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Enhanced *B2* transcription in simian virus 40-transformed cells is mediated through the formation of RNA polymerase III transcription complexes on previously inactive genes

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ABSTRACT **RNA polymerase III (pol III) transcribes the highly repeated murine *B2* elements. We showed previously that the B2 RNAs are induced 4-fold in normal growing cells and 20-fold in simian virus 40-transformed cells relative to the levels in normal confluent cells. By employing chromatin as a template in a partially purified pol III transcription system, we now demonstrate that the augmented expression results from the formation of pol III transcription complexes on previously inactive *B2* genes. Extracts prepared from normal growing cells and transformed cells transcribed cloned pol III templates 5-fold more efficiently than extracts from normal confluent cells. This increase was attributed to 5-fold greater levels of factor IIIC; the levels of pol III and factor IIIB were the same in all extracts. We discuss how the levels of IIIC and differing accessibility of this factor to repressed *B2* genes mediate the formation of pol III transcription complexes in normal growing and transformed cells.**

The *B2* elements are a highly repeated murine gene family present at 10^5 copies per genome (1). RNA polymerase III (pol III) transcribes these genes *in vivo* giving rise to a class of small heterogeneous RNAs, 200–600 nucleotides long (2, 3). The cytoplasmic levels of these transcripts are increased by a wide range of cellular conditions including oncogenic transformation (2, 4), growth (5), and heat shock (6). These RNAs are also induced during early mouse development (7) and in undifferentiated embryonal carcinoma cells (4). Although the function of the B2 RNAs is unknown, the induction provides a useful system to analyze how diverse phenomena effect similar changes in cellular gene expression.

We have demonstrated (2) that the B2 RNA levels are increased 20-fold in simian virus 40 (SV40)-transformed murine cell lines compared with normal confluent cells. These transcripts are also induced 4-fold in normal growing cells (2). We found that this enhanced expression was specific to highly repeated pol III gene families and that it occurred at the level of transcription (8).

B2 elements contain a split internal pol III promoter consisting of two conserved sequence motifs called the A and B boxes (for review, see ref. 9). Like the adenovirus *VA* and cellular tRNA genes, which contain a similar promoter, transcription of either cloned or genomic *B2* elements *in vitro* requires pol III and two partially purified accessory components (2, 8), designated factor IIIB and factor IIIC (10). On the tRNA and *VAI* genes factor IIIC binds specifically to the A and B boxes forming the minimum isolable complex observed *in vitro*. This binding permits factor IIIB to bind that in turn allows pol III to affix and initiate transcription (9).

Stable transcription complexes containing the factors necessary for specific initiation have been demonstrated on the 5S and tRNA genes in *Xenopus* chromatin (11). We wished to establish whether such complexes were associated with the *B2* genes in murine chromatin and whether formation of additional assemblies mediated the induction in normal growing and in SV40-transformed cells. We demonstrate that stable transcription complexes do exist on the *B2* genes in chromatin and that the amount of bound IIIC reflects the transcription rates previously observed in nuclei from normal confluent, normal growing, and SV40-transformed cells. Although we observe that factor IIIC activity is elevated 5-fold in normal growing and in transformed cell extracts, relative to normal confluent cell extracts, we find no evidence for a *B2*-specific factor.

MATERIALS AND METHODS

Preparation of Chromatin. Nuclei were prepared from SVT-2 and BALB/c 3T3 cell as described (8). Endogenous B2 RNA was removed by treating the nuclei with RNase A. Native and Bio-Rex 70-treated chromatin were prepared as described (11). The DNA concentration and size of the chromatin were monitored by absorbance at 260 nm and agarose gel electrophoresis, respectively.

Transcription of Chromatin *in Vitro*. Transcription factors IIIB and IIIC from HeLa cells and pol III from *Xenopus laevis* were fractionated as described (12). One unit of factor activity is the amount required to synthesize 50 fmol of specific RNA in a standard transcription reaction with the other components in excess (8). One unit of polymerase activity is the amount required to synthesize 1 pmol of RNA in a 20-min reaction at 30°C by using poly[(dA-dT)] as a template. The 20-μl reaction mixtures contained 20 mM Hepes (pH 7.5), 65 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 1.0 mM dithiothreitol, 7% (vol/vol) glycerol, 5 units of RNasin (Promega Biotec, Madison, WI), 200 μM ATP, 200 μM GTP, 200 μM CTP, 200 μM UTP, 1 μg of chromatin (DNA concentration), 0.5 unit of the indicated factors, and 10 units of pol III. After 1 hr at 30°C, the reactions were terminated and the RNA products were assayed by primer extension as described (8).

Transcription by the Extracts. Extracts were prepared as described by Manley *et al.* (13). BALB/c 3T3 cells were harvested at 0.5×10^6 cells per 100-mm dish for the normal growing cell extracts (3T3-G) or 3×10^6 cells per 100-mm dish for the confluent cell extracts. SVT-2 cells were collected between 1 and 3×10^7 cells per 100-mm dish. The transformed cells are smaller, are not contact inhibited, and thus grow to much higher densities than the normal cells.

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Abbreviations: pol III, RNA polymerase III; SV40, simian virus 40; TAg, tumor antigen.

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Protein was assayed with a Bio-Rad kit with bovine serum albumin as a standard.

The 20-μl transcription mixtures were incubated for 1 hr at 30°C and contained extract, 20 mM Hepes (pH 7.5), 65 mM KCl, 5 mM $MgCl_2$, 6% (vol/vol) glycerol, 200 μM ATP, 200 μM CTP, 200 μM UTP, and 0.3 μg of either pXltmet (14) or A18CL. Reactions with pXltmet, a plasmid containing a $tRNA^{Met}$ gene, also contained 10 μM GTP and 10 μCi of [α-^{32}P]GTP (1 Ci = 37 GBq). Reactions with A18CL, a plasmid containing a *B2* element, also contained 200 μM GTP. The *B2* element in A18CL is 95% similar to the *B2* consensus sequence. Transcription of the B2 element in A18CL proceeds into M13 vector DNA and transcripts were measured by primer extension analysis with the M13 reverse sequencing primer. The ^{32}P-labeled tRNA gene transcripts and the B2 primer extension products were fractionated by electrophoresis through 10% polyacrylamide sequencing gels and autoradiographed; the amount of specific product was quantitated by measuring the radioactivity of excised bands.

RESULTS

***B2* and tRNA Gene Transcription in Whole Cell Extracts.** By using a nuclear run-on assay we demonstrated (8) that the induction of B2 RNAs was due to increased transcription. To determine if augmented levels of either a *B2*-specific factor or a general pol III transcription component accompanied the increase in transcription, we prepared whole cell extracts from normal growing, confluent, and SV40-transformed cells and compared their ability to transcribe cloned pol III templates. Transcription of cloned *B2* and tRNA templates were compared to distinguish *B2*-specific effects from those on the general pol III transcription apparatus. *In vivo*, the levels of measured tRNAs do not change during growth or upon SV40 transformation (8). We have used primer extension analysis to measure the products of *in vitro* transcription of the *B2* template; pol III, factor IIIB, and factor IIIC were required and transcription was inhibited by α-amanitin at 200 but not at 2 μg/ml (Fig. 1, lanes 10–14). The lower level of drug inhibits RNA polymerase II whereas the higher level also inhibits pol III.

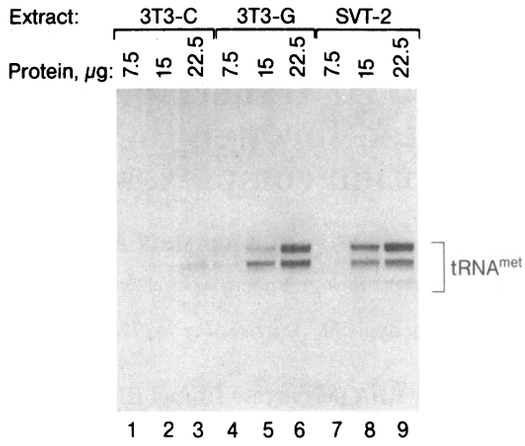

FIG. 2. *In vitro* transcription of a cloned $tRNA_1^{Met}$ gene with extracts prepared from normal and transformed cells. The transcription mixtures contained 0.3 μg of pXltmet and the indicated amounts of extract from normal confluent (3T3-C) cells (lanes 1–3), normal growing (3T3-G) cells (lanes 4–6), or SV40-transformed (SVT-2) cells (lanes 7–9). The ^{32}P-labeled RNAs were fractionated through 10% polyacrylamide/urea gels.

With this template we found 5-fold greater levels of specific transcription in extracts prepared from normal growing (3T3-G) and transformed (SVT-2) cells than in those made from normal confluent cells (3T3-C) (Fig. 1, lanes 1–9). Transcription of a tRNA gene by the extracts gave similar results (Fig. 2), demonstrating that the increase was not specific for *B2* genes but was instead a general effect on the specific pol III transcription activity.

The increased transcription capacity of the normal growing and transformed cell extracts could be due to increased levels of one of the pol III transcription components or the presence of an inhibitor in the normal confluent cell extracts. Transcription experiments in which the normal confluent cell

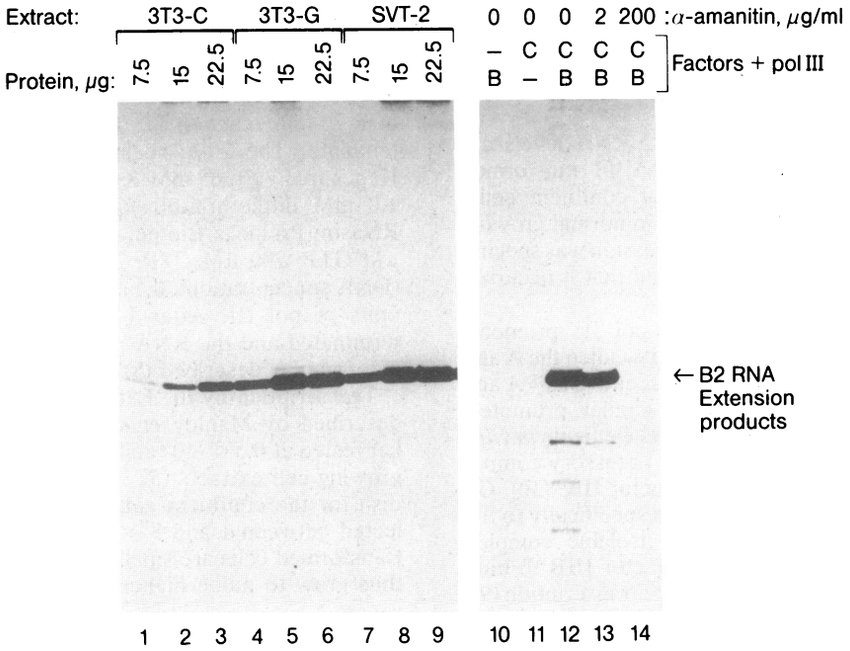

FIG. 1. *In vitro* transcription of a cloned *B2* element with extracts prepared from normal and transformed cells. The transcription mixtures contained 0.3 μg of a cloned *B2* element and the indicated amounts of extract prepared from normal confluent (3T3-C) cells (lanes 1–3), normal growing (3T3-G) cells (lanes 4–6), or SV40-transformed (SVT-2) cells (lanes 7–9), or 0.5 unit of the designated factors, 10 units of pol III, and the specified concentrations of α-amanitin (lanes 10–15). The RNAs were measured by primer-extension analysis.

extract was mixed with the other extracts failed to reveal the presence of an inhibitor. We, therefore, measured the levels of pol III, factor IIIB, and factor IIIC. When the levels of pol III were measured by a nonspecific template assay, we found less than a 2-fold difference between the three extracts (data not shown). We next measured factors IIIB and IIIC by complementation. In this assay, the levels of one factor were measured by placing the other factor and pol III in excess with a tRNA gene as template. Titrations with partially purified components were performed to ensure that the factor being measured was limiting. Transcription was assayed in the range in which the tRNA synthesized was roughly linear with the protein concentration in the extracts. We found nearly equivalent levels of factor IIIB in all three extracts (Fig. 3*A*). In contrast, with 22.5 μg of protein, for example, the levels of factor IIIC were 5-fold greater in the normal growing and transformed cell extracts than in the normal confluent extracts (Fig. 3*B*). Quantitatively similar results were observed with two other extracts prepared from each of the cell types.

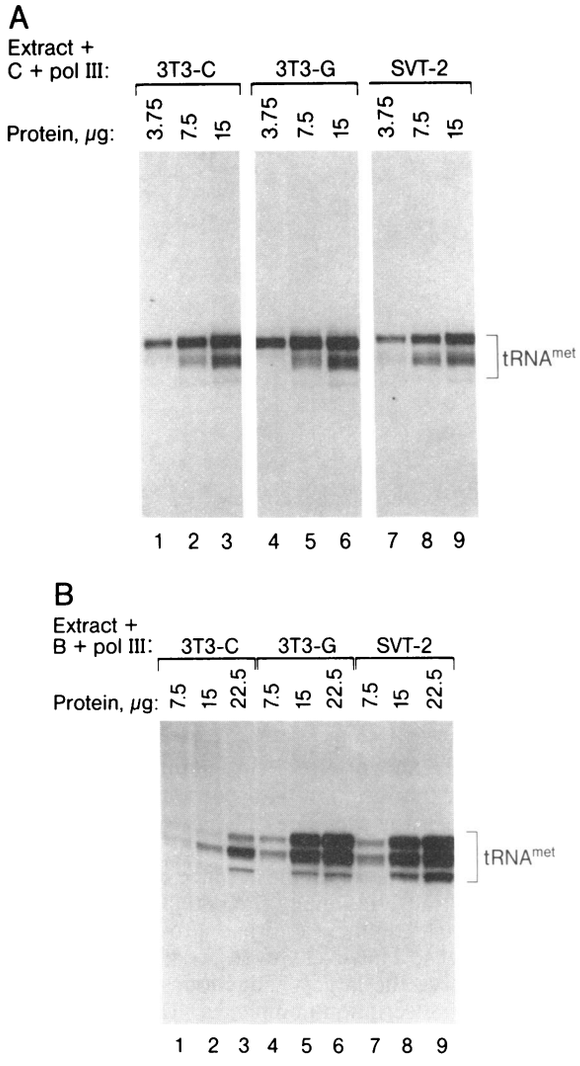

FIG. 3. (*A*) Complementation of extracts with pol III and factor IIIC. The transcription mixtures contained 0.3 μg of pXltmet, 10 units of *Xenopus* pol III, 0.5 unit of HeLa factor IIIC, and the indicated amounts of extract from normal confluent (3T3-C) cells (lanes 1–3), normal growing (3T3-G) cells (lanes 4–6), or SV40-transformed (SVT-2) cells (lanes 7–9). (*B*) Complementation of extracts with pol III and factor IIIB. The transcription mixtures contained 0.3 μg of pXltmet, 10 units of *Xenopus* pol III, 0.5 unit of HeLa factor IIIB, and the indicated amounts of extract from normal confluent (3T3-C) cells (lanes 1–3), normal growing (3T3-G) cells (lanes 4–6), or SV40-transformed cells (lanes 7–9).

***B2* Gene Transcription in Chromatin.** We wished to determine if stable transcription complexes existed on the *B2* genes, and if so, how these assemblies mediated the induction. Particularly interesting is the amount of IIIC bound to the *B2* genes in chromatin because of the elevated levels of this factor in normal growing and transformed cell extracts. The amount of IIIC bound to the *B2* genes was measured by complementing chromatin from normal confluent (3T3-C), normal growing (3T3-G), and SV40-transformed (SVT-2) cells with factor IIIB and pol III and quantitating the B2 RNA products by primer extension analysis (Fig. 4). Under these conditions, we measure the relative number of complexes containing either IIIC alone or paired with IIIB. We found that the relative levels of B2 RNA synthesized *in vitro* among the three chromatin preparations matched those observed in the cytoplasm (compare lanes 1–3 with lanes 7–9) and those produced in the nuclear transcription reactions (ref. 8 and unpublished data). In contrast, transcription of total tRNA genes in the chromatin was identical among all three preparations (unpublished data). Similar data were obtained with several preparations of chromatin. These results demonstrate that stable complexes exist on the *B2* genes and suggest that binding of IIIC mediates the transcriptional induction. If the levels of bound IIIC were identical among the preparations, then complementation with excess pol III and factor IIIB would have produced similar amounts of B2 RNA.

A crucial aspect of the IIIC binding model is that genes that are activated in normal growing and transformed cells must be repressed in normal confluent cells. A precedent for this type of regulation exists in the *Xenopus* 5S RNA gene system where the oocyte-type 5S genes are repressed by histone H1 in somatic cells (11). Treatment of the *Xenopus* chromatin with either high salt concentrations or the cation-exchange resin Bio-Rex 70 removes this repression and allows subsequent transcription of the oocyte genes by pol III and a full complement of transcription factors. To ascertain whether the *B2* genes in normal confluent cells are similarly repressed, we treated the three chromatin preparations with Bio-Rex 70 and employed them as templates in the fractionated pol III transcription system. We refer to these preparations as derepressed chromatin. Transcription with pol III and factor IIIB was used to determine the relative number of transcription complexes containing factor IIIC, and transcription with pol III, factor IIIB, and factor IIIC was used to assess the number of available but previously silent or repressed *B2* templates (Fig. 5). Although transcription from the complexes containing IIIC was reduced compared with native chromatin (Fig. 5, compare lane 8 with lane 2) the ratio of the B2 RNA products among the three derepressed preparations remained the same (compare Fig. 4, lanes 7–9 with Fig. 5, lanes 4, 6, and 8). Thus, treatment of chromatin with Bio-Rex 70 reduces the number of active transcription assemblies but affects each of the preparations similarly. When the derepressed chromatin was transcribed with pol III, factor IIIB, and factor IIIC, similar amounts of *B2* gene transcription were observed among all three preparations (Fig. 5, lanes 5, 7, and 9). This transcription was greatly increased relative to that observed with native chromatin as template (compare lane 3 with lane 9). No increase in the level of tRNA gene transcription was observed (unpublished results). Surprisingly, even in transformed cells, where we observe the largest induction, only 5% of the transcriptionally competent *B2* genes are active (compare lane 2 with lane 9). These data suggest that in all of the cells, the majority of *B2* genes are repressed; however, fewer are repressed in normal growing and transformed cells than in normal confluent cells. The results are consistent with the hypothesis that the increased transcription in normal growing and transformed cells is due

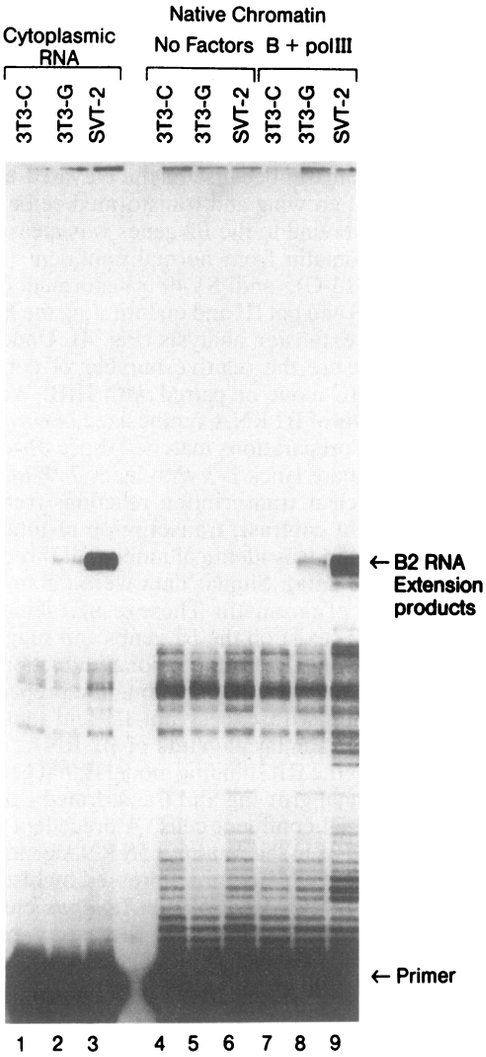

FIG. 4. *In vitro* transcription of *B2* genes in native chromatin. The transcription mixtures contained 1 μg of native chromatin (lanes 4–9) from normal confluent (3T3-C) cells (lanes 4 and 7), normal growing (3T3-G) cells (lanes 5 and 8), or SV40-transformed (SVT-2) cells (lanes 6 and 9) and either no factors (lanes 4–6) or 10 units of pol III and 0.5 unit of factor IIIB (lanes 7–9). The product RNAs were analyzed by primer extension. The primer extension mixtures contained a ^{32}P-labeled B2 synthetic oligonucleotide primer and either RNA synthesized *in vitro* (lanes 4–9) or 50 ng of cytoplasmic RNA (lanes 1–3) from normal confluent (3T3-C) cells (lane 1), normal growing (3T3-G) cells (lane 2), or SV40-transformed (SVT-2) cells (lane 3). The bands between the primer and the B2 RNA extension products appear to be due to extension of degraded endogenous B2 RNA resulting from pretreatment of the nuclei with RNase A.

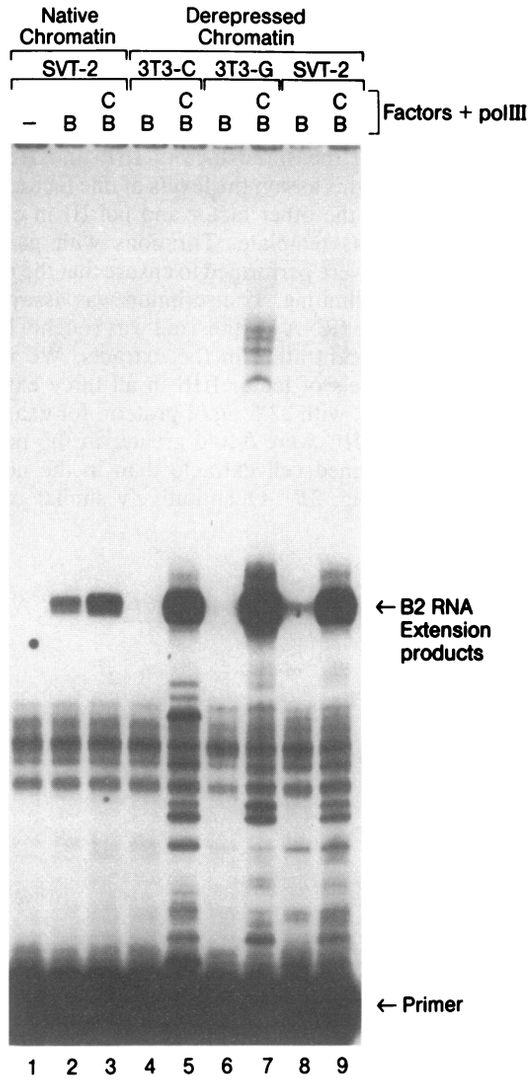

FIG. 5. *In vitro* transcription of *B2* genes in derepressed chromatin. The transcription reactions contained pol III, the indicated factors, and 1 μg of native transformed (SVT-2) cell chromatin (lanes 1–3) or Bio-Rex 70-derepressed chromatin (lanes 4–9) from normal confluent (3T3-C) cells (lanes 4 and 5), normal growing (3T3-G) cells (lanes 6 and 7), or SV40-transformed (SVT-2) cells (lanes 8 and 9). RNAs were analyzed by primer extension.

to the formation of transcription complexes on previously repressed *B2* genes but only a fraction of the transcriptionally competent genes become activated.

DISCUSSION

We have analyzed the induction of *B2* gene expression in normal growing and SV40-transformed cells. We found (8) that the relative amounts of B2 RNA synthesized in nuclear transcription reactions paralleled the levels observed in the cytoplasm, demonstrating that the induction was due to increased transcription. We observed the same result when we transcribed native chromatin in the presence of saturating amounts of pol III and factor IIIB, suggesting that these components are normally in excess in the cell and that binding of factor IIIC to the *B2* genes is the limiting step in the induction. Treatment of the chromatin with Bio-Rex 70 and subsequent transcription with pol III, factor IIIB, and factor IIIC reveals the presence of a large pool of transcriptionally competent genes among all three chromatin preparations that were not active in the absence of factor IIIC. The total number of transcriptionally competent *B2* genes was the same for normal confluent, normal growing, and SV40-transformed cells. However, even in transformed cells, where we observe the largest induction, only 5% of these genes contain transcription complexes. The other 95% are repressed either by histone H1 or some other component of the chromatin removed by Bio-Rex 70.

By using a complementation assay, we found a 5-fold increase in factor IIIC activity in extracts from normal growing compared with normal confluent cells. This correlates well with the B2 RNA induction in the growing cells and suggests that the increased levels of IIIC cause the augmentation. In contrast, the levels of factor IIIC alone cannot explain the induction in transformed cells because the increase in B2 RNA is 5-fold greater than in normal growing cells. This result suggests that there is a distinct effect of oncogenic transformation superimposed on the induction in

normal growing cells. It is possible that transformation results in a further increase in factor IIIC activity *in vivo*, perhaps by increasing its affinity for *B2* genes, but that this activity does not function *in vitro*. Alternatively, transformation increases the number of accessible *B2* genes, thereby driving the formation of additional transcription complexes in transformed cells. It is conceivable that large tumor antigen (TAg), the transforming protein of SV40, plays an active role in this process. TAg binds to specific sequences in cellular DNA that are similar to those present in the SV40 genome (S. Conrad and M. Botchan, personal communication) and has DNA helicase activity (15). Once bound, the helicase activity might allow TAg to proceed along the DNA disrupting inactive chromatin, thereby exposing previously repressed *B2* genes to the factors.

Other viral transforming genes have also been found to increase pol III transcription. Exogenous pol III templates transfected into cells harboring the adenovirus E1A or the pseudorabies IE protein are transcribed at much greater efficiencies than in control cells (16, 17). Extracts prepared from these cells also appear to contain greater levels of factor IIIC than those obtained from parental cell lines (17, 18). Unlike the adenovirus and pseudorabies systems, the levels of IIIC observed in cells harboring large TAg are no higher than those observed in normal growing cells.

It is surprising that only the *B2* genes and one other highly repeated gene family, designated *B1*, are affected by growth conditions and transformation. The 5-fold increase in the levels of factor IIIC in growing and transformed cells does not alter the steady-state levels of other pol III products including the 5S, 7SL, 7SK, $tRNA^{Met}$, $tRNA^{Pro}$, or $4.5S_1$ RNAs, nor does it affect the nuclear transcription rates of 5S RNA (8). These data suggest that even in the normal confluent cell, these genes are always being transcribed at their maximum rates. It is possible that these pol III templates contain stronger promoters than the *B1* and *B2* genes and are thus less sensitive to changes in the concentration of factor IIIC because they are already saturated. For example, the 7SL and tRNA genes are known to contain 5′ flanking elements that contribute significantly to their transcription efficiency (19, 20). In contrast, *B1* and *B2* genes have transposed throughout the genome by an RNA intermediate and, therefore, carry only their internal promoters (for review, see ref. 21). Thus, the increased levels of factor IIIC might be necessary for efficient transcription complex formation on the *B1* and *B2* genes.

The highly repetitive and interspersed nature of these elements might also contribute to the specificity of the system. One can envision a model whereby a variety of diverse stimuli unveil different regions of the genome for specific transcription by RNA polymerase II and that activation of nearby *B2* and *B1* genes is a fortuitous event.

The *B2* gene system shows many similarities with the *Xenopus* 5S gene system (for review, see ref. 22). Changes in the concentration of a limiting factor and accessibility of this factor to its target genes appear to be common strategies employed in the differential regulation of transcription in both systems. However, normal growing and transformed cells, which contain the same amounts of factor IIIC, show significant differences in *B2* gene transcription. This demonstrates that determinants of accessibility can operate independent of changes in the concentration of a limiting transcription factor.

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1. Krayev, A. S., Markusheva, T. V., Kramerov, D. A., Ryskov, A. P., Skryabin, K. G., Bayev, A. A. & Georgiev, G. P. (1982) *Nucleic Acids Res.* **10,** 7461–7475.
2. Singh, K., Carey, M., Saragosti, S. & Botchan, M. (1985) *Nature (London)* **314,** 553–556.
3. Kramerov, D., Grigoryan, A., Ryskov, A. & Georgiev, G. (1985) *Biochim. Biophys. Acta* **824,** 85–98.
4. Murphy, D., Brickell, P. M., Latchman, D. S., Willison, K. & Rigby, P. W. (1984) *Cell* **35,** 865–871.
5. Edwards, D. R., Parfelt, C. L. J. & Denhart, D. T. (1985) *Mol. Cell. Biol.* **5,** 3280–3288.
6. Fornace, A. J., Jr., & Mitchell, J. B. (1986) *Nucleic Acids Res.* **14,** 5793–5812.
7. Vasseur, M., Condamine, H. & Duprey, P. (1985) *EMBO J.* **4,** 1749–1753.
8. Carey, M. F., Singh, K., Botchan, M. & Cozzarelli, N. R. (1986) *Mol. Cell. Biol.* **6,** 3068–3076.
9. Geiduschek, E. P. & Tochini-Valentini, G. P. (1988) *Annu. Rev. Biochem.* **57,** 873–914.
10. Segall, J., Matsui, T. & Roeder, R. G. (1980) *J. Biol. Chem.* **255,** 11986–11991.
11. Schlissel, M. S. & Brown, D. D. (1984) *Cell* **37,** 903–913.
12. Carey, M. F., Gerrard, S. P. & Cozzarelli, N. R. (1986) *J. Biol. Chem.* **261,** 4309–4317.
13. Manley, J. L., Fire, A., Cano, A., Sharp, P. A. & Gefter, M. L. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 3855–3859.
14. Clarkson, S. G., Kurer, V. & Smith, H. O. (1978) *Cell* **14,** 713–724.
15. Stahl, H., Droge, P. & Knippers, R. (1986) *EMBO J.* **5,** 1939–1944.
16. Gaynor, R. B., Feldman, L. T. & Berk, A. J. (1985) *Science* **236,** 447–450.
17. Hoeffler, W. K. & Roeder, R. G. (1985) *Cell* **41,** 955–963.
18. Yoshimaga, S., Dean, N., Han, M. & Berk, A. J. (1985) *EMBO J.* **5,** 343–354.
19. Ullu, E. & Weiner, A. (1985) *Nature (London)* **318,** 371–374.
20. Morry, M. J. & Harding, J. D. (1986) *Mol. Cell. Biol.* **6,** 105–115.
21. Roger, J. H. (1985) *Int. Rev. Cytol.* **93,** 187–201.
22. Brown, D. D. (1984) *Cell* **37,** 359–365.