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Cell Cycle Regulation of RNA Polymerase III Transcription

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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Inactivation of the TATA-binding protein-containing complex TFIIIB contributes to the mitotic repression of RNA polymerase III transcription, both in frogs and in humans (J. M. Gottesfeld, V. J. Wolf, T. Dang, D. J. Forbes, and P. Hartl, Science 263:81–84, 1994; R. J. White, T. M. Gottlieb, C. S. Downes, and S. P. Jackson, Mol. Cell. Biol. 15:1983–1992, 1995). Using extracts of synchronized proliferating HeLa cells, we show that TFIIIB activity remains low during the early part of G_1 phase and increases only gradually as cells approach S phase. As a result, the transcription of all class III genes tested is significantly less active in early G_1 than it is in S or G_2 phase, both in vitro and in vivo. The increased activity of TFIIIB as cells progress through interphase appears to be due to changes in the TATA-binding protein-associated components of this complex. The data suggest that TFIIIB is an important target for the cell cycle regulation of RNA polymerase III transcription during both mitosis and interphase of actively proliferating HeLa cells.

The mitotic state is accompanied by a generalized cessation of nuclear gene expression (4–6, 26, 34, 35). In the case of RNA polymerase III (Pol III), this inhibition is associated with the specific inactivation of TFIIIB, a general transcription factor that is required for the expression of all class III genes (7, 40). Although human TFIIIB is known to be a multisubunit complex which contains the TATA-binding protein (TBP) (3, 22, 31, 33, 41, 42), its precise molecular architecture has yet to be established (reviewed in references 10, 27, and 39).

Gottesfeld et al. (7) found that fractionated *Xenopus* TFIIIB can be inactivated by incubation with affinity-purified mitotic kinase containing $p34^{cdc2}$ and cyclin B. This could account for the ability of recombinant cyclin B to cause a general repression of Pol III transcription when added to extracts of *Xenopus* eggs (9). We found that the activity of human TFIIIB also decreases substantially when proliferating HeLa cells pass through M phase (40). Thus, inactivation of TFIIIB may be an evolutionarily conserved mechanism for inhibiting Pol III transcription at mitosis. Although TBP is hyperphosphorylated in extracts of mitotic HeLa cells, adding unphosphorylated recombinant TBP to such extracts does not restore transcription (40). In contrast, affinity-purified fractions containing the TBP-associated components of TFIIIB can fully restore transcription to levels present in extracts of asynchronous cells (40). These observations imply that Pol III transcription in extracts of mitotic HeLa cells is restricted by a specific deficiency in the activity of one or more of the TBP-associated components of the TFIIIB complex. Such a deficiency is likely to contribute to the coordinate repression of Pol III transcription that occurs during mitosis.

Here we extend our investigation of the cell cycle regulation of Pol III transcription in proliferating HeLa cells to include the G_1, S, and G_2 phases. Unexpectedly, we find that Pol III activity remains low for some time after mitosis is finished. It increases gradually as G_1 progresses and reaches maximal levels during S and G_2 phases. Thus, extracts prepared from S or G_2 phase cells transcribe class III genes much more actively than do extracts prepared from early G_1 phase cells. Furthermore, the rate of Pol III transcription in vivo is greater during S and G_2 phases than it is during early G_1 phase. As with mitotic extracts (40), the low Pol III activity of G_1 phase extracts reflects a specific inactivation of one or more of the non-TBP components of TFIIIB. Transcription in G_1 phase extracts can be stimulated with affinity-purified fractions containing the TBP-associated constituents of TFIIIB. The same fractions have little or no effect when added to S or G_2 phase extracts. These results suggest that TBP-associated components of TFIIIB limit Pol III transcription during the early part of G_1 phase. By S and G_2 phases, this restriction has been removed and class III genes are expressed more actively.

MATERIALS AND METHODS

Plasmids and oligonucleotides. All the DNA templates have been described previously (20, 40, 46). The oligonucleotides were described by White et al. (43, 47).

Tissue culture. HeLa cells were maintained in log phase by passaging 1:6 every 3 days in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. A double-thymidine-arrest protocol was used to prepare S phase populations. For the first block, cells from a growing culture were maintained in 2 mM thymidine for 17 h and accumulated anywhere in S phase. The thymidine was removed, and the cells were incubated for 9 h to allow exit from S phase. Thymidine (2 mM) was then added again for 15 h, causing cells to accumulate near the G_1/S boundary. The cells were harvested 2 h after release from the second block, to give populations that were highly enriched in S phase. G_2 populations were obtained by reversing a double thymidine arrest and then incubating for 6 to 8 h until the majority of cells were in G_2 phase. After release of cells from the second thymidine block, nocodazole was added (0.04 μg/ml) to arrest in metaphase the most rapidly cycling cells, which would otherwise reach mitosis and pass into G_1. Plates were shaken to detach any mitotic cells, which were removed, leaving populations highly enriched in G_2 phase. To prepare mitotic populations, cells were first subjected to thymidine arrest to increase the subsequent mitotic yield. At 6 h after release from this block, cells were treated with nitrous oxide delivered at 80 lb/in^2 in a pressure vessel. Nitrous oxide causes much less microtubule disruption than do traditional mitotic arresting agents such as colcemid, but it induces metaphase arrest because the spindle fails to engage the condensed chromosomes correctly (1, 16). After 9 h, the cells were removed from the vessel. The majority were rounded up, with mitotic indices of at least 90%. Early G_1 phase cells were obtained by reversal of mitotic arrest and collection 2 h later. Any contaminating mitotic cells were removed by shaking, leaving fairly pure populations of early G_1 phase cells attached to the dish. Populations were examined microscopically as Cytospin preparations and also by flow cytometry, to verify that efficient synchronization had taken place.

* Corresponding author. Present address for Robert J. White: Institute of Biomedical and Life Sciences, Division of Biochemistry and Molecular Biology, University of Glasgow, Glasgow G12 8QQ, United Kingdom. Mailing address for Stephen P. Jackson: Wellcome/CRC Institute, University of Cambridge, Tennis Court Rd., Cambridge CB2 1QR, United Kingdom. Phone: 01223-334103. Fax: 01223-334089.

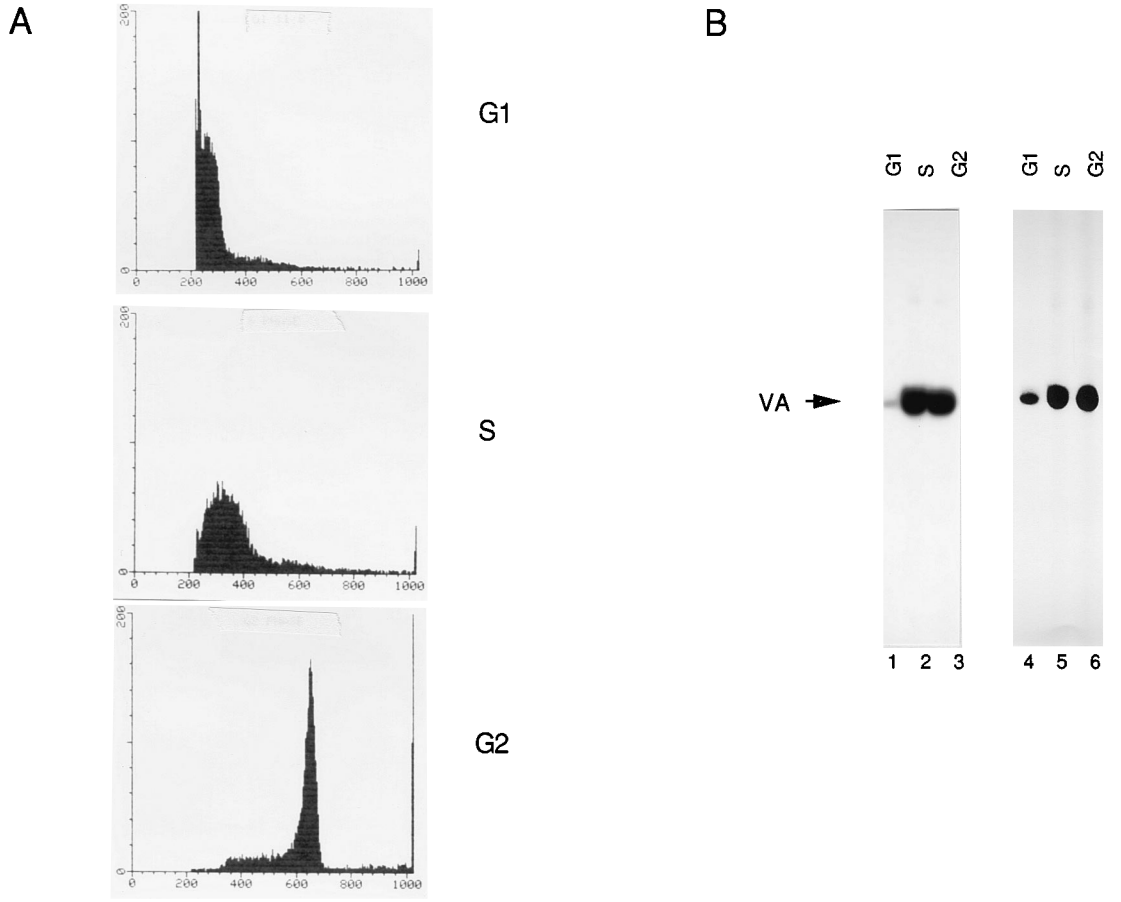

FIG. 1. Extracts of HeLa cells harvested in early G_1 phase have lower Pol III activity than do extracts of cells synchronized in S or G_2 phase. (A) Representative graphs from flow-cytometric analyses showing the relative DNA contents of cells synchronized in G_1, S, and G_2 phases. (B) Extracts of G_1 phase cells transcribe the VA_I gene less actively than do extracts of S or G_2 phase cells. Shown is transcription of pVA_I (500 ng) preincubated with 14 μg of extract prepared from G_1 (lanes 1 and 4), S (lanes 2 and 5), or G_2 (lanes 3 and 6) phase cells. The extract used in lane 1 was prepared from G_1 phase cells harvested 2 h after nitrous oxide treatment. The extracts used in lanes 2 to 6 were prepared from cells that were synchronized without nitrous oxide treatment.

Preparation of extracts and protein fractions. Whole-cell extracts were prepared as described previously (40). All the transcription factor fractions used in this study were the same as those used previously (40).

Transcription and band shift assays. In vitro transcription and band shift assays were carried out as described previously (46). Unless otherwise stated, preincubations were carried out for 15 min and transcriptions were performed for 40 min at 30°C. Quantitation was performed by PhosphorImager (Molecular Dynamics) analysis.

H1 kinase assay. H1 kinase assays were carried out as described previously (40). The $p34^{cdc2}$-cyclin B kinase was prepared by immunoprecipitation from extracted mitotic cells with an antibody against human cyclin B1, generously provided by J. Pines.

Nuclear run-on assays. Nuclear run-on assays were carried out by the method of Wright et al. (49). Quantitation was performed by PhosphorImager analysis.

Polymerase assays and Western blotting. Random RNA polymerization and Western immunoblot assays were carried out as described previously (40). 16E8 and 58C9 are monoclonal antibodies that recognize the C-terminal region of TBP. These were generous gifts from R. Weinzierl and R. Tjian. MTBP-6 is a monoclonal antibody that recognizes the N-terminal region of TBP, and was a generous gift from J. Flint.

RESULTS

Extracts of HeLa cells synchronized in G_1 phase are less active for Pol III transcription than are extracts of S or G_2 phase cells. Exponentially growing HeLa cells were synchronized near the G_1/S boundary by using a double thymidine block (16). They were then harvested 2, 7, or 10 h after release from this block, to provide populations of cells in S, G_2, and G_1, respectively. Flow-cytometric analyses revealed that the degree of synchrony of these populations decreased with time following release from thymidine (8). We therefore used a second blocking procedure to prepare more-synchronous populations of early G_1 phase cells. After release from thymidine, these were arrested in mitosis by treatment with nitrous oxide (16). They were then removed from the nitrous oxide and collected 2 h later in early G_1 phase. Flow-cytometric analyses of the DNA content of harvested cells revealed that efficient synchronization had taken place (Fig. 1A).

Whole-cell extracts were prepared from these populations of synchronized cells and tested for their ability to support Pol III transcription of the adenovirus VA_I gene (Fig. 1B). Whereas extracts of S and G_2 phase cells transcribed the VA_I gene efficiently, less active transcription was obtained with equal amounts of G_1 phase extracts. This was the case both with extracts prepared from nitrous oxide-treated G_1 phase cells (Fig. 1B, lanes 1 to 3) and with extracts of the less synchronous G_1 phase cells that were harvested without nitrous oxide treatment (Fig. 1B, lanes 4 to 6). These results were extremely reproducible. Six different matched sets of extracts were prepared from synchronized cells. In every case, the S and G_2 phase extracts gave similar levels of transcription to each other and were three- to sixfold more active than were the extracts of early G_1 phase cells (Table 1).

TABLE 1. Relative levels of specific transcription of class III genes determined by using extracts of G_1-, S-, and G_2-phase cells

Extract	Template[a]	S/G_1 ratio	G_2/G_1 ratio
Set 1	VA	3.1	4.9
Set 2	VA	3.2	4.1
Set 3	VA	6.2	5.4
Set 4	VA	3.1	4.2
Set 5	VA	4.3	6.2
Set 6	VA	4.4	4.0
Set 1	5S	3.7	6.3
Set 2	$tRNA^{Leu}$	3.9	4.4
Set 3	$tRNA^{Leu}$	4.7	4.7
Set 3	$tRNA^{Glu6}$	4.4	3.4
Set 2	U6	4.1	2.6

[a] VA, pVA_I; 5S, pXbs1; $tRNA^{Leu}$, pLeu; $tRNA^{Glu6}$, pGlu6. U6, pU6/Hae/RA.2.

We found previously that extracts of mitotic HeLa cells are three- to sevenfold less active in VA_I transcription than are extracts prepared from asynchronous cells (40). The magnitude of this difference is similar to that observed when early G_1 phase extracts are compared with S or G_2 phase extracts. We therefore prepared extracts, in parallel, from asynchronous cells and cells synchronized in early G_1, S, G_2, and M phases to compare directly their transcriptional capacities. We found that extracts of S or G_2 phase cells transcribe the VA_I gene as efficiently as does an extract of asynchronous cells, whereas the lower activity of an early G_1 phase extract is similar to that of an M phase extract. Similar results were obtained with 5S rRNA, tRNA, and U6 small nuclear RNA genes as templates (Fig. 2A; Table 1). This combination provides representatives of each of the three promoter types used by Pol III (reviewed in references 39 and 48).

Although early G_1 phase extracts have comparable Pol III activity to extracts prepared from mitotic cells, the flow-cytometric analyses demonstrate clearly that our early G_1 phase cell populations have the 2N DNA content characteristic of cells in G_1 phase (Fig. 1A). As an additional check, we tested the various extracts for their ability to phosphorylate histone H1 (Fig. 2B). As expected, mitotic extracts displayed highly elevated histone H1 kinase activity, reflecting the presence of active $p34^{cdc2}$ mitotic kinase. In contrast, early G_1 phase extracts phosphorylated histone H1 only weakly, at a level that was similar to that produced by the S and G_2 phase extracts and characteristic of interphase cells. This highly sensitive assay therefore confirms that the overwhelming majority of the cells in our early G_1 phase populations have left mitosis at the time of harvesting.

We carried out a time course experiment to assess the activity of the Pol III transcriptional apparatus as G_1 phase progresses. Following removal from metaphase arrest, cells were harvested either immediately while still mitotic or 2, 4, or 6 h later as they progressed through G_1 phase. Whole-cell extracts prepared from these populations were compared with an extract of S phase cells made in parallel (Fig. 3). As before, extracts of cells harvested in early G_1, 2 h after reversal of the metaphase block, transcribe VA_I no more efficiently than do extracts of mitotic cells. In contrast, extracts of cells harvested 4 h after release display a significant increase in VA_I transcription. A further increase occurs by 6 h after release from mitosis. However, even these late G_1 phase extracts were less active than S phase extracts. It therefore appears that the activity of the Pol III transcription machinery recovers slowly from mitosis and increases only gradually as cells progress through G_1 phase.

Endogenous Pol III templates in intact nuclei are transcribed less actively in early G_1 phase than in S or G_2 phase. The results above suggest that the Pol III transcription apparatus is less active in G_1 than in S or G_2 phase cells. We carried out nuclear run-on assays to discover whether the levels of expression of endogenous class III genes reflect these changes in the activity of Pol III factors. Using equal numbers of intact nuclei from synchronized HeLa cells, we found that each of the Pol III templates tested was transcribed less actively during early G_1 phase than it was during S and G_2 phases (Fig. 4A). After correction for nonspecific background hybridization to vector sequences, the transcription of endogenous U6 genes

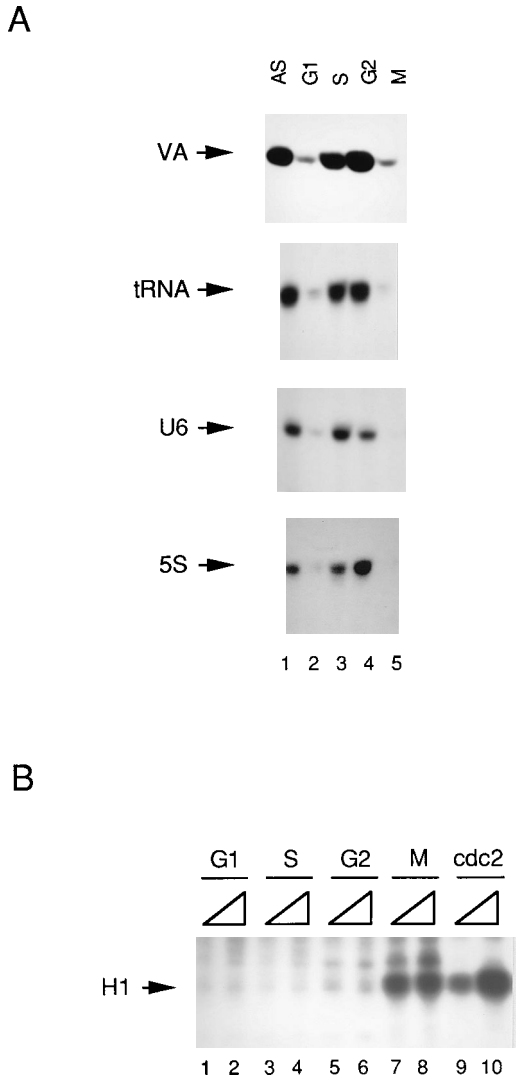

FIG. 2. Extracts of HeLa cells harvested in M phase or early G_1 phase have much less Pol III activity than do extracts prepared from asynchronous, S phase, or G_2 phase cells. (A) With all Pol III templates tested, extracts of HeLa cells harvested in M or early G_1 phase are much less active than are extracts prepared from asynchronous, S, or G_2 phase cells. Shown is transcription of pVA_I (VA), pLeu (tRNA), pU6/Hae/RA.2 (U6), and pXbs1 (5S) templates (500 ng) preincubated with 14 μg of extract from either asynchronous (lane 1), G_1 phase (lane 2), S phase (lane 3), G_2 phase (lane 4), or mitotic (lane 5) cells. (B) Mitotic kinase activity is absent from interphase extracts. Histone H1 was incubated for 10 min at 30°C in the presence of [γ-^{32}P]ATP and either 7 μg (lanes 1, 3, 5, and 7) or 21 μg (lanes 2, 4, 6, and 8) of G_1 phase (lanes 1 and 2), S phase (lanes 3 and 4), G_2 phase (lanes 5 and 6), or M phase (lanes 7 and 8) extract or with 1 or 2 μl of cyclin B-$p34^{cdc2}$ kinase (lanes 9 and 10, respectively).

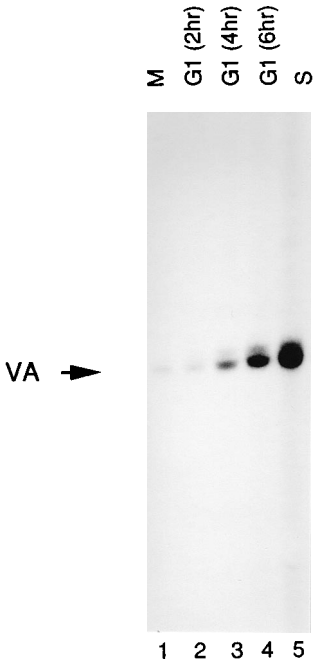

FIG. 3. Pol III transcriptional activity of extracts prepared from cells harvested at different times during passage from M to S phase. Shown is transcription of pVA_I (250 ng) preincubated with 7 μg of extract prepared from M (lane 1), G_1 phase (lanes 2 to 4), or S phase (lane 5) cells. The extracts in lanes 2, 3, and 4 were prepared from cells harvested 2, 4, or 6 h, respectively, after release from nitrous oxide.

was approximately three times more active in S and G_2 phase nuclei than in early G_1 phase nuclei, whereas two different endogenous tRNA genes were transcribed approximately twice as actively in S and G_2 as they were during early G_1. In contrast, transcription of a Pol II template control, the gene encoding Sp1, showed little variation between G_1, S, and G_2 phases. The synchrony of the cells was confirmed by flow cytometry (Fig. 4B). Similar results were obtained with a second batch of independently prepared cells (8). The data suggest that the transcription of Pol III templates in vivo increases as actively proliferating cells progress from early G_1 phase into S and G_2 phases.

The low transcription of class III genes in extracts of early G_1 phase HeLa cells is not due to an excess of dominant Pol III repressor. We carried out mixing experiments to test whether G_1 phase extracts contain an excess of dominant repressor. Adding increasing amounts of early G_1 phase extract to a constant amount of S phase extract caused no decrease in Pol III transcription (Fig. 5). Indeed, a mixture of the two types of extract gave higher expression than the sum of the levels obtained with each extract individually. Similar results were obtained on mixing G_1 and G_2 phase extracts (38). These results suggest that the low activity of G_1 phase extracts is not due to a dominant Pol III inhibitor that is present in stoichiometric excess relative to the general transcription factors.

Extracts of early G_1 phase cells are specifically deficient in TFIIIB activity. Because early G_1 phase cells appear not to contain an excess of dominant repressor, the low expression of class III genes during this part of the cell cycle may instead be due to a deficiency in some positive activity that is required for Pol III transcription. Since every Pol III template we tested shows the same pattern of cell cycle regulation, the positive activity that may be low during G_1 phase is likely to be either Pol III itself or one of the general factors that is required by it. We therefore assayed these components.

To determine the activity of the Pol III enzyme, we measured its ability to catalyze randomly initiated RNA synthesis independently of transcription factors, using a poly(dA-dT) template. No consistent difference in the level of random polymerization by Pol III was observed between G_1, S, and G_2 phase extracts (Table 2). We therefore turned our attention to the transcription factors that are required for sequence-specific initiation by Pol III.

A B-block internal promoter element was used as the probe in a band shift assay to compare the promoter-binding activity of TFIIIC during different phases of the cell cycle. That the complex detected in these synchronized cell extracts is indeed due to TFIIIC is indicated by two lines of evidence. First, this complex comigrates with a complex formed by affinity-purified

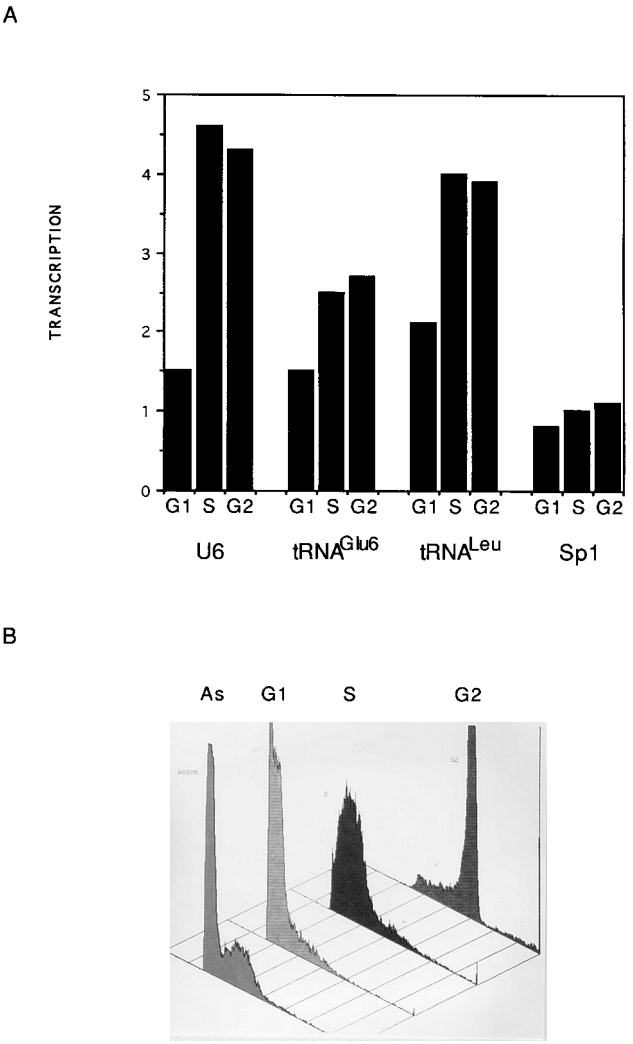

FIG. 4. Class III genes are transcribed less actively in intact nuclei of early G_1 phase cells than they are in nuclei of S or G_2 phase cells. (A) Graph displaying transcription levels of endogenous genes in nuclei of synchronized cells. Radiolabelled nuclear transcripts from equal numbers of cells synchronized in early G_1, S or G_2 phase were hybridized to nitrocellulose filters containing immobilized denatured plasmids. Transcription levels were determined by PhosphorImager quantitation and are displayed in arbitrary units after subtraction of background hybridization to vector DNA. (B) Graph displaying the results of flow-cytometric analysis of the relative DNA contents of cells used in this nuclear run-on assay.

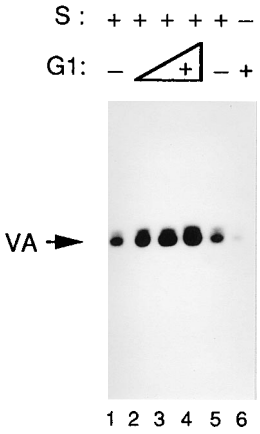

FIG. 5. Early G_1 phase extracts do not contain an excess of dominant repressor of Pol III transcription that is active when mixed with S phase extracts. Shown is transcription of pVA_I (500 ng) preincubated with 14 μg of S phase extract (lanes 1 to 5) and no additional extract (lanes 1 and 5) or 3.5 μg (lane 2), 7 μg (lane 3), or 14 μg (lanes 4 and 6) of early G_1 phase extract.

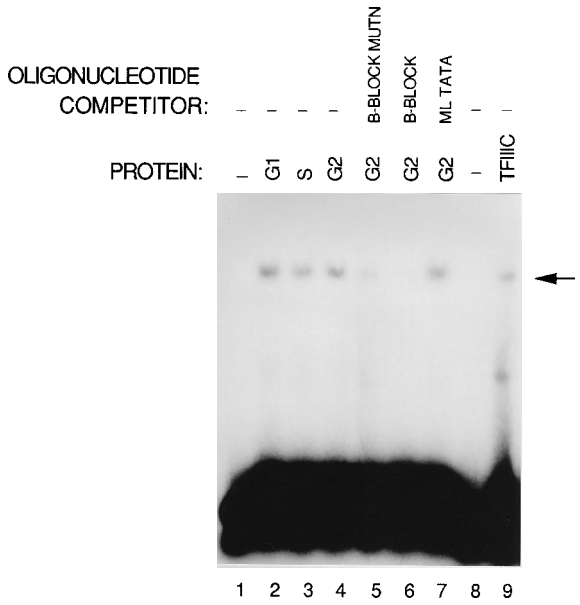

FIG. 6. Band shift assays reveal little change in the TFIIIC activity of early G_1, S, and G_2 phase cells. Reaction mixtures contained 1 ng of radiolabelled B-block oligonucleotide probe (all lanes), 1 μg (lanes 1 to 8) or 20 ng (lane 9) of poly(dI-dC), no protein (lanes 1 and 8), 25 μg of early G_1 phase (lane 2), S phase (lane 3), or G_2 phase (lanes 4 to 7) extract or 5 μl of affinity-purified TFIIIC (lane 9), and no oligonucleotide competitor (lanes 1 to 4, 8, and 9) or 100 ng of unlabelled B-block mutant (lane 5), B-block (lane 6), or TATA box (lane 7) oligonucleotide competitor. The predominant TFIIIC-containing complex is indicated by an arrow.

TFIIIC (Fig. 6, lane 9). Second, formation of this complex is inhibited efficiently by an unlabelled oligonucleotide containing a B-block sequence (lane 6) but not by an unrelated oligonucleotide (lane 7). Furthermore, a single base change in the B block at a residue conserved in all functional eukaryotic tRNA genes substantially reduces the ability to compete for this complex (lane 5). Comparison of lanes 2 to 4 suggests that the DNA-binding activity of TFIIIC is similar in extracts of G_1, S, and G_2 phase cells.

TFIIIB is required for the transcription of all class III genes (reviewed in references 39 and 48). Insufficient TFIIIB activity during early G_1 phase could therefore account for the observed regulation of all Pol III templates tested. Because an essential component of TFIIIB is TBP (2, 3, 18, 22, 31, 33, 41; reviewed in references 10, 27, and 39), we compared by Western blotting the levels of TBP at different phases of the cell cycle. Little or no variation was observed in the abundance of TBP (Fig. 7A). As described previously (40), we found that TBP is hyperphosphorylated during mitosis but that this modification has been removed by early G_1 phase and so cannot account for the observed regulation during interphase. Although we cannot exclude the possibility that the TBP content of individual complexes varies during the cell cycle, the overall level of this factor appears not to change appreciably.

TABLE 2. Extracts of G_1, S, and G_2 phase cells contain similar levels of Pol III polymerization activity

Extract	Pol III sp act (cpm)
Buffer	1,050
Extract set 1	
G_1	28,000
S	26,900
G_2	25,500
Extract set 2	
G_1	38,300
S	52,700
G_2	41,500
Extract set 3	
G_1	41,200
S	51,400
G_2	54,000

With the exception of TBP, the various components of TFIIIB are stable to heating at 47°C for 15 min (32, 40, 41, 43, 45). This property can be exploited in a complementation assay for measuring the activity of the non-TBP components of the TFIIIB complex (40, 41). Heating an extract in this way inactivates endogenous TBP and TFIIIC (19, 32, 43, 45). The activity of the non-TBP components of TFIIIB can therefore be assayed by adding the heated extract to a complementation system containing TBP, TFIIIC, and Pol III but no TFIIIB (40, 41). Figure 7B shows that transcription in such a system can be reconstituted efficiently with heat-treated TFIIIB that has been partially purified from asynchronous cells (lanes 1 and 2). When compared in this assay, extracts of S or G_2 phase cells were found to be six- to eightfold more active than extracts of early G_1 phase cells (lanes 3 to 5). These results suggest that one or more of the heat-stable components of TFIIIB increase in activity as HeLa cells pass from early G_1 into S and G_2. Since the levels of Pol III enzyme activity (Table 2), TFIIIC (Fig. 6), and TBP (Fig. 7A) appear to change little during interphase, this regulation of heat-stable TFIIIB components is a specific event. Furthermore, the magnitude of this regulation is sufficient to account for the observed increase in Pol III transcription.

If a lack of active TFIIIB components is responsible for the relatively low levels of class III gene expression during early G_1, the addition of TFIIIB to G_1 phase extracts should stimulate Pol III transcription. This proved to be the case. TFIIIB that had been partially purified by step fractionation on phosphocellulose followed by gradient chromatography on Mono Q strongly stimulated VA_I transcription when added to extracts

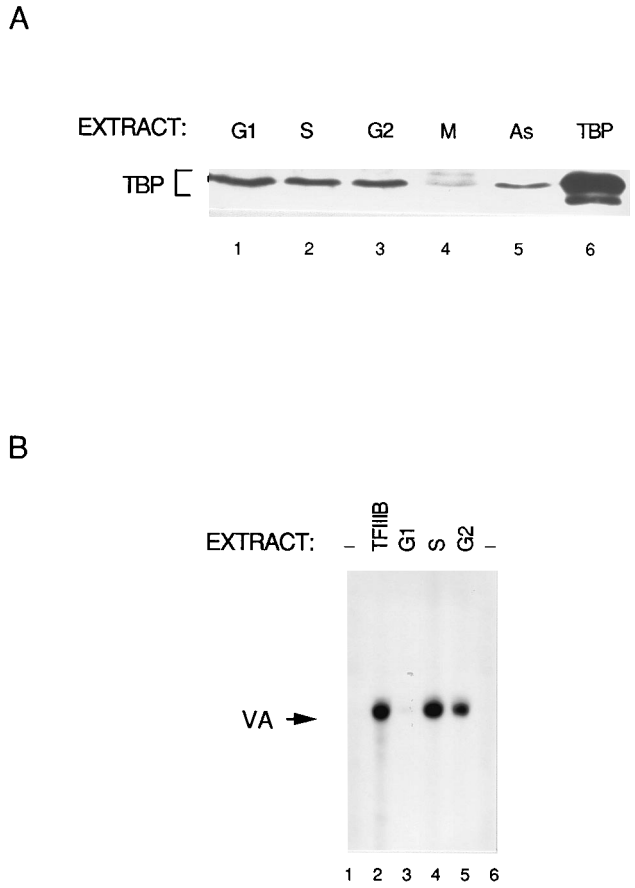

FIG. 7. Early G_1 phase extracts contain normal levels of TBP but are deficient in the activity of a heat-stable component of TFIIIB. (A) The abundance of TBP varies little during the cell cycle. A 25-μg sample of early G_1 phase (lane 1), S phase (lane 2), G_2 phase (lane 3), mitotic (lane 4), or asynchronous (As) (lane 5) cell extract and 40 ng of recombinant TBP (lane 6) were resolved on a sodium dodecyl sulfate–15% polyacrylamide gel and then analyzed by Western immunoblotting with anti-TBP antibodies 16E8 and 58C9. (B) The heat-stable component of TFIIIB has reduced activity in early G_1 phase extracts. Shown is transcription of pVA_I (500 ng) preincubated with 4 μl of PC-C, 5 ng of recombinant TBP, and no additional factors (lanes 1 and 6) or 6 μl of heat-treated Mono Q-fractionated TFIIIB (lane 2), 7.5 μg of heat-treated early G_1 phase extract (lane 3), 7.5 μg of heat-treated S phase extract (lane 4), or 7.5 μg of heat-treated G_2 phase extract (lane 5).

of early G_1 phase cells (Fig. 8A, lanes 1 to 4). This was a specific effect, since a TFIIIC fraction caused no stimulation in the same assay (Fig. 8A, lanes 5 to 9). Fractions containing Pol III or TFIIA also failed to activate transcription when added to these extracts (38). To provide evidence that the observed stimulation is indeed due to TFIIIB and not some copurifying contaminant, we used an anti-TBP antibody to immunodeplete TFIIIB from an activating fraction. Following immunodepletion with anti-TBP antibody, the ability of the fraction to increase transcription in a G_1 phase extract was severely diminished relative to that of a mock-immunodepleted control fraction (Fig. 8B). Thus, TBP and/or its associated factors are responsible for the ability of TFIIIB fractions to activate VA_I transcription in early G_1 phase extracts. We conclude that TFIIIB is the limiting component of the Pol III transcription apparatus during the early part of G_1. Indeed, by using these TFIIIB fractions, it was possible to raise levels of transcription in early G_1 phase extracts to those occurring in S phase extracts (Fig. 8C). This suggests that all the factors required for VA_I transcription are fully active in early G_1 phase with the exception of TFIIIB.

The activity of the TBP-associated components of TFIIIB limits Pol III transcription during the early part of G_1. Since the activity of the heat-stable components of TFIIIB increases during interphase (Fig. 7B), we tested whether these components are sufficient to activate transcription in an early G_1 phase extract. Mono Q-purified TFIIIB retained its stimulatory effect following heat treatment to inactivate TBP (Fig. 9A, lanes 1 to 3). In contrast, recombinant TBP alone was unable to increase transcription (lanes 4 to 7). This suggests that G_1 phase extracts contain sufficient TBP to support active Pol III transcription but are deficient in some other component of TFIIIB.

The TBP-associated subunits of TFIIIB can be purified by affinity chromatography with immobilized TBP (40, 41). Such fractions, which we refer to as B-TAFs, were added to synchronized cell extracts to determine if Pol III transcription is limited by the availability of active TBP-associated factors. B-TAF fractions were found to increase VA_I expression in early G_1 phase extracts in a dose-dependent manner (Fig. 9B, lanes 1 to 4). As a control, we prepared a fraction in the same way except that we used a column with no immobilized TBP. This CON-TAF fraction had no stimulatory effect (Fig. 9C). The TBP affinity-purified B-TAF fractions were able to raise VA_I transcription in early G_1 phase extracts by up to fivefold, to a level that was close to that of S phase extracts (Fig. 9B). This suggests that an increase in the activity of one or more TBP-associated components of TFIIIB is sufficient to account for the rise in Pol III transcription that accompanies passage from early G_1 phase into S phase.

The identity of the limiting Pol III factor varies during the course of interphase. Whereas B-TAF fractions produce a substantial increase in VA_I expression in early G_1 phase extracts, they have little or no effect when added to S phase extracts (Fig. 9B, lanes 5 to 8). This illustrates further the specificity of the response and suggests that by S phase the activity of the TBP-associated components of TFIIIB has increased to such an extent that it is now in relative excess. In contrast, VA_I expression in S phase extracts is stimulated strongly by the addition of TFIIIC (Fig. 10A). This suggests that TFIIIC has become limiting in S phase, albeit at a much higher level of overall transcription than is seen earlier in the cell cycle. As is the case in S phase extracts, transcription in G_2 phase extracts is stimulated by the addition of TFIIIC but not B-TAF fractions (Fig. 10B). We conclude that the identity of the limiting factor changes as cells progress through interphase. This can explain why mixtures of early G_1 and S phase extracts give more than additive levels of transcription (Fig. 5), since each extract provides a surplus of the factor that is limiting in the other.

DISCUSSION

We have shown that the activity of the Pol III transcription apparatus increases as proliferating HeLa cells pass through G_1 and into S phase. This appears to result from a specific increase in the activity of TBP-associated components of TFIIIB. The activity of these components seems to limit the overall level of class III gene expression during early G_1 but subsequently increases to such an extent that by S phase it is in relative excess. TFIIIC activity becomes limiting during S and G_2. As far as we can tell, this is not due to any change in TFIIIC itself but reflects the increase in TFIIIB activity that accompanies progression through interphase. However, we cannot rule out the possibility of changes in TFIIIC. We re-

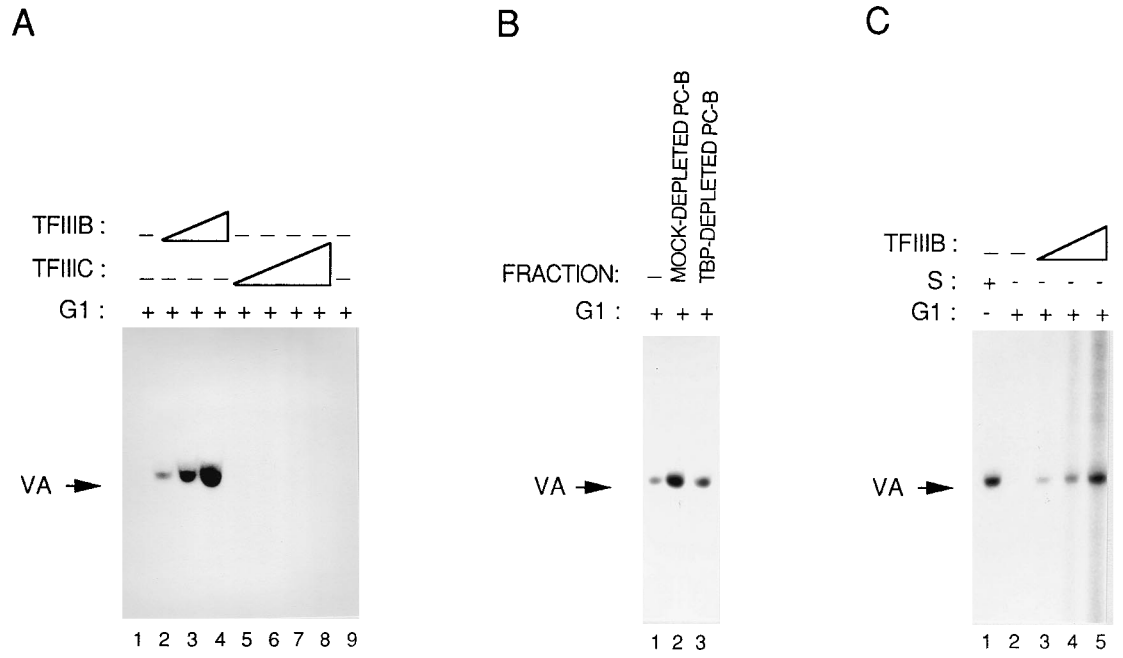

FIG. 8. TFIIIB is the limiting factor for Pol III transcription in early G_1 phase extracts. (A) A TFIIIB fraction stimulates transcription in early G_1 phase extracts, whereas a TFIIIC fraction does not. Shown is transcription of pVA_I (500 ng) preincubated with 10.8 μg of early G_1 phase extract and no additional fraction (lanes 1 and 9), 1.5, 3, or 6 μl of Mono Q-purified TFIIIB (lanes 2, 3, and 4, respectively), or 1.5, 3, 6, or 9 μl of affinity-purified TFIIIC (lanes 5, 6, 7, and 8, respectively). (B) The ability of a TFIIIB-containing fraction to stimulate transcription when added to an early G_1 phase extract is compromised by immunodepletion with anti-TBP antisera. Shown is transcription of pVA_I (500 ng) preincubated with 14.9 μg of early G_1 phase extract and no additional fraction (lane 1), 2 μl of mock-immunodepleted phosphocellulose B fraction (lane 2), or 2 μl of phosphocellulose B fraction that had been immunodepleted with the anti-TBP monoclonal antibody MTBP-6 (lane 3). (C) Mono Q-purified TFIIIB is sufficient to raise VA_I transcription in early G_1 phase extracts to the level present in S phase extracts. Shown is transcription of pVA_I (500 ng) preincubated with 14 μg of S phase (lane 1) or early G_1 phase (lanes 2 to 5) extract and no additional fraction (lanes 1 and 2) or 1.25, 2.5, or 5 μl of Mono Q-purified TFIIIB (lanes 3, 4, and 5, respectively).

ported previously that HeLa cells are deficient in TFIIIB during M phase, also because of the inactivity of TBP-associated components (40). It therefore seems likely that the same component is limiting in each case, undergoing inactivation at the start of mitosis and then recovering gradually during the course of G_1. It appears that the cell cycle of actively proliferating HeLa cells is characterized by a periodic fluctuation in the activity of TFIIIB. During mitosis and early G_1 phase, rela-

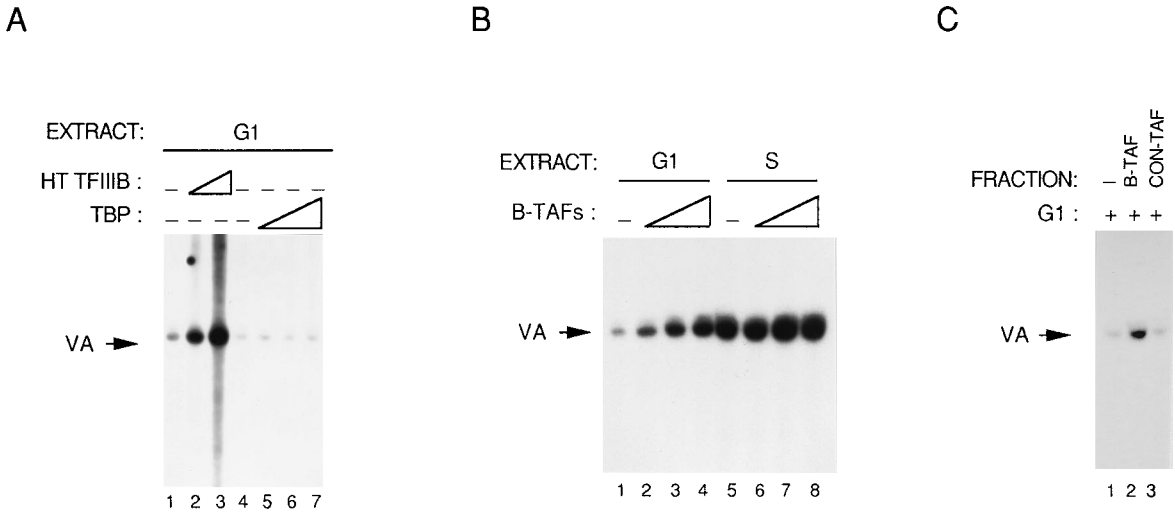

FIG. 9. The activity of a TBP-associated heat-stable component of TFIIIB is limiting for Pol III transcription in early G_1 phase extracts. (A) VA_I transcription in an early G_1 phase extract is stimulated by the addition of heat-treated Mono Q-purified TFIIIB but not by recombinant TBP. Shown is transcription of pVA_I (500 ng) preincubated with 13.2 μg of early G_1 phase extract and no additional fraction (lanes 1 and 4), 2 or 6 μl of Mono Q-purified TFIIIB that had been heated at 47°C for 15 min (lanes 2 and 3, respectively), or 5, 10, or 20 ng of recombinant TBP (lanes 5, 6, and 7, respectively). (B) TBP-associated TFIIIB components are limiting for Pol III transcription in early G_1 phase extracts but not in S phase extracts. Shown is transcription of pVA_I (500 ng) preincubated with 14 μg of early G_1 phase extract (lanes 1 to 4) or S phase extract (lanes 5 to 8) and no additional factors (lanes 1 and 5) or 3 μl (lanes 2 and 6), 6 μl (lanes 3 and 7), or 12 μl (lanes 4 and 8) of B-TAFs. (C) The ability of B-TAF fractions to stimulate Pol III transcription in early G_1 phase extracts is due to the presence of TBP-associated components. Shown is transcription of pVA_I (500 ng) preincubated with 14 μg of early G_1 phase extract and no additional factors (lane 1), 3 μl of B-TAFs (lane 2), or 3 μl of CON-TAFs (lane 3).

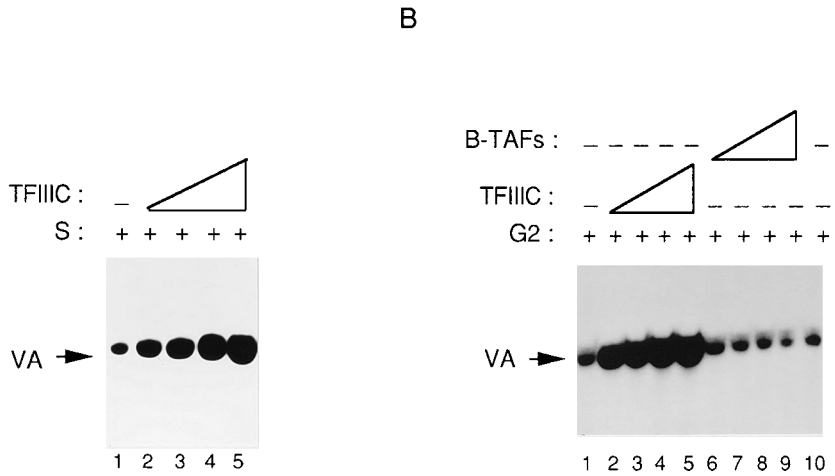

FIG. 10. TFIIIC is limiting for the transcription of class III genes in extracts of S and G_2 phase HeLa cells. (A) TFIIIC stimulates VA_I transcription in S phase extracts. Shown is transcription of pVAI (500 ng) preincubated with 14 μg of S phase extract and no added fraction (lane 1) or with 1.25 μl (lane 2), 2.5 μl (lane 3), 5 μl (lane 4), or 10 μl (lane 5) of purified TFIIIC. (B) TFIIIC stimulates VA_I transcription in G_2 phase extracts, whereas B-TAF fractions do not. Shown is transcription of pVAI (500 ng) preincubated with 9 μg of G_2 phase extract and no added fraction (lanes 1 and 10) or with 2 μl (lane 2), 4 μl (lane 3), 6 μl (lane 4), or 8 μl (lane 5) of purified TFIIIC or with 2 μl (lane 6), 4 μl (lane 7), 6 μl (lane 8), or 8 μl (lane 9) of B-TAF fraction.

tively little active TFIIIB is available and the overall capacity of the Pol III transcription apparatus is low. However, by S and G_2 phases, the activity of TFIIIB has increased substantially and class III genes are expressed at high levels.

We have yet to determine which polypeptides mediate the fluctuation in TFIIIB activity during the cell cycle. Indeed, the subunit composition of human TFIIIB remains to be established unequivocally (reviewed in references 10, 27, and 39). The complex begins to dissociate under relatively mild conditions (3, 22, 33), and this has hampered its characterization. Definitive identification of a specific polypeptide target for cell cycle regulation may require antibody or DNA probes against individual subunits of TFIIIB.

Both genetic and biochemical data have implicated a TBP-associated component of the TFIID complex, $TAF_{II}250$, as being involved in regulating class II genes that are required for passage through G_1 phase (11, 28–30, 37). Our data suggest that a TBP-associated component of TFIIIB controls the level of Pol III transcription during M and G_1 phases. Although it is not yet clear that this is a tightly bound and integral component of TFIIIB, comparable to $TAF_{II}250$ in the TFIID complex, this is certainly a possibility. This would provide a striking analogy between the two polymerase systems and their cell cycle control.

As yet, we can only speculate concerning the molecular mechanism(s) that regulates human TFIIIB during the cell cycle. In *Xenopus laevis*, TFIIIB is inactivated at mitosis through phosphorylation (7, 9). Indeed, cyclin B-associated $p34^{cdc2}$ can repress TFIIIB when added to a crude mixture of *Xenopus* factors (7). In this system, as in ours, a specific polypeptide substrate has yet to be identified, and it remains possible that $p34^{cdc2}$ inactivates TFIIIB via an intermediate factor. It seems highly probable that the cell cycle regulation of TFIIIB in humans also involves phosphorylation. If so, the restoration of TFIIIB activity may be catalyzed by a phosphatase that becomes active as G_1 progresses. Alternatively, TFIIIB might be inactivated at mitosis by a specific proteolytic event, which could itself be triggered by phosphorylation. In this case, the slow restoration of activity during G_1 might reflect the time required to synthesize and assemble new subunits. Another possibility is that repressors interact with TFIIIB during mitosis and early G_1 phase. This interaction might be controlled by phosphorylation of TFIIIB and/or the repressor. We have shown previously that TFIIIB can be inactivated by the TBP-binding protein Dr1 (44). We do not believe that Dr1 mediates the cell cycle regulation of Pol III transcription, because TBP is sufficient to overcome repression by Dr1 (14, 44), whereas it does not restore TFIIIB activity in M or G_1 phase extracts (40) (Fig. 9A). However, a repressor that binds to a TBP-associated component of TFIIIB could be involved. Mixing experiments reveal no large excess of dominant inhibitory activity in M or G_1 phase extracts (40) (Fig. 5) but do not rule out the possibility of a repressor(s) present in similar stoichiometry to TFIIIB. More than one mechanism may contribute to the cell cycle control of TFIIIB.

We have shown here that Pol III activity increases as proliferating HeLa cells progress from early G_1 into S phase. It is well known that Pol III transcription also increases when serum is added to growth-arrested G_0 phase cells (12, 15, 21, 24). Following serum stimulation of resting 3T6 fibroblasts, maximal rates of tRNA synthesis are achieved only when cells enter S phase (15, 24). The same is true of the transcription of B2 genes in serum-stimulated BHK cells (21). Thus, it appears that an increase in Pol III transcription accompanies passage into S phase whether the starting point is the G_1 phase of actively proliferating cells or the G_0 phase of growth-arrested cells. However, the mechanism of activation may differ in each case. Hoeffler et al. (12) showed that serum stimulation of HeLa cells causes an increase in the active form of TFIIIC. These workers did not measure TFIIIB activity, and so a contribution by this factor to the overall rise in transcription cannot be excluded. We find no consistent difference in the DNA-binding activity of TFIIIC when G_1, S, and G_2 phase extracts are compared (Fig. 6). Furthermore, transcription in G_1 phase extracts can be raised to S phase levels simply by adding TFIIIB (Fig. 8), which suggests that TFIIIC activity is not substantially deficient in G_1. It therefore appears that serum stimulation increases Pol III transcription by a mechanism that is not used once HeLa cells are already actively proliferating. It may be that when HeLa cells reenter the cycle, TFIIIC is activated during passage from G_0 into G_1 and then TFIIIB activity increases as they progress from G_1 into S phase. Con-

sistent with this possibility, Tower and Sollner-Webb (36) found in a murine system that both TFIIIB and TFIIIC are less active in stationary-phase cells than they are during active growth.

The overall rate of protein synthesis rises by about threefold when resting cells resume cycling (25). Activation of Pol III transcription may be required to provide sufficient tRNA and 5S rRNA to sustain the higher level of translation. Once cycling, cells are sensitive to external growth conditions only during G_1 phase (25). When the restriction point has been passed in late G_1, they are committed to replicate their DNA and then divide regardless of changes in environmental conditions (25). If the appropriate growth conditions are absent during G_1, they leave the cycle and go into G_0 (25). Since the requirement for tRNA and 5S rRNA is much lower in G_0 than in growing cells, it would be wasteful to resume high levels of synthesis as soon as mitosis is over. To defer high-level Pol III transcription until cells have made the decision between continued growth and quiescence is a far more economical strategy. Thus, Pol III activity is limited during early G_1 phase to a level that is sufficient for resting cells. Only once the decision is made to continue proliferating does the expression of class III genes increase to levels that are appropriate for sustaining active growth. This increase appears to be mediated by the regulation of TFIIIB.

The effects we have reported may not be restricted to human cells. When *Saccharomyces cerevisiae* accumulates in G_1 phase following exposure to *o*-phenanthroline, tRNA synthesis falls to 16% of the rate observed in actively cycling cells (17). In both yeast and hamster cells, genetic mutations in Pol III can cause arrest specifically in G_1 phase (13, 23). This indicates that the requirement for Pol III products or activity varies during the cell cycle. It also raises the possibility that Pol III activation is a necessary step for passage into S phase. The fact that this phenotype is obtained in both mammals and *S. cerevisiae* suggests that such regulation is of sufficient importance to have been conserved through evolution.

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