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PTEN Represses RNA Polymerase I Transcription by Disrupting the SL1 Complex†

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PTEN is a tumor suppressor whose function is frequently lost in human cancer. It possesses a lipid phosphatase activity that represses the activation of PI3 kinase/Akt signaling, leading to decreased cell growth, proliferation, and survival. The potential for PTEN to regulate transcription of the large rRNAs by RNA polymerase I (RNA Pol I) was investigated. As increased synthesis of rRNAs is a hallmark of neoplastic transformation, the ability of PTEN to control the transcription of rRNAs might be crucial for its tumor suppressor function. The expression of PTEN in PTEN-deficient cells represses RNA Pol I transcription, while decreasing PTEN expression enhances transcription. PTEN-mediated repression requires its lipid phosphatase activity and is independent of the p53 status of the cell. This event can be uncoupled from PTEN′s ability to regulate the cell cycle. RNA Pol I is regulated through PI3 kinase/Akt/mammalian target of rapamycin/S6 kinase, and the expression of constitutively activated S6 kinase is able to abrogate transcription repression by PTEN. No change in the expression of the RNA Pol I transcription components, upstream binding factor or SL1, was observed upon PTEN expression. However, chromatin immunoprecipitation assays demonstrate that PTEN differentially reduces the occupancy of the SL1 subunits on the rRNA gene promoter. Furthermore, PTEN induces dissociation of the SL1 subunits. Together, these results demonstrate that PTEN represses RNA Pol I transcription through a novel mechanism that involves disruption of the SL1 complex.

PTEN (phosphatase and tensin homolog deleted on chromosome 10) is one of the most frequently deleted or mutated genes in human cancer (23, 35). Germ line mutations in PTEN are associated with Cowden syndrome and related diseases in which affected individuals develop hyperplastic lesions in multiple organs, predisposing them to malignant transformation (25, 29). Disruption of the murine *Pten* locus further supports the importance of PTEN as a tumor suppressor (10, 31, 39). The homozygous deletion of *Pten* results in early embryonic lethality (39). Mice heterozygous for *Pten* develop hyperplastic and neoplastic changes in multiple organs as early as 4 weeks after birth. The loss of functional PTEN occurs in a wide range of sporadic human tumor types, including breast, lung, prostate, colon, endometrial, and brain. The overall frequency of loss of heterozygosity at the *PTEN* locus in these tumors is approximately 50%.

PTEN contains both protein and lipid phosphatase activities. Its lipid phosphatase activity has been shown to be responsible for its tumor suppressor function (23, 35). PTEN dephosphorylates the 3′ position of the second-messenger lipid phosphatidylinositol 3,4,5-triphosphate, PIP_3, a direct target of PI3 kinase (26). Binding of the serine/threonine kinase Akt to PIP_3 results in the translocation of Akt to the membrane and its subsequent phosphorylation/activation by PDK1 and PDK2 (2). This event triggers the activation of various downstream signaling events, which results in enhanced survival, proliferation, and growth of cells (42). Akt affects cell proliferation through both negative regulation of p27, an inhibitor of G_1 cyclin-dependent kinases, and positive regulation of cyclin D1 (19). The effect of Akt on cell size is brought about through the activation of the mammalian target of rapamycin (mTOR), a central regulator of cell growth that controls protein synthesis. This occurs through at least two downstream pathways, one that controls the phosphorylation of 4EBP1, which regulates eIF4E activity, and the other that controls phosphorylation of the ribosomal protein S6 by ribosomal S6 kinase (S6K) (34). These events increase the efficiency by which select mRNAs are translated. In tumors that have increased rates of metabolism, it is thought that mTOR may initiate a signal for increased ribosome biogenesis, which is commonly observed in cancer cells (9). Consistent with this idea, recent studies have shown that mTOR regulates the transcription of rRNAs (17, 20, 27). Thus, mTOR regulates translation efficiency as well as translation capacity.

RNA polymerase I (Pol I) is responsible for the transcription of the three large ribosomal RNAs, which are synthesized as a large 45S precursor from tandemly repeated units in the nucleolus (for reviews, see references 7 and 16). In humans, initiation of RNA Pol I transcription is mediated by the upstream binding factor (UBF) and the selectivity factor SL1. The binding of a UBF dimer to the promoter facilitates the recruitment of SL1 complex to the DNA and the subsequent association of RNA Pol I. SL1 is composed of the TATA-binding protein (TBP) and three associated factors, TAF_I110, TAF_I63, and TAF_I48.

The rate at which rRNA genes are transcribed dictates the

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number of ribosomes and, as a consequence, the translational capacity of cells. Consistent with this idea, oncogenic proteins and growth factors that induce cell growth and proliferation have been shown to enhance rRNA gene transcription. Stimulation of cells with epidermal growth factor (38, 49) or insulin-like growth factor-1 (20) was shown to enhance rRNA gene transcription. The activation of protein kinase C by the phorbol ester tetradecanoyl phorbol acetate (1) and the activation of Ras/Raf signaling (45) were shown to stimulate RNA Pol I transcription. The activation of extracellular signal-related kinase 1/2 (ERK1/2), a downstream signaling molecule in this cascade, has been shown to directly modulate the transcription machinery to enhance the transcription process (38). Recently, c-MYC and MAD1 were shown to regulate RNA Pol I transcription (32). In contrast to oncogenic agents that stimulate rRNA gene transcription, the tumor suppressor proteins p53 (47) and pRb (5) have been shown to decrease rRNA gene transcription. In these cases, both p53 and pRb repress transcription by directly binding to the transcription components to impair their function (5, 44, 47).

In light of its role as a tumor suppressor, we have examined the possibility that PTEN regulates rRNA synthesis. Here, we demonstrate that PTEN represses RNA Pol I transcription in a variety of different human cell lines. Consistent with our findings that the lipid phosphatase activity of PTEN is required for this response, RNA Pol I transcription can be regulated by the PI3 kinase/Akt/mTOR/S6K signaling pathway. In addition, activation of S6K1 was found to alleviate PTEN-mediated transcription repression, suggesting that this is the principal mechanism by which PTEN regulates rRNA gene transcription. Moreover, PTEN-mediated effects on transcription can be uncoupled from its ability to negatively regulate the cell cycle. Analysis of RNA Pol I transcription components revealed that PTEN selectively decreases the occupancy of SL1 components on rRNA gene promoters and facilitates dissociation of the SL1 complex.

MATERIALS AND METHODS

Plasmids and reagents. The DNA constructs were constructed as previously reported and were as follows: human rRNA gene reporter PrHuCAT (47); expression plasmids for PTEN, PTEN-G129E, and PTEN-G129R (41); cyclin D1 T286A (11), constitutively activated S6 kinase 1 (S6K1 E389); tuberous sclerosis complex 1 (TSC-1) and TSC-2 (40); and human TBP promoter-luciferase reporter plasmid, p−4500/+66 hTBP-luc (21). Constitutively activated Akt (Akt-myr) dominant negative Akt (Akt-DN) and dominant negative PI3 kinase (PI3K-DN) constructs were provided by David Ann (University of Southern California, Los Angeles). The sequences for the PTEN-specific small interfering RNA (siRNA) were as follows: sense, 5′-CAA AUC CAG AGG CUA GCA GTT-3′; antisense, 5′CUG CUA GCC UCU GGA UUU GTT-3′. The sequences for mismatch RNA were as follows: sense, 5′CAA AUC CGG ACG CUA GCA GTT-3′; antisense, 5′-CUG CUA GCG UCC GGA UUU GTT-3′. Dulbecco modified Eagle's was obtained from Cellgro, and OPTI-MEM I was obtained from Invitrogen.

Transient transfection and promoter assays. The LnCap, U-87, and LN18 cell lines were obtained from the American Type Culture Collection. PTEN-inducible U87 cells and PTEN-C124S-inducible U87 cells were obtained from Maria-Magdalena Georgescu (M. D. Anderson Cancer Center, Houston, Texas) and maintained in medium containing 1 mg of G418 per ml and 10 μg of blasticidin per ml. For transfection assays, 10^6 cells were seeded in 100-mm or 60-mm dishes. Fifteen hours later, cells were transfected using 2 μl of Lipofectin or Lipofectamine 2000 (Invitrogen)/μg of DNA following the manufacturer's instructions. For cells transfected with the TBP promoter construct, total cell lysates were prepared for measuring luciferase activity as previously described (49). At least three independent experiments were conducted for each determination. The values shown are the means ± the standard errors of the means (SEM).

For analysis of the transiently expressed RNA Pol I promoter, the primer, 5′-AGAGTTGAGAGGGTACGTACG-3′, was labeled following the protocol of Zhai and Comai (47). Briefly, the oligonucleotides (120 pmol; USC Microchemical Core Facility) were labeled by T4 nucleotide kinase. The labeled primer was purified by using a column, following the manufacturer's instructions. The primer extension assay was performed as described previously (48). Briefly, RNA (5 μg) was isolated 24 h after transfection and hybridized with the ^{32}P-labeled primers (550,000 cpm) at 42°C for 90 min followed by reverse transcription. The RNA was digested by RNase A (2 μg/ml; Ambion) at 37°C for 15 min followed by phenol-chloroform (24:1) extraction. The DNA was visualized by autoradiography after polyacrylamide gel electrophoresis (PAGE) with 8 M urea. Images were recorded and quantified using Un-Scan-It software (Silk Scientific). At least three independent experiments were conducted for each determination. The values shown are the means ± the SEM.

Immunoblot analysis. Total cell lysates were prepared and subjected to sodium dodecyl sulfate (SDS)-PAGE as previously described (49). Membranes were probed with rabbit polyclonal anti-human antibodies against TBP (Upstate Biotechnology), with UBF, or with TAF-$_I$110, TAF-$_I$ 63, and TAF-$_I$ 48. In addition, antibodies against phospho-Akt (Thr308) (Cell Signaling), total Akt (Cell Signaling), phospho-S6K1 (Thr389) (Cell Signaling), cyclinD1 (Santa Cruz), p27 (Santa Cruz), PTEN (Axel Schönthal, University of Southern California), anti-phosphoserine (Chemicon) or β-actin (Boehringer Mannheim) were used as indicated. Hybond-P membrane was used for protein transfer. Bound primary antibody was visualized using horseradish peroxidase-conjugated secondary antibody (Vector Laboratories) and enhanced chemiluminescence reagents (Amersham).

Immunoprecipitation assay. PTEN-inducible U87 cells were grown in the absence or presence of doxycycline (DOX). Immunoprecipitation assays were carried out as previously described (8, 36). Briefly, cells were incubated in immunoprecipitation buffer (400 μl per 100-mm plate) at 4°C for 15 min. Cells were sonicated for 15 s and centrifuged for 15 min at 10,000 × *g* and 4°C. The supernatant was collected, and protein concentrations of the resultant lysates were determined using Bio-Rad protein assay reagent. For each assay, 400 μg of cellular protein was incubated with 5 μl of antibodies overnight at 4°C. Protein A/G PLUS agarose beads (25 μl; Santa Cruz) were added and incubated for 3 h. The beads were centrifuged and washed with cold phosphate-buffered saline, and the samples subjected to SDS-PAGE.

S1 nuclease protection assay. The amount of 45S pre-rRNA was analyzed by using an S1 nuclease protection assay (6) with an oligonucleotide that is identical to −20 to +40 of the human rRNA gene template strand and complementary to the first 40 nucleotides of the 45S rRNA primary transcript. Total RNA was isolated 24 h after transfection. An excess of the 5′-end-labeled S1 oligonucleotide (0.1 pmol) was annealed for 12 h at 50°C to 0.5 μg or 1.5 μg of RNA and then digested with 350 U of S1 nuclease. Products were separated on 7.5 M urea-8% polyacrylamide gels. Signals were quantified with Un-Scan-It software (Silk Scientific).

ChIP assay. Chromatin immunoprecipitation (ChIP) was performed as described previously (8, 20). Briefly, PTEN-inducible U87 cells were treated with DOX (1 μg/ml) for various times as indicated. Cells were fixed with 1% formaldehyde at 24°C for 10 min. Immunoprecipitation of cross-linked chromatin was performed with antibodies coupled to protein A/G-Sepharose. Immunoprecipitated chromatin-derived DNA was analyzed by PCR with primer pairs specific for the ribosomal promoter (forward primer, rRNA gene promoter region from −182 [5′-TGTCCTTGGGTTGACCAG-3′]; reverse primer, +23 [5′-TCGCCA GAGGACAGCGTG-3′]). Reactions contained DNA, 50 pM concentrations of primers, 1.5 mM $MgCl_2$, 50 μM dNTPs, and 5 units of Taq and DNA polymerase. Cycling was for 5 min at 95°C, followed by 18 cycles of 45 s at 95°C, 45 s at 63°C, and 2 min at 72°C, and then a final 10 min at 72°C. The tRNA^{Leu} promoter was analyzed according to the method described by Crighton et al. (8). PCR products were resolved and visualized with ethidium bromide. Images were recorded and quantified using Un-Scan-It software (Silk Scientific).

RESULTS

PTEN represses RNA Pol I-dependent transcription through its lipid phosphatase activity. We first determined whether the expression of PTEN in PTEN-deficient cells would affect RNA Pol I transcription. The prostate cancer cell line, LnCap, and a glioblastoma cell line, U87, were transiently

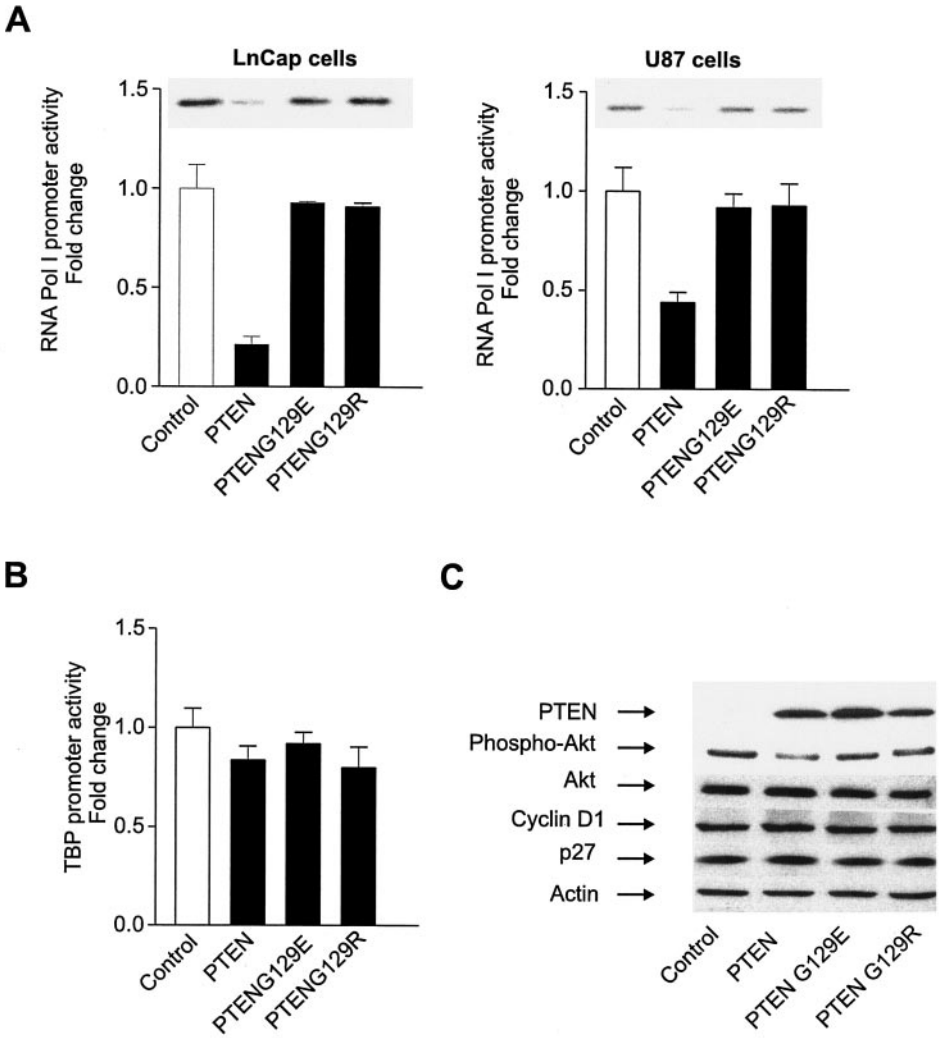

FIG. 1. PTEN repression of RNA Pol I-dependent transcription requires its lipid phosphatase activity. (A) RNA Pol I transcription is decreased by the expression of PTEN but not by a lipid phosphatase-defective mutant PTEN. PTEN and PTEN mutant expression vectors were cotransfected with the RNA Pol I reporter construct, PrHuCAT, into LnCap or U87 cells. RNA was isolated, and promoter activity was measured using a primer extension assay as described in Materials and Methods. An example of the resulting autoradiograph is shown above each graph. (B) TBP promoter activity is not affected by PTEN expression. A human TBP promoter-luciferase construct, p−4500/+66 hTBP-luc, was cotransfected with the PTEN or PTEN mutant plasmids into U87 cells as indicated. Lysates prepared from the transfected cells were assayed for luciferase activity. The values shown are the means ± SEM for four independent experiments. (C) Transcriptional repression is correlated with the down-regulated activation of AKT mediated by PTEN. U87 cells were transfected with the PTEN constructs as indicated, lysates were prepared, and immunoblot analysis was carried out using antibodies against the indicated proteins.

transfected with an RNA Pol I promoter construct together with expression plasmids for either PTEN or mutant PTEN proteins. The expression of PTEN reduced RNA Pol I transcription in both cell lines (Fig. 1A). However, expression of either PTEN-G129E, which is defective for lipid phosphatase activity, or PTEN-G129R, which is defective for both lipid and protein phosphatase activities, did not affect transcription activity. This was not due to differences in expression of the PTEN and PTEN mutant proteins, as they were present in comparable amounts in the transfected U87 cells (Fig. 1C) and LnCap cells (data not shown). The ability of PTEN to repress transcription was correlated with its ability to inhibit the phosphorylation or activation of Akt. Expression of the wild-type PTEN protein resulted in a decrease in the amount of phosphorylated Akt compared to cells expressing either PTEN-G129E or PTEN-G129R (Fig. 1C).

To assess whether PTEN was selective in its ability to regulate RNA Pol I transcription, we tested whether the TBP promoter would be responsive to PTEN expression. TBP promoter activity in the U87 cells was unchanged by the expression of PTEN or mutant PTEN (Fig. 1B). Together, these results indicate that PTEN-mediated repression of RNA Pol I transcription requires PTEN′s lipid phosphatase activity and correlates with its ability to inhibit the activation of Akt. These results further suggest that transcription of the TBP component of the RNA Pol I transcription machinery is not regulated by PTEN.

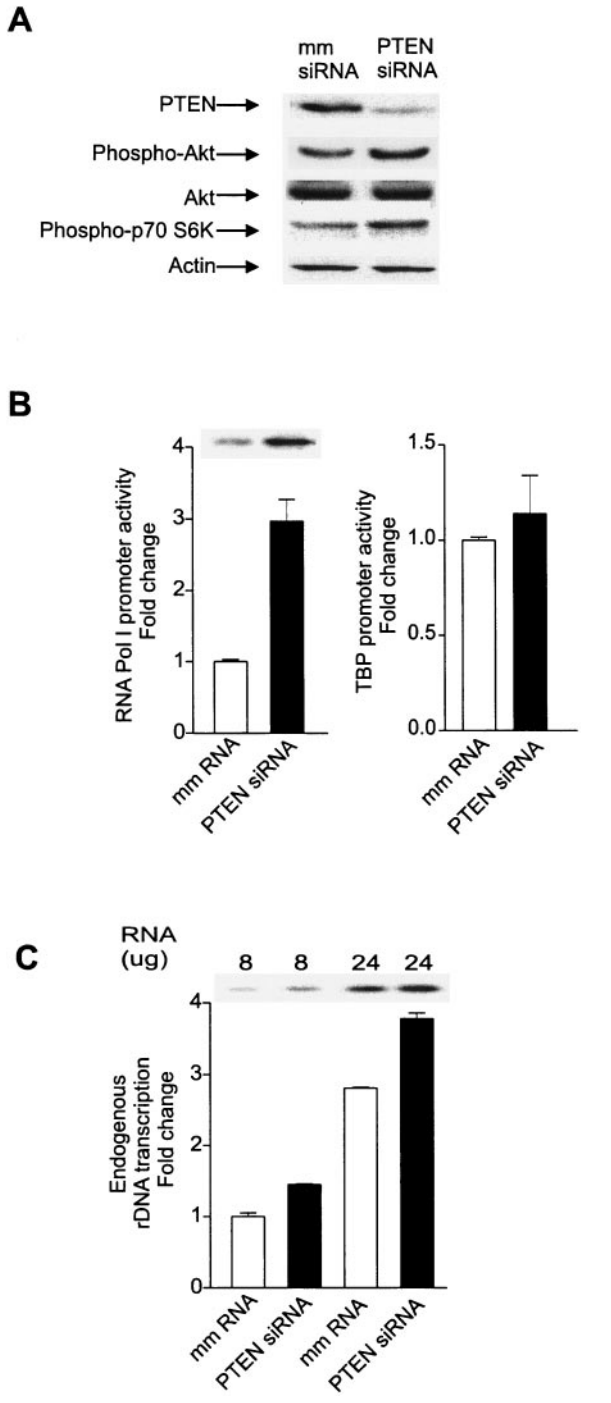

FIG. 2. Decreasing PTEN expression enhances RNA Pol I-dependent transcription in a p53-independent manner. (A) Reducing PTEN expression increases the phosphorylation status of Akt and S6K. LN18 cells were transfected with either an siRNA specific for PTEN or a mismatch siRNA, designated "mm RNA," at a final concentration of 100 nM as described in Materials and Methods. Protein lysates (80 μg) were subjected to SDS-PAGE and immunoblot analysis. Membranes were probed with antibodies against the indicated protein. (B) Reducing PTEN expression enhances RNA Pol I promoter activity. LN18 cells were cotransfected with PrHuCAT or −4500/+66 hTBP-luc, together with either PTEN siRNA or mm siRNA. Total RNA was isolated, and primer extension assays were performed to determine RNA Pol I promoter activity (left). Protein lysates were prepared, and luciferase activity was measured to determine TBP promoter activity (right). A representative autoradiograph is shown. The change (*n*-fold) was calculated based on the control (PrHuCAT or −4500/+66 hTBP-luc and mm siRNA). (C) Reducing PTEN expression enhances endogenous RNA Pol I-dependent transcription. LN18 cells were transfected with either PTEN siRNA or mm siRNA. Total RNA was isolated and was followed by a S1 nuclease protection assay to measure precursor 45S rRNA, using two concentrations of RNA as shown. An example of an autoradiograph is shown above the graph. The values shown are the means ± SEM for three independent experiments.

Reducing PTEN expression enhances RNA Pol I-dependent transcription in a p53-independent manner. Since the expression of PTEN in PTEN-deficient cells decreased RNA Pol I transcription, we examined whether reducing expression of PTEN in cells that normally express PTEN would result in an increase in transcription. As PTEN has been shown to positively regulate p53 (12, 28, 37) and RNA Pol I transcription has been previously shown to be negatively regulated by p53 (47), we further assessed whether the ability of PTEN to regulate transcription was dependent on its ability to regulate p53. The glioblastoma cell line, LN18, was used as it expresses PTEN but not functional p53. Cells were transfected with either siRNA specific for PTEN or a mismatched siRNA containing a 3-bp sequence change. PTEN expression was reduced in the LN18 cells transfected with the PTEN siRNA compared to cells transfected with the mismatched siRNA (Fig. 2A). Reduction of PTEN expression in these cells was shown to result in an increase in the amount of phosphorylated Akt as well as an increase in the amount of phosphorylated S6 kinase, a downstream target of Akt.

Transfection of the PTEN siRNA resulted in a selective increase in the activity of a cotransfected RNA Pol I promoter construct (Fig. 2B). To determine whether reducing PTEN expression could similarly enhance transcription of endogenous rRNA genes, the short-lived precursor 45S rRNA was measured in cells transfected with the siRNAs. Transcription of the endogenous 45S rRNA was enhanced in cells transfected with the PTEN-specific siRNA compared to cells containing the mismatch siRNA (Fig. 2C). Taking into account that the transfection efficiency of the siRNA in the LN18 cells is approximately 65%, we estimate that the overall endogenous 45S rRNA level was increased by approximately 70% in cells transfected with the PTEN siRNA. Together, these results indicate that reducing PTEN expression in cells that normally express PTEN enhances the transcription of both an exogenously introduced RNA Pol I promoter and the endogenously expressed genes. Furthermore, PTEN functions independently of p53 to regulate RNA Pol I transcription.

PTEN represses RNA polymerase I-dependent transcription through its ability to inhibit the activation of Akt/mTOR/S6 kinase signaling. The results above support a mechanism by which PTEN regulates RNA Pol I transcription through its ability to regulate PI3 kinase/Akt-dependent cellular signaling. To further test this possibility, we used other methods to inhibit the activation of PI3 kinase, Akt, or a downstream target of this signaling pathway, mTOR, and the effect on RNA Pol I transcription was determined. LN18 cells were transiently transfected with the RNA Pol I promoter construct, and cells were treated with the PI3 kinase inhibitor wortmannin or cotransfected with a dominant negative form of PI3 kinase. In-

hibiting PI3 kinase activation resulted in a decrease in RNA Pol I transcription (Fig. 3A). Furthermore, cotransfection of a dominant negative form of Akt or incubation of the cells with the mTOR-specific inhibitor rapamycin reduced transcription. The effect on RNA Pol I transcription was selective, since transcription from the TBP promoter was unaffected under these conditions.

To ensure that the results observed were not cell-type specific, PI3 kinase, Akt, and mTOR activation were inhibited in the U87 cells, and the effect on transcription was assessed. Blocking the activation of these signaling molecules also inhibited RNA Pol I transcription in these cells (Fig. 3B). Furthermore, the coexpression of TSC-1 and TSC-2, proteins that form a complex and inhibit the activation of mTOR (40), reduced RNA Pol I transcription. To determine if activation of Akt was sufficient for inducing transcription, LN18 cells were transfected with a constitutively activated form of Akt together with the RNA Pol I promoter construct. The expression of activated Akt selectively stimulated RNA Pol I transcription (Fig. 3C). Thus, activation of Akt is sufficient for inducing RNA Pol I transcription, whereas inhibiting the activation of Akt results in a decrease in transcription.

We analyzed whether the ability of PTEN to inhibit the activation of mTOR and its downstream target, S6K, was sufficient for PTEN-mediated repression of transcription. LN18 cells were cotransfected with PTEN siRNA or mismatched siRNA together with the RNA Pol I promoter construct. When PTEN expression was reduced, the increase in RNA Pol I transcription was abolished upon treatment of the cells with rapamycin (Fig. 4A). Consistent with these results, PTEN-mediated transcription repression in U87 cells could be alleviated by the coexpression of a constitutively activated mutant form of S6K1 (Fig. 4B). Together, these results support the idea that PTEN-mediated repression of RNA Pol I transcription occurs predominantly through its ability to inhibit mTOR/S6K signaling.

PTEN′s ability to repress RNA Pol I-dependent transcription can be separated from its ability to negatively regulate cyclin D1. Previous studies have shown that the overexpression of PTEN, under certain conditions, can induce G_1 phase cell-cycle arrest (13, 24, 46). This can occur by either a decrease in the expression and nuclear localization of cyclin D1 (33) or by enhanced expression of p27, an inhibitor of G_1 cyclin-dependent kinases (15). RNA Pol I transcription has been shown to be regulated in a cell cycle-dependent manner (43). In order to determine whether the effect of PTEN on the cell cycle could be separated from PTEN′s effect on transcription, we analyzed if there were discernible changes in the levels of cyclin D1 and p27 in U87 cells transfected with the PTEN expression vector (Fig. 1C). In contrast to changes in the amounts of phosphorylated or activated Akt, no apparent changes in the levels of either cyclin D1 or p27 proteins were observed upon PTEN expression, suggesting that under these conditions, PTEN was not promoting changes in the cell cycle but that RNA Pol I transcription was repressed (Fig. 1A).

To further assess whether PTEN-mediated effects on the cell cycle can be uncoupled from its ability to regulate RNA Pol I transcription, we obtained established tetracycline-inducible PTEN- and PTEN-C124S-expressing U87 cell lines (33). PTEN-C124S is defective for both lipid and protein phosphatase activities. These lines were induced to express PTEN or PTEN-C124S after incubation with DOX for 6 or 24 h. As expected, PTEN was not detected in the noninduced cells, and a time-dependent increase in PTEN or PTEN-C124S expression was observed upon DOX treatment (Fig. 5A). Previous studies showed that PTEN induces cell cycle arrest in these inducible cells by both negatively regulating cyclin D1 expression and nuclear localization (33). PTEN induction had little effect on cyclin D1 expression at 6 h. However, at 24 h after PTEN induction, there was an observable reduction in cyclin D1 expression (Fig. 5A). In contrast, the level of phosphorylated Akt was markedly decreased at 6 h after PTEN induction and further reduced at 24 h. The induction of PTEN-C124S expression had no effect on cyclin D1 expression or the phosphorylation state of Akt.

To determine the effect on RNA Pol I transcription, the PTEN and PTEN-C124S inducible cells were transfected with the RNA Pol I promoter construct, and the cells were incubated with DOX to induce the expression of PTEN or PTEN-C124S (Fig. 5B). A decrease in RNA Pol I transcription was observed after 6 h of induction, yet a more significant decrease in transcription was observed after 24 h of induction. In contrast, no change in transcription was observed with the PTEN-C124S-expressing cells following DOX treatment. To determine whether PTEN′s ability to repress RNA Pol I transcription was mediated by PTEN′s ability to negatively regulate cyclin D1, a nucleus-persistent mutant form of cyclin D1, cyclin D1 T286A, was coexpressed in the PTEN-inducible cells. The expression of this protein was previously shown to abrogate PTEN-mediated growth arrest in these cells (33). The expression of cyclin D1 T286A had no effect on transcription in the absence of PTEN (Fig. 6A). At 6 h following PTEN induction, the decrease in transcription was unaffected by coexpression of cyclin D1 T286A. However, the expression of cyclin D1 T286A at 24 h following PTEN induction resulted in an increase in transcription. While expression of cyclin D1 T286A did not fully restore transcription to the level observed with the noninduced cells, transcription was comparable to that observed with cells induced for 6 h. This was not due to differences in the levels of cyclin D1 T286A, because cyclin D1 T286A was expressed at comparable levels in noninduced and PTEN-induced cells (Fig. 6B). These results indicate that RNA Pol I transcription is initially repressed after PTEN induction in a cyclin D1-independent manner. However, prolonged and increased expression of PTEN results in a more substantial reduction in RNA Pol I transcription by a mechanism that is cyclin D1 dependent.

PTEN alters the ability of SL1 to bind to endogenous rRNA gene promoters. To decipher the mechanism for PTEN-mediated repression, we examined whether PTEN affected the expression of the RNA Pol I transcription components. No changes in the expression of either UBF or the four SL1 subunits were observed upon PTEN induction in the U87 cells (Fig. 7A). ChIP assays were used to identify potential changes in the occupancy of these transcription components on chromosomal genes in vivo. Binding of UBF to the rRNA gene promoter was not appreciably changed at 6 h following PTEN induction (Fig. 7B). At 24 h following PTEN induction, there was an approximately 25% decrease in binding of UBF to the promoter. Examining the relative occupancy of the SL1 sub-

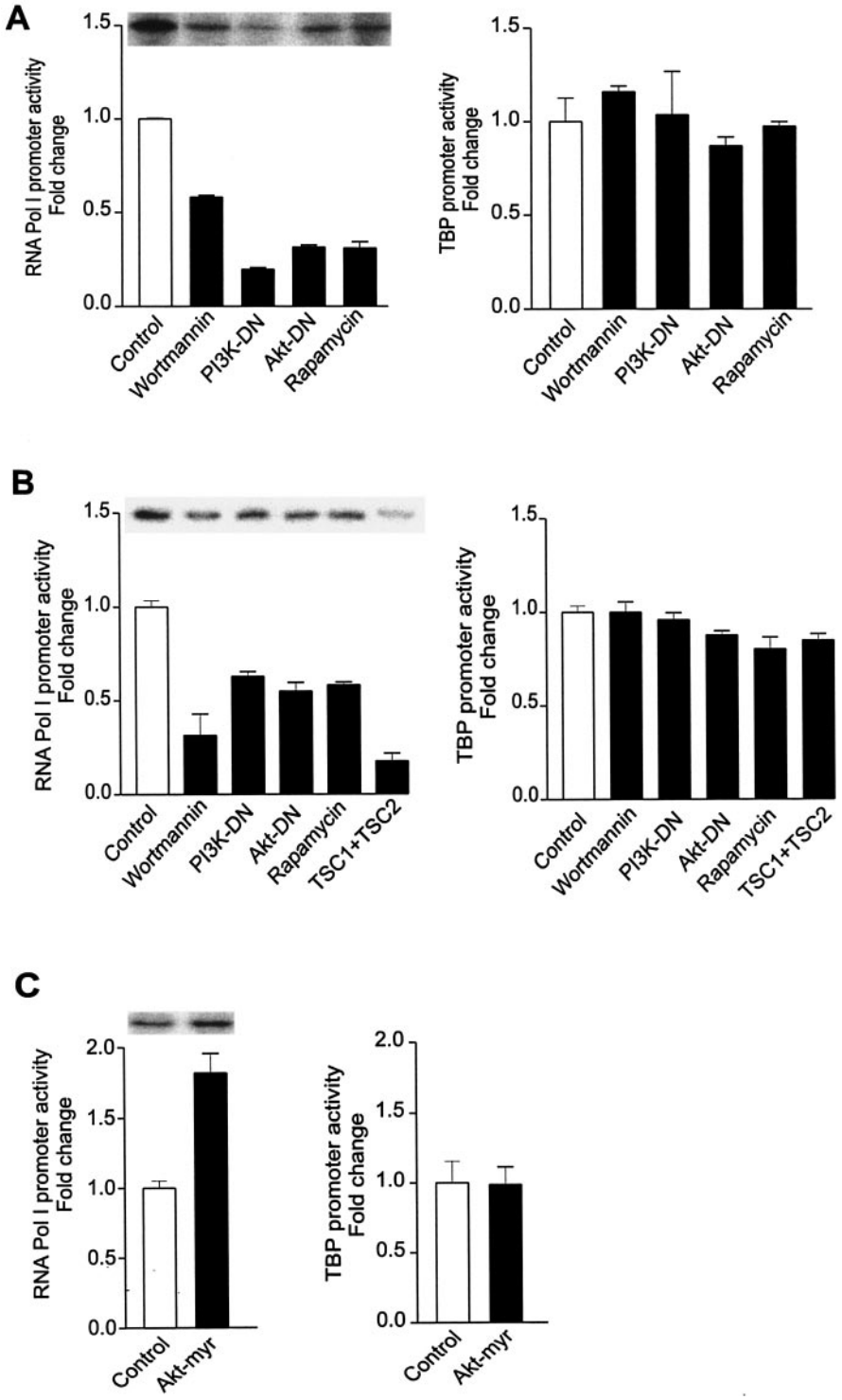

FIG. 3. RNA Pol I-dependent transcription is regulated through the PI3 kinase/Akt/mTOR signaling pathway. (A) Repressing the activation of PI3 kinase/Akt/mTOR inhibits RNA Pol I-dependent promoter activity in LN18 cells. LN18 cells were transiently transfected with PrHuCAT (left panel) or −4500/+66 hTBP-luc (right panel) together with plasmids expressing dominant negative forms of PI3 kinase or Akt, as designated. Where designated, cells were treated with either wortmannin (5 μM) or rapamycin (100 nM) for 3 h. The promoter assays were conducted as described in Materials and Methods. (B) Repression of PI3 kinase/Akt/mTOR signaling inhibits RNA Pol I-dependent transcription in U87 cells. U87 cells were transiently transfected with PrHuCAT (left panel) or −4500/+66 hTBP-luc (right panel) together with plasmids expressing dominant negative forms of PI3 kinase or Akt or TSC1 and TSC2, as designated. Where designated, cells were treated wither wortmannin or rapamycin as described for panel A. The promoter assays were conducted as described for Fig. 1. (C) RNA Pol I transcription is induced by the activation of Akt. LN18 cells were transiently transfected with PrHuCAT (left panel) and −4500/+66 hTBP-luc (right panel), together with a plasmid that expresses a constitutively activated form of Akt or empty vector (control). RNA Pol I and TBP promoter activities were measured as described for panel A. The values shown are the means ± SEM for three independent experiments.

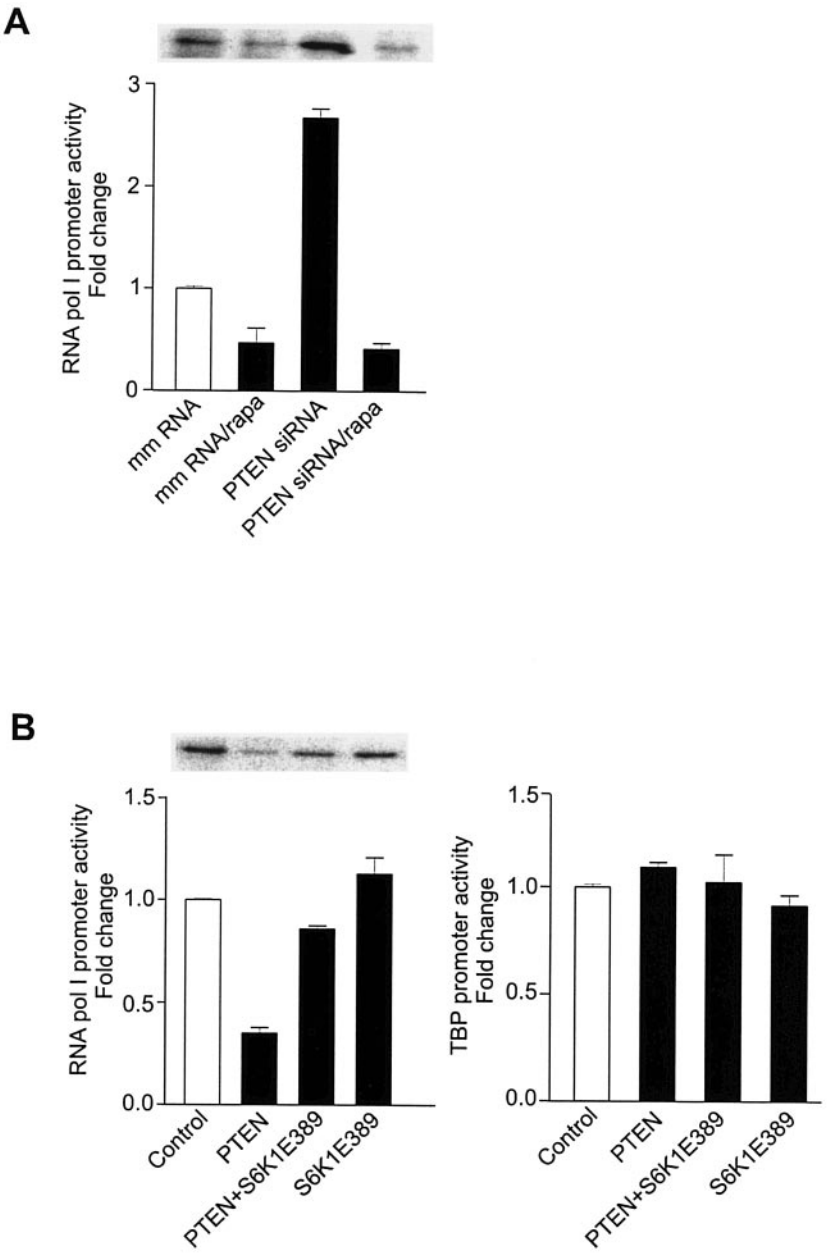

FIG. 4. Repression of mTOR/S6 kinase signaling is responsible for PTEN-mediated repression of RNA Pol I-dependent transcription. (A) Decreasing PTEN expression does not affect RNA Pol I-dependent transcription in cells treated with the mTOR inhibitor rapamycin. LN18 cells were transiently transfected with PrHuCAT together with either PTEN siRNA or mm siRNA (mm RNA) as designated. Twenty-one hours thereafter, cells were treated with rapamycin (100 nM) for 3 h. The RNA Pol I promoter assays were conducted as described in Materials and Methods. (B) Expression of constitutively activated S6K1 alleviates PTEN-mediated repression of RNA Pol I-dependent transcription. U87 cells were transiently transfected with PrHuCAT (left panel) or −4500/+66 hTBP-luc (right panel), together with either PTEN expression vectors, empty vector, or plasmids expressing constitutively active S6K1 as designated. RNA Pol I and TBP promoter assays were conducted as described for Fig. 1. An example of an autoradiograph is shown above the graphs. The values shown are mean ± SEM for three independent determinations.

units, we found that, in contrast to UBF binding, TBP binding was significantly decreased following PTEN induction (Fig. 7C). Importantly, this effect is apparent at 6 h, when PTEN mediates transcription repression in a cyclin D1-independent manner. Further reduction in TBP binding was observed after 24 h of induction of PTEN. Interestingly, no change in the binding of any of the TAFs to the promoter was observed following PTEN induction for 6 h. PTEN induction for 24 h resulted in a decrease in the occupancy of TAF_I110 and TAF_I48, while no change in the binding of TAF_I63 was observed. These results suggest that PTEN expression initially produces a selective reduction the binding of TBP to the rRNA gene promoter. This subsequently results in the loss of both TAF_I110 and TAF_I48 binding to the promoter. In contrast,

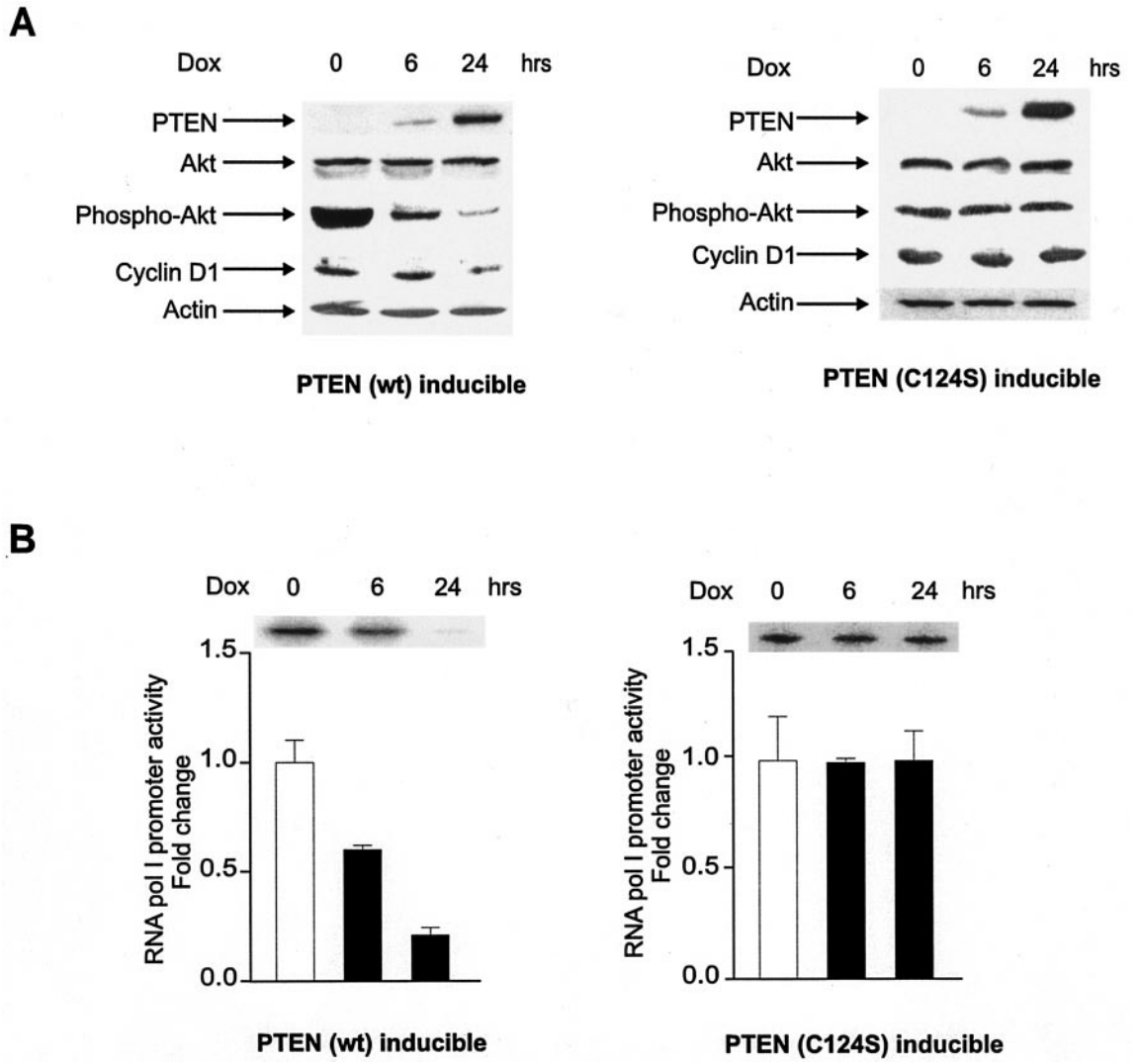

FIG. 5. Induction of PTEN expression in U87 cells represses RNA Pol I transcription and concomitantly decreases the activation of Akt. (A) Induction of wild-type (wt) PTEN expression, but not PTEN-C124S, decreases the activation state of Akt. PTEN-inducible U87 cells (left panel) or PTEN-C124S-inducible U87 cells (right panel) were transfected with PrHuCAT. Cells were treated with vehicle or 1 μg/ml DOX for 6 or 24 h prior to harvesting the cells, as designated. Protein lysates were prepared, and 80 μg of protein was subjected to immunoblot analysis. Membranes were probed with antibodies as designated. (B) Wild-type PTEN, but not PTEN-C124S, decreases RNA Pol I-dependent transcription. Cells were transfected as described for panel A, total RNA was isolated, and RNA Pol I promoter activity was measured. An example of an autoradiograph is shown above the graphs. The values shown are means ± SEM from four independent determinations.

relatively little change in the binding of UBF and TAF_I63 to the promoter was observed within the same time period of PTEN induction.

The differential reduction in binding of the SL1 components to the promoter upon PTEN expression suggested that PTEN might disrupt the SL1 complex. We therefore examined whether the relative association of the SL1 components would change upon the expression of PTEN. TBP was immunoprecipitated from the U87 cell lysates, and the amounts of TBP and associated TAFs were measured by immunoblot analysis (Fig. 8A). The induction of PTEN expression resulted in a marked decrease in the association of TBP with both TAF_I110 and TAF_I63. This was observed at 6 h after PTEN induction and was more pronounced at 24 h following PTEN induction. Similarly, immunoprecipitation of TAF_I110 revealed a decrease in the amount of associated TBP upon PTEN expression (Fig. 8B). Together, these results indicate that the selective reduction of TBP occupancy on the chromosomal rRNA promoter upon PTEN expression reflects disruption of the SL1 complex.

To determine how PTEN facilitates the disruption of the SL1 complex, we considered that changes in the phosphorylation state of these proteins might impair their association. Previous studies have shown that both TBP and TAF_I110 are phosphorylated and that the phosphorylation state of these proteins changes during the cell cycle (18). We therefore examined whether changes in the phosphorylation state of serine residues within these proteins correlated with their decreased association after PTEN induction. Immunoprecipitation of TAF_I110 and analysis of the serine phosphorylation state of

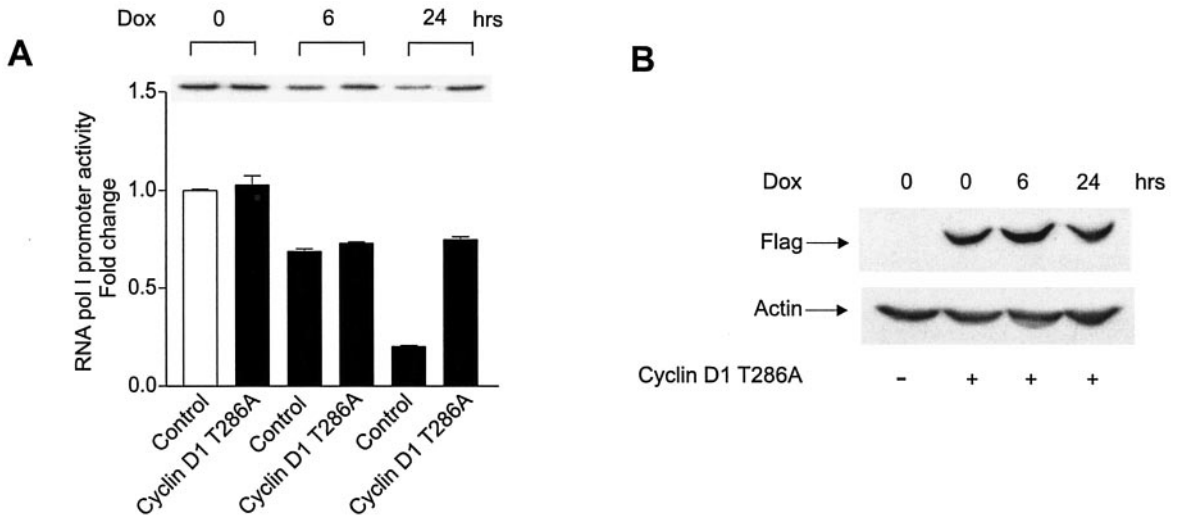

FIG. 6. Expression of cyclin D1 T286A does not completely alleviate the PTEN-mediated repression of RNA Pol I-dependent transcription. (A) The expression of cyclin D1 T286A does not reverse the repression of RNA Pol I-dependent transcription by PTEN. PTEN-inducible U87 cells were cotransfected with cyclin D1 T286A expression vector or empty vector and PrHuCAT. Cells were treated with 1 μg/ml DOX as designated. Total RNA was isolated, and RNA Pol I promoter activity was measured. The values shown are means ± SEM from three independent determinations. (B) Expression of cyclin D1 T286A in transfected U87 cells. Cells were transfected as described for panel A, and protein lysates were prepared and subjected to immunoblot analysis. Membranes were probed with either actin or Flag antibodies to detect the Flag-tagged cyclin D1 T286A, as designated.

the protein revealed that a relatively low degree of phosphorylation was observed in noninduced cell lysates and that no change in the level of TAF_I110 phosphorylation was observed after 6 h of PTEN induction (see Fig. S1 in the supplemental material). Similarly, the serine phosphorylation state of TBP remained the same in noninduced and PTEN-induced cells. We were unable to detect any phosphorylation at tyrosine residues within either TBP or TAF_I110 (data not shown). These results suggest that changes in the phosphorylation state of serine residues within TBP and TAF_I110 are not responsible for the PTEN-mediated dissociation of these proteins. Interestingly, however, prolonged PTEN induction produced a marked increase in the level of phosphorylation of TAF_I110, while no change was observed in the phosphorylation state of TBP (see Fig. S1 in the supplemental material).

DISCUSSION

Our studies have uncovered a novel function of the tumor suppressor PTEN in regulating RNA Pol I-dependent transcription. Changes in PTEN expression in a variety of human cell lines regulate the transcription of the large rRNAs in a p53-independent manner, and this regulation is inversely correlated with the activation state of Akt. We provide several lines of evidence that demonstrate that the ability of PTEN to inhibit Akt signaling is the major mechanism responsible for PTEN-mediated repression of RNA Pol I transcription. First, a lipid phosphatase-defective PTEN mutant that is unable to regulate Akt signaling does not affect RNA Pol I transcription. Second, the activation or repression of Akt is sufficient for modulating RNA Pol I transcription. Third, the expression of a constitutively activated form of S6K, a downstream target of Akt, alleviates PTEN-mediated repression. These results indicate that PTEN represses RNA Pol I transcription through its ability to regulate the PI3 kinase/Akt/mTOR/S6K pathway. However, we cannot rule out the possibility that other mechanisms may contribute to PTEN-mediated repression. Since PTEN is also present and functional in the nucleus (12, 14, 30), PTEN could also directly interfere with the transcription process.

The rate of RNA Pol I transcription varies significantly throughout the cell cycle, being lowest during mitosis (22, 43). Ectopic overexpression of PTEN has been shown to induce G_1 phase cell-cycle arrest (4, 15, 46), which can be reversed by the expression of a nucleus-persistent cyclin D1 mutant, cyclin D1 T286A (33). We examined whether the ability of PTEN to repress RNA Pol I transcription could be kinetically uncoupled from PTEN-mediated changes in the cell cycle. At 6 h following PTEN induction, transcription is decreased, correlating with a reduction in the amount of activated Akt, but with no discernible change in cyclin D1 levels. Importantly, under these conditions, transcription repression cannot be abrogated by the expression of cyclin D1 T286A. However, at 24 h following PTEN induction, the expression of cyclin D1 T286A partially relieves PTEN-mediated transcription repression, restoring it to the level observed after 6 h of induction. These results indicate that PTEN is able to elicit changes in the RNA Pol I transcription machinery independently from its effect on cyclin D1 in U87 cells early after induction, but at 24 h, there are both cell cycle-independent and cell cycle-dependent changes that contribute to the PTEN-mediated reduction in RNA Pol I transcription. Previous work revealed that mTOR regulates RNA Pol I transcription in nonproliferating primary cardiomyocytes (17). Together, these results demonstrate that PTEN, and its ability to regulate mTOR signaling, can produce effects on RNA Pol I transcription that are separable from those regulating the cell cycle.

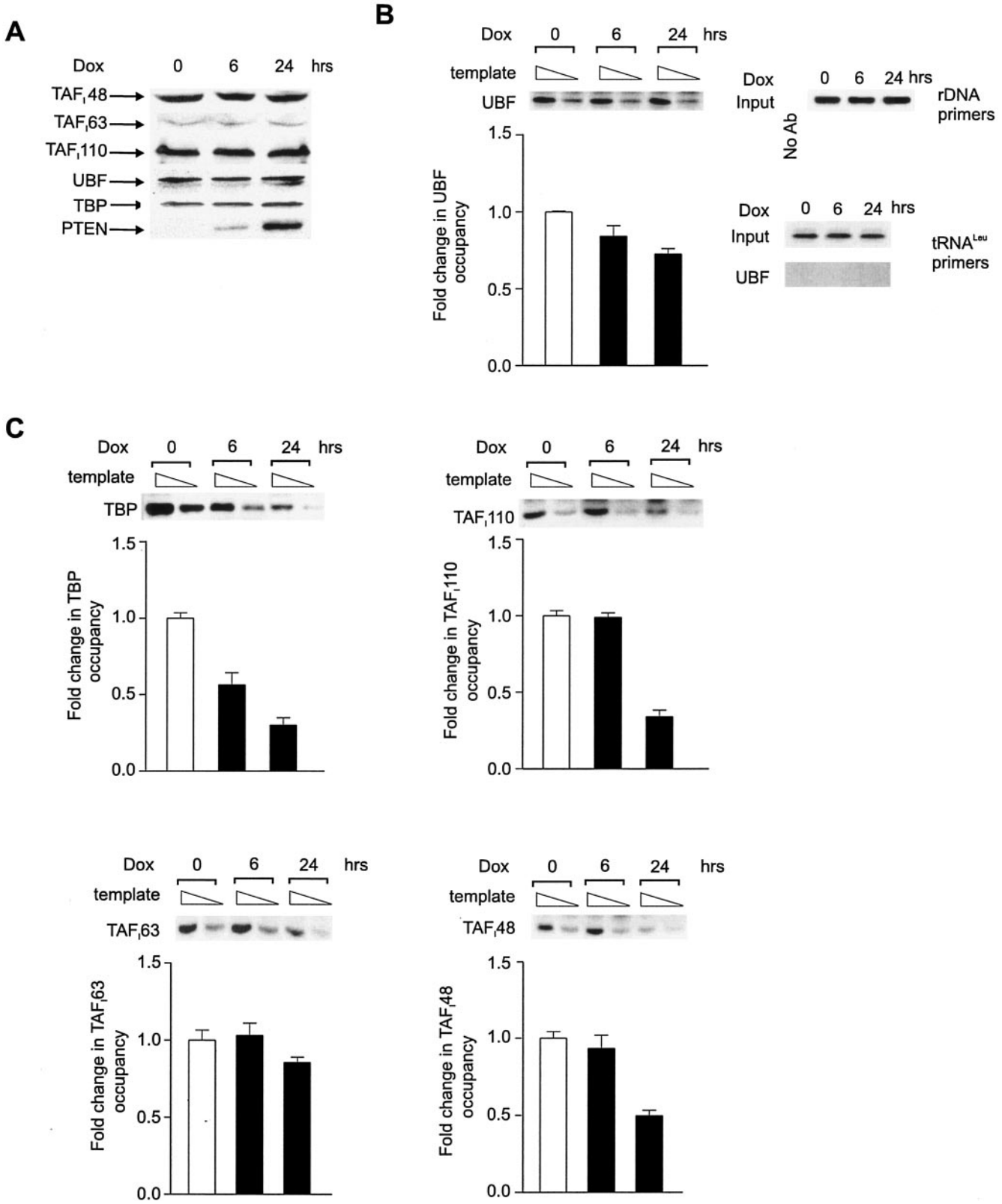

FIG. 7. PTEN expression selectively decreases the occupancy of SL1 subunits on the rRNA gene promoter. (A) The levels of UBF and SL1 are not changed upon PTEN expression. PTEN-inducible cells were treated with DOX as designated. Resultant protein lysates were subjected to immunoblot analysis. Membranes were probed with antibodies against the indicated proteins. (B) The occupancy of UBF on the promoter of rRNA genes is not changed by PTEN expression. PTEN-inducible U87 cells were treated with DOX as designated, and ChIP assays were carried out as described in Materials and Methods. For input DNA, 10% of the chromatin preparation that was not incubated with antibodies was

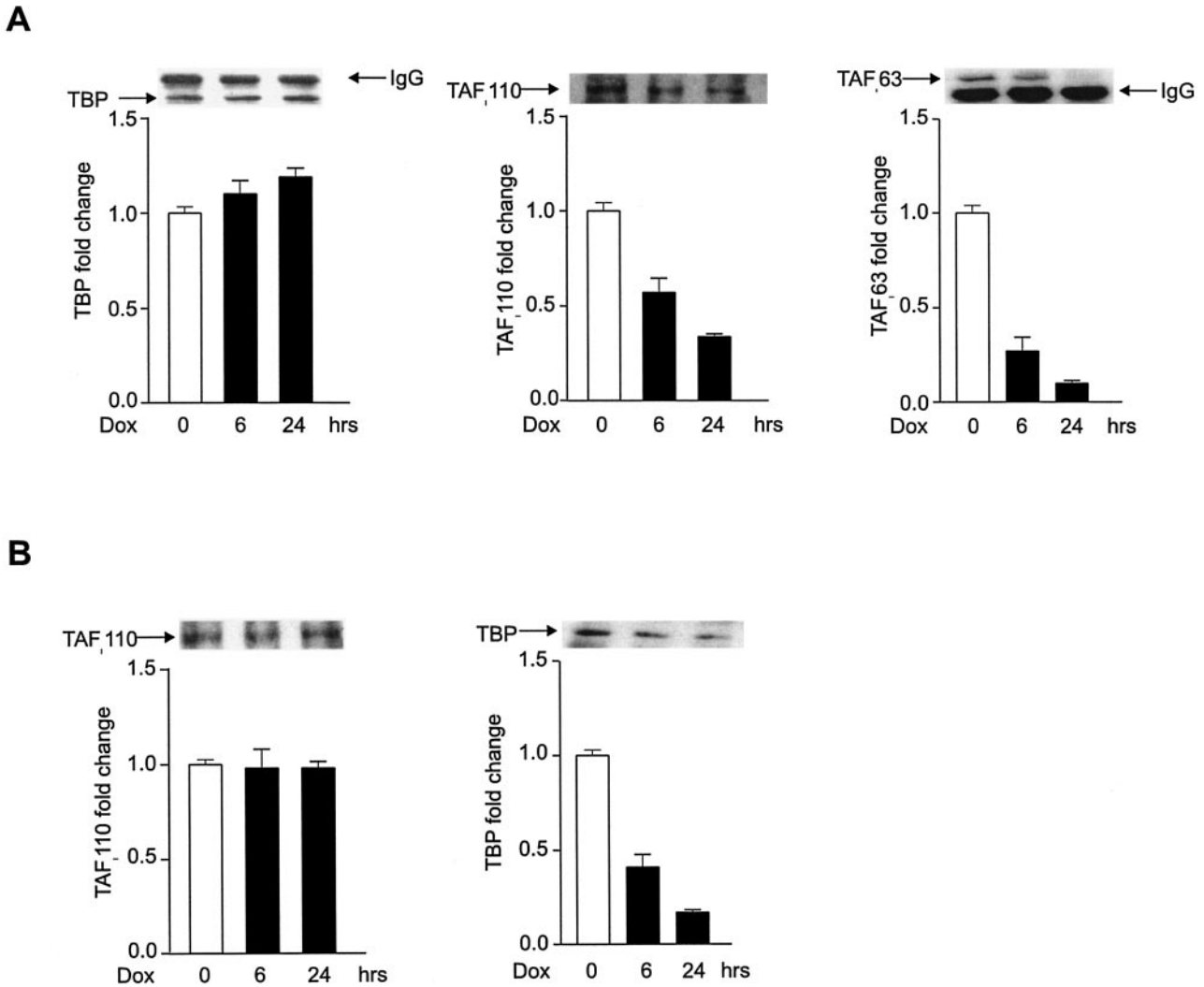

FIG. 8. PTEN expression disrupts the association of TBP with RNA Pol I-specific TAFs. (A) Immunoprecipitation of TBP-TAF_I110 and TBP-TAF_I63 complexes. Protein lysates were prepared from PTEN-inducible U87 cells treated with 1 μg/ml DOX as designated. Immunoprecipitation assays were carried out using anti-TBP antibodies and 400 μg of protein lysate as described in Materials and Methods. Bound proteins were then subjected to immunoblot analysis, and antibodies against TBP, TAF_I 110, or TAF_I 63 were used to detect the proteins. The values shown are means ± SEM from three independent determinations where the amounts of TBP, TAF_I110, and TAF_I63 coimmunoprecipitated with TBP were calculated relative to control noninduced cells. (B) Immunoprecipitation of TAF_I110-TBP complexes. Protein lysates were prepared from PTEN-inducible U87 cells as designated. Immunoprecipitation assays were carried out using 400 μg of protein lysate by using antibodies against TAF_I110. Bound proteins were then subjected to immunoblot analysis, and antibodies were used to detect TAF_I110 and TBP. The values are the means ± SEM from four independent determinations where the amounts of TAF_I110 and TBP coimmunoprecipitated with TAF_I110 were calculated relative to control noninduced cells.

We found no apparent change in the expression of SL1 or UBF proteins upon PTEN induction. Therefore, we kinetically examined potential alterations in the RNA Pol I transcription components and SL1/UBF interactions at the rRNA gene promoter responsible for the reduction in transcription. At 6 h following PTEN induction, we observed a selective reduction in the occupancy of TBP on the endogenous rRNA gene promoter. These results are consistent with a previous study showing that induction of RNA Pol I transcription by insulin-like growth factor-dependent activation of PI3 kinase signaling was associated with enhanced occupancy of TBP on rRNA gene promoters (20). Surprisingly, however, we find that the de-

subjected to PCR. As a negative control, immunoprecipitated chromatin was examined for the binding of UBF to $tRNA^{Leu}$ genes. PCR products were resolved and visualized with ethidium bromide. Two amounts of the diluted immunoprecipitated products (0.25 μl and 0.5 μl) were used as template for PCR. Representative PCR products generated from reactions are shown. (C) The occupancy of subunits of the SL1 complex is differentially reduced by PTEN expression. The chromatin was isolated from U87 cells treated with DOX as designated. ChIP assays were conducted as described for panel A. The SL1 subunits bound to DNA were immunoprecipitated by antibodies against the subunits as designated. The values shown are mean ± SEM from determinations using three different chromatin preparations.

crease in promoter occupancy of TBP is not correlated with a decrease in binding of the other SL1 components, suggesting that PTEN expression induces the dissociation of the SL1 complex. Coimmunoprecipitation assays further support this idea, indicating that PTEN leads to a decreased association of TBP with the SL1 TAFs. This initial event is independent of the phosphorylation state of TBP or TAF_I110. Thus, our studies provide new evidence that the formation or stability of the SL1 complex itself can limit the rate of rRNA gene synthesis.

At 24 h following PTEN induction, RNA Pol I transcription repression is more pronounced than that observed at 6 h and accompanied by further dissociation of the SL1 complex. Moreover, a decrease in binding of TAF_I110 and TAF_I48 to the promoter is now observed. A pronounced increase in serine phosphorylation of TAF_I110 accompanies its dissociation from the rRNA gene promoter. The phosphorylation states of both TBP and TAF_I110 are increased during mitosis, correlating with the inactivation of SL1 and the decreased interaction between SL1 and UBF (18). Since PTEN begins to induce cell cycle changes 24 h after induction, it is possible that the increase in TAF_I110 phosphorylation is cell cycle dependent. This event likely facilitates the dissociation of TAF_I110 from UBF and the rRNA gene promoter. Interestingly, TAF_I63 appears to remain bound to the rRNA gene promoter. Since TAF_I63 is in close contact with the DNA (3), it is possible that this allows it to remain stably associated with the promoter for a prolonged period of time, even in the absence of the other TAFs. Thus, our ability to kinetically examine the effect of PTEN expression on the transcription process suggests that SL1 is targeted for inactivation through a two-step process. The initial event, which is independent of the phosphorylation state of TBP or TAF_I110, triggers the selective dissociation of TBP from the rRNA gene promoter and disruption of the SL1 complex. The second step, induced by increased phosphorylation of TAF_I110, decreases its association with UBF, facilitating its release from the promoter.

Recent studies have shown that RNA Pol I transcription can be regulated by PI3 kinase/mTOR signaling through inactivation of TIF-1A/RRN3, a regulatory factor associated with initiation-competent RNA Pol I (27). In contrast, studies by Hannan et al. (17) showed that mTOR/S6K signaling regulated RNA Pol I transcription by modulating the phosphorylation state of UBF, preventing its association with SL1. These results, which used in vitro systems to analyze the transcription components, are potentially compatible with our results that examined changes in rRNA gene occupancy in vivo. Our results support the idea that PTEN works through mTOR/S6K to regulate RNA Pol I transcription through multiple, kinetically separable, events. The early PTEN-induced loss of TBP from the promoter could be initiated by dissociation of the SL1 complex coinciding with the inability of RNA Pol I-RRN3 complexes to be recruited to the promoter. Increased phosphorylation of TAF_I110 could disrupt its interaction with UBF, as previously suggested (18), leading to its dissociation from the promoter. Modification of UBF could further lead to its reduced association with TAF_I110 or TAF_I48 and their loss from the promoter. Thus, it is likely that multiple events occur to facilitate the sequential dissociation of the transcription components from the rRNA gene promoter, leading to the repression of transcription that was observed.

Regulation of RNA Pol I-dependent transcription is achieved by a variety of mechanisms involving changes in the amounts or activities of each of the transcription components. While changes in TIF-1A/RRN3-RNA Pol I interactions, UBF-DNA interactions, or UBF-SL1 interactions have been shown extensively to regulate the RNA Pol I transcription process, our studies provide new evidence that the formation of the SL1 complex itself can contribute to the rate of rRNA synthesis. The rate of transcription of rRNA is a limiting factor in the production of ribosomes. Controlling the synthesis of rRNAs therefore serves to regulate the translational capacity of cells. Thus, the ability of PTEN to repress RNA Pol I-dependent transcription is likely an important contributor to its function as a tumor suppressor.

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