Regulation of RNA Polymerase III Transcription during Cell Cycle Entry*

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Received for publication, June 21, 2000, and in revised form, October 4, 2000
Published, JBC Papers in Press, October 9, 2000, DOI 10.1074/jbc.M005417200

Increased rates of RNA polymerase (pol) III transcription constitute a central feature of the mitogenic response, but little is known about the mechanism(s) responsible. We demonstrate that the retinoblastoma protein RB plays a major role in suppressing pol III transcription in growth-arrested fibroblasts. RB knockout cells are compromised in their ability to down-regulate pol III following serum withdrawal. RB binds and represses the pol III-specific transcription factor TFIIIB during G0 and early G1, but this interaction decreases as cells approach S phase. Full induction of pol III coincides with mid- to late G1 phase, when RB becomes phosphorylated by cyclin D- and E-dependent kinases. TFIIIB only associates with the underphosphorylated form of RB, and overexpression of cyclins D and E stimulates pol III transcription in vitro. The RB-related protein p130 also contributes to the repression of TFIIIB in growth-arrested fibroblasts. These observations provide insight into the mechanisms responsible for controlling pol III transcription during the switch between growth and quiescence.

The retinoblastoma protein RB is a highly abundant tumor suppressor that can bind and regulate a variety of transcription factors (reviewed in Refs. 1–4). One example that has been added recently to the growing list of RB-binding proteins is the RNA polymerase (pol)1 III-specific factor TFIIIB (5, 6). Recombinant RB was shown to bind to TFIIIB in vitro and repress its activity (5, 6). Furthermore, coimmunoprecipitation and cofractionation experiments demonstrated a stable association between endogenous cellular RB and TFIIIB (6). The functional significance of this interaction was shown in studies of RB knockout mice, since primary fibroblasts from Rb−/− mice display elevated TFIIIB activity relative to fibroblasts derived from their wild-type siblings (6). These results establish TFIIIB as a bona fide target for repression by RB. Similar approaches have shown that TFIIIB is also bound and repressed by the RB-related proteins p107 and p130 (7).

TFIIIB is required for the expression of all pol III templates (reviewed in Refs. 8 and 9). It serves to recruit the polymerase to a promoter and position it over the transcription start site (10). By interacting with this general factor, RB appears able to regulate the expression of all pol III-transcribed genes, including tRNA, 5 S rRNA, U6 small nuclear RNA, VAI, and Alu elements (5, 6, 11). Since a high rate of tRNA and rRNA synthesis is required to sustain rapid growth, it has been speculated that the inhibition of pol III transcription may contribute to the growth suppression capacity of RB (12–14).

RB function is regulated by cyclin-dependent kinases (reviewed in Refs. 2 and 3 and Ref. 15). The cyclin D-dependent kinases CDK4 and CDK6 phosphorylate RB partially and the process is completed by cyclin E-CDK2 (16, 17). The action of cyclin E-CDK2 appears to depend on prior phosphorylation by the cyclin D-dependent kinases (17). At least 10 serine and threonine residues can become phosphorylated in RB (2, 16). Once hyperphosphorylated, RB loses its ability to bind to many of its targets and function as a growth suppressor (2, 15). This occurs at the G1/S phase transition, in parallel with the synthesis of cyclins D and E (2, 15). The cyclin D-dependent kinases become active in mid- to late G1, at a stage called the restriction, or R, point when cells lose their serum dependence (18). Cyclin E-CDK2 is activated shortly afterward, as cells leave G1 phase (18). RB is then maintained in the hyperphosphorylated state throughout S, G2, and M phases, until it is dephosphorylated by protein phosphatase 1 at the end of mitosis (15). In cycling cells, therefore, the underphosphorylated form of RB is only present during the early period of G1. However, it is also found in resting G0 cells, which do not express significant levels of cyclins D and E (3).

The level of pol III transcription decreases significantly when growing fibroblasts are deprived of serum (19, 20). This is likely to reflect a diminished requirement for protein production. Although the switch between G0 and G1 phases is the principle determinant of proliferation rate in mammalian cells, the molecular mechanism(s) responsible for regulating pol III activity during this transition are largely uncharacterized. This constitutes an important gap in our current understanding, since the rate of pol III transcription will undoubtedly have a major influence on the growth and proliferation of cells. One study concluded that a specific reduction in TFIIIB activity was responsible for down-regulating pol III transcription in growth-arrested cells, although the molecular details were not determined (21). In contrast, another laboratory demonstrated that HeLa cells down-regulate pol III transcription when grown in low serum due to a decrease in the activity of TFIIIC2 (22, 23).
orthophosphate either in the absence of FCS, to give G0 phase cells, from repression by RB at the G1/S transition due to hyperphosphorylation of pol III activity. Only the underphosphorylated form of RB substantially diminished after cells have passed the R point. The dissociation of RB from TFIIIB coincides with an increase in pol III activity. Only the underphosphorylated form of RB associates with TFIIIB. This suggests that TFIIIB is released from repression by RB at the G1/S transition due to hyperphosphorylation of the latter by the cyclin-dependent kinases. Indeed, overexpression of cyclins D and E activates pol III transcription. We also demonstrate that RB knockout cells are compromised in their ability to down-regulate pol III following serum deprivation. In addition, the RB-related pocket protein p130 is shown to interact with TFIIIB during G0 and early G1 phases and contributes to the repression of pol III in serum-starved cells. We conclude that RB, p130, and the cyclin-dependent kinases play a major role in controlling pol III transcription during the switch between growth and quiescence.

EXPERIMENTAL PROCEDURES

Cell Culture—Balb/c 3T3 (A31), SV3T3 (C138), and mouse embryonic fibroblast cells were all grown in Dulbecco’s modified Eagle’s medium (DMEM; Life Technologies, Inc.) supplemented with 10% fetal calf serum (FCS), 100 units/ml penicillin, and 100 µg/ml streptomycin and were harvested when subconfluent. An insulin-transferrin-selenium supplement (Life Technologies) was added to the medium used to grow mouse embryonic fibroblasts. Unless otherwise specified, cell growth was arrested by reducing the serum concentration to 0.5%; mitogenic stimulation was then induced with 20% serum.

Flow Cytometry—Cells to be analyzed by flow cytometry were harvested in dissociation buffer (Sigma) and fixed in phosphate-buffered saline/ethanol (1:1, v/v). Propidium iodide (40 µg/ml) was added, and the DNA content of cell samples was measured using a Becton Dickinson FACScan (10,000 events/sample). Data were analyzed using Cell Quest software.

Thymidine Incorporation—[3H]Thymidine (0.1 µCi/ml) was added to serum-stimulated or quiescent cells 3 h prior to harvesting. Cells were then washed twice in phosphate-buffered saline, three times in 5% trichloroacetic acid, and twice in ethanol. Samples were solubilized in 0.5 m NaOH, and the incorporation of [3H]thymidine into DNA was then measured by liquid scintillation counting.

Phosphate Labeling in Vivo—Subconfluent Balb/c 3T3 cells were cultured for 24 h in DMEM containing 0.5% FCS. They were then incubated for 15 h in phosphate-free DMEM containing 100 µCi/ml [32P]orthophosphate either in the absence of FCS, to give G0 phase cells, or the presence of 10% FCS, to give S phase cells; early G1 phase cells were generated by adding 10% FCS for the final 3 h of the incubation. Cells were then lysed in RIPA buffer (64 mM Hepes, pH 7.4, 150 mM NaCl, 50 mM Tris, 10 mM EDTA, 1.2% Triton X-100, 0.1% sodium deoxycholate, 0.128% SDS, 10 mM β-glycerophosphate, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 0.5 µg/ml leupeptin, 1.0 µg/ml trypsin inhibitor, 0.5 µg/ml aprotinin, 40 µg/ml bovine serum albumin) and centrifuged at 1 h at 4 °C, and centrifuged at 15 min at 4 °C.

Northern Blotting and Nuclear Run-on—Total cellular RNA was extracted using TRI reagent (Sigma), according to the manufacturer’s instructions. It was then analyzed by primer extension using primers for VA1 (5’-CACCAGGGCCTAAACATGCGATGCTCGGATGGATTGCGATC-3’) and CAT (5’-CATG- GCGCTGCGTTTCTTATACATATACTCATAT-3’), as described previously (11).

Plasmids—The pVA1 plasmid contains the adenovirus VA1 gene (27). pHu5S3.1, pLeu, and pU6/Hae/RA2 contain human S 5S rRNA, tRNAasu, and U6 gene promoters, respectively (25, 31). Expression vectors RC-CDK2, RC-CDK4, Re-cycD1, and Re-cycE contain CDK2, CDK4, cyclin D1, and cyclin E cDNAs, respectively, cloned into the pRC-CMV vector (In Vitrogen) downstream of the CMV immediate early promoter (28). pCMVp16 contains the p16 cDNA fused to a CMV polyadenylation signal and cloned downstream of the CMV immediate early promoter (29). Re-89-12 contains a ribozyme against murine p16 mRNA subcloned into the pEX expression vector (30). pCAT (Promega) contains the pol III gene driven by the SV40 promoter and enhancer.

Transfection—Transfection transfections used the calcium phosphate precipitate method. DNA precipitates were left on the plates overnight, and then the cells were washed with phosphate-buffered saline and cultured for 24 h before harvesting. Total RNA was extracted using TRI reagent (Sigma), according to the manufacturer’s instructions. It was then analyzed by primer extension using primers for VA1 (5’-CACCAGGGCCTAAACATGCGATGCTCGGATGGATTGCGATC-3’) and CAT (5’-CATG-GCGCTGCGTTTCTTATACATATACTCATAT-3’), as described previously (11).

Immunoprecipitation—Whole cell extracts were prepared using a freeze-thaw procedure described previously (31). HeLa nuclear extracts were purchased from the Computer Cell Culture Center (Mons). PC-B and PC-C phosphocellulose step fractions were prepared as described previously (31). A250/0.15 fraction containing TFIIIB was prepared by chromatography on phosphocellulose and DEAE-Sephadex, as previously shown (31). Chep-1.0 fraction containing TFIIIC and pol III was prepared by sequential chromatography on phosphocellulose and heparin-Sepharose, as previously shown (31). Recombinant TBP was purchased from Promega.

Transcription reactions were carried out as previously described (25), except that pBR322 was not included, and the incubations were for 60 min at 30 °C.

Reverse Transcription-PCR Analysis—RNA was extracted using TRI Reagent (Sigma), according to the manufacturer’s specifications. Reverse transcription reactions were performed for 1 h at 42 °C using 3 µg of RNA, 200 ng of Random Hexamers (Promega), and 400 units of Superscript II Reverse Transcriptase (Life Technologies) in a total volume of 40 µl of 1× First Strand Buffer (Life Technologies) containing 10 mM dithiothreitol and a 0.5 mM concentration of each dNTP. PCRs were carried out using a PTC-100 programmable thermal controller (MJ Research Inc). 2 µl of cDNA was amplified with 20 pmol of either TFIIIC primers (5’-CGACAAGGGCTTCGAAAAGTTG-3’ and 5’-CTTCCTTCAGAGATGGTCAAAAAG-3’ to give a 303-base pair product, or ARPP P0 primers (5’-GACCCTGAACTCACAATCTCTC-3’ and 5’-TGGAGGGCTTCCTGTGGTGAAACAC-3’) to give a 268-base pair product. Amplification reactions contained 0.5 units of Taq DNA Polymerase (Promega) in a total volume of 1× Taq DNA polymerase buffer (Promega) containing 1.5 mM MgCl2 and a 0.2 µM concentration of each dNTP. PCR was performed under the following cycling parameters: 1) TFIIIC, 94 °C for 3 min, six cycles of 95 °C for 1 min, 66 °C for 40 s, and 72 °C for 40 s; 22 cycles of 95 °C for 1 min, 62 °C for 40 s, and 72 °C for 40 s; 72 °C for 5 min; 2) ARPP P0, 95 °C for 2 min, 25 cycles of 95 °C 1 min, 58 °C for 30 s, and 72 °C for 1 min; 72 °C for 3 min.
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RESULTS

Pol III Transcription Decreases When Fibroblasts Are Deprived of Serum—Actively growing Balb/c 3T3 cells were made quiescent by serum withdrawal. The majority of cells had arrested in a G0/G1 phase state after 1 day of culture under serum-free conditions, as indicated by flow cytometric analyses of their DNA content (Fig. 1A, 0 h serum stimulation). This conclusion was supported by measurements of thymidine incorporation into newly synthesized DNA (Fig. 1B). The abundance of pol III transcripts derived from the B2 middle repetitive gene family was substantially reduced in the growth-arrested cells, as revealed by Northern blotting (Fig. 1C). The expression of a pol II transcript encoding ARPP P0 did not diminish following serum deprivation (Fig. 1C, lower panel).

The growth-arrested fibroblasts were stimulated to reenter the cell cycle by the addition of medium containing 20% serum. Flow cytometric analysis and thymidine incorporation measurements demonstrated that S phase was reached between 12 and 15 h after serum stimulation (Fig. 1, A and B). Northern blotting with a B2 gene probe revealed a slight increase in pol III transcript levels by mid-G1 phase, 6–9 h after the addition of serum, and revealed that near maximal expression was reached by 12 h after mitogenic stimulation, shortly before S phase entry (Fig. 1C). We conclude that Balb/c 3T3 cells undergo growth arrest within 24 h of serum withdrawal and that this is accompanied by a significant reduction in pol III activity; when these fibroblasts resume cycling, pol III activity is restored during late G0 phase. These observations are consistent with previous studies of 3T6 and BHK cells (19, 20, 35).

The Level of TFIICβ Remains Relatively Constant in Growth-arrested 3T3 Cells—To begin to investigate the mechanism responsible for the growth control of pol III transcription in Balb/c 3T3 fibroblasts, we prepared whole cell extracts after various periods of culture in 10% serum or serum-free medium. Although little or no apoptosis was detected after 24 or 48 h without serum, flow cytometry suggested that a fraction of the cells undergo apoptosis after 72 h in serum-free medium.2

Extracts of fibroblasts maintained without serum for 24 h or more were found to transcribe the adenovirus VA1 gene significantly less actively than extracts prepared from proliferating cells that had been cultured in 10% serum (Fig. 2A). Similar results were obtained with other pol III templates, including tRNA, 5 S rRNA, and U6 small nuclear RNA genes (Fig. 2A). The extracts therefore mimic the serum-responsiveness of pol III transcription that is observed in vivo.

Sinn et al. (23) reported previously that growth of HeLa cells in 0.5% serum results in a specific decrease in the abundance of the β subunit of TFIIC2. If this is true in untransformed 3T3 cells, then it may account for the down-regulation of pol III transcription under quiescent conditions. To address this possibility, we carried out Western blots with extracts to test whether the decrease in pol III transcription correlated with a down-regulation of TFIICβ. However, little or no change was detected in the level of TFIICβ when growing and arrested 3T3 cells were compared (Fig. 2B). Indeed, TFIICβ levels were maintained even after culture for 72 h without serum. This result was confirmed using two additional antisera raised against different regions of TFIICβ.3 Reverse transcriptase-PCR analysis was employed to compare the levels of the mRNA encoding TFIICβ, as an independent method to investigate the expression of this essential component of TFIIC2. This approach also provided no evidence that TFIICβ is sensitive to serum availability (Fig. 2C). We conclude that changes in the

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2 P. H. Scott, unpublished data.

3 H. M. Alzuherri, unpublished data.
abundance of TFIIICβ are unlikely to be responsible for regulating pol III transcription in growth-arrested Balb/c 3T3 cells.

**TFIIB Activity Is Down-regulated and Limiting in Serum-starved 3T3 Cells**—Add-back experiments were carried out to determine which factor is limiting for pol III transcription in extracts of 3T3 cells. A fraction containing partially purified TFIIB was found to stimulate transcription when titrated into extracts of either growing or serum-starved cells (Fig. 3A). This effect was highly specific, since little or no stimulation was observed in response to a fraction containing TFIIIC and pol III. The activity of all fractions was confirmed using complementation assays. The data suggest that under the conditions used, TFIIB is limiting, while TFIIIC and pol III are in relative excess. This implies that the rate of pol III transcription in 3T3 cells may be dictated by the availability of active TFIIB.

We therefore carried out complementation assays to compare directly the activity of TFIIB in extracts prepared from cells harvested either before or after serum withdrawal. In these assays, the extracts are subjected to mild heat treatment, which selectively inactivates endogenous TFIIIC; they are then tested for their ability to support transcription when mixed with a complementing system containing excess TFIIIC, TBP, and pol III (31). Extracts of growing 3T3 cells were found to contain sufficient TFIIB activity to allow robust transcription. This implies that the rate of pol III transcription in extracts prepared from cells that had been grown continuously in 10% serum (lanes 1 and 5) or cultured without serum for 24, 48, or 72 h (lanes 2–4, respectively). Heat treatment was at 47 °C for 15 min.

**Serum Deprivation Produces Little or No Change in the Levels of BRF and TBP**—We investigated whether the abundance of TFIIB changes in response to serum deprivation. Western blotting showed that 72 h of serum deprivation resulted in little or no change in the level of the BRF subunit of TFIIIB (Fig. 4A). Levels of the TFIIB subunit TBP are also maintained after extended periods without serum (Fig. 4B). As an internal control, we also monitored the pol II-specific factor TFIIB in these extracts.  

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*Fig. 3. TFIIB activity is limiting for pol III transcription in 3T3 cell extracts and decreases in response to serum deprivation. A, plasmid pVA1 (500 ng) was transcribed using whole cell extract prepared from 3T3 cells that had been grown continuously in 10% serum (lanes 1–6) or cultured without serum for 72 h (lanes 7–13). Extracts were supplemented with 1.5 (lanes 2 and 8), 3 (lanes 3 and 9), or 4.5 μl (lanes 4 and 10) of the CHePl.0 fraction containing active TFIIIC and pol III or with 1.5 (lane 11), 3 (lanes 5 and 12), or 4.5 μl (lanes 6 and 13) of the A25/0.15 fraction containing active TFIIB. B, pVA1 (500 ng) was transcribed using 2 μl of CHePl.0 fraction and 1 μl of recombinant TBP supplemented with 10 μg of heat-treated extract prepared from 3T3 cells that had been grown continuously in 10% serum (lanes 1 and 5) or cultured without serum for 24, 48, or 72 h (lanes 2–4, respectively). Heat treatment was at 47 °C for 15 min.  

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4 R. J. White, unpublished data.
Down-regulation of pol III activity in serum-starved 3T3 cells is not due to a decrease in the abundance of the TFIIIB subunits TBP and BRF. A, partially purified TFIIIB (10 μg of PC-B fraction; lane 1) and whole cell extract (100 μg) prepared from 3T3 cells that had been grown continuously in 10% serum (lane 2) or cultured without serum for 24, 48, or 72 h (lanes 3–5, respectively) were resolved on an SDS-7.8% polyacrylamide gel and then analyzed by Western immunoblotting with antibody 128 against BRF. B, partially purified TFIIIB (10 μg of PC-B fraction; lane 1) and whole cell extract (100 μg) prepared from 3T3 cells that had been grown continuously in 10% serum (lane 2) or cultured without serum for 24, 48 or 72 h (lanes 3–5, respectively) were resolved on an SDS-7.8% polyacrylamide gel and then analyzed by Western immunoblotting with monoclonal antibody SL30 against TBP. C, whole cell extract (100 μg) prepared from 3T3 cells that had been grown continuously in 10% serum (lane 1) or cultured without serum for 24, 48, or 72 h (lanes 2–4, respectively) were resolved on an SDS-7.8% polyacrylamide gel and then analyzed by Western immunoblotting with antibody SI-1 against TFIIIB.

extracts and found that this too remained unchanged (Fig. 4C). Although we cannot exclude the possibility that unidentified components of TFIIIB become less abundant in growth-arrested fibroblasts, the available evidence suggests that a decrease in the amount of TFIIIB is not responsible for its loss of activity when Balb/c 3T3 cells are cultured in the absence of serum.

RB Knockout Fibroblasts Are Compromised in Their Ability to Down-regulate pol III Transcription following Exit from the Cell Cycle—Genetic experiments have demonstrated previously that RB plays an important role in regulating pol III transcription in murine fibroblasts (11). We therefore addressed the possibility that RB may contribute to the control of pol III during cell cycle withdrawal. Fibroblasts derived from either wild-type or RB knockout mice were maintained in 20% serum or made quiescent by transfer to 0.5% serum. Previous studies have demonstrated that specific disruption of the RB gene does not prevent fibroblasts from withdrawing from cycle following serum deprivation (26, 36). This was confirmed in the current study by measuring the incorporation of thymidine into newly synthesized DNA. Northern blot analysis revealed that tRNA levels are elevated in the Rb−/− cells when compared with the wild-type cells (Fig. 5A, upper panel). Transfer of the Rb−/− cells into 0.5% serum results in a 2.4-fold decrease in the level of tRNA, as expected from previous studies (19, 20, 25). However, when the Rb−/− cells are transferred to low serum, the abundance of tRNA shows only a 1.3-fold decrease. Indeed, quiescent Rb−/− cells maintain a level of tRNA expression that is close to that seen in actively growing wild-type cells. A similar pattern is shown by the pol III transcripts derived from B2 genes, which drop by 5.5-fold when wild-type cells are deprived of serum but show only a 1.4-fold decrease when the RB knockout cells are treated in the same way (Fig. 5A, middle panel). These effects are specific, since the pol II transcript encoding ARPP P0 gene is not deregulated in the RB knockout cells (Fig. 5A, bottom panel).

To reinforce the above data, we carried out nuclear run-on assays to measure directly the effect of serum on rates of pol III transcription in matched Rb−/− and Rb−/− cells (Fig. 5B). The synthesis of tRNA decreased by 2.1-fold when wild-type fibroblasts were cultured in 0.5% serum. In contrast, only a 1.2-fold decrease was observed in tRNA gene transcription when the Rb−/− cells were cultured in the same way. In keeping with the Northern blots, the nuclear run-ons showed that tRNA synthesis is maintained in serum-starved Rb−/− cells at a level very close to what is observed in the actively proliferating wild-type cells. These data provide clear genetic evidence that RB plays a major role in controlling pol III transcription during the switch between growth and quiescence. It is nevertheless apparent that RB is not solely responsible for this control, since some down-regulation is still observed when Rb−/− cells are cultured in low serum; we shall return to this point later.
RB Binds to TFIIIB in Growth-arrested Fibroblasts, but This Interaction Diminishes following Mitogenic Stimulation—It has been shown that TFIIIB is a specific target for repression by RB in murine fibroblasts (6). Since the experiments above using knockout cells provide evidence that RB contributes to the repression of pol III following growth arrest, we carried out immunoprecipitation assays to compare the level of interaction between RB and TFIIIB during quiescence with that seen in fibroblasts that have been stimulated to reenter the cell cycle. An antiserum against RB was used to immunoprecipitate proteins from 3T3 cells that had been starved of serum for 36 h or starved for 24 h and then refed with 20% serum for various times prior to harvesting; the precipitates were then probed by Western blotting for both RB (lower panel) and the BRF subunit of TFIIIB (upper panel). A substantial amount of BRF was found to coprecipitate with RB from the quiescent cells (Fig. 6A). This coprecipitation reflects a specific interaction with RB, since BRF was not detected in material immunoprecipitated with a control antiserum against the TAF,48 subunit of the pol I factor SL1. The association between RB and BRF was maintained in cells harvested in early G1 phase, 3 or 6 h after mitogen stimulation. However, the interaction had begun to diminish after 9 h and was substantially reduced by late G1 phase, 12 h after the serum addition. Fig. 6B shows quantitation of this and two similar experiments, in which the amount of coprecipitated BRF at each time point has been normalized to the total amount of RB in each immunoprecipitation. It is apparent that the down-regulation of pol III transcription that accompanies growth arrest correlates with an interaction between TFIIIB and its repressor RB; when cells resume cycling, this interaction is maintained during early G1 phase but decreases late in G1, in parallel with the activation of pol III. We estimate that RB is bound to between 66 and 74% of TFIIIB present in growth-arrested fibroblasts but that binding has diminished by 6-fold or more by 15 h after serum stimulation, when most cells are in S phase.

TFIIIB Interacts Specifically with the Underphosphorylated Form of RB—Western blotting revealed no change in the abundance or electrophoretic mobility of RB that might account for its increased binding to RB in quiescent fibroblasts (Fig. 4A). In contrast, RB becomes phosphorylated at multiple sites when resting cells are stimulated to proliferate (16, 17, 37–39). More specifically, RB is found in an underphosphorylated state during G0 and early G1 phases but becomes heavily phosphorylated by cyclin D- and cyclin E-dependent kinases shortly before entry into S phase (reviewed in Refs. 2 and 3 and Ref. 15). Evidence of this can be seen in the lower panel of Fig. 6A, where hyperphosphorylation leads to a decrease in the electrophoretic mobility of RB, beginning 9 h after mitogenic stimulation. The effect is seen more clearly in Fig. 6C, which shows a Western blot carried out using an antibody that only recognizes RB when it is phosphorylated at serine 780. Since phosphorylation of RB correlates temporally with its dissociation from TFIIIB, we looked for a causal link between these two phenomena.

Experiments were carried out to test whether pretreatment of RB with cyclin-dependent kinases could influence its ability to coimmunoprecipitate with TFIIIB. Radiolabeled RB was synthesized in vitro by translation with a reticulocyte lysate in the presence of [35S]Met and [35S]Cys. Pretreatment of this RB with a mixture of recombinant CDK2 and CDK4 and their partner cyclins caused a decrease in its electrophoretic mobility, consistent with hyperphosphorylation (Fig. 7A, lanes 1 and 2). These forms of RB were incubated with a cell-free extract, to allow interaction with the endogenous TFIIIB; immunoprecipitations were then carried out using an anti-BRF antiserum. Whereas the underphosphorylated RB was found to coimmunoprecipitate with BRF, no interaction was observed with the RB that had been preincubated with cyclin-dependent kinases (Fig. 7A, lanes 4 and 5). These data suggest that hyperphosphorylation of RB can prevent it from binding to TFIIIB.

To address this issue further, we examined the phosphorylation state of the RB that is associated with TFIIIB. A population of RB molecules copurifies extensively with TFIIIB because of the physical interaction between these proteins (6). We compared the electrophoretic mobility of the RB present in an unfraccionated cell extract with that found associated with partially purified TFIIIB (Fig. 7B). The RB present in extracts of asynchronous cells migrates as a doublet in SDS-polyacrylamide gels, because the hyperphosphorylated protein has a retarded mobility compared with the underphosphorylated form (37–39). In contrast, the RB present in the TFIIIB fraction runs as a single tight band that comigrates with the higher mobility form detected in crude extracts. This suggests that it is only the underphosphorylated form of RB that copurifies

![Fig. 6. The interaction between RB and TFIIIB is maximal during G0 and early G1 phase.](http://www.jbc.org/content/101/3/1010/F6.large.jpg)
with TFIIIB. To test this further, we used a panel of antibodies that exclusively recognize RB that is phosphorylated at particular sites (34). Serine 780 of RB is phosphorylated in vivo specifically by the cyclin D-dependent kinases (34, 40). An antibody that only reacts with RB that is phosphorylated at serine 780 gives a strong signal when used to probe crude extracts of asynchronous cells (Fig. 7C). In contrast, virtually no signal was obtained when this antibody was tested in parallel with partially purified TFIIIB, as shown in Fig. 7B. We conclude that the RB which copurifies with TFIIIB is not phosphorylated at serine 780. A similar result was obtained using an antibody against RB that has been phosphorylated at threonine 373, a site that is preferentially targeted by cyclin E- and cyclin A-dependent kinases (40). Again, the antibody recognized the RB present in crude extracts but did not react with the RB that cofractionates with TFIIIB (Fig. 7D). We also tested antibodies that are specific for RB phosphorylated at serine 795, serines 807 and 811, or threonine 252; each reacted with RB in a crude extract but did not recognize the RB associated with TFIIIB.4 These results suggest strongly that it is only the underphosphorylated form of RB that cofractionates with TFIIIB, despite the abundance of hyperphosphorylated RB in the crude extracts used as starting material for chromatography.

Overexpression of Cyclin D-CDK4 and Cyclin E-CDK2 Stimulates pol III Transcription in Vivo—The above results provide evidence that the RB that interacts with TFIIIB is not phosphorylated at sites that are targeted by the cyclin D- and cyclin E-dependent kinases. This is consistent with the data in Fig. 7A, which suggest that phosphorylation of RB by these kinases may prevent it from binding to TFIIIB. We therefore investigated how the expression of a transfected VA1 gene would respond in vivo to the overexpression of cyclin-dependent kinases. Transfecting 3T3 cells with expression vectors encoding cyclin D1 and its associated kinase CDK4 resulted in a slight increase in VA1 transcription by pol III (Fig. 8A). In contrast, cyclin E and its associated kinase CDK2 produced little or no effect when tested in parallel. However, coexpression of cyclin D1-CDK4 with cyclin E-CDK2 resulted in a dramatic activation of VA1. This stimulation can be blocked by the coexpression of p16, a specific inhibitor of cyclin D-dependent kinases.

The substantial response obtained when cyclin D1-CDK4 is combined with cyclin E-CDK2 is unlikely to be an indirect consequence of accelerated passage through the G1/S transition, since cyclin D1 alone or cyclin E alone is sufficient to shorten G1 phase when overexpressed in rodent fibroblasts (18, 41, 42). A more direct effect is consistent with the observation that TFIIIB only associates with the underphosphorylated form of RB (Fig. 7). To investigate further the idea that the CDKs can activate pol III transcription by overcoming repression by RB, we made use of an SV40-transformed derivative of the 3T3 line, called SV3T3 CI38. These SV3T3 cells express the large T antigen of SV40, which binds and neutralizes RB (43, 44). We have shown recently that the interaction between RB and TFIIIB is 3- or 4-fold diminished in these cells, although not completely abolished (45). When cyclin D1-CDK4 and cyclin E-CDK2 were cotransfected into the SV3T3 cells, VA1 transcription increased by less than 3-fold, a much weaker response than the 11-fold activation obtained in the parental 3T3 cells (Fig. 8B). Although several explanations could account for this difference, the data are consistent with the possibility that the cyclin-CDKs stimulate pol III by overcoming the effect of RB on TFIIIB, since most RB is inactive in SV3T3 CI38 cells. The G1 cyclins also had little effect on VA1 transcription when they were transfected into a 3T3 cell line that was transformed with the E7 oncoprotein of human papillomavirus, which binds and neutralizes RB.5

Overexpression of protein kinases can sometimes result in reduced substrate specificity. Indeed, at higher levels of transfection we observed activation of VA1 by the cyclin E-CDK2 pair alone.5 Clear evidence that some specificity is maintained in the overexpression experiments shown in Fig. 8A is provided by the fact that VA1 is activated by cyclin D1-CDK4 but not by

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5 C. A. Cairns, unpublished data.
Regulation of pol III during Cell Cycle Entry

Fig. 8. Overexpression of cyclin D1-CDK4 together with cyclin E-CDK2 activates pol III transcription in vivo. A, 3T3 cells growing in 10% serum were transfected with pVA1 (2 μg, all lanes), pCAT (2 μg, all lanes), pRc-CMV vector (6 μg in lane 1, 4 μg in lanes 2 and 3, 3 μg in lane 4, and 1.5 μg in lane 5), Rc-CDK4 (1 μg in lanes 2, 4, and 5), Rc-cyclin D1 (1 μg in lane 2, 0.5 μg in lanes 4 and 5), Rc-CDK2 (1 μg in lanes 3–5), Rc-cyclin E (1 μg in lane 3, 0.5 μg in lanes 4 and 5), and pCMV16 (1.5 μg in lane 5). VA1 and CAT RNA levels were assayed by primer extension and then quantitated using a PhosphorImager. Values shown are for VA1 expression after normalization to the levels of CAT RNA to correct for transfection efficiency; they are given relative to the value obtained with pX vector alone (designated 1.0) and represent the mean of two experiments. B, 3T3 cells (lane 1) and SV3T3 Cl38 cells (lane 2) growing in 10% serum were transfected with pVA1 (2 μg), pCAT (2 μg), and pRc-CMV vector (6 μg) alone or pRc-CMV vector (3 μg), Rc-CDK4 (1 μg), Rc-cyclin D1 (0.5 μg), Rc-CDK2 (1 μg), and Rc-cyclin E (0.5 μg). VA1 and CAT RNA levels were assayed by primer extension and then quantitated using a PhosphorImager. VA1 expression was then normalized to the level of CAT RNA to correct for transfection efficiency. The values shown give the increase in normalized VA1 expression in response to CDK4-cyclin D1 and CDK2-cyclin E. C, 3T3 cells growing in 10% serum were transfected with pVA1 (2 μg), pCAT (2 μg), and 2 μg of pX vector (lane 1) or Rz 89-12 antip16 ribozyme (lane 2). VA1 and CAT RNA levels were assayed by primer extension and then quantitated using a PhosphorImager. Values shown are for VA1 expression after normalization to the levels of CAT RNA to correct for transfection efficiency; they are given relative to the value obtained with pX vector and represent the mean of four experiments.

cyclin E-CDK2. Nevertheless, we wanted to test whether endogenous CDK4 and/or CDK6 are involved in controlling pol III transcription when they are present at physiological concentrations within the cell. To address this, we employed a ribozyme that cleaves p16 mRNA specifically at nucleotide 89, just after the translation start site (30). 3T3 cells were transfected with a vector encoding this ribozyme or with the empty vector, and pol III transcription of a cotransfected VA1 gene was monitored by primer extension. The anti-p16 ribozyme was reproducibly found to increase the level of VA1 expression (Fig. 8C). This is consistent with the stimulation obtained with cyclin D1-CDK4 and the ability of p16 to block this effect when it is overexpressed. These data support the contention that the endogenous p16/CDK/RB pathway is involved in regulating pol III activity in vivo.

p130 Contributes to the Serum Response of pol III Transcription—Although the down-regulation of pol III following serum withdrawal is substantially compromised in RB knockout fibroblasts, some decrease is nevertheless observed (Fig. 5). This indicates that the serum responsiveness of pol III activity is not mediated solely by RB. Recent work has shown that the RB-related pocket proteins p107 and p130 can bind to TFIIB and repress pol III transcription both in vitro and in vivo (7). That study demonstrated that the effect on pol III activity of deleting p107 and p130 is most pronounced in quiescent cells; thus, p107/p130 double knockout fibroblasts show much less of a decrease in B2 transcript levels after serum deprivation than the matched cells expressing a full complement of pocket proteins (7). These observations suggest that p107 and/or p130 play a significant role in suppressing pol III transcription during G1 phase. Relatively little p107 is found in serum-starved fibroblasts, but p130 is much more abundant (3, 26, 46, 47). Not only does mitogenic stimulation cause the level of p130 to decrease, but it also becomes inactivated at the G1/S transition through hyperphosphorylation by the cyclin D- and cyclin E-dependent kinases (3, 26, 47). This phosphorylation can be seen clearly in Fig. 9A, where endogenous p130 is immunoprecipitated from cells that are metabolically labeled with [32P]orthophosphate. Although Western blotting reveals that p130 is present in G0, G1, and S phases (bottom panel), autoradiography shows that p130 only becomes phosphorylated once the cells have passed the G1/S transition (upper panel). We therefore investigated whether the high levels of active unphosphorylated p130 present during G1 and early G2 phases might contribute to the repression of pol III transcription.

Coprecipitation experiments were carried out to monitor the interaction between p130 and TFIIB. An antisera against p130 was used to immunoprecipitate proteins from 3T3 cells that had been starved in low serum or starved and then refed with 20% serum for various times prior to harvesting; the precipitated material was then probed by Western blotting for the BRF subunit of TFIIB and for p130 (Fig. 9B, upper and lower panels, respectively). Fig. 9C shows quantitation of this and a similar experiment, in which the amount of coprecipitated BRF at each time point has been normalized to the total amount of p130 in each immunoprecipitation. A substantial amount of BRF coprecipitated with p130 from the quiescent cells. This reflects a specific interaction, since BRF was not coprecipitated with a control antiserum against TAF4B (lane 1). The association between p130 and TFIIB was maintained during early G1 phase, being undiminished 6 h after mitogenic stimulation, but had decreased by late G1 and early S phase, 12 h and 15 h after refeeding, respectively. This dissociation correlated with the hyperphosphorylation of p130, as indicated by phosphate labeling and a decrease in its electrophoretic mobility (Fig. 9, A and B, lower panel). Thus, the interaction between TFIIB and p130 is maximal during the G1 and early G2 phases, when pol III transcription is repressed. We estimate that p130 is bound to 29–34% of TFIIB in extracts of serum-starved cells, but this value falls to 4–7% once the cells have entered S phase. These observations, combined with the previous knockout data, suggest that p130 may make a significant contribution toward suppressing pol III transcription in quiescent fibroblasts.
The data suggest that the related pocket proteins RB and p130 play a major role in down-regulating pol III transcription when untransformed murine fibroblasts withdraw from the cell cycle following serum deprivation. They appear to achieve this by interacting with TFIIIB, thereby derepressing it and allowing a surge in the level of pol III transcription.

Our data allow, for the first time, a continuous pathway to be traced from the growth factors at the cell surface to the pol III machinery in the nucleus. Ras-mediated signaling pathways are responsible for inducing the expression of cyclin D1 and for promoting its assembly into functional complexes with CDK4 and CDK6 (48–51). This involves the sequential action of Raf1, the mitogen-activated protein kinase/extracellular signal-regulated kinase kinases, and the extracellular signal-regulated protein kinases (48–51). Ras also controls the nuclear accumulation and proteolytic turnover of cyclin D1 through a distinct signaling pathway involving phosphatidylinositol-3-OH kinase, protein kinase B (Akt), and glycogen synthase kinase-3β (52). Thus, when mitogens activate Ras via their cell surface receptors, one net result is the nuclear accumulation of functional complexes containing cyclin D1 bound to activated CDK4 and CDK6. These, in turn, phosphorylate RB at specific sites, inducing conformational rearrangements that allow the remaining phosphoacceptor residues to be phosphorylated by cyclin E-CDK2 (53). As we have shown, the hyper phosphorylated RB dissociates from TFIIIB, thereby derepressing it and allowing a surge in pol III transcriptional activity. It is clear that Ras plays a key role in this process, which is consistent with a previous study that showed that constitutively active Ras will stimulate pol III transcription in Rat-1 fibroblasts (54). We have confirmed that this is also true of the murine Balb/c 3T3 fibroblast system that was employed in the current study. Furthermore, inhibitors of Ras, mitogen-activated protein kinase/extracellular signal-regulated kinase kinases, or phosphatidylinositol-3-OH kinase can all compromise the mitogenic response of pol III in Balb/c 3T3 cells.2 We therefore believe that growth factors can regulate class III genes in this cell type via TFIIIB, RB, cyclin D, and the signal transduction pathways that connect these to Ras.

Previous studies have shown that TFIIIC2 is down-regulated when HeLa cells are cultured under low serum conditions (22, 23). This correlates with a specific reduction in the abundance of its second largest subunit TFIIICβ (23). We have found no evidence for such an effect in Balb/c 3T3 cells, where the level of TFIIICβ polypeptide remains undiminished after 3 days of serum deprivation. Expression of the mRNA encoding TFIIICβ is also maintained under low serum conditions. Several possible explanations could account for the distinct behavior of TFIIICβ in these two systems. HeLa cells are highly transformed and were derived from human cervical epithelium, whereas Balb/c 3T3 cells are untransformed murine fibroblasts; any one (or more) of these differences between the two cell types might be responsible for the distinct response of TFIIICβ to serum deprivation. Given the malignant nature of HeLa cells and their continued growth under low serum con-
ditions, the 3T3 cell system is much more likely to reflect a normal physiological response to mitogen withdrawal.

Although we have identified the pocket proteins as key players in coordinating pol III activity with growth factor availability, it is extremely likely that additional control mechanisms contribute to the overall effect. Some stimulation of pol III is observed at early time points, prior to the major increase that occurs at the R point (19, 20, 35). It is not yet clear what is responsible for this rapid and limited initial activation. An obvious possibility is that one or more of the pol III transcription factors, such as TFIIIB or TFIIIC, is itself a direct target for phosphorylation in response to mitogens. All five subunits of TFIIIC2 are phosphorylated in HeLa cells (55), although it is unknown whether this is constitutive or regulated, and the kinases responsible have yet to be identified. In Saccharomyces cerevisiae, the TOR signaling pathway is involved in coordinating pol III activity with nutrient availability (56). Casein kinase II has been shown to regulate TFIIIB in the same organism (57, 58). It therefore seems plausible that mammalian TOR and/or casein kinase II are also involved in controlling pol III transcription in higher organisms. We are currently investigating these possibilities. Given the complex mix of mitogens that are present in serum, the full response is likely to involve multiple pathways that may feed into several components of the pol III machinery.

An increased rate of protein synthesis is an essential feature of the mitogenic response. Conversely, a 50% reduction in translation rate causes cells to withdraw from cycle and quiesce (59). The availability of tRNA and rRNA is clearly an important determinant of protein synthetic capacity. Accord-
