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Mitotic Regulation of a TATA-Binding-Protein-Containing Complex

ROBERT J. WHITE,[1,2]* TANYA M. GOTTLIEB,[1,2] C. STEPHEN DOWNES,[2] AND STEPHEN P. JACKSON[1,2]*

Wellcome/CRC Institute[1] and Department of Zoology,[2] University of Cambridge, Cambridge CB2 1QR, United Kingdom

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The mitotic state is associated with a generalized repression of transcription. We show that mitotic repression of RNA polymerase III transcription can be reproduced by using extracts of synchronized HeLa cells. We have used this system to investigate the molecular basis of transcriptional repression during mitosis. We find a specific decrease in the activity of the TATA-binding-protein (TBP)-containing complex TFIIIB. TBP itself is hyperphosphorylated at mitosis, but this does not appear to account for the loss of TFIIIB activity. Instead, one or more TBP-associated components appear to be regulated. The data suggest that changes in the activity of TBP-associated components contribute to the coordinate repression of gene expression that occurs at mitosis.

Nuclear transcription is repressed during mitosis (4, 7, 9, 31, 45, 46). In mammalian cells, all RNA synthesis stops by midprophase before any disintegration of the nuclear membrane is apparent, and it does not resume again until late in telophase (31). This repression may be necessary to allow chromosomal condensation and division to occur without interference from the transcriptional apparatus. However, very little is known about the molecular mechanisms responsible for transcriptional inhibition at mitosis. An important step toward characterizing this process came with the recent demonstration that mitotic repression can be mimicked in vitro by using *Xenopus* egg extracts (15). Hartl et al. (15) showed that extracts shifted to a mitotic state by the addition of recombinant cyclin B exhibit a marked decrease in ability to transcribe tRNA or 5S RNA genes relative to untreated interphase extracts. This happens even if active transcription complexes are preassembled on the promoters of these genes (15). The in vitro inhibition does not require nucleosome deposition on the template and occurs in the presence of the topoisomerase II inhibitor VM-26, which prevents the complete assembly of chromosomes into metaphase structures. It appears to involve phosphorylation-dependent inactivation of one or more components of the transcription machinery that are required for tRNA and 5S RNA synthesis (15).

tRNA and 5S RNA genes are transcribed by RNA polymerase III (Pol III). The proteins involved in this process have been studied extensively (reviewed in references 51 and 59). Recruitment of Pol III to specific promoter sites requires a factor called TFIIIB, which is a multisubunit complex containing the TATA-binding protein (TBP) and associated polypeptides (reviewed in references 16 and 34). Since most Pol III promoters contain no TATA sequence and cannot be recognized directly by TBP (19, 22, 53), TFIIIB is normally recruited via protein-protein interactions with an assembly factor called TFIIIC that binds downstream of the initiation site (reviewed in references 16, 51, and 59).

We have investigated how the human Pol III transcription apparatus behaves at mitosis by comparing extracts made from asynchronous HeLa cells with those of cells synchronized in M phase of the active growth cycle. The mitotic changes that occur in growing HeLa cells may differ significantly from those observed in metaphase-arrested *Xenopus* eggs, given the substantial differences that exist between the *Xenopus* early embryonic cell cycle (with only S and M phases) and the more complex somatic cell cycle (with G_1, S, G_2, and M phases). Nevertheless, we find that extracts made from mitotic human cells display a dramatic decrease in Pol III transcriptional activity, as reported for the *Xenopus* egg system (15). The generality of this effect suggests that it may be a common feature of the mitotic response. We show that the diminished Pol III transcriptional capacity reflects a specific decrease in TFIIIB activity. The abundance of TBP changes little at mitosis, although it becomes hyperphosphorylated. However, TBP is not limiting for the transcription of TATA-less Pol III templates in mitotic extracts. Instead, it is the activity of one or more of the other components of TFIIIB that is specifically deficient. This inactivation of TBP-associated components is likely to contribute to the coordinate repression of Pol III transcription that accompanies mitosis.

* Corresponding author. Mailing address: Wellcome/CRC Institute, University of Cambridge, Tennis Court Rd., Cambridge CB2 1QR, United Kingdom. Phone: 0223-334103. Fax: 0223-334089.

MATERIALS AND METHODS

Plasmids and oligonucleotides. pVAI contains the adenovirus VA_I gene (5). pXbs1 contains a *Xenopus* somatic 5S gene (38). pHu5S3.1 is a 638-bp *Bam*HI-*Sac*I fragment of human genomic DNA containing a 5S gene, subcloned into pBluescript SK+. pU6/Hae/RA.2 contains a human U6 promoter (24). pRH5.7 contains a human *Alu* gene (57). pGlu6 contains a human $tRNA^{Glu}$ gene (11). pTI contains the TATA box and initiator element of the adenovirus major late promoter (33).

Tissue culture. Asynchronous HeLa cells were maintained in log phase by passaging 1:6 every 3 days in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 1 U of penicillin per ml, and 0.1 mg of streptomycin per ml. The mitotic arresting agents used were nitrous oxide and nocodazole {methyl-(5-[2-thienylcarbonyl]-1H-benzimidazol-2-yl)-carbamate}. Cells were first subjected to a thymidine block in order to increase the subsequent mitotic yield. The effects of these agents are reversible upon their removal. Proliferating asynchronous HeLa cells were plated in 2 mM thymidine and left for 24 h. The thymidine was then washed out. Six hours later, the cells were treated with nitrous oxide delivered at 80 lb/in^2 in a pressure vessel. After 9 h, the cells were removed from the vessel and examined microscopically. The majority of cells were either floating or loosely attached but rounded up and mitotic. Those that had not detached were released by gentle shaking. Alternatively, 5 h after release from the thymidine block, nocodazole was added to a final concentration of 0.04 μg/ml, and mitotic cells were collected 7 to 8 h later. Samples of the mitotic cells were examined microscopically as a Cytospin preparation and also by flow cytometry, to verify that efficient synchronization had taken place. Cytospin preparations revealed mitotic indices usually greater than 90%.

H1 kinase assay. Extract or purified kinase was incubated for 10 min at 30°C in 10 mM Tris-HCl (pH 7.4)–5 mM $MgCl_2$–1 mM dithiothreitol (DTT) with 200 ng of histone H1 (Boehringer Mannheim) and 5 μCi of [γ-^{32}P]ATP. Samples were then resolved on a sodium dodecyl sulfate (SDS)–polyacrylamide gel and visualized by autoradiography. The p34^{cdc2}-cyclin B kinase was prepared by immunoprecipitation from extracted mitotic cells, using an antibody against human cyclin B1 that was generously provided by J. Pines.

Preparation of extracts. Whole cell extracts were prepared by a method

developed by Partridge and La Thangue (30). A frozen cell pellet (0.5×10^7 to 3×10^7 cells) was thawed on ice and resuspended in 75 to 125 μl of freshly made extraction buffer (50 mM NaF, 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.8], 450 mM NaCl, 25% glycerol, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 μg of leupeptin per ml, 0.5 μg of protease inhibitor per ml, 1.0 μg of trypsin inhibitor per ml, 0.5 μg of aprotinin per ml, 40 μg of bestatin per ml) before being sequentially snap frozen on dry ice and thawed at 30°C three times. After 7 min of microcentrifugation at 4°C to pellet cell debris, the supernatant was aliquoted, snap frozen, and stored at −70°C. Asynchronous and mitotic extracts were also prepared by the method of Dignam et al. (6). These extracts gave results very similar to those for the whole cell extracts.

Protein fractions. The starting material for all fractionations was nuclear extract (6) of asynchronous HeLa cells that was depleted of glycosylated proteins by wheat germ agglutinin affinity chromatography as described previously (21). The extracts were then chromatographed on phosphocellulose by the method of Segall et al. (40). Direct assay confirmed that PC-B fractions (0.1 to 0.35 M KCl step) contained TFIIIB and Pol III and PC-C fractions (0.35 to 0.6 M KCl step) contained TFIIIC and Pol III (50).

TFIIIC was purified further from the PC-C fraction by chromatography on heparin-Sepharose. PC-C was loaded onto heparin Sepharose CL-6B in BC buffer (25 mM Tris-HCl [pH 7.9], 10% glycerol, 10 mM β-mercaptoethanol) plus 100 mM KCl (BC-100). The column was washed with BC-280 and eluted with BC-1000 to generate the CHep-1.0 fraction containing TFIIIC. Peak fractions were dialyzed into LDB buffer (20 mM HEPES-KOH [pH 7.9], 17% glycerol, 100 mM KCl, 12 mM $MgCl_2$, 0.1 mM EDTA, 2 mM DTT). TFIIIC was affinity purified on a B-block oligonucleotide resin, prepared as described previously (21). The oligonucleotides had the sequences 5′-GGATCCGGGGTTCGAACCCC-3′ and 5′-ATCCGGGGTTCGAACCCCGG-3′, corresponding to the B-block region of the adenovirus VA_I gene. PC-C fraction in BC-70 was incubated on ice with 30 mg of poly(dI-dC) per mg of protein for 10 min and then passed through the affinity resin over 75 min at 4°C. After being washed with BC-350, TFIIIC was eluted in BC-2000 and dialyzed into LDB.

Pol III enzyme was further purified from the PC-B fraction by chromatography on DEAE-Sephadex. PC-B was applied to A25 DEAE-Sephadex in buffer A (20 mM HEPES-KOH [pH 7.9], 20% glycerol, 5 mM $MgCl_2$, 3 mM DTT, 0.2 mM phenylmethylsulfonyl fluoride) plus 50 mM $(NH_4)_2SO_4$. After being washed with this buffer, TFIIIB was eluted in buffer A plus 150 mM $(NH_4)_2SO_4$. Pol III was then eluted without detectable TFIIIB contamination, using buffer A plus 1 M $(NH_4)_2SO_4$. Peak fractions were dialyzed into LDB.

TFIIIB was purified from the PC-B fraction by Mono Q gradient chromatography under the conditions described by Chiang et al. (2). TBP and TFIIIB activity eluted between 480 and 560 mM KCl. Fractions were dialyzed into LDB.

TFIIIB was immunopurified by using the anti-TBP monoclonal antibody MTBP-6. This antibody was cross-linked to protein A-Sepharose by the method of Harlow and Lane (14). A 0.2-ml amount of this resin was added to 1.3 mg of PC-B in 1 ml of LDB. After mixing by rotation for 90 min at 4°C, the supernatant was collected. The resin was washed with 4 ml of LDB followed by 1.4 ml of LDB containing 190 mM KCl. Immunoaffinity-purified TFIIIB was then eluted by using LDB containing 2 M urea. Peak fractions were dialyzed into LDB. We estimate that the specific activity of TFIIIB is approximately 11-fold greater in immunoaffinity-purified TFIIIB than in PC-B, as assayed by its ability to restore VA_I transcription in a nuclear extract that has been immunodepleted with anti-TBP antibody MTBP-6 (50). The purity of TFIIIB is likely to have increased by more than this, but some activity is lost as a result of denaturation and dilution. Silver staining of immunoaffinity-purified TFIIIB reveals a complex mixture of polypeptides (50).

Extracts were immunodepleted by incubation with anti-TBP antibody MTBP-6 linked to protein A-Sepharose for 1 h on ice with frequent mixing. The beads were then pelleted by centrifugation, and the supernatant was used for transcription. Mock depletions were carried out in the same way, using protein A-Sepharose without antibody.

Highly purified TFIIA was a generous gift from L. Zawel and D. Reinberg.

Recombinant human TBP was expressed in bacteria and purified to greater than 95% homogeneity as described previously (55).

TBP-associated factors (TAFs) purified from a PC-B fraction by affinity chromatography (B-TAF fractions) were purified from PC-B by affinity chromatography on a nickel-nitrilotriacetic acid resin containing immobilized, histidine-tagged human TBP. The TBP was expressed in bacteria from construct pET-HisIID#9 and purified by binding to nickel-NTA (Qiagen) according to the manufacturer's specifications. A 2.9-mg amount of PC-B was loaded at room temperature in buffer B (20 mM HEPES-KOH [pH 7.9], 20% glycerol, 1.5 mM β-mercaptoethanol) containing 100 mM KCl. The flowthrough was collected and reloaded onto the resin twice more. Flow was then stopped, and the fraction was incubated with the resin for 15 min at 30°C with constant gentle agitation. Flow was resumed, and the column was washed at room temperature with 4 ml of buffer B containing 100 mM KCl. B-TAF fractions were then eluted from the resin by using buffer B containing 2.5 M urea. Peak protein fractions were dialyzed into LDB. The CON (control)-TAF fraction was prepared in exactly the same way, using nickel-nitrilotriacetic acid resin without TBP bound to it. The CON-TAF fraction contained no detectable transcription activity (50). We estimate that the specific activity of TBP-associated Pol III factors is approximately 12-fold greater in B-TAF fractions than in PC-B, as assayed by their ability to reconstitute VA_I transcription in the presence of PC-C and recombinant TBP (50). The purity of TFIIIB polypeptides is likely to have increased by far more than this, but much activity will have been lost as a result of denaturation and dilution. Furthermore, reassembly of the TFIIIB complex from renatured purified components and recombinant TBP may be very inefficient. Silver staining reveals multiple polypeptides in the B-TAF fractions (50).

Transcription. Pol III transcription was carried out as described previously (58). Pol II transcription was carried out as described elsewhere (33). Unless otherwise stated, preincubations were for 15 min and transcriptions were for 40 min at 30°C. Quantitation was by PhosphorImager (Molecular Dynamics) analysis.

Polymerase assays. Random RNA polymerization assays were conducted as described previously (35). Each reaction mixture contained 5 μg of poly(dA-dT) template and 10 μg of extract protein. Pol III activity was calculated by subtracting the polymerization obtained in the presence of 200 μg of α-amanitin per ml (due to Pol I) from that obtained in the presence of 1 μg of α-amanitin per ml (due to Pol III plus Pol I).

Western blotting (immunoblotting). Western immunoblot analyses were performed as described previously (14). Blots were developed by the enhanced chemiluminescence method (Amersham) according to the manufacturer's specifications. MTBP-6, a monoclonal antibody that recognizes the N-terminal region of TBP (32), was the generous gift of J. Flint. 16E8 and 58C9 are monoclonal antibodies that recognize the C-terminal region of TBP. These were the generous gift of R. Weinzierl and R. Tjian.

RESULTS

Extracts of mitotic HeLa cells are impaired for Pol III transcription. Exponentially growing HeLa cells were synchronized in S phase with a thymidine block. After release from this block, they were arrested in M phase by treatment with nitrous oxide. Samples of the mitotic cells were analyzed microscopically and also by flow cytometry to verify that efficient synchronization had taken place. Such analyses revealed mitotic indices usually greater than 90%. In contrast, 96 to 98% of unsynchronized log-phase HeLa cells are in interphase (46). Representative flow cytometric analyses of the DNA content of harvested cells are shown in Fig. 1A. Whole cell extracts were prepared from these cells. The mitotic extracts displayed highly elevated histone H1 kinase activity (Fig. 1B), thereby providing an independent confirmation of the mitotic state of the cells used to prepare them.

Six different matched pairs of extracts prepared from asynchronous and mitotic cells were compared for the ability to transcribe Pol III templates. In each case, the mitotic extracts were significantly less active than asynchronous extracts (Fig. 2A). Representatives of each of the three promoter types used by Pol III were tested: 5S genes have type I promoters, with internal A and C blocks and no TATA box; tRNA, VA and *Alu* genes have type II promoters, with internal A and B blocks and no TATA box; and human U6 genes have type III promoters, which are entirely external and include a TATA box (reviewed in references 51 and 59). Each type gives significantly less transcription in mitotic extracts than in asynchronous extracts.

We wanted to ensure that the reduced Pol III transcription in mitotic extracts is not an artifactual response to the drug treatment used in synchronizing the cells. Whereas a thymidine block followed by nitrous oxide treatment synchronizes cells in M phase, a thymidine block alone arrests cells in S phase, and nitrous oxide treatment without a prior thymidine block results in a largely asynchronous population with an elevated proportion of mitotic cells. Extracts were prepared from cells cultured under these conditions and tested for transcriptional activity (Fig. 2B). Whereas mitotic extracts are severely compromised in the ability to transcribe a tRNA gene, extracts of cells that had been treated with thymidine alone or nitrous oxide alone have activity similar to that of asynchronous extracts. Thus, neither thymidine nor nitrous oxide alone leads to a significantly lower transcriptional capacity. However, combining these treatments so as to synchronize cells in M phase pro-

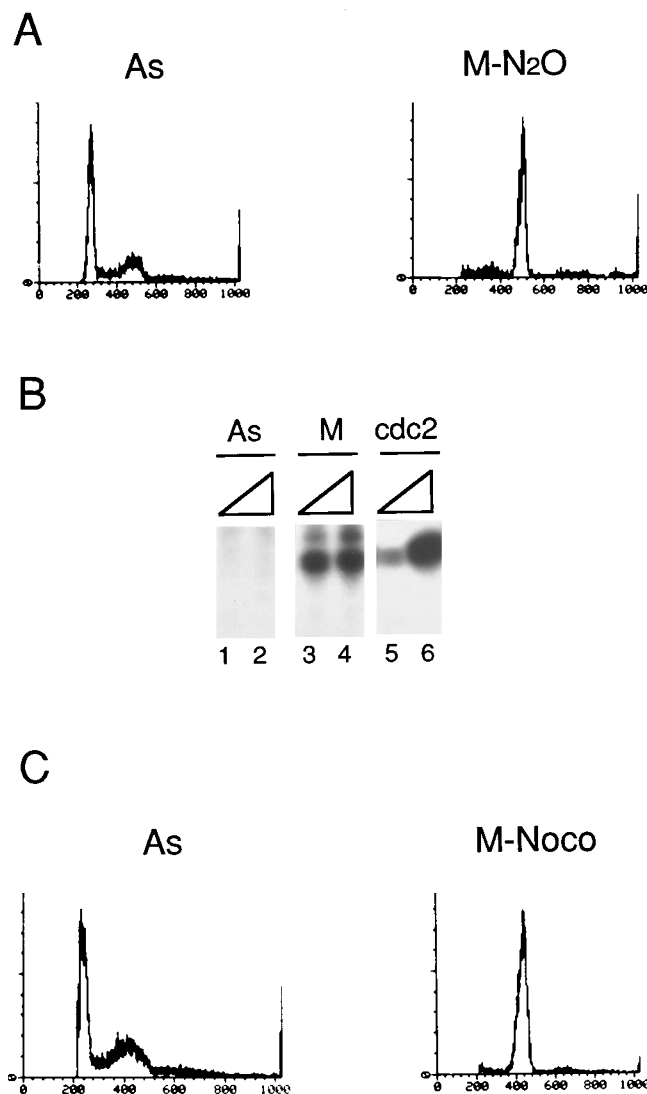

FIG. 1. Synchronization analyses of cells used to prepare extracts. (A) Representative graphs from flow cytometric analyses showing the relative DNA contents of asynchronous cells (As) and cells synchronized in mitosis by nitrous oxide treatment (M-N_2O). (B) Mitotic extracts display elevated histone H1 kinase activity. Histone H1 was incubated for 10 min at 30°C in the presence of [γ-^{32}P]ATP and either 7 μg (lanes 1 and 3) or 21 μg (lanes 2 and 4) of As (lanes 1 and 2) or M-N_2O extract (lanes 3 and 4) or with 1 or 2 μl of cyclin B-p34^{cdc2} kinase (lane 5 or 6, respectively). (C) Representative graphs from flow cytometric analyses showing the relative DNA contents of asynchronous cells (As) and cells synchronized in mitosis by nocodazole treatment (M-Noco).

duces a marked decrease in Pol III activity. This finding suggests that the transcriptional apparatus responds to the mitotic state rather than to either of the drugs themselves.

To test this idea further, we also prepared mitotic cells by culturing in the presence of nocodazole. Flow cytometric analyses again confirmed the phase of these populations (Fig. 1C). Two matched pairs of extracts were prepared from asynchronous cells and nocodazole-treated mitotic cells. Once again, Pol III templates were transcribed more efficiently in the asynchronous extracts than in the mitotic extracts (Fig. 2C). Thus, synchronization in M phase by treatment with nocodazole also results in diminished Pol III activity. The fact that this effect is seen with two different strategies for synchronizing human cells, as well as upon cyclin B addition to *Xenopus* egg extracts (15), suggests that it is a bona fide response to the mitotic state.

Given the changes in nuclear structure that accompany mitosis, the low Pol III transcription in mitotic extracts might be explained if factors were less readily extracted from cells in M phase. To test for a general lack of factors in mitotic extracts, we assayed levels of Pol II transcription. We found that pTI, a basal Pol II template composed of an initiator element and a TATA box (33), is transcribed as efficiently in mitotic extracts as in asynchronous extracts (Fig. 2D). This finding suggests that there is no general change in factor extractability as cells undergo mitosis and demonstrates that the Pol III response is a specific one.

Extracts of mitotic HeLa cells do not contain a dominant Pol III repressor. The low Pol III transcription in mitotic extracts could be due to the presence of a repressor during M phase. Mixing experiments were carried out to test this. Adding increasing amounts of mitotic extract to a constant amount of asynchronous extract resulted in no decrease in Pol III transcription (Fig. 3). Indeed, a mixture of the two types of extract gave expression higher than the sum of the levels obtained with each extract individually. This implies that mitotic extracts do not contain a dominant Pol III repressor. Instead, the low expression may be due to a deficiency in some positive activity that is required for Pol III transcription. We therefore assayed these components.

TFIIIB activity is specifically diminished in mitotic extracts. Since every Pol III template tested is expressed less actively in mitotic extracts, the positive activity that may be low at mitosis is likely to be either Pol III itself or one of the general Pol III factors. To determine the activity of Pol III itself, we measured its ability to catalyze randomly initiated RNA synthesis independently of transcription factors, using a poly(dA-dT) template. Two matched pairs of extracts were tested. In neither case was there any significant difference in the level of random polymerization by Pol III between the mitotic and the asynchronous extracts (Table 1). We therefore turned our attention to the transcription factors required for sequence-specific initiation by Pol III.

TFIIIB is required for the transcription of all class III genes (reviewed in references 51 and 59). Its regulation at mitosis could therefore account for the observed repression of all Pol III templates tested. Consistent with this possibility, we found that a phosphocellulose step fraction (PC-B) containing TFIIIB strongly stimulates transcription when added to mitotic extracts (Fig. 4A, lanes 1 to 3). This finding suggests that TFIIIB may be the component of the Pol III transcription apparatus that is deficient at mitosis. However, a PC-B fraction contains many proteins besides TFIIIB. For example, Pol III enzyme is enriched in PC-B. We therefore fractionated samples further in order to identify which component of PC-B is limiting the number of active transcription complexes that are formed in mitotic extracts.

Pol III was separated from TFIIIB by chromatography on DEAE-Sephadex. The more purified Pol III fraction had no stimulatory effect when added to a mitotic extract (Fig. 4A, lanes 4 to 6). Therefore, Pol III itself is not limiting at M phase, consistent with the direct assays that showed little change in the activity of this enzyme (Table 1). The PC-B was subjected to gradient chromatography on Mono Q, and each fraction was analyzed by Western blotting with an anti-TBP antibody in order to locate the TBP subunit of TFIIIB. Almost all TBP was found in fractions 41 and 42 (Fig. 4B, upper panel). Each fraction was also assayed for its ability to stimulate Pol III transcription when added to mitotic extract. The stimulatory activity eluted in a sharp peak, again centered at fractions 41

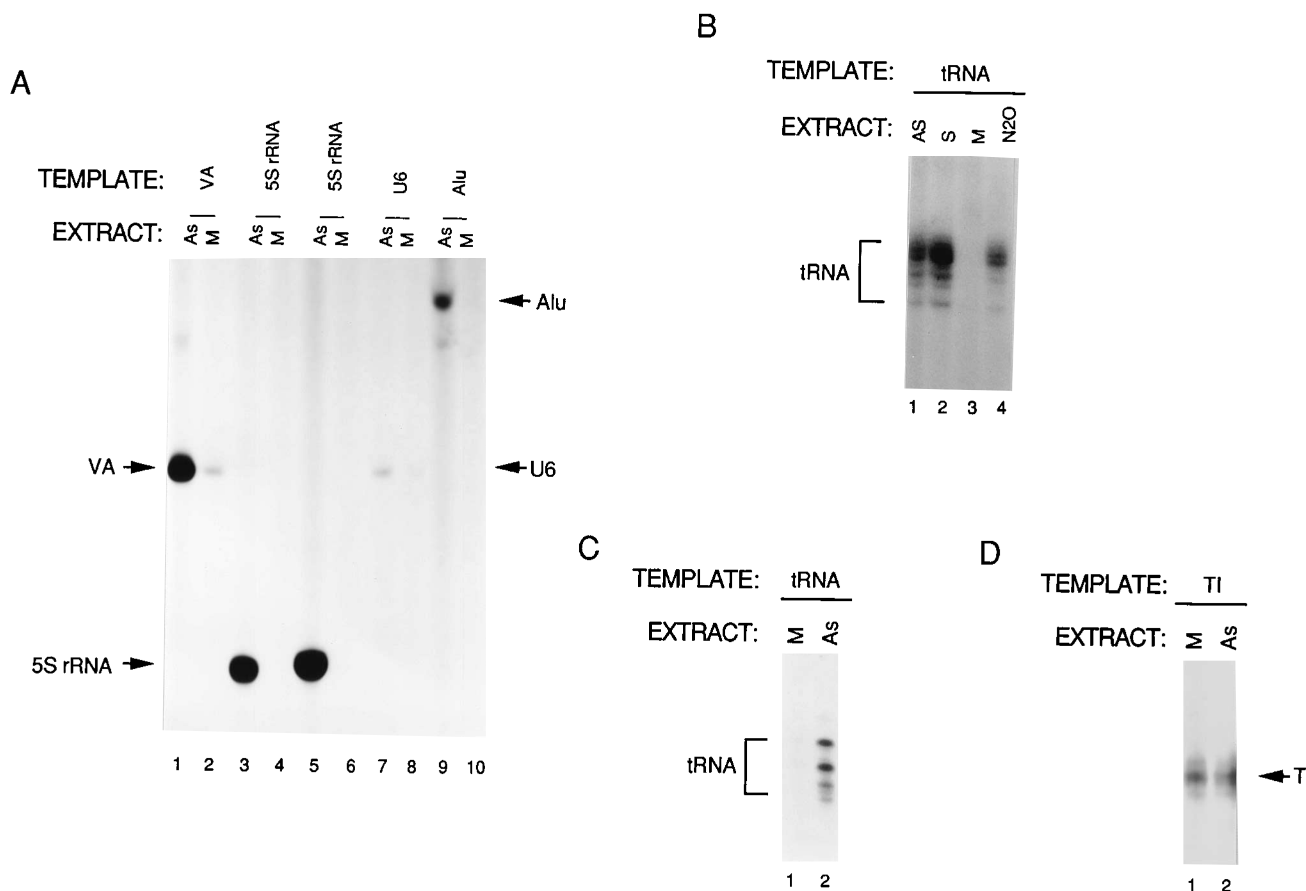

FIG. 2. Extracts of mitotic cells are less active in Pol III transcription than extracts of asynchronous or S-phase cells. (A) Class III genes are transcribed more efficiently by asynchronous (As) extracts than by mitotic extracts. Five hundred-nanogram samples of pVA1 (lanes 1 and 2), pXbs1 (lanes 3 and 4), pHu5S3.1 (lanes 5 and 6), pU6/Hae/RA.2 (lanes 7 and 8), and pRH5.7 (lanes 9 and 10) were preincubated with 14 μg of either As (lanes 1, 3, 5, 7, and 9) or M-N_2O extract (M; see legend to Fig. 1; lanes 2, 4, 6, 8, and 10). Nucleotides were then added to assay transcription. (B) Extracts prepared from thymidine- or nitrous oxide-treated cells do not show the decrease in Pol III transcription that is displayed by mitotic extracts. pGlu6 (500 ng) was preincubated with 14 μg of extract prepared from As (lane 1), thymidine-treated (lane 2), M-N_2O (lane 3), or nitrous oxide-treated (lane 4) cells. Nucleotides were then added to assay transcription. (C) Extracts of cells synchronized in M phase by using nocodazole (M-Noco) have reduced Pol III transcriptional activity. pGlu6 (500 ng) was preincubated with 30 μg of M-Noco (lane 1) or As (lane 2) extract. Nucleotides were then added to assay transcription. (D) Extracts of cells synchronized in M phase by using nocodazole have basal Pol II transcriptional activity similar to that of extracts of asynchronous cells. Linearized pTI (200 ng) was preincubated for 30 min at 30°C with 35 μg of M-Noco (lane 1) or As (lane 2) extract. Nucleotides were then added, and transcription was allowed to proceed for 15 min at 30°C.

and 42 (Fig. 4B, lower panel). Therefore, the factor that is limiting for Pol III transcription at mitosis cofractionates on Mono Q with TFIIIB. Indeed, this TFIIIB-containing fraction is able to raise the levels of VA_I transcription in mitotic extracts to those occurring in asynchronous extracts (Fig. 4C).

The fact that Mono Q-purified TFIIIB is sufficient to overcome mitotic repression (Fig. 4C) suggests that TFIIIB is the only general component of the Pol III transcription apparatus that is deficient at mitosis. To test this directly, we determined the effect of adding other Pol III factors. No increase in VA_I transcription was observed when partially purified TFIIIC was added to a mitotic extract (Fig. 5, lanes 4 to 7). In the same assay, Mono Q fraction 42 produced a strong activation (Fig. 5, lanes 1 to 3). This finding suggests that TFIIIC is in relative excess during M phase. Since TFIIA has been reported to stimulate Pol III transcription in vitro (26, 48), changes in this factor could potentially contribute to the mitotic repression. However, inactivation of this factor at mitosis might be expected to produce a concomitant reduction in basal Pol II transcription, which is not observed (Fig. 2D). We nevertheless tested whether TFIIA is limiting for Pol III transcription in mitotic extracts. Adding highly purified TFIIA to mitotic extracts did not increase VA_I expression (Fig. 5, lanes 8 to 10). This finding suggests that the low Pol III activity at mitosis is not due to a lack of TFIIA. The combined data demonstrate that the mitotic state in HeLa cells is characterized by a specific deficiency in a factor that cofractionates with TFIIIB.

To confirm that the limiting factor in mitotic extracts is TFIIIB, a monoclonal antibody against TBP was used to immunoaffinity purify TBP and those components of the TFIIIB complex that are tightly associated with it. The affinity-purified fraction was able to reconstitute Pol III transcription in an extract that had been immunodepleted with an anti-TBP antibody (Fig. 6A). This result shows that it contains active TBP and TAFs. The same fraction stimulated transcription when added to a mitotic extract (Fig. 6B). These results strongly implicate TBP or an associated factor in the TFIIIB complex as the component of the Pol III transcription apparatus that is deficient and limiting in mitotic extracts.

The identity of the limiting Pol III factor is cell cycle dependent. In contrast to these results with mitotic extracts, previous studies using extracts prepared from asynchronous HeLa

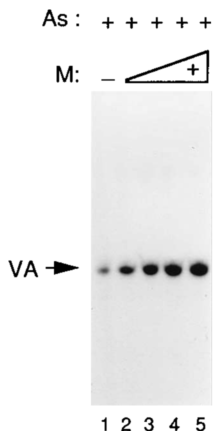

FIG. 3. Mitotic extracts do not contain a dominant repressor of Pol III transcription. pVAI (500 ng) was preincubated with 12 μg of asynchronous cell (As) extract and 0 (lane 1) 3 (lane 2), 6 (lane 3), 12 (lane 4), or 24 (lane 5) μg of M-N_2O extract (M; see legend to Fig. 1). Nucleotides were then added to assay transcription.

cells found that TFIIIC is the limiting factor for transcription of TATA-less class III genes (10, 18, 53, 60). We have confirmed this observation. Thus, VA_I transcription in our asynchronous extracts is stimulated by a fraction containing TFIIIC (Fig. 7A, lanes 1 to 4). In contrast, Mono Q-purified TFIIIB (Fig. 7A, lanes 5 to 7) and affinity-purified TFIIIB (Fig. 7B) have little or no stimulatory effect. This result implies that the relative amounts of active class III factors vary according to the phase of the cell cycle. TFIIIB is in excess during interphase but becomes limiting at mitosis. The fact that different factors are limiting in asynchronous and mitotic extracts explains why mixtures give more than additive levels of transcription (Fig. 3), since each extract provides a surplus of the factor that is limiting in the other.

TBP is hyperphosphorylated at mitosis. Since the activity of the TBP-containing complex TFIIIB becomes limiting at mitosis, Western blotting experiments were carried out to test for changes in TBP. Asynchronous and mitotic extracts were found to contain similar amounts of TBP (Fig. 8A, lanes 1 to 4). However, a variable proportion of the TBP in mitotic extracts has a reduced electrophoretic mobility relative to the TBP in asynchronous extracts. The amount of TBP with this reduced mobility increased substantially when phosphatase inhibitors were included in the cell extraction buffer (Fig. 8A, lanes 5 and 6). This result suggests that the change in mobility in mitotic extracts results from phosphorylation. This interpretation was confirmed by the fact that treating mitotic extracts with alkaline phosphatase causes an increase in TBP mobility, such that it comigrates with the TBP present in asynchronous extracts (Fig. 8B). This treatment has no effect in the presence

TABLE 1. Mitotic and asynchronous extracts contain similar levels of Pol III polymerization activity

Prepn	Pol III sp act (cpm)	Asynchronous cell/ mitotic cell ratio
Buffer	1,050	
Asynchronous 1	23,800	1.13
Mitotic 1	21,100	
Asynchronous 2	42,400	1.08
Mitotic 2	39,400	

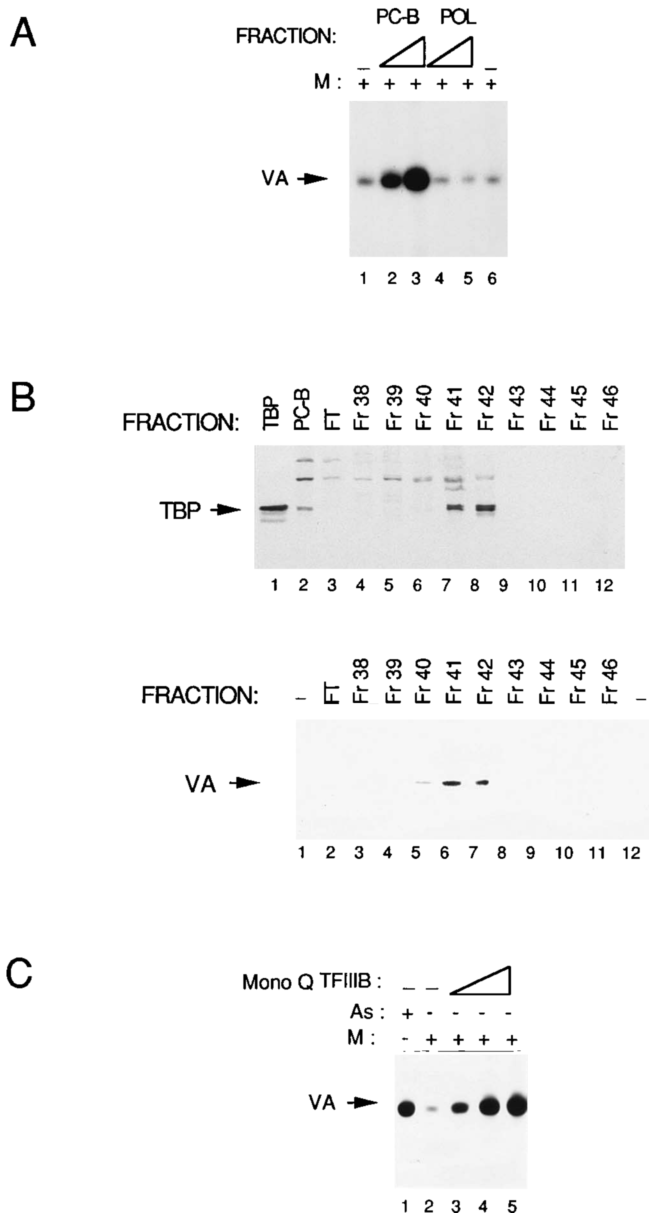

FIG. 4. The Pol III factor that is limiting in mitotic extracts cofractionates with TFIIIB. (A) A PC-B fraction stimulates Pol III transcription when added to mitotic extract, whereas more purified Pol III does not. pVAI (500 ng) was preincubated with 13.2 μg of M-N_2O extract (M; see legend to Fig. 1) and no additional fraction (lanes 1 and 6), 1 or 3 μl of PC-B (lane 2 or 3, respectively), or 1 or 3 μl of DEAE-purified Pol III (lane 4 or 5, respectively). Nucleotides were then added to assay transcription. (B) The activity that is limiting in mitotic extracts copurifies with TFIIIB on a Mono Q gradient. For the upper panel, 10 ng of recombinant TBP (lane 1) and 9 μl of PC-B input (lane 2), Mono Q flowthrough (FT; lane 3), and Mono Q gradient fractions (Fr; lanes 4 to 12) were resolved on an SDS–10% polyacrylamide gel and then analyzed by Western immunoblotting with anti-TBP antibody MTBP-6. For the lower panel, 500 ng of pVAI was preincubated with 10 μg of M-N_2O extract and no added fraction (lanes 1 and 12), 2 μl of Mono Q flowthrough (lane 2), or 2 μl of Mono Q gradient fractions (lanes 3 to 11). Nucleotides were then added to assay transcription. (C) Mono Q-purified TFIIIB is sufficient to raise levels of VA_I transcription in mitotic extracts to those occurring in asynchronous extracts. pVAI (500 ng) was preincubated with 14 μg of asynchronous (As; lane 1) or M-N_2O (lanes 2 to 5) extract and no additional fraction (lanes 1 and 2) or 2, 4, or 8 μl of Mono Q fraction 42 (lane 3, 4, or 5, respectively). Nucleotides were then added to assay transcription.

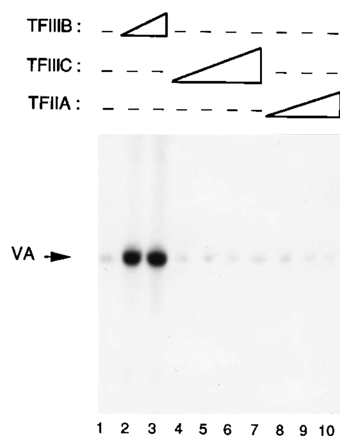

FIG. 5. A TFIIIB fraction stimulates transcription in mitotic extracts, whereas TFIIIC and TFIIA fractions do not. pVAI (500 ng) was preincubated with 14 μg of M-N_2O extract (see legend to Fig. 1) and no additional fraction (lane 1), 2 or 4 μl of Mono Q fraction 42 (lane 2 or 3, respectively), 2, 4, 6, or 8 μl of purified TFIIIC (lane 4, 5, 6, or 7, respectively), or 2, 4, or 6 μl of purified TFIIA (lane 8, 9, or 10, respectively). Nucleotides were then added to assay transcription. PhosphorImager analysis of specific transcripts reveals the following quantitation after correction for background (in arbitrary units): lane 1, 4.88; lane 2, 23.9; lane 3, 24.7; lane 4, 4.57; lane 5, 4.33; lane 6, 3.93; lane 7, 4.56; lane 8, 4.35; lane 9, 3.82; lane 10, 4.19.

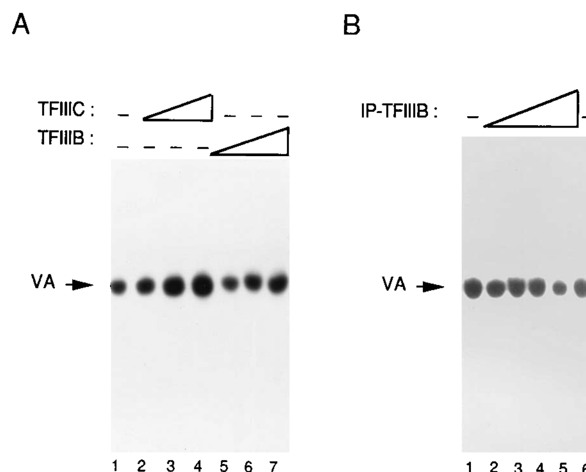

FIG. 7. TFIIIB is in relative excess for the transcription of TATA-less class III genes in extracts of asynchronous HeLa cells. (A) TFIIIC stimulates VA_I transcription in asynchronous extracts specifically. pVAI (500 ng) was preincubated with 10 μg of asynchronous extract and no added fraction (lane 1), with 2 μl (lane 2), 6 μl (lane 3), or 8 μl (lane 4) of purified TFIIIC, or with 4 μl (lane 5), 6 μl (lane 6), or 8 μl (lane 7) of Mono Q fraction 42. Nucleotides were then added to assay transcription. PhosphorImager analysis of specific transcripts reveals the following quantitation after correction for background (in arbitrary units): lane 1, 31.2; lane 2, 42.1; lane 3, 62.0; lane 4, 69.8; lane 5, 29.8; lane 6, 33.3; lane 7, 35.7. (B) Affinity-purified TFIIIB does not stimulate VA_I transcription when added to asynchronous extracts. pVAI (500 ng) was preincubated with 10 μg of asynchronous extract and no added fraction (lanes 1 and 6) or with 90 ng (lane 2), 180 ng (lane 3), 360 ng (lane 4), or 720 ng (lane 5) of immunoaffinity-purified TFIIIB (IP-TFIIIB). Nucleotides were then added to assay transcription. PhosphorImager analysis of specific transcripts reveals the following quantitation after correction for background (in arbitrary units): lane 1, 51.7; lane 2, 42.7; lane 3, 45.1; lane 4, 43.0; lane 5, 27.4; lane 6, 40.5.

of the phosphatase inhibitor sodium vanadate. These results indicate that TBP is phosphorylated as cells undergo mitosis.

The mitotic reduction in Pol III transcription is seen whether or not phosphatase inhibitors are included during extract preparation and therefore does not correlate with the phosphorylation state of TBP. Nevertheless, we tested the effect of adding TBP to mitotic extracts to find out whether this factor is limiting for Pol III transcription. Recombinant TBP produced no increase in VA_I expression and at high levels of input had an inhibitory squelching effect (Fig. 8C). The fact that added TBP can squelch transcription suggests that it is not inactivated when mixed with the mitotic extract. The available evidence therefore suggests that TBP is not limiting for Pol III transcription in extracts made from mitotic HeLa cells.

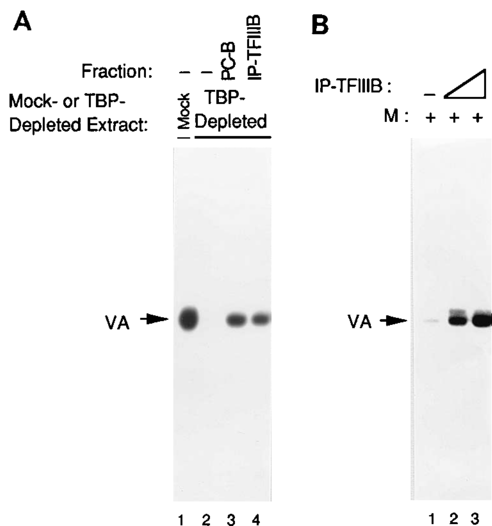

FIG. 6. TFIIIB activity is deficient at mitosis. (A) Affinity-purified TFIIIB restores Pol III transcription in extracts depleted with anti-TBP antibodies. pVAI (500 ng) was preincubated with 4 μl of either mock-immunodepleted extract (lane 1) or TBP-immunodepleted extract (lanes 2 to 4). Reactions 3 and 4 also contained 1.34 μg of PC-B (lane 3) or 120 ng (lane 4) of immunoaffinity-purified TFIIIB (IP-TFIIIB). Nucleotides were then added to assay transcription. (B) Immunoaffinity-purified TFIIIB stimulates Pol III transcription when added to mitotic extracts. pVAI (500 ng) was preincubated with 14 μg of M-N_2O extract (M; see legend to Fig. 1) and 0 (lane 1), 90 (lane 2), or 720 (lane 3) ng of immunoaffinity-purified TFIIIB. Nucleotides were then added to assay transcription.

TBP-associated components of TFIIIB have reduced activity in mitotic extracts. Apart from its TBP subunit, TFIIIB is stable to heating at 47°C for 15 min (43, 55, 57). This property can be exploited to devise a complementation assay for measuring the activity of the non-TBP components of the complex. Heating an extract in this way inactivates endogenous TBP and TFIIIC (23, 43, 55, 57). The activity of the non-TBP components of TFIIIB can then be assayed by adding the heated extract to a complementation system containing TBP, TFIIIC, and Pol III but no TFIIIB (53). Figure 9A shows that heat-treated Mono Q-purified TFIIIB is able to reconstitute transcription in such a system (lanes 1 and 2). Furthermore, asynchronous extracts are substantially more active in this assay than mitotic extracts (lanes 3 and 4). These results suggest that one or more of the heat-stable components of TFIIIB are deficient in mitotic cells. Since the levels of Pol II basal transcription (Fig. 2D), Pol III enzyme activity (Table 1), and TBP (Fig. 8A) change little at mitosis, the reduced activity of heat-stable TFIIIB components is a specific event.

We have shown previously that TBP-associated subunits of TFIIIB can be purified from a PC-B fraction by affinity chromatography on a column containing immobilized TBP (53). Such fractions, which we refer to as B-TAF fractions, can reconstitute VA_I transcription when added to a mixture of recombinant TBP and partially purified TFIIIC and Pol III

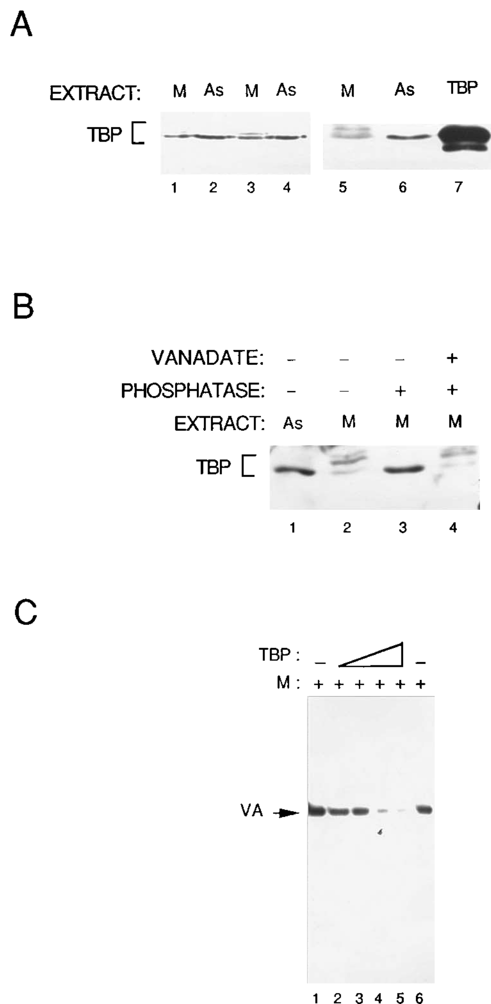

FIG. 8. TBP becomes phosphorylated at mitosis. (A) Mitotic (M) and asynchronous (As) extracts contain similar amounts of TBP. Twenty-five micrograms of M (lanes 1, 3, and 5) or As (lanes 2, 4, and 6) extract and 40 ng of recombinant TBP (lane 7) were resolved on an SDS–15% polyacrylamide gel and then analyzed by Western immunoblotting with anti-TBP antibodies 16E8 and 58C9. Three different matched pairs of extracts are shown. Those in lanes 5 and 6 were prepared in the presence of NaF, whereas those in lanes 1 to 4 were not. (B) The altered electrophoretic mobility of TBP at mitosis is due to phosphorylation. Thirteen micrograms of As (lane 1) or M-N_2O (lanes 2–4) extract was incubated for 1 h at 37°C in either the presence (lanes 3 and 4) or absence (lanes 1 and 2) of 6 U of calf intestinal alkaline phosphatase. The reaction in lane 4 also included 1 mM sodium vanadate to inhibit phosphatase activity. Samples were then resolved on an SDS–12% polyacrylamide gel and analyzed by Western immunoblotting with antibodies 16E8 and 58C9. (C) TBP is not limiting for VA_I transcription in mitotic extracts. Five hundred nanograms of pVAI was preincubated with 14 μg of M-N_2O extract (M; see legend to Fig. 1) and 0 (lanes 1 and 6), 2.5 (lane 2), 5 (lane 3), 10 (lane 4), or 20 (lane 5) ng of recombinant TBP. Nucleotides were then added to assay transcription.

(Fig. 9B). We used these B-TAF fractions to test whether a TBP-associated factor is limiting for Pol III transcription in mitotic extracts. Addition of the TBP affinity-purified B-TAFs results in a marked increase in VA_I expression (Fig. 9C, lanes 2 to 4). Three different independently prepared B-TAF preparations were found to have this effect (50). As a control, we prepared a fraction in the same way, using a column with no bound TBP. This CON-TAF fraction produced no stimulation of VA_I transcription when added to mitotic extracts but instead had a slightly inhibitory effect (Fig. 9C, lanes 5 to 7). These add-back experiments therefore demonstrate that a TBP-associated component of TFIIIB is limiting for Pol III transcription in extracts prepared from mitotic HeLa cells. Furthermore, by using affinity-purified B-TAF fractions it is possible to raise transcription in mitotic extracts to levels approaching those in asynchronous extracts (Fig. 9C; compare lanes 1 and 4). This finding suggests that the regulation of TBP-associated components of TFIIIB may be sufficient to account for the overall difference in Pol III transcription between asynchronous and mitotic extracts.

DISCUSSION

We have found that mitotic repression of Pol III transcription can be reproduced by using extracts prepared from synchronized HeLa cells. We used this system to investigate molecular changes in the transcription machinery that occur during M phase. We find that the balance of active Pol III factors changes during the cell cycle. In interphase, TFIIIC activity limits the level of transcription of TATA-less class III genes. However, during mitosis, TFIIIC is no longer limiting because of a specific decrease in TFIIIB activity. TFIIIB is required for transcription of all class III genes (reviewed in references 51 and 59). Its inactivation can therefore bring about a coordinate repression of Pol III transcription. TFIIIB activity is also regulated in response to the growth state of Ehrlich ascites cells (47), the differentiation state of F9 embryonal carcinoma cells (58), and tetradecanoylphorbol acetate stimulation of *Drosophila* Schneider cells (10a). It may therefore serve as a key control point through which the transcriptional machinery can respond to changes in the cellular environment.

TFIIIB is a multisubunit complex comprising TBP and at least two associated components (reviewed in references 16 and 34). TBP itself is involved in transcription by all three nuclear RNA polymerases (3, 39). As such, it would provide an ideal target for coordinately repressing the activities of Pol I, Pol II, and Pol III, thereby achieving the universal inhibition of transcription that is observed in mitotic cells. Although we find that TBP becomes hyperphosphorylated specifically at mitosis, we have so far been unable to ascribe any functional consequences to this phosphorylation. Mitotic extracts are not deficient in TBP activity for Pol III transcription, as shown by TBP add-back experiments, and TATA-dependent transcription by Pol II occurs normally. The G_2-M transition is associated with a dramatic and general increase in protein phosphorylation (1, 37). Indeed, one study found an 8- to 10-fold increase in the incorporation of phosphate groups into nonhistone proteins in mitotic HeLa cells (37). Much of this may result from the mixing of nuclear proteins with cytoplasmic kinases as the nuclear membrane breaks down. As such, a significant proportion of mitotic phosphorylation events may be entirely fortuitous and without functional consequences. It may be that the mitotic phosphorylation of TBP falls into this category. However, given the pleiotropic effects of TBP upon nuclear transcription (reviewed in references 16, 34, and 54), a functional response to phosphorylation cannot be ruled out.

Add-back experiments and direct assays suggest that a heat-stable TBP-associated component of TFIIIB is inactivated at mitosis. However, we have yet to identify a specific polypeptide that is targeted. The subunit composition of human TFIIIB is far from well characterized (reviewed in references 16 and 34). Several candidate subunits have been detected by immunoprecipitation with anti-TBP antibodies (2, 25, 44), but molecular probes against these are not yet available. Immunoprecipitation requires relatively large amounts of material which must be fractionated in order to separate the various TBP-contain-

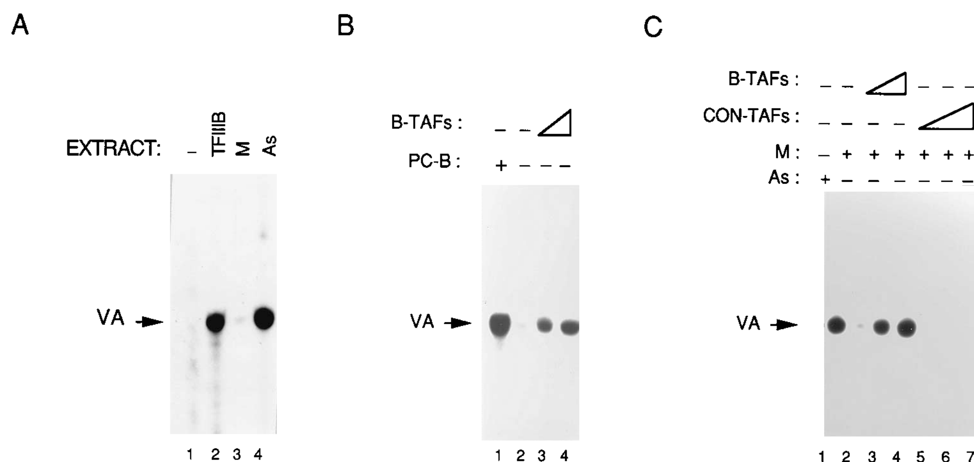

FIG. 9. The activity of a TBP-associated component of TFIIIB is deficient in mitotic extracts. (A) The heat-stable component of TFIIIB has reduced activity in mitotic extracts. pVAI (500 ng) was preincubated with 4 μl of PC-C, 5 ng of recombinant TBP, and no additional factors (lane 1) or 6 μl of heat-treated Mono Q fraction 42 (lane 2), 15 μg of heat-treated M-N_2O (M; see legend to Fig. 1) extract (lane 3), or 15 μg of heat-treated asynchronous cell (As) extract (lane 4). Nucleotides were then added to assay transcription. (B) The TBP-associated components of TFIIIB can be affinity purified by using immobilized TBP. pVAI (500 ng) was preincubated with 3 μl of PC-C, 5 ng of recombinant TBP, and 2 μl of PC-B (lane 1), no additional factors (lane 2), 1.5 μl of B-TAFs (lane 3), or 3 μl of B-TAFs (lane 4). Nucleotides were then added to assay transcription. (C) TBP-associated TFIIIB components are limiting for Pol III transcription in mitotic extracts. pVAI (500 ng) was preincubated with 14 μg of As extract (lane 1) or M-N_2O extract (lanes 2 to 7) and no additional factors (lanes 1 and 2), 1 μl (lane 3) or 3 μl (lane 4) of B-TAFs or 1 μl (lane 5), 3 μl (lane 6), or 10 μl (lane 7) of CON-TAFs. Nucleotides were then added to assay transcription.

ing complexes. It is extremely difficult to maintain good cell cycle synchrony in large populations of HeLa cells. Indeed, our best synchronization has been achieved with populations of $\sim10^7$ nitrous oxide-treated cells. Although this number is sufficient to produce an active transcription extract, it is clearly not enough for biochemical fractionation. As a consequence, the definitive identification of a specific polypeptide target for mitotic regulation of Pol III transcription may have to await the availability of antibody or DNA probes against individual subunits of TFIIIB.

Once a specific target has been determined, we shall then be able to address the mechanism of its control. We have demonstrated recently that TFIIIB activity can be negatively regulated by the repressor protein Dr1, which targets TBP (56). Several considerations suggest that Dr1 is not responsible for the mitotic repression of TFIIIB. First, Dr1 coordinately represses transcription by Pol II and Pol III (56), whereas basal Pol II transcription is unchanged in mitotic extracts (Fig. 2D). Second, elevated levels of a dominant repressor such as Dr1 were not revealed by the mixing experiments (Fig. 3). Third, repression by Dr1 can be overcome by adding TBP (20, 56), whereas the mitotic inhibition of Pol III transcription is not overcome by TBP (Fig. 8C). It therefore seems unlikely that Dr1 is responsible for the reduced activity of TFIIIB at mitosis. However, the phase-specific association of other regulatory proteins with TFIIIB remains a potential mechanism for achieving cell cycle control. Alternative mechanisms which may be used include stage-specific phosphorylation and/or turnover. Certain cyclins undergo rapid proteolysis during mitosis (8, 27–29). It is possible that one or more subunits of TFIIIB could also be subject to phase-specific turnover. Such an effect might be triggered by mitotic phosphorylation. Alternatively, phosphorylation may be sufficient in itself to neutralize the activity of TAFs. Indeed, Gottesfeld et al. (12) have shown recently that purified mitotic kinase, containing cyclin B and $p34^{cdc2}$, can inactivate TFIIIB in a fractionated *Xenopus* transcription system, although these workers did not distinguish between TBP and the other subunits. Our conclusion that TFIIIB is a target for mitotic repression coincides with theirs and suggests that this aspect of cell cycle control is conserved evolutionarily.

We have, however, been unable to confirm that TFIIIB is a direct target of cyclin B-associated $p34^{cdc2}$ in humans. We have prepared this kinase from mitotic extracts by using either anti-cyclin B1 antibodies or $p13^{suc1}$-agarose beads. Although both preparations phosphorylated histone H1 actively, neither repressed Pol III transcription when preincubated with purified factors (52). Direct inactivation by $p34^{cdc2}$ is also inconsistent with the fact that transcription in mitotic human extracts can be stimulated by the addition of purified TFIIIB or B-TAF fractions (Fig. 4 to 6 and 9). These extracts contain active mitotic kinases, as shown by histone H1 phosphorylation assays (Fig. 1B), but they do not inactivate added TFIIIB. The data suggest that TFIIIB is not a direct target of cyclin B-associated $p34^{cdc2}$ in HeLa cells. Indeed, $p34^{cdc2}$ and related kinases can be removed from mitotic *Xenopus* egg extracts without any effect on the repression of Pol III transcription (15). Thus, after its primary role in establishing the mitotic state, $p34^{cdc2}$ may not be required directly for inactivating TFIIIB.

Genes that are transcribed during interphase become repressed as cells enter M phase. As such, the mechanism of inhibition is clearly dominant. However, our mitotic extracts do not contain a dominant repressor of Pol III transcription. This is shown clearly both by mixing experiments (Fig. 3) and by the fact that purified TFIIIB or B-TAF fractions can activate transcription when added to these extracts (Fig. 4 to 6 and 9). These observations suggest that once TFIIIB has been inactivated at the onset of mitosis, the continued presence of an inhibitor is not required to maintain repression. Cells harvested after the beginning of M phase would therefore not contain any dominant inhibitory activity. Consistent with this interpretation, Hartl et al. (15) reported that once $p34^{cdc2}$ kinase has fully converted frog extracts into a mitotic state, transcriptional repression is maintained even if kinase activity is blocked by using dimethylaminopurine. Thus, TFIIIB inactivation at mitosis appears to be a “hit-and-run” event.

The Pol II TAFs in the TFIID complex are believed to be involved in cell cycle control. Thus, genetic and biochemical

data have implicated human $TAF_{II}250$ in regulating the expression of class II genes required for progression through G_1 phase (17, 36, 41, 42, 49). Our data suggest that a TBP-associated component of TFIIIB is involved in repressing Pol III transcription during M phase. Although it is not yet clear that this is a tightly bound TAF subunit of TFIIIB, this is certainly a distinct possibility. Given this precedent, it seems plausible that TFIID TAFs may be involved in mediating mitotic repression of activator-dependent Pol II transcription and that SL1 TAFs may perform a similar function for Pol I. This would provide a unifying mechanism of control, despite using class-specific components of the TBP-containing complexes.

The decreased activity of the Pol III machinery observed in vitro may not be sufficient to account for the apparently absolute silencing of gene expression that occurs during M phase in vivo. It is likely that additional levels of regulation are involved in mitotic repression in the cell. The most obvious of these is the condensation of chromatin into compacted metaphase chromosomes. Class III genes that have been assembled into highly condensed solenoid-like structures are incapable of supporting significant levels of transcription (13). The inactivation of specific transcription factors such as TFIIIB may combine with the masking of genetic templates in compacted chromosomes to produce an efficient repression of gene expression at mitosis.

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