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Polymerase III Transcription Factor B Activity Is Reduced in Extracts of Growth-Restricted Cells

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Extracts of cells that are down-regulated for transcription by RNA polymerase I and RNA polymerase III exhibit a reduced in vitro transcriptional capacity. We have recently demonstrated that the down-regulation of polymerase I transcription in extracts of cycloheximide-treated and stationary-phase cells results from a lack of an activated subform of RNA polymerase I which is essential for rDNA transcription. To examine whether polymerase III transcriptional down-regulation occurs by a similar mechanism, the polymerase III transcription factors were isolated and added singly and in pairs to control cell extracts and to extracts of cells that had reduced polymerase III transcriptional activity due to cycloheximide treatment or growth into stationary phase. These down-regulations result from a specific reduction in TFIIIB; TFIIIC and polymerase III activities remain relatively constant. Thus, although transcription by both polymerase III and polymerase I is substantially decreased in extracts of growth-arrested cells, this regulation is brought about by reduction of different kinds of activities: a component of the polymerase III stable transcription complex in the former case and the activated subform of RNA polymerase I in the latter.

The synthesis of the eucaryotic translational apparatus involves the coordinate production of the 28S, 18S, and 5.8S ribosomal RNAs, which are products of transcription by RNA polymerase I, and the 5S ribosomal RNA and tRNAs, which are synthesized by RNA polymerase III. Accumulation of cellular rRNA is known to be inhibited by a number of treatments, including administration of cycloheximide or glycocorticoids, growth into stationary phase, and starvation for an essential nutrient (reviewed in reference 18). Nuclear run-on analyses have shown that this regulation occurs in large part at the transcriptional level (9, 11, 19), and extracts prepared from such treated cells are accordingly inactive in catalyzing in vitro transcription of cloned rDNA (2–4, 10, 14). We have recently demonstrated that this down-regulation is due to the lack of an activated subform of RNA polymerase I that is required for ribosomal gene transcription (factor C), whereas the activities of the other required rDNA transcription factor (factor D) and of total bulk RNA polymerase I remain relatively constant (19). Parallel studies have shown that the accumulation of 5S RNA and tRNA is also down-regulated under the growth-inhibiting condition of cycloheximide treatment, while polymerase II transcription continues unaffected (9). As was the case for polymerase I, synthesis in isolated nuclei has indicated that this down-regulation of polymerase III-transcribed RNAs is exerted at the transcriptional level, and extracts prepared from these down-regulated cells were found to exhibit a reduced polymerase III transcriptional capacity (9). Since polymerase III transcription and polymerase I transcription appear to be subject to similar growth-state regulation and since these polymerase classes evidently share common subunits (13; reviewed in reference 16), we were intrigued by the possibility that the regulation of polymerase III transcription might also be caused by a modification of the polymerase itself. To address this question, we have examined the basis for the reduced polymerase III transcriptional capacity of extracts of both stationary-phase cells and cycloheximide-treated cells.

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Extracts of cells treated with cycloheximide and cells in stationary phase are down-regulated for transcription by RNA polymerase III. S-100 extracts were prepared from cell cultures that had been treated for 1.5 h with 100 μg of cycloheximide per ml and from cell cultures that had been grown into stationary phase, as well as from parallel control cultures of untreated log-phase mouse tissue culture cells. These preparations derived from the same numbers of starting cells and resulted in extracts that contained the same (± ~10%) protein concentration; duplicate extracts prepared in parallel exhibited virtually identical transcriptional activities. To verify that the down-regulation was successful, the extracts of the cycloheximide-treated cells (Fig. 1A) and the stationary-phase cells (data not shown) were assayed for polymerase I transcriptional capacity by using a cloned mouse rRNA gene template. As expected (19), the down-regulated extract (Fig. 1A, lane 2) was inactive relative to the parallel control extract (lane 1) or to a mixture of the two extracts (lane 3), and its transcriptional capacity was restored by supplementing with a highly purified preparation of the activated subform of RNA polymerase I (factor C; lanes 4 and 5).

To ascertain the capacity of the down-regulated S-100 extracts to transcribe 5S RNA, they were assayed alone and in combination by using the cloned *Xenopus borealis* 5S gene derivative, 5S maxigene (1). As shown in Fig. 1B, both the extract of the cycloheximide-treated cells (lane 2) and the extract of the stationary-phase cells (lane 3) were reduced in polymerase III transcriptional capacity relative to the parallel control cell extract (lane 1). The fact that neither the cycloheximide-treated cell extract (lane 4) nor the stationary-phase cell extract (lane 5) caused a diminution in transcriptional capacity when added to a control cell extract suggests that the reduced activity was not due to the presence of a diffusible inhibitor; rather, the down-regulated extracts evidently lack a positive-acting factor, as was previously suggested (9).

Transcription of the 5S gene is known to require at least

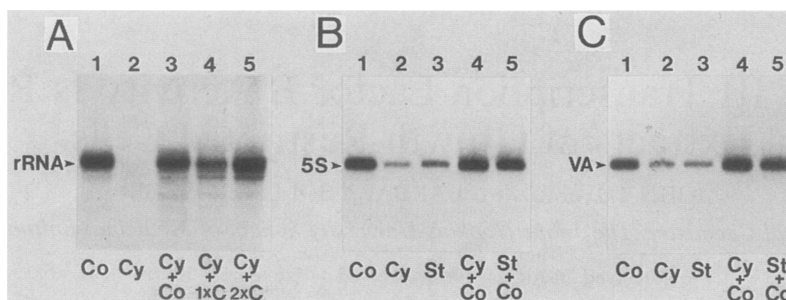

FIG. 1. Transcriptional activity of cycloheximide-treated and stationary-phase cell extracts. Transcription reactions containing S-100 extracts (0.15 M KCl soluble, nuclear plus cytoplasmic material) of control log-phase mouse tissue culture cells (Co), cells treated for 1.5 h with 100 μg of cycloheximide (Cy) per ml, and cells in stationary phase (St) were performed as described (5, 19). (A) Extracts were assayed alone and in combination for RNA polymerase I transcription of the mouse rDNA template (clone p5'*Sal-Pvu* [6], truncated at residue +292) as indicated. In lanes 4 and 5, the cycloheximide-treated cell extract was supplemented with 2.5 and 5 μl, respectively, of sucrose-gradient-purified polymerase I transcription factor C (19). (B) The extracts were assayed (6) alone and in combination for polymerase III transcription of the 5S maxigene (1) as indicated. (C) The extracts were assayed as in panel B but with the adenovirus 2 VA-I gene (plasmid pHK, which consists of the adenovirus DNA *Hin*dIII fragment from 17 to 32 map units cloned into the *Hin*dIII site of pBR322, kindly provided by Gary Ketner).

four components: the 5S gene-specific factor TFIIIA, two general polymerase III transcription components (TFIIIB and TFIIIC), and RNA polymerase III (7, 17). However, the low activity of the down-regulated extracts cannot be attributed to a lack of TFIIIA. This is shown in Fig. 1C, in which the experiment of Fig. 1B was repeated, except with the adenovirus VA-I gene template rather than the 5S gene template. VA-I transcription requires TFIIIB, TFIIIC, and polymerase III but not TFIIIA, yet the same results were obtained with the VA-I as with the 5S template. Thus, extracts of stationary-phase cells, like those of cycloheximide-treated cells, were down-regulated for polymerase III transcription because of their decreased abundance of activity of TFIIIB, TFIIIC, or RNA polymerase III.

TFIIIB is required to restore transcriptional activity to stationary-phase and cycloheximide-treated cell extracts. We next wished to identify the putative positive-acting transcriptional component that is depleted in the inactive extracts. To this end, we first isolated the known polymerase III transcription factors, starting with an S-100 extract of Erlich ascites cells (19) which is active for polymerase III transcription (~10 transcripts per gene per h under our usual reaction conditions of template excess; data not shown). By using minor modifications of published procedures (17), the S-100 extract was fractionated by chromatography on a phosphocellulose column, eluting with a linear KCl gradient from 0.1 to 1.0 M. The TFIIIB, polymerase III, and TFIIIC components eluted as peaks of activity centered at ~0.34, 0.45, and 0.60 M KCl, respectively. The polymerase III peak and the TFIIIC peak were essentially free of contamination by the other factors, and these fractions were dialyzed, stored at −70°C, and used without further purification. The TFIIIB-containing fractions were somewhat contaminated with polymerase III activity, so they were further purified by chromatography on DEAE-cellulose. The DEAE column was eluted with a linear $(NH_4)_2SO_4$ gradient from 50 to 350 mM, and the TFIIIB and polymerase III activities eluted as partially overlapping peaks centered at ~100 and ~125 mM $(NH_4)_2SO_4$, respectively. Fractions from the leading edge of the TFIIIB peak were essentially free of polymerase III contamination and were pooled, dialyzed, and stored at −70°C. To confirm our identification of TFIIIB and TFIIIC, we showed that TFIIIC but not TFIIIB forms a stable complex with the VA-I template, and that TFIIIB, the limiting factor in mouse S-100 and whole-cell extracts, associates slowly with a 5S DNA-TFIIIA-TFIIIC complex (6; data not shown). The TFIIIB, TFIIIC, and polymerase III fractions were all required to reconstitute significant levels of transcription of the VA-I gene (Fig. 2A, lanes 1 to 4).

To determine which of the polymerase III transcriptional components was required to restore transcriptional activity to the stationary-phase and cycloheximide-treated cell extracts, these down-regulated extracts were supplemented with each of the partially purified polymerase III transcription factors and assayed for transcription of the VA-I gene. Supplementation with RNA polymerase III had virtually no stimulatory effect (Fig. 2A, lanes 5 versus 8 and 9 versus 12). This is unlike rDNA transcription, in which activated RNA polymerase I is required to restore activity to the down-regulated extracts (19; Fig. 1A). Similarly, addition of TFIIIC to either of the kinds of down-regulated extracts had a negligible effect on the level of VA-I transcription (Fig. 2A, lanes 7 and 11). However, addition of TFIIIB restored activity to control levels (lanes 6 and 10). Thus, it is low quantity of the polymerase III transcription factor B that is limiting VA-I synthesis in extracts of both stationary-phase and cycloheximide-treated cells.

In accordance with the results obtained with the VA-I template, only TFIIIB, and not polymerase III or TFIIIC, was able to stimulate transcription of the 5S gene in both the stationary-phase cell extract (Fig. 2B) and the cycloheximide-treated cell extract (data not shown). Thus, TFIIIA is not limiting in the down-regulated extracts. Indeed, polymerase III transcription in the control cell extract was also limited by the availability of TFIIIB activity (Fig. 2A, lanes 13 to 16).

Since the S-100 extracts examined in Fig. 2A and B consisted of the material from lysed nuclei plus cytoplasm that was soluble in isotonic salt, it remained conceivable that merely the extractability and not the level of TFIIIB was decreased in the down-regulated cells. To address this possibility, high-salt whole-cell extracts were prepared from control cells and from cells that had been treated with cycloheximide for 2 h. This method of extract preparation has previously been shown to recover template-bound, as well as free, polymerase III transcription components (12). Confirming the results obtained with the isotonic extracts in Fig. 1 and 2, the high-salt extract of cycloheximide-treated cells (Fig. 2C, lane 2) was inactive relative to the parallel

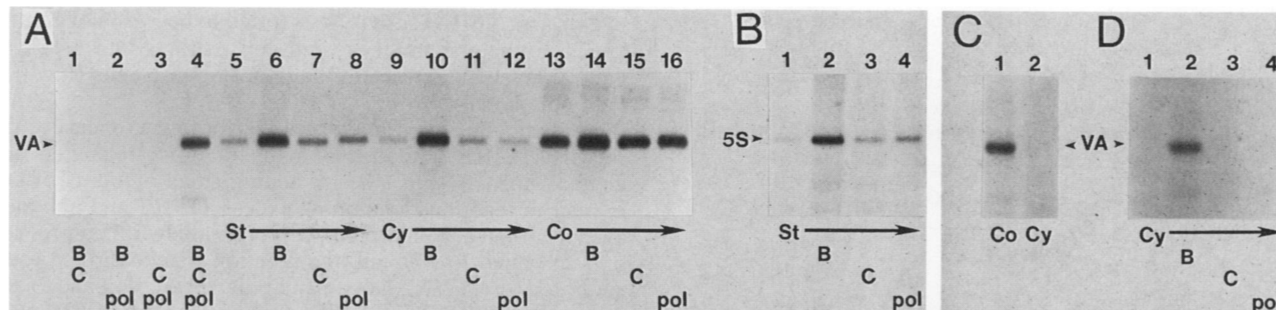

FIG. 2. Addition of partially purified polymerase III transcription factors to the down-regulated extracts. (A) The isolated TFIIIB (B), TFIIIC (C), and polymerase III (pol) fractions were assayed for their ability to support VA-I transcription in various combinations, as indicated (lanes 1 to 4). In lanes 5 to 16, stationary-phase cell extract (St), cycloheximide-treated cell extract (Cy), and control cell extract (Co) were assayed for transcriptional activity alone and in combination with the same amounts of the indicated fractions as were used in lanes 1 to 4. (The shorter transcript faintly visible in lane 4 is VA-II RNA). These same results were obtained with three extracts of cycloheximide-treated cells and two extracts of stationary-phase cells. (B) Experiments shown in lanes 5 to 8 of panel A were repeated with the 5S maxigene template. (C) 1.1 M ionic strength extracts (12) of control cells (lane 1) and cycloheximide-treated cells (lane 2) were assayed for VA-I transcriptional capacity. Similar results were obtained with various amounts of extract and various template concentrations. (D) The high-salt extract of cycloheximide-treated cells was assayed for transcription alone and in combination with isolated TFIIIB, TFIIIC, and polymerase III.

extract of control cells (Fig. 2C, lane 1). Furthermore, transcriptional activity was restored to the high-salt extract of cycloheximide-treated cells by supplementing with TFIIIB (Fig. 2D, lanes 1 and 2); supplementing with TFIIIC (lane 3) or polymerase III (lane 4) had a negligible effect. We also have noted no preferential instability of TFIIIB in this or other down-regulated extracts (data not shown). We thus conclude that the down-regulated cells have reduced polymerase III transcriptional capacity because they truly have reduced TFIIIB levels, not merely because this factor is sequestered in the cells or labile in the extracts.

Down-regulated extracts contain control amounts of polymerase III activity. Since transcriptional activity in both of the down-regulated cell extracts is limited by TFIIIB availability, the experiments shown in Fig. 2 are not sensitive to the amount of active polymerase III present in these extracts. It thus remained possible that polymerase III activity could be substantially reduced in the down-regulated extracts relative to that in the control extracts. To address this possibility, we measured the relative polymerase III activity of a number of control, cycloheximide-treated, and stationary-phase cell extracts by adding small amounts of each extract to reactions containing saturating amounts of TFIIIB and TFIIIC (Fig. 3). The assays were indeed limited by polymerase availability, for supplementation of reactions containing the largest amount of extract with additional TFIIIB (lane 7) or TFIIIC (lane 8) had no effect, whereas supplementation with additional polymerase III stimulated transcription (lane 9). Reactions containing increasing amounts of control extract produced increasing transcriptional signals (lanes 2 to 6). By performing this analysis with the various control and down-regulated cell extracts, we were able to measure the amount of transcriptionally active polymerase III in these extracts, independent of their TFIIIC and TFIIIB activities. Densitometric quantitation of the control lanes 2 to 6 provided a standard curve against which the relative amount of active polymerase III in the various extracts was quantitated.

Three pairs of S-100 extracts prepared in parallel from control and cycloheximide-treated cells, as well as two extracts prepared from stationary-phase cells, were assayed for their relative polymerase III activity (Fig. 3, lanes 6 and 10 to 15; data not shown). By comparing the signals obtained with the pairs of parallel control and cycloheximide-treated cell extracts (lane 6 versus 10, 11 versus 12, or 13 versus 14) or stationary-phase cell extract (lane 13 versus 15), RNA polymerase III activity was seen to remain at or near control levels upon transcriptional down-regulation. In multiple assays of these five pairs of extracts, polymerase III activity was found to remain at ~100% of control levels upon cycloheximide treatment, and at ~80% of control levels in stationary-phase cell extracts (Fig. 3; data not shown; the relevant quantitation is summarized in the legend to Fig. 3). Thus, unlike the situation with polymerase I transcription (19; Fig. 1A), stationary-phase and cycloheximide-treated

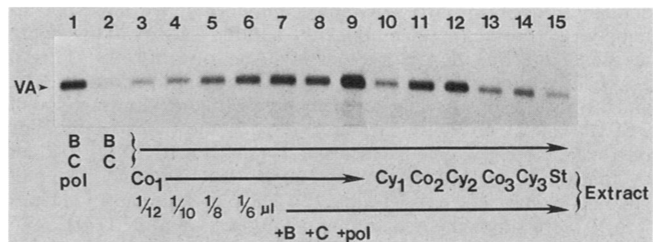

FIG. 3. Measurement of transcriptionally competent polymerase III activity in various control and down-regulated cell extracts. Reactions containing 4 μl of TFIIIB, 3 μl of TFIIIC, and the VA-I template were supplemented with the following: 7 μl of polymerase III (lane 1), no additional factors (lane 2), the indicated fractions of 1 μl of control extract no. 1 (lanes 3 to 9), 1/6 μl of cycloheximide-treated cell extract no. 1 (lane 10), 1/6 μl of control and cycloheximide-treated cell extracts no. 2 (lane 11 and 12), 1/6 μl of control and cycloheximide-treated cell extracts no. 3 and stationary-phase cell extract (lanes 13 to 15, respectively). The reactions in lanes 7 to 9 were supplemented with an additional 2 μl of TFIIIB, 1.5 μl of TFIIIC, and 3.5 μl of polymerase III, respectively. Densitometric quantitation of these and duplicate experiments, relative to the standard curves (as in lanes 2 to 6), demonstrated that the cycloheximide-treated cell extract no. 1 (Cy_1) exhibited 69%, 87%, and 113% (average, 90%) the polymerase III activity of its control extract, cycloheximide-treated cell extract no. 2 (Cy_2) exhibited 104% and 110% (average, 107%) the polymerase III activity of its control extract, and cycloheximide-treated cell extract no. 3 (Cy_3) exhibited 120% and 92% (average, 106%) the polymerase III activity of its control extract; in each case, the first activity measurement represents the data of this figure. The stationary-phase cell extract shown in this figure had 78% of control polymerase III activity, and a second stationary-phase cell extract exhibited 81% activity. For other abbreviations, see the legend to Fig. 2.

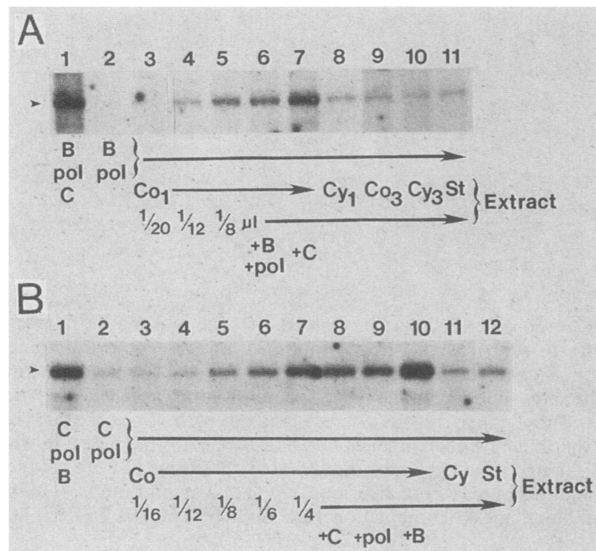

FIG. 4. Measurement of TFIIIC and TFIIIB activities in control and down-regulated cell extracts. (A) TFIIIC assay. Reactions containing 7 µl of polymerase III, 4 µl of TFIIIB, and the VA-I template were supplemented with the following: 3 µl of TFIIIC (lane 1), no additional fractions (lane 2), the indicated fractions of 1 µl of control cell extract no. 1 (lanes 3 to 7), 1/8 µl of cycloheximide-treated cell extract no. 1 (lane 8), and 1/8 µl each of control and cycloheximide-treated cell extracts no. 3 and stationary-phase cell extract (lanes 9 to 11, respectively). The reaction in lane 6 was supplemented with an additional 2 µl of TFIIIB and 3.5 µl of polymerase III, and the reaction in lane 7 was supplemented with an additional 1.5 µl of TFIIIC. While this manuscript was in preparation, four reports have been published (15, 20, 21, 23) which indicate that the activity that has traditionally been identified as TFIIIC actually consists of two separable components; we have not attempted to resolve these components, and the C assay in this figure measures the one that is present in lower quantity. (B) TFIIIB assay. Reactions containing 3 µl of TFIIIC, 7 µl of polymerase III, and the VA-I template were supplemented with the following: 4 µl of TFIIIB (lane 1), no additional factors (lane 2), the indicated fractions of 1 µl of control cell extract no. 1 (lanes 3 to 10), 1/4 µl of cycloheximide-treated cell extract no. 1 (lane 11), and 1/4 µl of stationary-phase cell extract (lane 12). The reactions shown in lanes 8 to 10 were supplemented with an additional 1.5 µl of TFIIIC, 3.5 µl of polymerase III, and 2 µl of TFIIIB, respectively. See legend to Fig. 2 for other abbreviations.

cell extracts are not depleted in transcriptionally competent RNA polymerase III.

By repeating the experiment of Fig. 3, but using different pairs of isolated polymerase III transcription factors, one is able to measure the relative activities of TFIIIB and TFIIIC in the various extracts. To assay for TFIIIC (Fig. 4A), small amounts of extract were added to reactions saturated in TFIIIB and polymerase III. As in Fig. 3, assaying various amounts of control extract produced a standard curve (lanes 2 to 5), and the assay was shown to be TFIIIC limited, for supplementation with additional polymerase III and TFIIIB had no effect (lane 6), whereas supplementation with TFIIIC further stimulated transcription (lane 7). Relative activity was again densitometrically quantitated relative to the standard curve provided by the various dilutions of the control extract in lanes 2 to 5. The two illustrated cycloheximide-treated cell extracts contained ~70% and ~95% of the amount of TFIIIC activity present in the control cell extracts that had been prepared in parallel (lane 5 versus 8 and 9 versus 10, respectively). Upon multiple assays of a number of extracts, TFIIIC activity was found to be ~80% of control levels in cycloheximide-treated cell extracts and ~75% of control levels in extracts of stationary-phase cells (Fig. 4A; data not shown).

An analogous experiment was performed to measure the relative TFIIIB activities of pairs of down-regulated and control extracts (Fig. 4B) by adding small amounts of cell extract to reactions containing excess TFIIIC and polymerase III. Again a standard curve was created with control cell extract (lanes 2 to 7), and the reaction was confirmed to be saturated for TFIIIC (lane 8) and polymerase III (lane 9) and limited by TFIIIB (lane 10). Comparison of the level of transcription from the control extract (lane 7) with that from the parallel cycloheximide-treated cell extract (lane 11) and from stationary-phase cell extract (lane 12) demonstrates that these down-regulated cell extracts were reduced in TFIIIB activity (to 38% and 44% of control levels, respectively), thus confirming the conclusion drawn from the data of Fig. 2. Multiple assays of a number of cycloheximide-treated and stationary-phase cell extracts yielded an average TFIIIB activity of ~40% of control levels for both kinds of down-regulated extracts (Fig. 4B; data not shown).

While it may initially appear surprising that an ~60% decrease in TFIIIB activity is sufficient to explain the decreased transcriptional activity of the cycloheximide-treated and stationary-phase cell extracts (Fig. 1 and 2), data from other laboratories (12, 22), as well as our own (Fig. 4B; data not shown), indicates that the response of the in vitro transcription reaction to the amount of TFIIIB can be quite nonlinear. Thus, the ~60% decrease in TFIIIB activity is sufficient to account for the ~80% decrease in overall transcriptional activity of the down-regulated cell extracts.

Changes in extract activity may mimic cellular regulatory mechanisms. The preferential decrease in TFIIIB activity in extracts of mouse stationary-phase cells and cycloheximide-treated cells was unexpected from three lines of reasoning. First, the decrease in cellular RNA polymerase I transcription in these down-regulated cell extracts correlates with a decrease in activity of the activated subform of RNA polymerase I (19; Fig. 1A), and it had seemed reasonable to hypothesize that polymerase III transcription might be regulated in an analogous fashion. Second, the increase in polymerase III transcriptional activity of HeLa cells upon adenovirus infection has been reported to correlate with an increase in the amount of extractable TFIIIC activity (12, 22). Finally, while the final revisions were being added to this paper, it was reported that polymerase III transcription is depressed upon poliovirus infection, and this is evidently due primarily to a reduction in TFIIIC levels (although TFIIIB levels were also somewhat reduced [8]). However, our data argue that the reduced transcriptional activity in the down-regulated mouse cell extracts is due to reduced TFIIIB activity and does not correlate with either decreased polymerase III or TFIIIC activity. In summary, the selective reduction of TFIIIB activity we observed in extracts of stationary-phase cells and cycloheximide-treated cells is unprecedented and suggests that changes in TFIIIB activity may be involved in the growth rate regulation of cellular RNA polymerase III transcription.

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