

## Activation of RNA Polymerase III Transcription in Cells Transformed by Simian Virus 40

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**RNA polymerase (Pol) III transcription is abnormally active in fibroblasts that have been transformed by simian virus 40 (SV40). This report presents evidence that two separate components of the general Pol III transcription apparatus, TFIIB and TFIIC2, are deregulated following SV40 transformation. TFIIC2 subunits are expressed at abnormally high levels in SV40-transformed cells, an effect which is observed at both protein and mRNA levels. In untransformed fibroblasts, TFIIB is subject to repression through association with the retinoblastoma protein RB. The interaction between RB and TFIIB is compromised following SV40 transformation. Furthermore, the large T antigen of SV40 is shown to relieve repression by RB. The E7 oncoprotein of human papillomavirus can also activate Pol III transcription, an effect that is dependent on its ability to bind to RB. The data provide evidence that both TFIIB and TFIIC2 are targets for activation by DNA tumor viruses.**

A broad range of transformed cell types display abnormally elevated levels of RNA polymerase (Pol) III transcripts (reviewed in references 53 and 54). This was first discovered with murine fibroblast lines that have been transformed by simian virus 40 (SV40) (5, 36, 42). When different SV40-transformed clones are compared, those which most efficiently induce tumors in nude mice also display the highest abundance of Pol III transcripts, whereas lower levels are detected in the less tumorigenic lines (36, 60). A tight link between transformation and Pol III induction is suggested by analyses of two cell lines that were transformed with temperature-sensitive mutants of the SV40 oncoprotein large T antigen; these cells down-regulate Pol III products at the nonpermissive temperature while reverting to normal morphology and phenotype (36). Large T antigen has also been shown to activate Pol III transcription in transient transfection assays (9, 28).

The activity of a general Pol III factor called TFIIC2 has been shown to be greater in the SV40-transformed cell lines SV3T3 Cl38 and SV3T3 Cl49 than in the untransformed parental 3T3 line A31 (60). TFIIC2 is a large DNA-binding factor that is required for the expression of most class III genes (reviewed in reference 53). It can relieve chromatin-mediated repression of Pol III transcription and has been shown to display histone acetyltransferase activity (18). TFIIC2 can be detected in at least two forms, distinguishable by their differential migrations in electrophoretic mobility shift assays (EMSAs) (8, 15, 17, 43, 60). Whereas the high-mobility form predominates in untransformed A31 cell extracts, the SV3T3 derivatives contain a greater proportion of TFIIC2 in the slowly migrating form (60). Chromatographic fractionation of HeLa cells has revealed that the low-mobility form is transcriptionally active, whereas the higher-mobility species can bind DNA but is unable to support transcription (15, 17). Following purification, the inactive form was found to be missing a 110-

kDa subunit, which was named TFIIC $\beta$  (17, 43). The abundance of TFIIC $\beta$  in HeLa cells has been shown to increase substantially following serum stimulation or adenovirus infection, two conditions which activate TFIIC2 (43). In contrast, the level of TFIIC $\alpha$ , which is the DNA-binding subunit of TFIIC2, remains constant under these conditions (43).

The large T antigen of SV40 can bind and neutralize RB, the protein product of the retinoblastoma susceptibility gene (11, 13, 31). Mutations in T antigen that interfere with RB binding also abrogate its transforming activity (11, 13, 31). Gene disruption experiments have shown that endogenous RB represses Pol III transcription approximately fivefold in murine fibroblasts (61). The increased synthesis of tRNA and 5S rRNA that is observed in RB-knockout cells correlates with the deregulation of the general Pol III factor TFIIB (24). TFIIB is required for the expression of all class III genes; it is recruited via TFIIC2 and then serves to position the polymerase over the initiation site (reviewed in reference 53). TFIIB activity has been shown to be inhibited specifically by recombinant RB *in vitro* (7, 24). Furthermore, immunoprecipitation, cofractionation, and pull-down experiments have demonstrated that TFIIB interacts with both endogenous and recombinant RB (7, 24). The ability of large T antigen to neutralize RB therefore raises the possibility that TFIIB is released from repression following SV40 transformation. We present evidence that this is the case. We also demonstrate that SV40-transformed Cl38 and Cl49 cells express much higher levels of TFIIC $\alpha$  and TFIIC $\beta$  than the untransformed parental A31 line. The data suggest that at least two components of the general Pol III transcription apparatus become activated following transformation by SV40.

### MATERIALS AND METHODS

**Tissue culture.** SV3T3 Cl38 and Cl49 cell lines were generated by infection of BALB/c 3T3 A31 cells with SV40 (wt830 strain) and selected by focus formation in low serum (35). All cell lines were grown in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% fetal calf serum, 100 U of penicillin per ml, and 100  $\mu$ g of streptomycin per ml and were harvested when subconfluent.

**Antibodies and Western blotting.** Antibodies used were C-15 (Santa Cruz Biotechnology) and G99-549 (PharMingen) against RB, M-19 (Santa Cruz) against TAF<sub>48</sub>, 330 and 128 against BRG (1, 4), SL30 against TATA-binding

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protein (TBP) (27), clone 46 (Transduction Laboratories) against TFIIC $\beta$ , and Ab4 against TFIIC $\alpha$  (38). Western immunoblot analysis was performed as previously described (56).

**Reverse transcription-PCR (RT-PCR) analysis.** RNA was extracted from subconfluent A31, C138, and C149 cells by using TRI reagent (Sigma) according to the manufacturer's specifications. Reverse transcription reactions were performed for 1 h at 42°C, using 3  $\mu$ g of RNA, 200 ng of random hexamers (Promega), and 400 U of Superscript II reverse transcriptase (Life Technologies) in a total volume of 40  $\mu$ l of 1 $\times$  First Strand Buffer (Life Technologies) containing 10 mM dithiothreitol (DTT) and 0.5 mM each deoxynucleoside triphosphate.

PCRs were carried out in a PTC-100 programmable thermal controller (MJ Research Inc.); 2  $\mu$ l of cDNA was amplified with 20 pmol of either glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers (5'-TCCACCACCTGTGCTGTA-3' and 5'-ACCACAGTCCATGCCATCAC-3') to give a 452-bp product, TFIIC $\beta$  primers (5'-CCAGAAGGGGTCTCAAAGTCC-3' and 5'-CTTCTTACAGAGATGTCAAAGG-3') to give a 303-bp product, TFIIC $\alpha$  primers (5'-TCCAGCGAGACCTTACACC-3' and 5'-GGATTGAGTGTGCTGGGCT-3') to give a 144-bp product, or BRF primers (5'-AAATTCGTGAGCCTCTTCGGTAGTG-3' and 5'-AGACCATGCTGTACATTCCACG-3') to give a 260-bp product. Amplification reaction mixtures contained 0.5 U of *Taq* DNA polymerase (Promega) in a total volume of 20  $\mu$ l of 1 $\times$  *Taq* DNA polymerase buffer (Promega) containing 1.5 mM MgCl<sub>2</sub> and 0.2 mM each deoxynucleoside triphosphate. PCR was performed under the following cycling parameters: for GAPDH, (i) 94°C for 3 min, (ii) 18 cycles of 95°C for 30 s, 66°C for 40 s, and 72°C for 1 min, (iii) 72°C for 5 min; for TFIIC $\beta$ , (i) 94°C for 3 min, (ii) 6 cycles of 95°C for 1 min, 66°C for 40 s, and 72°C for 40 s, (iii) 22 cycles of 95°C for 1 min, 62°C for 40 s, and 72°C for 40 s, (iv) 72°C 5 min; for TFIIC $\alpha$ , (i) 95°C for 3 min, (ii) 30 cycles of 94°C for 20 s, 62°C for 30 s, and 72°C for 30 s, (iii) 72°C 10 min; for BRF, 95°C for 2 min, 26 cycles of 95°C for 1 min, 60°C for 30 s, and 72°C for 1 min, (iv) 72°C 5 min. Reaction products were resolved on a 2% agarose gel and visualized by ethidium bromide staining. Gels were scanned, and the intensity of each band was quantitated by using a UVP image analysis system and Adobe Photoshop 4.0 software.

**Plasmids.** pVAI contains the adenovirus VA<sub>1</sub> gene (10). pCAT (Promega) contains the chloramphenicol acetyltransferase (CAT) gene driven by the SV40 promoter and enhancer. Expression constructs encoding the E7 oncoprotein of human papillomavirus type 16 (HPV-16) and its mutant derivatives  $\Delta$ 21-35, PRO2, and GLY24 have been described elsewhere (21).

**Transfection assays.** Cell lines were transiently transfected by the calcium phosphate precipitation 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 by using TRI reagent (Sigma) according to the manufacturer's instructions and then analyzed by primer extension using both VA<sub>1</sub>-specific (5'-CACGCGGGCGGTAACCGCATG-3') and CAT-specific (5'-CGATGCCATTGGGATATATCA-3') labeled primers. Primer extension reactions were conducted as previously described (61).

**Transcription.** Pol III transcription was carried out as described previously (59) except that pBR322 was not included and the incubations were for 60 min at 30°C.

**Preparation of extracts and protein fractions.** Whole-cell extracts were prepared from exponentially growing cells by the method of Manley et al. (29). Nuclear extracts were purchased from the Computer Cell Culture Center (Mons, Belgium). PC-B is the 0.1 to 0.35 M KCl step fraction from a phosphocellulose column and contains both TFIIB and Pol III (37). PC-C is the 0.35 to 0.6 M KCl step fraction from a phosphocellulose column and contains both TFIIC and Pol III (37). PC-D is the 0.6 to 1.0 M KCl step fraction from a phosphocellulose column and contains TFIID (37). The CHep-1.0 fraction was generated as described previously (56) by loading PC-C onto heparin-Sepharose CL-6B in BC buffer (25 mM Tris-HCl [pH 7.9], 10% glycerol, 10 mM  $\beta$ -mercaptoethanol) plus 100 mM KCl (BC-100). The column was washed with BC-280 and eluted with BC-1000 to produce the CHep-1.0 fraction containing TFIIC. The A25(0.15) fraction containing TFIIB was generated as described previously (56) by applying PC-B to an A25 DEAE-Sephadex column in buffer A (20 mM HEPES-KOH [pH 7.9], 20% glycerol, 5 mM MgCl<sub>2</sub>, 3 mM DTT, 0.2 mM phenylmethylsulfonyl fluoride) plus 50 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>. After extensive washing with this buffer, the A25(0.15) fraction containing TFIIB was eluted in buffer A plus 150 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>. Mono Q gradient-purified TFIIB was prepared as described previously (27).

Recombinant glutathione *S*-transferase (GST) and GST-RB fusion proteins were expressed in bacteria and purified on glutathione-agarose as previously described (24). GST-RB contains residues 379 to 928 of RB. Immunoaffinity-purified recombinant SV40 large T antigen was a generous gift from David Lane.

**Immunoprecipitation.** Whole-cell extract (150  $\mu$ g) was incubated at 4°C on an orbital shaker with 20  $\mu$ l of protein A-Sepharose beads carrying equivalent amounts of prebound immunoglobulin G. Samples were then pelleted, supernatants were removed, and the beads were washed five times with 150  $\mu$ l of LDB buffer (20 mM HEPES-KOH [pH 7.9], 17% glycerol, 100 mM KCl, 12 mM MgCl<sub>2</sub>, 0.1 mM EDTA, 2 mM DTT). The bound material was analyzed by Western blotting. In the experiments shown in Fig. 5, reticulocyte lysate (15  $\mu$ l) containing BRF translated in the presence of [<sup>35</sup>S]Met and [<sup>35</sup>S]Cys was mixed with nuclear extract (150  $\mu$ g) during immunoprecipitation. In this case, the precipitated material was analyzed by autoradiography rather than Western blotting.

## RESULTS

**TFIIC2 subunits are more abundant in SV3T3 C138 and C149 cell extracts than in parental 3T3 A31 cell extracts.** Previous studies have shown that the SV40-transformed cell lines C138 and C149 express high levels of Pol III transcripts relative to the untransformed parental line A31 (36, 60). Nuclear run-on assays demonstrated that this up-regulation occurs at the transcriptional level (60). EMSA analysis revealed that the DNA-binding activity of TFIIC2 is significantly higher in C138 and C149 cell extracts than in extracts of the A31 cells (60). This is a specific effect, since Sp1 activity is not elevated in the transformed cell lines (60). In addition, the C138 and C149 extracts were found to contain a greater proportion of TFIIC2 in the slowly migrating transcriptionally active form (60). It was proposed that these changes might account for the abnormally high rates of Pol III transcription that are observed in these SV3T3 cell lines (60).

Since the time of this previous analysis, the two forms of TFIIC2 have been purified to homogeneity and shown to differ in the presence of a 110-kDa subunit that is found only in the active form and is referred to as TFIIC $\beta$  (17, 43). The availability of antibodies that recognize TFIIC $\beta$  (43) and the ~240-kDa DNA-binding subunit TFIIC $\alpha$  (20, 26) has provided the opportunity to investigate further the changes in TFIIC2 that accompany SV40 transformation. We therefore carried out Western blot analyses to compare the levels of these subunits in A31, C138, and C149 cell extracts. The level of TFIIC $\alpha$  was found to be higher in C138 and C149 cell extracts than in A31 cell extracts (Fig. 1A), consistent with the increased DNA-binding activity detected by EMSA following SV40 transformation (60). A striking change was also detected with the TFIIC $\beta$  subunit, which is substantially more abundant in the C138 and C149 cell extracts than in A31 cell extracts (Fig. 1B). This increase in TFIIC $\beta$  may account for the higher proportion of TFIIC2 that is found in the transcriptionally active form following SV40 transformation (60).

To investigate the specificity of these effects, we also examined the abundance of two subunits of TFIIB. Human TFIIB has been shown to contain TBP and an associated polypeptide of ~90 kDa called TFIIB90 or human BRF (27, 30, 40, 45, 51, 57). Neither of these subunits was found to be significantly more abundant in C138 cell extracts than in A31 cell extracts (Fig. 1C and D). We conclude that the up-regulation of TFIIC $\alpha$  and TFIIC $\beta$  in the SV40-transformed cells is a specific phenomenon.

**SV40-transformed C138 and C149 cells express elevated levels of mRNA encoding TFIIC $\alpha$  and TFIIC $\beta$ .** To determine the level at which TFIIC $\alpha$  and TFIIC $\beta$  become up-regulated, we carried out semiquantitative RT-PCR analyses of their mRNAs. The transcript encoding TFIIC $\beta$  was found to be significantly more abundant in C138 and C149 cells than in A31 cells (Fig. 2A, top). Again, this is a specific phenomenon, since the mRNA encoding GAPDH is expressed at similar levels in these three cell lines (Fig. 2A, bottom). The TFIIC $\alpha$  transcript is also more abundant in C138 and C149 cells than in A31 cells (Fig. 2B, top). However, the mRNA for BRF shows little or no increase following SV40 transformation (Fig. 2B, bottom), consistent with the Western blotting data. After normalization to the level of GAPDH mRNA, the levels of the BRF transcript in C138 and C149 cells were 90 and 114%, respectively, of that seen in A31 cells. In contrast, expression of TFIIC $\alpha$  transcript was elevated four- to fivefold, whereas that of TFIIC $\beta$  was elevated seven- to eightfold in C138 and C149 cells, relative to the parental A31 cells. The observed specific increases in the mRNAs encoding TFIIC $\alpha$  and TFIIC $\beta$  may

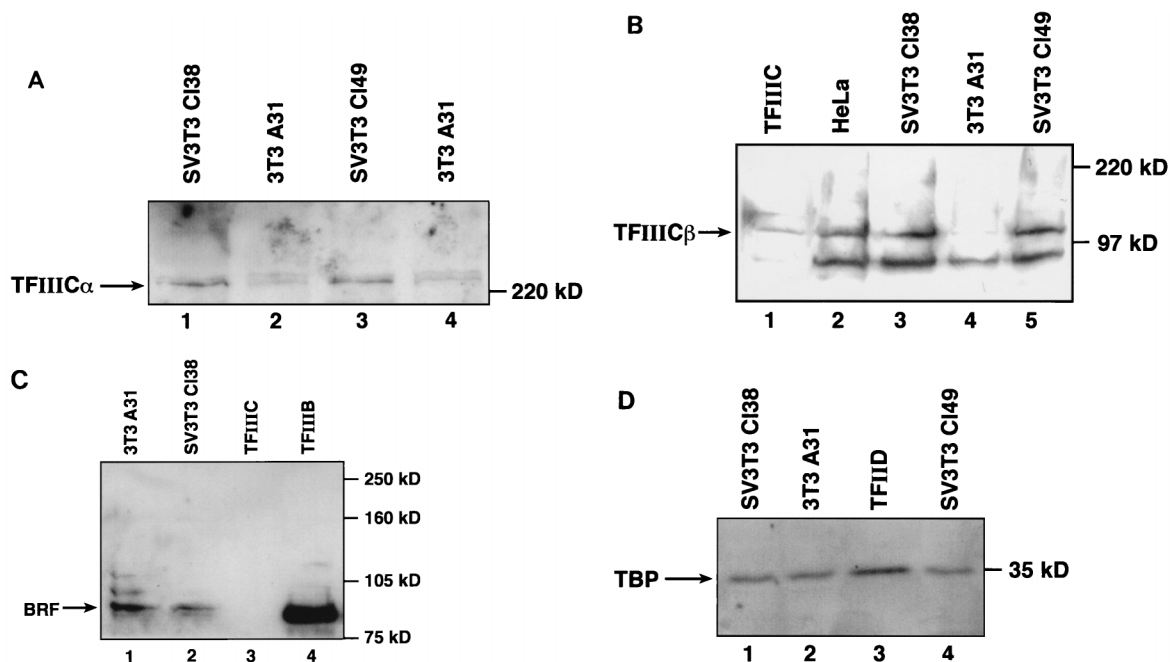


FIG. 1. The SV40-transformed cell lines Cl38 and Cl49 express elevated levels of TFIIIC2 subunits. (A) Whole-cell extracts (57  $\mu$ g of protein) prepared from Cl38 (lane 1), A31 (lanes 2 and 4), and Cl49 (lane 3) cells were resolved on an SDS-7.8% polyacrylamide gel and then analyzed by Western immunoblotting with anti-TFIIIC $\alpha$  antibody Ab4. Lanes 2 and 4 show extracts prepared from two separate batches of A31 cells. (B) CHep-1.0 fraction (15  $\mu$ g of protein) containing TFIIIC2 (lane 1) and whole-cell extracts (38  $\mu$ g of protein) prepared from HeLa (lane 2), Cl38 (lane 3), A31 (lane 4), and Cl49 (lane 5) cells were resolved on an SDS-7.8% polyacrylamide gel and then analyzed by Western immunoblotting with anti-TFIIIC $\beta$  monoclonal antibody clone 46. (C) Whole-cell extracts (50  $\mu$ g of protein) prepared from A31 (lane 1) and Cl38 (lane 2) cells, CHep-1.0 (12  $\mu$ g of protein) TFIIIC2 fraction (lane 3), and A25(0.15) (1.6  $\mu$ g of protein) TFIIIB fraction (lane 4) were resolved on an SDS-7.8% polyacrylamide gel and then analyzed by Western immunoblotting with anti-BRF antibody 330. (D) Whole-cell extracts (50  $\mu$ g of protein) prepared from Cl38 (lane 1), A31 (lane 2), and Cl49 (lane 4) cells and PC-D (5.6  $\mu$ g of protein) TFIIID fraction (lane 3) were resolved on an SDS-7.8% polyacrylamide gel and then analyzed by Western immunoblotting with anti-TBP antibody SL30.

therefore account for the elevated abundance of these subunits in the SV40-transformed cell lines.

**SV40 large T antigen can activate Pol III transcription by overcoming the repressive effects of RB.** The transforming protein of SV40 is its large T antigen, and this has been shown to be sufficient to stimulate Pol III transcription in transient transfection assays (9, 28). The ability of T antigen to transform cells is dependent on its capacity to bind and inactivate RB (11, 13, 31). Since RB is a potent repressor of Pol III transcription in murine fibroblasts (61), we investigated whether T antigen can release Pol III from repression by RB (Fig. 3). When recombinant T antigen was added to reactions that had been reconstituted with partially purified factors, it caused no increase in the level of VA<sub>1</sub> transcription by Pol III (lanes 1 to 3). However, a different response was obtained after a recombinant GST-RB fusion protein had been added to depress transcription. In the presence of this added RB, titration of T antigen resulted in a significant stimulation of VA<sub>1</sub> expression (lanes 4 to 6). The fact that T antigen increases Pol III transcription in the presence of excess RB but not in its absence suggests that its stimulatory effect in this system results from its ability to neutralize RB.

**TFIIIB activity is abnormally elevated in the SV40-transformed cell lines Cl38 and Cl49.** Previous studies have shown that the general Pol III factor TFIIIB is a target for repression by RB (7, 24). Since the T antigen present in SV40-transformed cells might be expected to counteract the effects of RB, we investigated whether this results in an increase in TFIIIB activity. Extracts of 3T3 A31 cells and SV3T3 Cl38 cells were subjected to chromatography on phosphocellulose. Equivalent TFIIIB fractions were then compared for their abilities to reconstitute transcription when mixed with a fraction contain-

ing TFIIIC and Pol III from HeLa cells (Fig. 4A). No transcription was observed with this system in the absence of added TFIIIB (lane 2). However, a robust transcription signal was obtained following the addition of TFIIIB fractions derived

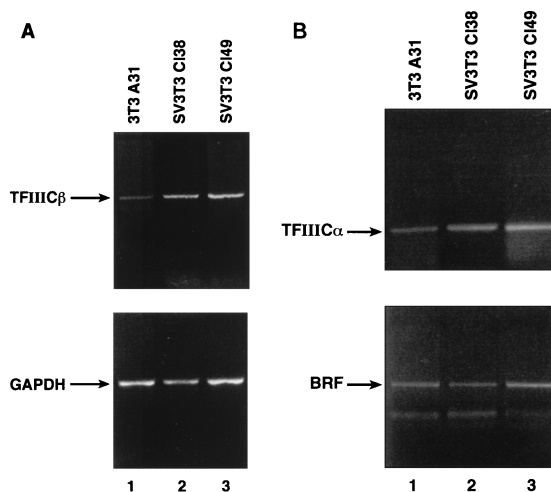


FIG. 2. The SV40-transformed cell lines Cl38 and Cl49 express elevated levels of transcripts encoding TFIIIC $\alpha$  and TFIIIC $\beta$ . (A) cDNAs generated by reverse transcription of 3  $\mu$ g of RNA from A31 (lane 1), Cl38 (lane 2), and Cl49 (lane 3) cells were PCR amplified by using TFIIIC $\beta$  (top) and GAPDH (bottom) primers. Amplification products were resolved on a 2% agarose gel. (B) cDNAs generated by reverse transcription of 3  $\mu$ g of RNA from A31 (lane 1), Cl38 (lane 2), and Cl49 (lane 3) cells were PCR amplified by using TFIIIC $\alpha$  (top) and BRF (bottom) primers. Amplification products were resolved on a 2% agarose gel.

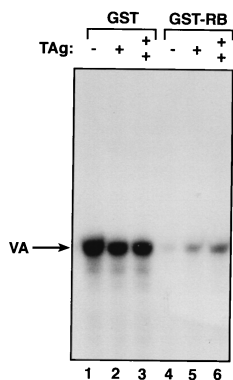


FIG. 3. SV40 large T antigen can counteract the repressive effect of RB on Pol III transcription. Template pVAI (250 ng of DNA) was transcribed by using 2  $\mu$ l of Mono Q-purified TFIIB and 2  $\mu$ l of CHep-1.0 fraction in the presence of 100 ng of either GST (lanes 1 to 3) or GST-RB (lanes 4 to 6) and 20 ng (lanes 2 and 5) or 40 ng (lanes 3 and 6) of immunoaffinity-purified recombinant large T antigen (TAg).

from HeLa or Cl38 cells (lanes 1 and 3, respectively). In contrast, an equal amount of the equivalent TFIIB fraction derived from 3T3 A31 cells gave much lower levels of Pol III transcription (lane 4).

The reduced activity of A31 cell-derived TFIIB fractions could conceivably result from differential recovery or inactivation during chromatography. We therefore also conducted

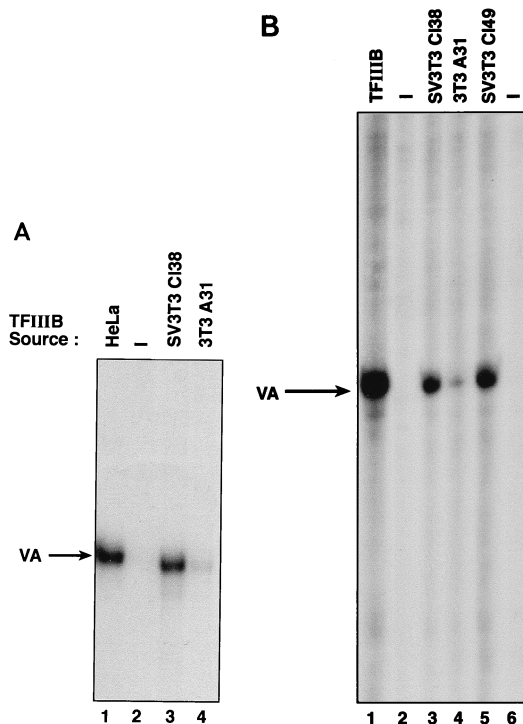


FIG. 4. Cl38 and Cl49 cell extracts contain higher TFIIB activity than A31 cell extracts. (A) Template pVAI (250 ng of DNA) was transcribed by using 2  $\mu$ l of HeLa-derived PC-C fraction and buffer alone (lane 2) or PC-B fraction (6.4  $\mu$ g of protein) derived from HeLa (lane 1), Cl38 (lane 3), or A31 (lane 4) cell extracts. (B) Template pVAI (500 ng DNA) was transcribed by using 4  $\mu$ l of HeLa-derived PC-C fraction supplemented with 10 ng of recombinant TBP and 6  $\mu$ l of heat-treated Mono Q-purified TFIIB (lane 1), buffer alone (lanes 2 and 6), or 7.5  $\mu$ g of heat-treated extract from Cl38 (lane 3), A31 (lane 4), or Cl49 (lane 5) cells.

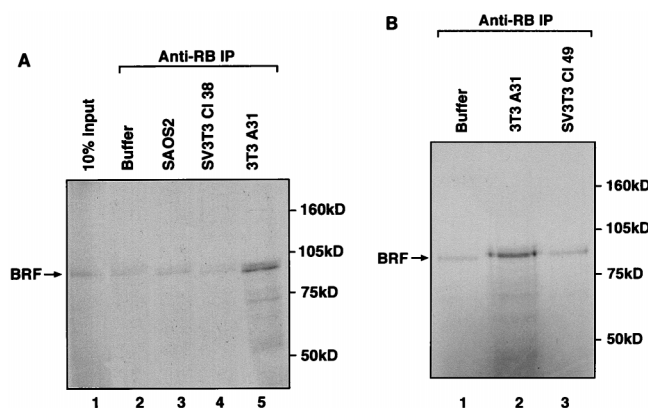


FIG. 5. The BRF subunit of TFIIB coimmunoprecipitates with endogenous RB present in A31 extracts, but this interaction is compromised in extracts of Cl38 and Cl49 cells. (A) Reticulocyte lysate (15  $\mu$ l) containing in vitro-translated BRF was immunoprecipitated (IP) with anti-RB antibody G3-245 in the presence of buffer alone (lane 2) or 150  $\mu$ g of whole-cell extract prepared from SAOS2 (lane 3), Cl38 (lane 4), or A31 (lane 5) cells. Proteins retained after extensive washing were resolved on an SDS-7.8% polyacrylamide gel and then visualized by autoradiography. Lane 1 shows 10% of the input reticulocyte lysate containing in vitro-translated BRF. (B) Reticulocyte lysate (15  $\mu$ l) containing in vitro-translated BRF was immunoprecipitated with anti-RB antibody C-15 in the presence of buffer alone (lane 1) or 150  $\mu$ g of whole-cell extract prepared from A31 (lane 2) or Cl49 (lane 3) cells. Proteins retained after extensive washing were resolved on an SDS-7.8% polyacrylamide gel and then visualized by autoradiography.

complementation assays to measure TFIIB activity in unfractionated extracts. These assays make use of the differential sensitivity of Pol III transcription factors to inactivation by mild heat treatment. When an extract is heated at 47°C for 15 min, TFIIC and TBP become inactivated whereas the other components of the Pol III machinery are not compromised (17, 41, 57, 58). The activity of TFIIB in an unfractionated extract can therefore be assayed by heat treating the extract and then measuring its ability to reconstitute transcription when added to a complementing fraction containing TFIIC, TBP, and Pol III (1, 24, 55, 56). Using such assays, we found that A31 cell extracts contain significantly less TFIIB activity than extracts of Cl38 or Cl49 cells (Fig. 4B). We conclude that SV40 transformation of A31 cells is accompanied by an increase in TFIIB activity, even though the levels of TBP and BRF do not increase.

**The interaction between RB and TFIIB is compromised following SV40 transformation.** Previous genetic analyses have shown that TFIIB is subject to repression by RB in murine fibroblasts (24). Coimmunoprecipitation experiments were therefore carried out to test whether the physical interaction between RB and TFIIB is diminished following SV40 transformation. The BRF subunit of the TFIIB complex was synthesized by using a reticulocyte lysate in the presence of [<sup>35</sup>S]cysteine and [<sup>35</sup>S]methionine. The radiolabeled protein was then incubated with whole-cell extracts under conditions which allow complex assembly (4). The mixtures were subjected to immunoprecipitation using a monoclonal antibody that is specific for RB, and the precipitates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography (Fig. 5A). Significant amounts of radiolabeled BRF were coprecipitated with RB from an A31 cell extract (lane 5). This was not due to cross-reaction with the antibody, since only background levels of BRF were detected when buffer was used in place of the whole-cell extract (lane 2). Similar background levels were also obtained using extracts of SAOS2 osteosarcoma cells (lane 3), which express an inactive trun-

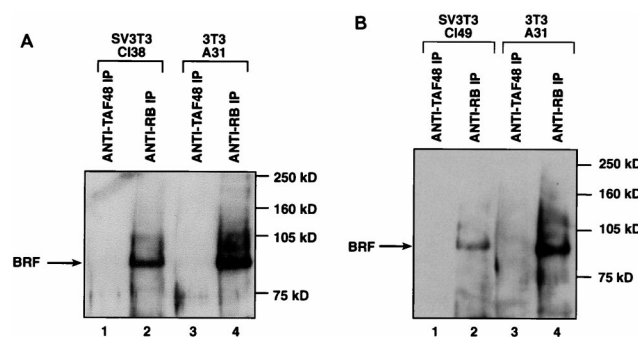


FIG. 6. The association between endogenous RB and endogenous TFIIB is greater in A31 cells than in Cl38 and Cl49 cells. (A) Cl38 (lanes 1 and 2) and A31 (lanes 3 and 4) cell extracts (150  $\mu$ g of protein) were immunoprecipitated (IP) with anti-TAF<sub>48</sub> antibody M-19 (lanes 1 and 3) or anti-RB antibody C-15 (lanes 2 and 4). Precipitated material was resolved on an SDS-7.8% polyacrylamide gel and then analyzed by Western blotting with anti-BRF antiserum 128. (B) Cl49 (lanes 1 and 2) and A31 (lanes 3 and 4) cell extracts (150  $\mu$ g of protein) were immunoprecipitated with anti-TAF<sub>48</sub> antibody M-19 (lanes 1 and 3) or anti-RB antibody C-15 (lanes 2 and 4). Precipitated material was resolved on an SDS-7.8% polyacrylamide gel and then analyzed by Western blotting with anti-BRF antiserum 128.

cated mutant form of RB (39). Furthermore, very little BRF was coimmunoprecipitated with RB when it was mixed with an extract of SV3T3 Cl38 cells (lane 4), despite the fact that Cl38 cells contain full-length RB that is no less abundant than the RB present in A31 cells (25). A similar analysis using a different RB antibody revealed that the interaction between BRF and RB is also compromised in SV3T3 Cl49 cell extracts (Fig. 5B). Identical results were obtained in assays using a third antibody against a different region of RB (44). In contrast, irrelevant control antibodies failed to coprecipitate BRF (44).

The previous experiments suggest that the BRF subunit of TFIIB is much less able to associate with cellular RB following SV40 transformation. As these results were obtained in studies using exogenous radiolabeled BRF, we also carried out coimmunoprecipitation analyses to compare the extent to which endogenous TFIIB is bound by RB in 3T3 and SV3T3 cells. Extracts of A31 and Cl38 cells were immunoprecipitated with either an anti-RB antibody or an irrelevant control antibody against the TAF<sub>48</sub> subunit of the Pol I factor SL1. Immunoprecipitated material was then resolved by SDS-PAGE and probed for the presence of BRF by Western blotting (Fig. 6A). Substantial amounts of endogenous BRF were found to coprecipitate with RB from A31 cell extracts, an effect that was specific since none was detected in the control immunoprecipitates. In contrast, significantly less BRF was coprecipitated with RB from the Cl38 cell extracts. Similarly, anti-RB immunoprecipitates from Cl49 cell extracts contained much less BRF than those obtained in parallel from A31 cell extracts (Fig. 6B). These results reinforce the data obtained in assays using radiolabeled BRF and indicate that the interaction between endogenous RB and TFIIB is diminished following SV40 transformation.

**The E7 oncoprotein of HPV-16 can also activate Pol III transcription.** The data described above suggest that T antigen can activate TFIIB by releasing it from the repressive influence of RB. The RB-binding site within T antigen has been mapped to an LXCXE motif, where X can be any amino acid (11, 64, 65). HPV-16 encodes an oncoprotein called E7 that has also been shown to bind and neutralize RB by means of an LXCXE motif (12, 32, 48). We therefore tested whether E7 resembles T antigen in being able to activate Pol III transcription.

The untransformed murine fibroblast cell line NIH 3T3 was transfected with expression vectors encoding either wild-type E7 or several mutant forms of E7 (Fig. 7). Primer extension analysis was used to determine the levels of Pol III transcription of a cotransfected VA<sub>1</sub> gene; a cotransfected control plasmid, in which the CAT gene is driven by the SV40 early promoter, was used to normalize for transfection efficiency. Specific VA<sub>1</sub> expression was stimulated substantially by the wild-type E7 protein (lane 2) relative to empty vector (lane 1). The ability of E7 to activate VA<sub>1</sub> transcription was severely compromised by deletion of residues 21 to 35, which removes the LXCXE motif that is located between amino acids 22 and 26 (lane 4). Indeed, substitution of the C24 residue to glycine is sufficient to abrogate the ability of E7 to stimulate VA<sub>1</sub> expression (lane 5). In contrast, mutant PRO2, which carries a proline substitution in its second residue and has an intact LXCXE motif, remains fully capable of activating Pol III transcription (lane 3). The PRO2 mutant is significant, because it has lost the ability to transform although it can still bind and neutralize RB (21). The fact that the PRO2 mutant can activate VA<sub>1</sub> expression indicates that the effect of E7 on Pol III

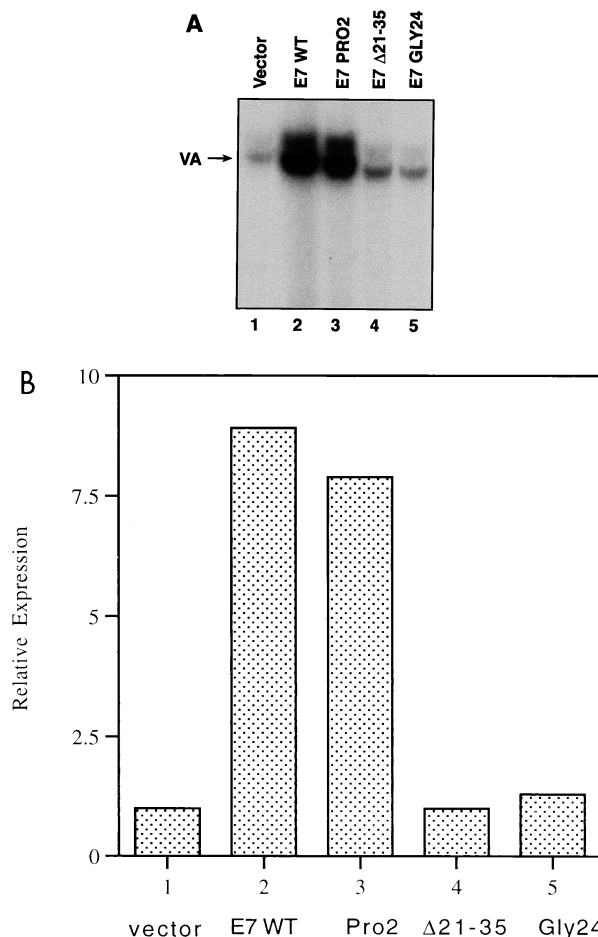


FIG. 7. Transfected HPV E7 activates Pol III transcription *in vivo* in a manner that is dependent on its RB-binding domain. NIH 3T3 cells were transfected with pVAI (2  $\mu$ g), pCAT (2  $\mu$ g), and 6  $\mu$ g of empty vector (lane 1) or vector encoding wild-type E7 (E7 WT; lane 2), PRO2 mutant E7 (lane 3),  $\Delta$ 21-35 mutant E7 (lane 4), or GLY24 mutant E7 (lane 5). (A) VA<sub>1</sub> primer extension products. (B) VA<sub>1</sub> transcription levels that have been normalized to the levels of CAT expression to correct for transfection efficiency and are expressed relative to the value obtained for the empty vector control (designated 1.0).

transcription is not an indirect consequence of cell transformation. These E7 proteins are all expressed at comparable levels in NIH 3T3 cells (21). We conclude that E7 can activate Pol III transcription *in vivo* in a manner that is dependent on its RB-binding LXCXE motif.

## DISCUSSION

Abnormally high levels of Pol III transcripts are observed in SV40-transformed cells (5, 36, 42, 60). The present study has provided evidence that more than one mechanism contributes to this effect and that two distinct Pol III factors, TFIIB and TFIIC2, are both targeted for activation by SV40.

The deregulation of TFIIC2 in SV3T3 cells was first detected by EMSA, which revealed an overall increase in DNA-binding activity and a greater proportion of the transcriptionally active form (60). These observations can be explained by our new data which show that SV40 transformation is accompanied by an increase in the abundance of both TFIIC $\alpha$  and TFIIC $\beta$ , two of the principal components of the TFIIC2 complex. Cross-linking has demonstrated that the TFIIC $\alpha$  subunit is responsible for contacting promoter DNA (3, 17, 63). In contrast, the presence of TFIIC $\beta$  is not required for DNA binding but is necessary for transcriptional activity (17, 43). Both the  $\alpha$  and  $\beta$  subunits have been reported to display histone acetyltransferase activity (18). TFIIC2 also contains three other subunits (17, 63), but the cloning of cDNAs encoding these subunits has not yet been reported and their roles have yet to be established. In the absence of molecular reagents that recognize the uncharacterized components of TFIIC2, we cannot determine whether these are also up-regulated in SV3T3 cells. However, it seems probable that they would be, in order to allow an overall increase in the level of functional TFIIC2. Our RT-PCR analyses have demonstrated that the transcripts encoding TFIIC $\alpha$  and TFIIC $\beta$  are more abundant in C138 and C149 cells than they are in the parental A31 cells. This is likely to account, at least in part, for the elevated levels of the TFIIC $\alpha$  and TFIIC $\beta$  polypeptides.

SV40 transformation of A31 cells also results in the activation of TFIIB. In contrast to TFIIC2, this does not appear to result from an increase in the abundance of TFIIB subunits. Thus, Western blotting reveals little or no change in the levels of TBP and BRF when A31 extracts are compared with C138 and C149 extracts. Human TFIIB is incompletely characterized at present but is believed to contain at least one additional subunit besides TBP and BRF (27, 30, 46, 47). We cannot exclude the possibility that the abundance of one or more uncharacterized component(s) of the complex becomes elevated in response to SV40 transformation. However, it is not necessary to invoke such a contingency in order to explain the observed activation of TFIIB. Both biochemical and genetic data have shown that TFIIB is subject to negative regulation in untransformed murine fibroblasts through a physical interaction with RB (24, 61). Coimmunoprecipitation analyses reveal that much less of the TFIIB in SV3T3 cells is associated with RB (Fig. 5 and 6). The higher specific activity of TFIIB that is observed in C138 and C149 cells (Fig. 4) is therefore likely due, at least in part, to a partial release from repression by RB. This interpretation is supported strongly by the fact that recombinant SV40 T antigen can stimulate Pol III transcription in a partially purified system in an RB-dependent fashion (Fig. 3). These data are consistent with a model in which T antigen counteracts the inhibitory influence of RB on TFIIB.

This study has described two distinct mechanisms which allow SV40-transformed cells to maintain abnormally high lev-

els of Pol III transcription, namely, the increased abundance of TFIIC2 and the release of TFIIB from repression by RB. Given the complexity of gene regulation by SV40, it is entirely possible that additional levels of control are also involved. For example, T antigen has been shown to bind and neutralize the tumor suppressor p53, and this capability is important for the transformation of some cell types (22, 48, 65). Among its many functions, p53 has been found to interact with TFIIB and repress Pol III transcription (4, 6). Inactivation of p53 might therefore provide an additional mechanism whereby T antigen is able to deregulate TFIIB. However, this may not have a major effect in the 3T3 system investigated here, since p53 levels are extremely low in unstressed A31 cells (25).

Damania et al. have reported recently that T antigen can be found in a complex with TFIIB, a conclusion based on both cofractionation and coimmunoprecipitation experiments with extracts prepared from HeLa cells that had been infected with a recombinant adenovirus which overexpresses SV40 large T antigen (9). The authors suggested that binding to TFIIB may allow T antigen to activate Pol III transcription directly, although no evidence was presented to establish this model (9). Our experiments with partially purified factors are not consistent with a direct effect on TFIIB, since T antigen was found to activate Pol III transcription only in the presence of added RB (Fig. 3). It is possible that T antigen acts directly on TFIIB under different experimental conditions. However, we have been unable to detect T antigen associated with TFIIB in extracts of SV3T3 cells. Thus, T antigen is not detected in anti-BRF immunoprecipitates, nor is BRF detected in anti-T immunoprecipitates from C138 or C149 cells, even though both cell lines express high levels of T antigen (44). We can therefore find no evidence that T antigen acts directly on TFIIB in the SV3T3 cell system.

The up-regulation of both TFIIB and TFIIC2 is likely to ensure that SV40-transformed cells maintain high rates of Pol III transcription. At present it is not clear which target is of greater importance in determining the overall level of class III gene expression. Add-back experiments carried out previously with crude fractions have suggested that TFIIC activity is limiting in extracts prepared from A31, C138, and C149 cells (60). If TFIIC is also limiting for Pol III transcription *in vivo*, then the up-regulation of this factor may make the principal contribution to the activation observed in SV3T3 cells. However, much of the complexity of the situation is likely to be concealed when asynchronous cell populations are analyzed. Thus, experiments with synchronized HeLa populations have revealed that alternative Pol III factors can be limiting during different phases of the cell cycle (55). Whereas TFIIC activity limits the rate of VA<sub>1</sub> expression in extracts of S- or G<sub>2</sub>-phase HeLa cells, TFIIB is the limiting factor in extracts of cells that were harvested during early G<sub>1</sub> phase (55). Under these circumstances, stimulation of TFIIB or TFIIC alone might influence the transcriptional output only during a restricted interval of the cell cycle. If a similar situation pertains in 3T3 cells, then the activation of both TFIIB and TFIIC might allow SV40 to sustain elevated rates of Pol III transcription throughout interphase.

Like SV40, adenovirus appears able to use more than one mechanism to stimulate the production of Pol III products. Adenovirus infection results in a marked increase in TFIIC2 activity (15, 16, 43, 62). Western blotting revealed that the infected cells express elevated levels of TFIIC $\beta$ , although TFIIC $\alpha$  was not induced under these circumstances (43). Induction of TFIIC $\beta$  requires the adenoviral transforming protein E1A (43). In addition to its effect on TFIIC $\beta$ , E1A has also been shown to relieve Pol III transcription from repres-

sion by RB, both *in vitro* and *in vivo* (61). Adenovirus therefore resembles SV40 in employing at least two distinct mechanisms to activate the Pol III machinery. The present study has provided evidence to suggest that a third DNA tumor virus, HPV, can also stimulate Pol III transcription by overcoming the repressive influence of RB. We have shown that the E7 oncoprotein of HPV activates VA<sub>1</sub> expression in transfected fibroblasts (Fig. 7). This capacity is abolished by mutations that specifically prevent E7 from binding and neutralizing RB. Previous work has shown that hepatitis B virus and human T-cell leukemia virus type 1 can also stimulate Pol III transcription by activating TFIIB (2, 14, 19, 34, 49, 50). It therefore seems that TFIIB is a frequent target for transforming viruses (reviewed in reference 54). High rates of Pol III transcription are a prerequisite of rapid growth, in order to sustain the production of tRNA and 5S rRNA. It has therefore been suggested that repression of the Pol III system may provide a mechanism for restraining cell growth (23, 33, 52). The fact that this system is targeted by two unrelated tumor suppressors, RB and p53, provides strong support for this contention. Additional support is provided by the range of oncogenic viruses that have evolved mechanisms to stimulate Pol III transcription.

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