A Role for TAF3B2 in the Repression of Human RNA Polymerase III Transcription in Nonproliferating Cells*

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RNA polymerase III (Pol III) synthesizes various small RNA species, including the tRNAs and the 5 S ribosomal RNA, which are involved in protein synthesis. Here, we describe the regulation of human Pol III transcription in response to sustained cell cycle arrest. The experimental system used is a cell line in which cell cycle arrest is induced by the regulated expression of the tumor suppressor protein p53. We show that the capacity of cells to carry out Pol III transcription from various promoter types, when tested in vitro, is severely reduced in response to sustained p53-mediated cell cycle arrest. Furthermore, this effect does not appear to be due to direct inhibition by p53. By using complementation assays, we demonstrate that a subcomponent of the Pol III transcription factor IIIB, which contains the proteins TATA-binding protein and TAF3B2, is the target of repression. Moreover, we reveal that TAF3B2 levels are markedly reduced in extracts from cell cycle-arrested cells because of a decrease in TAF3B2 protein stability. These findings provide a novel mechanism of Pol III regulation and yield insights into how cellular biosynthetic capacity and growth status can be coordinated.

Cellular growth and proliferation are two distinct processes. Proliferation is defined as the increase in the number of cells, which occurs when cells progress through a new cell division cycle. Cell growth, on the other hand, is defined as the increase in mass of an individual cell. Growth is related to the biosynthetic capacity of a cell, a reliable indicator of which is the level of protein synthesis (1). Conceptually, it seems requisite that the processes of growth and proliferation are linked and that sensitive fine tuning exists between them. At present, however, very little is known about the molecular mechanisms that ensure that this crucial cross-regulation is achieved.

After neoplastic transformation of cells, uncontrolled proliferation is only sustainable if it is linked to deregulated cell growth (2). Major determinants of protein synthesis are the ribosomes and the machinery that delivers activated amino acids to the ribosomes during translation. Growth is therefore dependent on the availability of adequate supplies of rRNAs and tRNAs. It has been shown that, as cells enter quiescence, existing ribosomes disassemble into their subunits, the levels of rRNAs and tRNAs decrease, and net protein synthesis is down-regulated. These events are reversed upon mitogenic stimulation (2). But how are regulation of growth and regulation of proliferation jointly accomplished? The prevailing model is that tumor suppressor proteins play a crucial role in eliciting this control (1, 3, 4). For example, the retinoblastoma protein regulates entry into S phase by controlling E2F, a transcription factor involved in the expression of S phase-inducing genes (5, 6). It may also affect cell growth potential by repressing the activities of RNA polymerases I (7) and III (8), which synthesize rRNAs and tRNAs, respectively. Multi-functional proteins such as retinoblastoma protein may therefore be vital for adjusting cellular growth potential to match proliferative activity. Conversely, as a consequence of losing retinoblastoma protein function, cells may lose control over both proliferation and growth and be put on a fast track toward neoplasia (1).

RNA polymerase (Pol) III synthesizes a number of small RNA species, including the 5 S rRNA, tRNAs, the splicosomal U6 small nuclear RNA, the 7SL RNA of the signal recognition particle, and the adenovirus VA1 RNA (9). The promoters of genes transcribed by Pol III are subdivided into three groups, type 1, type 2, and type 3 promoters, based on promoter structure and their requirements for basal Pol III transcription factors (10). TFIIIA is a monomeric protein factor specific for the promoters of the 5 S rRNA genes (type 1 promoters) (11–13). TFIIIC is a multimeric protein required for both type 1 and type 2 promoters. Human TFIIIC consists of at least nine polypeptides (14) and can be split into two subcomponents, TFIIIC1 and TFIIIC2 (15). For type 3 promoters, only the C1 component of TFIIIC is required as an initiation factor (16). Like TFIIIC, TFIIIB is an important Pol III transcription factor involved in initiating transcription on type 1 and type 2 promoters. According to the sequential recruitment model, TFIIIB is assembled into the preinitiation complex by TFIIIC on type 2 promoters or by the TFIIIC-TFIIIA complex on type 1 promoters and is positioned upstream of the transcription start site (17). In vitro studies have shown that TFIIIB then recruits Pol III to the promoter to direct accurate initiation of transcription and can do so even after TFIIIC has been stripped off the promoter (18). TFIIIB contains the TATA-binding protein (TBP) and various TBP-associated factors (TAFs) (19–22). Although the number and identity of TAFs in human TFIIIB have not been fully established, a TAF that has been characterized in some detail is TAF3B2 (previously named BRF or hTFIIIB90) (23, 24). Recently, two additional TAFs that form part of TFIIIB have been identified: hB*, which is the human homologue of the yeast TFIIIB component, B*, and hBRFU/TFIIIB50 (25, 26).

The aim of the work presented here was to investigate how
Pol III transcription is regulated during changes in cell growth and proliferation. Toward this end, we used the human fibroblast cell line TR9–7, derived from a patient with Li-Fraumeni syndrome, in which both copies of the endogenous gene for p53 are inactivated (27). TR9–7 cells are stably transfected with a recombinant construct that allows for the inducible expression of p53 from a tetracycline-controlled promoter. Thus, whereas p53 expression is barely detectable in TR9–7 cells grown in the presence of 1 μg/ml of tetracycline, decreasing the tetracycline concentration in the growth medium results in an increase in both p53 expression and function, as determined by the induction of the p53-responsive p21/Waf1 gene (27). Furthermore, the levels of p53 expression in TR9–7 cells upon tetracycline withdrawal are comparable with those induced in a wild-type cell line in response to DNA damage (27). This degree of p53 induction in the TR9–7 cells results in a slow onset, reversible cell cycle arrest in both the G1 and G2/M stages of the cell cycle. Complete cell cycle arrest is achieved 4–6 days after the onset of p53 induction. TR9–7 cells can be maintained in this arrested state for up to 20 days and yet can still return to a normal cycling state afterward (27). The TR9–7 cell line therefore provides a versatile experimental system in which cell cycle arrest can be manipulated. As discussed below, by use of this system, we have gained insights into the mechanisms by which sustained cell cycle arrest leads to a reduced capacity for Pol III transcription.

MATERIALS AND METHODS

Plasmids—The following plasmids were used as templates for Pol III in vitro transcription reactions. pGlu6 contains a HindIII fragment including a human tRNA58 gene inserted into pAT153 (28). pHuS3.1 contains a 638-base pair BamH1-SacI fragment from human genomic DNA including a 5 S RNA gene, inserted into pBlueScript SK+ from Stratagene (29). pBSK-Leu was constructed by subcloning a 240-base pair EcoRI-HindIII fragment containing a human tRNA58 gene (30) into pBlueScript SK+. pBSKV-VA was constructed by subcloning a 232-base pair XbaI-KpnI fragment including the adenoviral VA1 gene into pBlueScript SK+. The V1 promoter from this construct contains a point mutation in the A block (G-23 changed to A), which brings it closer to the TATA box. A block consensus is found in eukaryotic tRNA genes and increases the promoter’s strength (31, 32).

The following plasmids were used as templates for the synthesis of RNA probes for RNase protection analysis. pGEM-TAF3B2 contains a sequence comprising nucleotides 97–999 of the coding sequence of the human TAF3B2 cDNA (24) cloned into pGEM-T (Promega); a clone was selected in which the 5′-end of the insert was oriented toward the bacteriophage T7 promoter. pGEM-ACT contains a sequence comprising the first 217 base pairs of the third expressed exon of the β-actin gene cloned into pGEM-T; the 5′-end of the insert was oriented toward the bacteriophage T7 promoter of the vector.

Western Blotting—Western immunoblot analyses were performed as described by Coligan et al. (33). Primary antibodies were mouse monoclonal anti-β-actin antibody (Sigma), mouse monoclonal anti-p53 antibody (DO-1; Santa Cruz Biotechnology), and mouse anti-human p21 monoclonal antibody (Pharmingen). Rabbit anti-human TAF3B2 polyclonal antibody was a gift from Nouria Hernandez (Cold Spring Harbor Laboratories, Cold Spring Harbor, NY; Ref. 24). Mouse monoclonal anti-human TBP antibody (MBP-6) was a gift from S. J. Flint (34). The secondary antibodies (goat anti-mouse antibody from Sigma and goat anti-rabbit antibody from Pierce) were both horseradish peroxidase conjugates. Bound antibodies were detected by enhanced chemiluminescence using the ECL system (Amersham Pharmacia Biotech). In some cases, nitrocellulose membranes were stripped by incubation in 62.5 mM Tris-HCl, pH 6.8, 1% (v/v) SDS, and 0.14% (v/v) β-mercaptoethanol at 60 °C for 30 min with gentle shaking, followed by extensive washing before reprobing.

Tissue Culture—The TR9–7 cell line was a gift from George Stark (Department of Molecular Biology, Cleveland Clinic Foundation, Cleveland, OH), and were grown as described previously (27). To suppress p53 expression, cells were grown in the presence of 1 μg/ml tetracycline. To induce p53 expression, cells at about 80% confluence were replated at a 1:6 dilution, grown in the presence of tetracycline for 1 day, and then washed twice with 1× phosphate-buffered saline. Growth was continued for various periods of time in the absence of tetracycline before harvesting. Proteasome inhibition in TR9–7 cells was induced by adding the calpain/proteasome inhibitor N-acetyl-Leu-Leu-norleucinal (LLnL; Sigma) to the growth medium at a final concentration of 20 μM, as described by Shieh et al. (35). Cells were grown in the presence of LLnL for 1 h before harvesting.

Extract Preparation—TR9–7 cells were washed in 1× phosphate-buffered saline and incubated with 2.5 ml/dish of 0.5× trypsin/EDTA solution (Sigma) for 5 min at 37 °C. Detached cells were collected, transferred into a 15-ml centrifugation tube, centrifuged for 5 min at 500 × g, and washed twice in 1× phosphate-buffered saline. To prepare trypsin extracts, cell pellets were resuspended in an equal volume of freshly prepared whole cell extraction buffer (50 mM NaF, 20 mM HEPES, pH 7.8, 450 mM NaCl, 25% (v/v) glycerol, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml leupeptin, 0.5 μg/ml protease inhibitor, 1.0 μg/ml trypsin inhibitor, 0.5 μg/ml aprotinin, 40 μg/ml bestatin). Cells were subjected to three cycles of freeze-thawing (quick freezing on dry ice and thawing at 30 °C) and then centrifuged in a microcentrifuge at top speed for 7 min at 4 °C. The supernatant containing the protein extract was divided into samples, frozen on dry ice, and stored at −80 °C. Nuclear extracts from HeLa cells were prepared according to Dignam et al. (36).

Protein Fractionation—HeLa cell nuclear extracts were fractionated on Whatman P11 phosphocellulose according to Segall et al. (37). Chromatography was done at 4 °C. Buffer D-100 (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.5 mM MgCl2, 0.2 mM EDTA, 20 mM ζ-Amanitin, 2 μM DAPI, 0.5 mM dithiothreitol, 1 mM sodium metaphosphite, and 0.5 mM phenylmethylsulfonyl fluoride; adjusted to pH 7.9) at flow rates of 1.0–1.5 column volumes/h. 150–200 μg of nuclear extract were loaded on 15–20 ml of equilibrated phosphocellulose resin (binding capacity of 10 mg of protein/ml of resin). The flow-through fraction was collected as phosphocellulose fraction (PC) A. Proteins were eluted in a stepwise fashion with buffer D containing increasing concentrations of KCl: with buffers D-350, D-600, and D-1000 containing KCl at 350, 600, and 1000 mM, respectively. Fractions that eluted between 350 and 600 mM KCl were collected as PC-B fractions, those between 350 and 600 mM KCl were collected as PC-C, and those between 600 and 1000 mM KCl were collected as PC-D. For each elution, fractions with the highest protein concentration were pooled and dialyzed against buffer D-100. Pooled PC-B and PC-C fractions used for transcription assays and further purification had protein concentrations of approximately 1.5 and 0.5 mg/ml, respectively. The PC-B fraction was further purified and fractionated as described by Lobo et al. (20), except that a BioCAD Sprint perfusion chromatography system (PerSeptive Biosystems) was used. The Poros HR 500 column was eluted with a linear KCl gradient from 100 to 600 mM in buffer Q (same as buffer D except that it contained additional protease inhibitors: aprotinin, pepstatin A, and leupeptin all at 1 μg/ml and benzamidine at 1 mM). 5 ml of PC-B (7.5 mg of protein) was loaded onto the HQ resin and eluted with fractions of 750 μl. Fractions that contained peak amounts of the TBP and TAF3B2 were pooled and loaded on the HR resin. Chromatography on the HR resin was done as described for the HQ resin.

Immunodepletion of Extracts—Antibodies were adsorbed onto protein G-Sepharose beads (Amersham Pharmacia Biotech) according to Harlow and Lane (38). For TBP depletions, 400 μl of beads were incubated with 1 ml of tissue culture supernatant of MBP-6 cells, a hybridoma cell line expressing a mouse monoclonal anti-human TBP antibody (34). 300 μl of antibody-coated beads were equilibrated with Pol III transcription buffer (20% (v/v) glycerol, 20 mM HEPES, 100 mM KCl, 5 mM MgCl2, 0.2 mM EDTA, 20 mM ζ-Amanitin, and 2 mM dithiothreitol; adjusted to pH 7.9) and incubated with the same volume of a PC-C fraction for immunodepletion of TBP-containing protein complexes from PC-C. For p53, 10 μg of DO-1 antibody were linked to 100 μl of protein G-Sepharose beads, and antibody-coated beads were equilibrated with 1× phosphate-buffered saline. 70 μg of whole cell extract from TR9–7 cells were depleted with 10 μl of antibody-coated beads.

Transcription Assay—Transcriptional activity was assayed in vitro by monitoring the direct incorporation of [3H]uridine into synthesized RNA. The reactions were carried out in a final volume of 25 μl. As a source of transcription factors and Pol III, we used whole cell extract from TR9–7 cells (generally 0.8–1.2 μl; corresponding to 20 μg of protein) and/or partly purified factors that had been dialyzed into buffer D-100. The reactions were adjusted with buffer D-100 and other reagents such that the final salt concentrations were equivalent to 60%
FIG. 1. Regulation of protein expression in TR9–7 cells in response to tetracycline withdrawal. Whole cell extracts were prepared from cells that had been grown either in the presence of tetracycline (day 0) or from cells that had been incubated after tetracycline had been withdrawn for between 2 and 24 h (A) or between 1 and 7 days (B). 25 μg of protein extract were loaded and analyzed by Western blotting for p53, p21, or human β-actin as indicated. As a control, 1 ng of recombinant p53 was loaded into the seventh lane of (A).

FIG. 2. Levels of RNA Pol III-dependent transcription of various promoters in response to cell cycle arrest. A–D, total protein extracts were prepared from proliferating cells (lanes P) or from cells arrested for 6 days (lanes A) following the withdrawal of tetracycline. Equal amounts of protein (20 μg) were used to transcribe various RNA Pol III promoters (as indicated). Reactions were carried out at 1 or 200 μg/ml α-amanitin to verify that the main transcript was a genuine product of RNA Pol III. The sizes of selected nucleic acid marker bands that were run alongside the samples are indicated. E, levels of transcription in A–D were quantitated by PhosphorImager analysis; in each case, the amount of transcript from proliferating cells was assigned a value of 100%.

RESULTS

Pol III Transcription Is Repressed during Sustained Cell Cycle Arrest—After withdrawal of tetracycline from the growth medium of TR9–7 cells, we found that they stopped proliferating with similar kinetics to those published previously (27) and became enlarged and flattened compared with cells grown in the continual presence of tetracycline (data not shown). To investigate the expression of p53 and p21/Waf1 in response to tetracycline withdrawal over a 7-day period, we carried out Western blot analysis. As shown in Fig. 1, p53 and p21 were barely detectable when TR9–7 cells were grown in the presence of tetracycline. Their levels, however, increased markedly when tetracycline was withdrawn, reaching a peak after about 1 day and then slowly declining (blots were also probed for β-actin, which was used as a loading control). Notably, at peak levels of expression, the concentration of p53 was less than 1 ng/25 μg of total soluble cellular protein, as judged by the intensity of a Western blot signal to that of 1 ng of recombinant p53 loaded on a separate lane (Fig. 1A).

To assay for Pol III activity, transcription assays were carried out in vitro with TR9–7 whole cell extracts and DNA templates containing the promoter regions and coding sequences of various Pol III target genes, including the human 5 S rRNA gene (type 1 promoter) and the human tRNA^Leu^,
tRNA\(^{\text{Glu}}\), and the adenoviral V\(\text{A}_1\) genes (all type 2 promoters). Fig. 2 (A–D) shows that all four Pol III promoters were transcribed efficiently by extracts from proliferating TR9–7 cells (lanes P). Analysis of the assays performed at 1 or 200 \(\mu\)g/ml of \(\alpha\)-amanitin (Fig. 2) reveals that the observed transcripts were indeed generated by Pol III (40, 41). In striking contrast, transcription from the same promoters was greatly reduced in extracts from TR9–7 cells that had been cell cycle-arrested for 6 days (lanes A). Indeed, PhosphorImager analysis revealed that transcription was reduced by between 86 and 94%; Fig. 2E). Thus, overall Pol III transcription capacity of extracts derived from TR9–7 cells is markedly reduced following tetracycline withdrawal.

To assess in more detail the kinetics of repression of Pol III transcription, we analyzed the transcriptional capacity of extracts generated from cells every day over a 7-day period of tetracycline withdrawal (Fig. 3A). These studies revealed that levels of V\(\text{A}_1\) transcription declined progressively throughout the time course, with virtually no transcription being observed in days 6 and 7. Similar results were obtained using the human tRNA\(^{\text{Leu}}\) promoter (Fig. 3B).

Repression of Pol III Transcription in TR9–7 Extracts Is Not Directly Mediated by p53—Full repression of Pol III transcription in TR9–7 cell extracts only takes place 5–6 days after p53 levels have been fully induced (Figs. 1B and 3), suggesting that p53 is unlikely to be directly mediating the repression. To see whether this was indeed the case, we carried out several types of analysis. First, we tested whether transcription in extracts from proliferating TR9–7 cells was sensitive to exogenously added recombinant p53 that had been expressed and purified from baculovirus-infected insect cells (Fig. 4A). Two different amounts of p53, 150 and 450 ng, were added to 10 \(\mu\)g of protein of whole cell extract from proliferating TR9–7 cells (second and third \(P\) lanes, respectively), and levels of transcription were then compared with those of extracts in which no p53 had been added (first \(P\) lane) and of extracts from cell cycle-arrested TR9–7 cells (lane A) in which endogenous p53 had been inactivated in the presence of tetracycline; day 0) or from cells that had been incubated in the absence of tetracycline for 1–7 days (as indicated). Transcription assays with 20 \(\mu\)g of protein extract were carried out with the V\(\text{A}_1\) promoter (A) or the tRNA\(^{\text{Leu}}\) promoter (B); nt, nucleotides.

FIG. 3. Time course of effects of growth arrest on Pol III transcription. Extracts were prepared from proliferating cells (grown in the presence of tetracycline; day 0) or from cells that had been incubated in the absence of tetracycline for 1–7 days (as indicated). Transcription assays with 20 \(\mu\)g of protein extract were carried out with the V\(\text{A}_1\) promoter (A) or the tRNA\(^{\text{Leu}}\) promoter (B); nt, nucleotides.

FIG. 4. Linkage between p53 and Pol III transcription capacity in the TR9–7 cell system. A, effect of adding exogenous p53 to extracts of TR9–7 cells. Whole cell extract (10 \(\mu\)g of protein) from proliferating TR9–7 cells (lanes P) was complemented with two different amounts of baculovirus-expressed purified human p53: 150 ng (second lane) or 450 ng (third lane). The effect of p53 complementation on transcription was measured in vitro using the V\(\text{A}_1\) promoter. For comparison, assays were also carried out in the absence of exogenous p53 by using extracts from proliferating (first lane) or cell cycle-arrested cells (lane A). B, effect on transcription of immunodepletion of p53 from TR9–7 cell extracts. Extract from cell cycle-arrested cells was immunodepleted with an anti-p53 antibody (DO-1) coupled to protein G-Sepharose beads, and the efficiency of depletion was analyzed by Western blotting (50 \(\mu\)g of total protein loaded in each lane). As a control, the blot was also probed with an antibody against \(\beta\)-actin. C, 20 \(\mu\)g of protein extract from either proliferating or cell cycle-arrested cells was used for in vitro transcription with the V\(\text{A}_1\) promoter. One set of the two extracts had been immunodepleted with an antibody against p53 as described above, and the other had not. nr, nucleotides.

FIG. 4A. p53 (ng) — 150 450 —
Extract P P P A

FIG. 4B. p53-depletion: — + +
Extract P A A P

produced by incubation in the absence of tetracycline for 6 days. Notably, 150 ng of recombinant p53, equating to \(>\)1% of the total protein in the assay, had no significant effect on transcription from the V\(\text{A}_1\) promoter by proliferating TR9–7 cell extract (Fig. 4A, first and second lanes). By contrast, transcription was greatly reduced in extracts from cell cycle-arrested TR9–7 cells (Fig. 4A, lane 4), even though only 0.5 ng of endogenous p53 or less was present per 10 \(\mu\)g of cellular protein (for rough estimate of endogenous p53 levels, see Fig. 1A). The endogenous p53 in such reactions thereby constitutes roughly 0.005% of the total protein, which is 200–300 times less than the amount of the recombinant p53 used in the above experiment. Nevertheless, the addition of 450 ng of p53 (\(\sim\)4.5% of the total protein in the reaction) led to repression of V\(\text{A}_1\) transcription in TR9–7 extracts (Fig. 4A, lane 3). One possibility is that this is due to a "squelching" effect, similar to that observed by Mack et al. (42) when studying the p53-dependent repression of Pol II transcription at high levels of exogenously added p53.

As a second approach to assessing the mechanism of p53
repression in the TR9–7 system, we tested whether repression of Pol III transcription could be sustained after selectively removing p53 from extracts of growth-arrested cells. Cairns and White (43) reported that RNA Pol III preinitiation complexes are resistant to the repressive effects of p53 when they are preassembled on the promoter before adding p53 but not when p53 is added to extracts before the DNA template. We therefore reasoned that if p53 was indeed responsible for Pol III repression in TR9–7 cells, extracts should regain their transcriptional activity if p53 was removed by immunodepletion before carrying out transcription assays. To see whether this was the case, we coupled the p53-specific mouse monoclonal antibody DO-1 to protein G-Sepharose beads and incubated these with extracts from TR9–7 cells. Fig. 4B shows that p53 was efficiently removed from extracts by use of this procedure. Notably, and as shown in Fig. 4C, extracts from growth-arrested cells (lanes A) did not regain transcriptional activity when p53 was depleted (compare lanes 2 and 4). As a control for these experiments, the immunodepletion procedure did not impair the transcriptional capacity of extracts from proliferating cells (lanes P; compare lanes 1 and 3). Taken together with the other data described below, these findings strongly suggest that repression of transcription upon sustained p53-mediated cell cycle arrest in TR9–7 cells is not caused directly by p53.

A Subunit of TFIIIB Is a Target for Transcriptional Repression—The next question we investigated was which of the factors involved in Pol III transcription is the target for repression in cell cycle-arrested TR9–7 cells. First, we tested for the activity of the Pol III enzyme itself to see whether it might become inactivated upon cell cycle arrest. The method used for these experiments was a random Pol III initiation assay on a synthetic template consisting only of adenine and thymidine (poly(dA-dT); Ref. 39). The assay was carried out in the presence of different concentrations of a-amanitin to allow us to distinguish between the activities of the three nuclear RNA polymerase enzymes (40, 41). Table I shows that the activities of the various factors and tested whether the addition of incorporation radioactivity.

|                | Pol I + III (1 μg/ml a-amanitin) | Pol I only (200 μg/ml a-amanitin) | Pol III (by subtraction) |
|----------------|----------------------------------|----------------------------------|-------------------------|
| Proliferating cells | 6614                             | 4526                             | 2088                    |
| Growth-arrested cells | 6614                             | 4550                             | 2264                    |

We therefore purified the 0.38M-TFIIIB subcomponent first by anion and then by cation exchange chromatography. After verifying that TFDP and TAF3B2 co-fractionated over both columns (Fig. 6A and data not shown), we used these fractions in complementation assays. Notably, the fractions eluted from the cation exchange column that contained peak levels of TFDP and TAF3B2 (fractions 14 and 15) also contained peak levels of the activity that reconstituted transcription to extracts from cell cycle-arrested TR9–7 cells (Fig. 6B; this activity also co-fractionated with both TFDP and TAF3B2 on the anion exchange column; data not shown). None of the other fractions, including those that contained 0.48M-TFIIIB, contained any reconstituting transcriptional activity in the complementation assays (data not shown). The co-fractionation over three columns of the activity that reconstitutes transcription to cell cycle-arrested TR9–7 extracts with TFIIIB activity, and in particular the 0.38M-TFIIIB component, therefore suggests strongly that this is the factor whose activity is regulated in response to cell cycle arrest.

The TAF3B2 Subunit of TFIIIB Is Destabilized during Sustained Cell Cycle Arrest—We next determined which component of 0.38M-TFIIIB (TFDP or TAF3B2) might be regulated by investigating the levels of expression of these proteins in TR9–7 cells both before and after the induction of cell cycle arrest. Western blot analysis of whole cell extracts from proliferating and cell cycle-arrested cells revealed that TAF3B2 levels were greatly decreased in response to proliferation arrest, whereas TFDP levels were not markedly affected (Fig. 7; the identity of the additional band seen when probing with the TAF3B2 antibody is not known, but this serves as a useful
reaction was also carried out with 20 μg of extract in each reaction. A, extracts from cell cycle-arrested cells (lanes A) were complemented with increasing amounts of partially purified TFIIIB (PC-B fraction); 0 μl (second lane), 1 μl (third lane), 2 μl (fourth lane), or 4 μl (fifth lane). As a control, a reaction was also carried out with 20 μg of extract from proliferating cells (lane P). B, the same assay was carried out as above, except that extracts were complemented with increasing amounts of partly purified TFIIIC (anti-TBP-depleted PC-C fraction (PC-C(TBP⁺)) fraction; same amounts as above). nt, nucleotides.

FIG. 5. Transcriptional activity of extracts from cell cycle-arrested TR9–7 cells complemented with partly purified Pol III transcription factors. Transcriptional activity was tested with the VAI promoter and 20 μg of extract (PC-B fraction) in each reaction. A, extracts from cell cycle-arrested cells (lanes A) were complemented with increasing amounts of partially purified TFIIIB (PC-B fraction); 0 μl (second lane), 1 μl (third lane), 2 μl (fourth lane), or 4 μl (fifth lane). As a control, a reaction was also carried out with 20 μg of extract from proliferating cells (lane P). B, the same assay was carried out as above, except that extracts were complemented with increasing amounts of partly purified TFIIIC (anti-TBP-depleted PC-C fraction (PC-C(TBP⁺)) fraction; same amounts as above). nt, nucleotides.

loading control for the two cell extracts). Because TAF3B2 is essential for TFIIIB activity and for Pol III transcription of the VAI promoter being tested (23, 24), the decrease in TAF3B2 levels could thus at least partly account for the reduction in Pol III transcription capacity in extracts of cell cycle-arrested TR9–7 cells.

To gain insights into the level(s) at which TAF3B2 was being regulated, we first carried out an RNase protection analysis of the TAF3B2 mRNA (Fig. 8). This indicated that TAF3B2 mRNA levels were not significantly reduced in extracts from cell cycle-arrested TR9–7 cells, meaning that modulation of RNA levels were unlikely to account for the dramatic reduction at the protein level. We wondered therefore whether TAF3B2 levels might be controlled at the level of protein stability. In this regard, we noted that a PEST sequence (a sequence rich in Pro, Glu, Ser, and Thr) exists toward the extreme C-terminal end of TAF3B2 (data not shown). PEST sequences are a common feature of many proteins that can be rapidly degraded by the ubiquitin-dependent proteasome pathway (45, 46). Strikingly, addition of the proteasome inhibitor LLLNl to the growth medium of cell cycle-arrested TR9–7 cells led to a dramatic increase in TAF3B2 protein levels within 1 h (Fig. 9A). Moreover, when we carried out transcription assays with extracts from LLLNl-treated cell cycle-arrested cells, we found that transcriptional activity was fully restored to levels observed with extracts from proliferating cells (Fig. 9B). These data therefore support the conclusion that loss of Pol III transcription capacity in cell cycle-arrested TR9–7 cells is mediated primarily by destabilization of TAF3B2.

Because LLLNl has previously been used by others to rescue uninduced p53 protein from proteasome-mediated degradation and thereby bring about its stabilization (35), we thought it unlikely that it would lead to loss of p53 from the cell cycle-arrested TR9–7 cells. Indeed, as shown in Fig. 9A, p53 remains stable and is present at high levels in cell cycle-arrested TR9–7 cells following incubation with LLLNl. Therefore, Pol III transcription is restored in this system (Fig. 9B) despite high p53 levels. Taken together, these data provide strong additional support for the conclusion that repression of transcription in cell cycle-arrested TR9–7 cells is brought about by loss of TAF3B2 and RNA Pol III Repression.
were loaded in nondigested probe and the RNase protection product, 2000 cpm of probe B RNase protection product was 190 nucleotides. Reactions was carried out with a labeled probe specific for the mRNA. Because of the abundance of yeast tRNA instead of cellular RNA (lane 5). To distinguish between the nondigested probe and the RNase protection product, 2000 cpm of probe were loaded in lane 2. The probe size was 277 nucleotides; the size of RNase protection product was 190 nucleotides. B, to show that the two preparations contained equivalent amounts of RNA, the same set of reactions was carried out with a labeled probe specific for the β-actin mRNA. Because of the abundance of β-actin mRNA, only 0.2 μg of total RNA were used. The size of the probe was 307 nucleotides; the size of the product was 217 nucleotides. nt, nucleotides.

TAF3B2 rather than via p53 directly inhibiting the Pol III transcription apparatus.

DISCUSSION

The aim of this study was to investigate whether RNA Pol III transcription is regulated in response to sustained cell cycle arrest. RNA Pol III transcription is responsible for the synthesis of various small RNA species involved in maintaining cellular biosynthetic capacity. Under conditions of prolonged cell cycle arrest, when cells do not require high levels of protein synthesis, it might therefore be assumed that cells can afford to down-regulate Pol III transcriptional activity. We tested this hypothesis in the human TR9–7 tissue culture cell line in which cell cycle arrest can be initiated by the inducible expression of p53 (27). These studies revealed that Pol III capacity for a range of type 1 and type 2 Pol III promoters is indeed reduced greatly as a consequence of sustained cell cycle arrest. Furthermore, we showed that this was not the result of decreased activity of the RNA polymerase III enzyme itself nor decreased TFIIIC activity. Instead, we showed that TFIIIB activity was the target for repression and that it was the 0.38M-TFIIIB component but not the 0.48M-TFIIIB component, of this factor that was specifically repressed. Finally, we revealed that this repression was accompanied by a dramatic destabilization of the 0.38M-TFIIIB component TAF3B2 and showed that reversing this destabilization by treating cells with proteasome inhibitors led to restored Pol III transcriptional capacity. Because TAF3B2 is essential for Pol III transcription (23, 24), we conclude that its destabilization is likely to play a major role in effecting the loss of Pol III capacity in the TR9–7 system. However, it should be noted that not all human TFIIIB subunits have so far been cloned; it therefore remains possible that down-regulation of another TFIIIB component may also contribute to the reduced Pol III transcription that we observe. It will clearly be of great interest to determine the features of TAF3B2 that allow it to be targeted for degradation upon sustained cell cycle arrest and to identify and characterize the trans-acting factors that mediate this control.

Two previous studies reported that p53 can act as a direct repressor of Pol III transcription through inhibitory interactions with TFIIIB (43, 47). Furthermore, the promoters we used in our study, namely those of the human 5S rRNA gene, two different tRNA genes, and the adenoviral VA1 gene, were inhibited directly by p53 in the study of Cairns and White (43). In light of these findings, we considered the possibility that the repressive effects on Pol III transcription that we observed in TR9–7 cells could be mediated directly by p53. However, several lines of evidence indicate that this is not the case. First, the kinetics of Pol III repression in TR9–7 cells correlated best with the onset of cell cycle arrest, not the kinetics of p53 induction. Second, the addition of recombinant p53 to extracts of proliferating TR9–7 cells, in amounts similar to those used by Cairns and White (43), did not reproduce the repression observed in extracts from cell cycle-arrested cells. The amount of exogenous p53 added in these experiments was at least 100-fold higher than the amounts present endogenously in extracts of cell cycle-arrested cells. Third, transcriptional repression was sustained after removing p53 selectively from extracts of cell cycle-arrested cells by immunodepletion. Finally, and most compelling, we showed that p53 protein was maintained at high levels when cell cycle-arrested cells were treated with a proteasome inhibitor, yet there was full recovery.

FIG. 8. RNase protection analysis of TAF3B2 mRNA from TR9–7 cells. Total RNA was isolated from proliferating and cell cycle-arrested cells. A, RNase protection of TAF3B2 mRNA, 3 × 10⁸ cpm of a labeled TAF3B2 antisense probe were combined for RNase protection with 20 μg of total cellular RNA (lanes 3 and 4). As a control for the specificity of the probe, the reaction was also carried out with 10 μg of yeast tRNA instead of cellular RNA (lane 5). To distinguish between the nondigested probe and the RNase protection product, 2000 cpm of probe were loaded in lane 2. The probe size was 277 nucleotides; the size of RNase protection product was 190 nucleotides. B, to show that the two preparations contained equivalent amounts of RNA, the same set of reactions was carried out with a labeled probe specific for the β-actin mRNA. Because of the abundance of β-actin mRNA, only 0.2 μg of total RNA were used. The size of the probe was 307 nucleotides; the size of the product was 217 nucleotides. nt, nucleotides.

FIG. 9. Effects of proteasome inhibition on protein stability and Pol III transcription in cell cycle-arrested TR9–7 cells. Cell cycle-arrested TR9–7 cells (7 days after tetracycline withdrawal) were incubated for 1 h with the proteasome inhibitor LLnL (at 20 μM). The cells were harvested, and the extracts were prepared. The extracts from proliferating cells and from arrested cells that had not been treated with LlnL were also prepared at the same time. A, Western blot of the extracts probe for the TAF3B2 protein (100 μg of each extract was loaded in this instance) or for p53 and actin (25 μg of extract was used).

For the TAF3B2 analysis, 20 μl of partly purified 0.38M-TFIIIB was also analyzed alongside. B, transcription assays were carried out with extracts from proliferating cells (lane P, lane 1), from cell cycle-arrested cells (A) that had not been treated with proteasome inhibitor (lane 2), and from cell cycle-arrested cells that had been treated with 20 μg of the inhibitor LlnL (lane 3). In each case, 20 μg of extract was used with the VA1 promoter as template.
of Pol III transcriptional activity. It therefore seems clear that p53 is not directly responsible for repression of Pol III transcription capacity following sustained cell cycle arrest in the TR9–7 cell system.

The data we present here are in agreement with the model that there is an intimate linkage between cellular biosynthetic capacity and the activity of RNA Pol III transcription apparatus (1). We describe a novel pathway by which Pol III transcription may be negatively regulated during times when cells do not require elevated levels of active protein synthesis, such as during sustained cell cycle arrest. Taken together with other work, our results suggest that down-regulation of Pol III transcription can be elicited by multiple mechanisms: direct inhibitory interactions between the retinoblastoma protein and TBP antibodies.

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