting the rate of increase in cellular mass (Nasmyth, 1996; White, 1997; Larminie *et al.*, 1998). Indeed, a 2-fold reduction in the level of initiator tRNA is sufficient to cause a 3-fold increase in doubling time in *Saccharomyces cerevisiae* (Francis and Rajbhandary, 1990). The plausibility of pol III regulation as a means of controlling growth is strengthened further by the fact that TFIIB is targeted by two different major tumour suppressors, RB and p53, both of which perform important physiological roles in restraining growth under specific environmental conditions.

Materials and methods

Plasmids

Plasmids pU6/Hae/RA.2, pRH5.7, pHu5S3.1, Mcet1, pTB14, E2-160, pVAI and pCTub have all been described by White *et al.* (1989, 1992, 1995a). pG₆I was described by Pugh and Tjian (1991).

Cell lines

The jps-1 and jps-13 cells are p53^{-/-} immortalized 3T3 fibroblast lines that were established from p53 knock-out mice. The j-140 cells are p53^{+/+} immortalized 3T3 fibroblasts that were established in parallel from wild-type siblings. These lines were generously provided by Drs Erwin Wagner and Martin Schreiber. The cells were grown in high glucose Dulbecco's modified Eagle's medium with 10% fetal calf serum.

Protein extracts and fractions

HeLa cells harvested in exponential phase were obtained from the Computer Cell Culture Center. Nuclear extracts were prepared from HeLa cells by the method of Dignam *et al.* (1983). Whole-cell extracts were prepared from fibroblast lines jps-1, jps-13 and j-140 using the method described by White *et al.* (1995a).

PC-B is the 0.1–0.35 M KCl step fraction from a phosphocellulose column, and contains both TFIIB and pol III. PC-C is the 0.35–0.6 M KCl step fraction from a phosphocellulose column, and contains both TFIIC and pol III. The conditions used for phosphocellulose chromatography, Mono Q-purification of TFIIB, affinity-purification of TFIIC,

and the generation of CHep-1.0, A25(0.15) and A25(1.0) fractions have been described previously (White *et al.*, 1995a; Larminie *et al.*, 1997). CHep-1.0 contains TFIIC1, TFIIC2 and pol III; A25(0.15) contains TFIIB; and A25(1.0) contains pol III. BHeP-52 is a peak TFIIB fraction (fraction 52) from the heparin gradient described by Larminie *et al.* (1997). To prepare QS-PC-B fractions, nuclear extract was applied to a Q-Sepharose column in 20 mM HEPES pH 7.6, 100 mM KCl, 20% glycerol, 2 mM MgCl₂, 0.5 mM EDTA, 0.5 mM PMSF, 0.1 mM Na metabisulfite. The bound material was then eluted in the same buffer with a salt gradient up to 500 mM KCl. Fractions that eluted between 350 and 500 mM KCl were dialyzed against LDB buffer (20 mM HEPES-KOH pH 7.9, 17% glycerol, 100 mM KCl, 12 mM MgCl₂, 0.1 mM EDTA, 2 mM DTT). These were then pooled and chromatographed on phosphocellulose. The 0.1–0.35 M KCl step fraction was found to contain TFIIB activity and is referred to as QS-PC-B.

The GST-p53 proteins, their expression in bacteria and purification with glutathione-agarose have been described previously (Sorensen *et al.*, 1996). Baculovirus-expressed p53 was a generous gift from Drs David Lane and Ted Hupp.

Transcription and band-shift assays

Transcription and band-shift assays were conducted as previously described (White *et al.*, 1989). The B-block and MSV oligonucleotides are described by White *et al.* (1989).

Immunoprecipitation and Western blotting

Immunoprecipitation and Western immunoblotting were performed as previously described (Larminie *et al.*, 1997). The antibodies used were DO-1 (Santa Cruz) and pAb421 (Calbiochem) against p53, C-21 (Santa Cruz) against Oct-1, 6B3 (Santa Cruz) against TAF_{II}250, pAb108 (Santa Cruz) against SV40 large T antigen, MTBP-6 against TBP and 128 against BRF. Anti-BRF antibody 128 was raised by immunising rabbits with synthetic peptide KISSKINYSVLRGLS (human BRF residues 533–547) coupled to keyhole limpet haemocyanin. MTBP-6, a monoclonal antibody that recognizes the N-terminal region of TBP, was a generous gift from S.J. Flint. *In vitro* translation of BRF was carried out according to the manufacturer's specifications (Promega).

Northern blotting

Total cellular RNA was extracted using TRI reagent (Sigma), according to the manufacturer's instructions. Agarose gel electrophoresis, Northern transfer and hybridization were carried out according to the methods of Maniatis *et al.* (1982). The B2 gene probe was a 240 bp *EcoRI*–*PstI* fragment from pTB14. The α -tubulin probe was a 1.4 kb *PstI* fragment from p α Tub.

Nuclear run-on assay

Nuclear extraction, run-on reactions and electrophoresis of labelled RNAs were performed as previously described (White *et al.*, 1996).

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