MOLECULAR AND CELLULAR BIOLOGY, Sept. 1986, p. 3068–3076
0270-7306/86/093068-09$02.00/0
Copyright © 1986, American Society for Microbiology
Vol. 6, No. 9

Induction of Specific Transcription by RNA Polymerase III in Transformed Cells

MICHAEL F. CAREY, KARAMBIR SINGH, MICHAEL BOTCHAN, AND NICHOLAS R. COZZARELLI*
Department of Molecular Biology, University of California, Berkeley, California 94720

Received 13 March 1986/Accepted 19 May 1986

RNA polymerase III (pol III) transcripts of the highly repeated mouse B2 gene family are increased in many oncogenically transformed murine cell lines. In cells transformed by simian virus 40, the small, cytoplasmic B2 RNAs are present at 20-fold-higher levels than in normal cells (M. R. D. Scott, K. Westphal, and P. W. J. Rigby, Cell 34:557–567, 1983; K. Singh, M. Carey, S. Saragosti, and M. Botchan, Nature [London] 314:553–556). We found that transcripts of the highly repeated B1 gene family are also increased 20-fold upon simian virus 40 transformation and showed that these RNAs result from pol III transcription. In contrast, transcripts from less highly repeated pol III templates such as the 5S, 7SL, 7SK, 4.5S$_I$, tRNA^{Met}, and tRNA^{Pro} genes are unaffected. The expression of the B2 RNAs in isolated nuclei shows that the augmentation is due mainly to an increased rate of transcription by pol III. There is thus specific transformation-inducible pol III transcription. We developed an in vitro transcription assay which utilizes genomic DNA as a template to study the transcription of all members of a repetitive gene family in their native context. This assay reproduces the low cytoplasmic levels of B1 compared with B2 RNAs suggesting that this ratio is dictated by intrinsic signals in the DNA.

While studying transformation-induced changes in cellular gene expression, we found that the cytoplasmic levels of the small B2 RNAs were significantly increased in cell lines transformed by simian virus 40 (SV40), methylcholanthrene, and bovine papilloma virus (34). Others have also reported increased levels of B2 RNAs in a number of transformed cell lines and tumors (12, 21, 25, 32), although there are exceptions (12, 32). These RNAs are also abundant in undifferentiated embryonic carcinoma cells, but not in their differentiated derivatives (2, 27), and in serum-stimulated quiescent mouse Swiss 3T3 cells (7). Thus, the level of the small B2 RNAs is high not only in transformed cells but in other situations in which the cells are actively proliferating. The function of the small B2 RNAs and the mechanism by which their levels are increased are unknown.

The B2 transcripts are encoded by a highly repetitive short DNA sequence. A number of such repeat families are interspersed throughout the vertebrate genome (for a review, see reference 29); the prototype is the *Alu* sequence in primates (16). There are approximately 100,000 genomic copies of B2 genes, and the average size is 190 base pairs (21). B2 repeats, like many short-gene families, contain sequences homologous to the consensus RNA polymerase III (pol III) split promoter. This promoter is contained within the gene and consists of two conserved sequence elements, the A- and B-boxes; each are approximately 10 base pairs in size and separated by about 35 base pairs (for a review, see reference 5). Initiation of transcription occurs 10 to 20 nucleotides upstream from the A-box (5). Previously, we concluded that the B2 RNAs are transcribed by pol III because the 5′ ends of the cytoplasmic RNAs were located 12 and 13 nucleotides upstream from the A-box and the transcription of cloned B2 templates in vitro required pol III and the specific transcription factors for this enzyme (34). Kramerov et al. (19) reached similar conclusions from the α-amanitin sensitivity of B2 transcription in isolated nuclei.

We proposed the existence of a pol III expression system whose activity is enhanced by oncogenic transformation (34). We have now studied whether this system is operating at the level of transcription and whether other pol III genes respond similarly. We found that the system is operating mainly at the level of transcription and is specific thus far to just two highly repeated gene families.

MATERIALS AND METHODS

Cell culture. SVT-2 (1) and BALB/c 3T3 cells were grown in Dulbecco modified Eagle minimal essential medium containing 10% calf serum. Only early-passage BALB/c-3T3 cells were used.

Isolation of genomic DNA and cytoplasmic RNA. Genomic DNA from the BALB/c 3T3 and SVT-2 cell lines was prepared by standard procedures (26), based on a modification of the method of Thomas et al. (36). The isolation of cytoplasmic RNA and Northern blot analysis were as previously described (34).

Isolation of nuclei and transcription reactions. Nuclei were prepared from confluent BALB/c 3T3 and SVT-2 cells as described by Greenberg and Ziff (11). Nuclei (10^7) were preincubated in 100 μl of 50 mM Tris (pH 8.3)–5 mM $MgCl_2$–0.1 mM EDTA–40% glycerol and twice the indicated concentrations of α-amanitin. After 5 min at 0°C, 100 μl of 10 mM Tris (pH 8.0)–5 mM $MgCl_2$–0.15 M KCl–0.5 mM ATP–0.5 mM CTP–0.5 mM GTP–100 μCi of [α-^{32}P]UTP (400 Ci mmol^{-1}; Amersham Corp., Arlington Heights, Ill.) was added, and the mixtures were incubated for 15 min at 30°C. Pulse-chase studies were carried out exactly as described above except that both the pulse and the chase were for 7 min and the chase reaction was in the presence of 0.5 mM UTP.

Isolation of labeled nuclear transcripts and hybridization conditions. Two procedures were used to isolate ^{32}P-labeled

* Corresponding author.

nuclear transcripts. The first was a modification of the method of Vannice et al. (38). After the 15-min transcription reaction, the nuclei were pelleted by centrifugation at 12,000 × *g* for 15 s, and the supernatant was decanted. The nuclei were suspended in 1 ml of 10 mM Tris (pH 7.5)–1 mM EDTA (TE)–0.3% sodium dodecyl sulfate (SDS)–100 μg of carrier tRNA per ml–100 μg of proteinase K. After 15 min at 37°C, protein and high-molecular-weight DNA were removed by extraction with 1 volume of H_2O-saturated phenol. The interphase was reextracted with 500 μl of 0.1 M sodium acetate–20 mM EDTA. The aqueous phases were combined, and RNA was collected by precipitation with ethanol. Hybridization reactions were performed essentially as described by Greenberg and Ziff (11). Briefly, the ^{32}P-labeled RNA was suspended at a concentration of 2×10^6 cpm/ml in 10 mM TES [*N*-tris-(hydroxymethyl)methyl-2-aminoethane sulfonic acid; pH 7.4]–0.2% SDS–10 mM EDTA. An equal volume of 10 mM TES (pH 7.4)–0.2% SDS–10 mM EDTA–0.6 M NaCl was added, and 1.5 ml of this solution was incubated for 36 h at 65°C with the indicated DNAs immobilized on nitrocellulose strips. The preparation of the filters is described below. The filters were washed for 2 h with 2× SSC (0.3 M NaCl plus 0.025 M sodium citrate, pH 7.0), followed by incubation with 10 μg of RNase A per ml in 2× SSC at 37°C for 30 min. The filters were washed again in 2× SSC at 65°C for 1 h, air dried, and autoradiographed by exposure to Kodak XAR-5 film with a Du Pont Cronex intensifying screen.

The results shown in Fig. 1 were obtained by this procedure which has the advantage of rapidly removing most of the unincorporated ^{32}P immediately after the transcription reactions. However, we were concerned that some of the small B2 transcripts might diffuse into the supernatant and would be lost upon pelleting of the nuclei.

We therefore used a modification of the procedure of Linial et al. (23), which did not involve spinning out the nuclei from the reaction mixture to isolate the labeled RNA. After the 15-min transcription reaction, the mixture was adjusted to 10 mM $CaCl_2$ and incubated with 75 μg of DNase I for 15 min at 30°C. The mixture was then adjusted to 1% SDS, 5 mM EDTA, 10 mM Tris (pH 7.5), and 200 μg of proteinase K per ml, heated for 30 s at 65°C, and then incubated at 37°C for 45 min. The solution was extracted with an equal volume of phenol-chloroform, and the interphase was back-extracted with 100 μl of TE. The combined aqueous phases were adjusted to 2.3 M ammonium acetate, and the RNAs were precipitated with an equal volume of isopropyl alcohol. The RNA was suspended in 100 μl of TE and centrifuged through a Sephadex G-25 (medium) spin column. The eluate was adjusted to 0.3 M sodium acetate, and the RNA was precipitated with ethanol. The RNA was suspended in hybridization buffer (10 mM TES [pH 7.4], 0.2% SDS, 10 mM EDTA, 0.3 M NaCl, 1× Denhardt solution [26], 250 μg of *Escherichia coli* RNA per ml) to 10^6 cpm/ml, and 1.5 ml was hybridized at 65°C for 36 h to the indicated DNAs immobilized on nitrocellulose strips. The filters were washed and autoradiographed as described above. The data in Fig. 2 were obtained with this procedure. We found the same increase in B2 transcription with the two procedures.

Preparation of nitrocellulose filters. Plasmid DNAs were linearized by restriction endonuclease digestion and denatured in 0.3 M NaOH for 10 min at room temperature. After neutralization with 1 volume of 2 M ammonium acetate, the DNA was filtered onto nitrocellulose with a Schleicher & Schuell dot-blot apparatus. Either 3 or 5 μg of DNA per dot was used and gave the same increase in B2 transcription, thus showing that our experiments were in DNA excess. The plasmid DNAs used were pBR322, a control plasmid; pDES, a human 28S rDNA clone provided by R. Tjian; pJYM, an SV40 clone (24); a rat 5S clone provided by R. Reddy; and p10λ5, a B2-containing clone (34).

In vitro transcription reactions with genomic DNA. HeLa cell transcription factors IIIB and IIIC and RNA pol III were purified as described previously (4). The 50-μl reaction mixtures contained 20 mM HEPES (*N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid) (pH 7.5), 65 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM dithiothreitol, 7% glycerol, 20 μg of deproteinized genomic DNA per ml, 200 μM each ATP, GTP, CTP, and UTP, 100 U of RNA pol III per ml, and the indicated factors at 15 U/ml. (A unit of transcription factor activity is the amount of protein required to yield 50 fmol of transcript per h in a standard reaction with other factors and DNA template in excess.) After 1 h at 25°C, the reaction was stopped by the addition of 150 μl of a solution containing 0.2% SDS, 0.4 M sodium acetate, 10 mM EDTA, and 50 μg of carrier tRNA per ml. The products were extracted with phenol, precipitated with ethanol, and washed with 80% ethanol. The pellets were suspended in 10 μl of TE and analyzed by primer extension.

Primer extension reactions. The 20-μl hybridization mixtures contained 10 mM Tris (pH 7.5), 1 mM EDTA, 0.3 M NaCl, 0.1 pmol of ^{32}P-labeled oligonucleotide primer, and RNA. The mixtures were heated at 85°C for 10 min to denature the RNA after which the primers were annealed for 2.5 h at 55°C. The hybridized primers were extended by the addition of 80 μl of a mixture containing 12.5 mM Tris (pH 8.1), 10 mM $MgCl_2$, 12.5 mM dithiothreitol, 1.25 mM each dATP, dGTP, dCTP, and TTP, 62.5 μg of actinomycin D per ml, 125 U of RNasin per ml, and 125 U of avian reverse transcriptase (Life Sciences Inc., St. Petersburg, Fla.) per ml. After 1 h at 42°C, the products were extracted with phenol, precipitated with ethanol along with 5 μg of carrier tRNA, suspended in 95% formamide–0.1% xylene cyanol–0.05% bromphenol blue, and fractionated by electrophoresis through 10% polyacrylamide gels (40-cm long, 0.4-mm thick) containing 7 M urea. The gels were autoradiographed by exposure of Kodak XAR-5 film with a Du Pont Cronex intensifying screen. Gels were also autoradiographed in the absence of an intensifying screen for use in quantitative laser densitometry. Titrations were performed to assure that the primer was in excess and that the extension product label was linearly proportional to the input RNA concentrations.

End labeling of primers with [γ-^{32}P]ATP. The 30-μl reaction mixtures contained 50 mM Tris (pH 7.5), 10 mM $MgCl_2$, 5 mM dithiothreitol, 0.1 mM spermidine, 0.1 mM EDTA, 10 pmol of oligonucleotide primer, 100 μCi of [γ-^{32}P]ATP (5,000 Ci/mmol; Amersham), and 3 U of T4 polynucleotide kinase. After 30 min at 37°C, the mixtures were heated to 65°C for 10 min to inactivate the kinase; an equal volume of 5 M ammonium acetate was then added, and the products were precipitated with ethanol. The pellets were washed with 80% ethanol, dried, and suspended in 100 μl of 10 mM Tris (pH 7.5)–1 mM EDTA.

RESULTS

Transcription of B2 RNAs in isolated nuclei. To determine whether the increased cytoplasmic levels of the B2 RNAs in SV40-transformed cells were the result of increased transcription, we performed nuclear run-on reactions with nuclei

isolated from a normal BALB/c 3T3 cell line and its SV40-transformed derivative, SVT-2 (1). In the nuclear run-on assay, previously initiated RNA chains are elongated by incubating the nuclei in the presence of ^{32}P-labeled nucleoside triphosphates. This technique allows a quantitative measure of the rate of initiation in vivo (6). In our experiments, the labeled RNAs were then used to probe B2 DNA and four other control DNA sequences immobilized on nitrocellulose filters. We used cloned 28S rDNA and 5S DNA as internal standards to normalize changes in B2 transcription. The 5S DNA clone is particularly well suited for this because 5S RNA is a pol III transcript, and our studies (see below) show that the cytoplasmic levels of 5S RNA are identical in the BALB/c 3T3 and SVT-2 cells.

An important factor to consider when assaying for differences in pol III transcription of B2 elements is that many of the 10^5 copies are in the introns and noncoding sequences of genes transcribed by RNA polymerase II (pol II) (29). Therefore, transcription of such genes gives rise to a significant amount of heterogeneous nuclear RNA containing B2 sequences (21). Because these RNAs might obscure changes that are occurring in the pol III transcription of B2 genes, we also performed nuclear run-on experiments in the presence of low levels of α-amanitin which selectively inhibit pol II transcription (39).

The results of one such experiment are shown in Fig. 1. Normal and transformed nuclei were preincubated with 0, 2, 6, or 18 μg of α-amanitin per ml. After a 15-min incubation in the presence of [α-^{32}P]UTP, transcription was terminated, and the labeled RNA transcripts were used to probe nitrocellulose-immobilized DNAs. A clone containing the SV40 early genes was used to show that 2 μg of α-amanitin per ml inhibits pol II transcription in transformed nuclei (Fig. 1, lanes 1 and 2); we infer that the same applies to the normal nuclei. The transcription of the rDNA (pol I) and 5S (pol III) genes was similar in the normal and transformed nuclei and was not affected by low levels of α-amanitin within experimental error. Transcription of B2 RNA is more complicated. In the absence of α-amanitin we found only a small increase in total B2 transcription (pol II and pol III) in the transformed nuclei compared with normal (Fig. 1, lanes 1 and 5). This augmentation was more pronounced when pol II transcription was inhibited (Fig. 1, lanes 2 to 4 and 6 to 8). From the results of several different experiments, we estimate that there is a 5- to 10-fold increase in the transcription of B2 elements by pol III in the transformed nuclei. We discuss below possible explanations for why this is less than the 20-fold enhancement observed in the cytoplasmic levels of these RNAs.

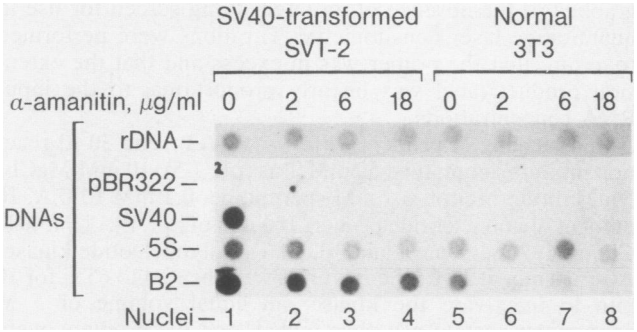

FIG. 1. B2 gene transcription in isolated nuclei from normal and transformed cells. The 100-μl preincubation mixtures contained nuclei from 10^7 cells of either SV40-transformed SVT-2 (lanes 1 to 4) or normal BALB/c 3T3 (lanes 5 to 8) cells and twice the indicated concentrations of α-amanitin. After 5 min at 0°C, 100 μl of a mixture containing ^{32}P-labeled nucleoside triphosphates was added, and the reactions were incubated for 15 min at 30°C. The ^{32}P-labeled nuclear transcripts (10^6 cpm) were then hybridized to 5 μg each of the indicated DNAs immobilized on nitrocellulose strips. The rDNA hybrids were exposed for less time than the others so the signal intensity would be linearly proportional to the radioactivity.

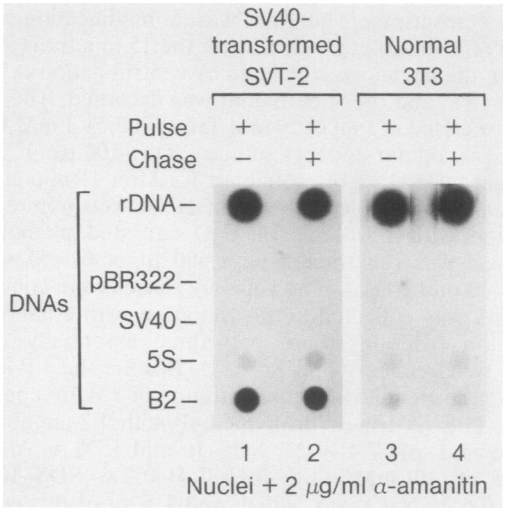

FIG. 2. Pulse-chase studies on B2 nuclear transcripts from normal and transformed cells. Two sets of transformed (lanes 1 and 2) and normal (lanes 3 and 4) nuclei were preincubated for 5 min in the presence of 2 μg of α-amanitin per ml. Nuclear transcription reactions were then performed in the presence of [α-^{32}P]UTP for 7 min. Transcription was terminated in nuclei 1 and 3 and continued in nuclei 2 and 4 for 7 min more in the presence of a vast excess (0.5 mM) of unlabeled UTP. The ^{32}P-labeled nuclear transcripts were then isolated, and 10^6 cpm were used in each hybridization reaction to 5 μg of the indicated DNAs immobilized on nitrocellulose strips.

To assure that the differences we observed were at the level of transcription and not due to differential stability of the transcripts, we performed pulse-chase experiments. Two sets of transformed and normal nuclei were incubated with [α-^{32}P]UTP in the presence of 2 μg of α-amanitin per ml. After 7 min, transcription in one set of nuclei was terminated, a vast excess of unlabeled UTP was added to the second set, and transcription was allowed to proceed for 7 min more. The labeled RNAs were then isolated and used to probe identical filters. The results (Fig. 2) indicate that the amount of labeled B2 RNA is not affected by the chase in either the transformed or the normal nuclei. We conclude that the increase of B2 RNAs occurs at the level of transcription and is not due to differential stability of the nuclear transcripts.

Levels of other pol III transcripts in transformed cells. To determine whether the augmented levels of B2 RNA are a specific or general feature of pol III transcription in SV40-transformed cells, we measured the amounts of the pol III transcripts encoded by the 5S, 7SL, tRNA^{Met}, tRNA^{Pro}, 7SK, 4.5S$_I$, and B1 genes. The 5S, 7SL, and tRNA genes encode well-characterized RNAs of known function that are present in all eucaryotic cells. The 7SK gene gives rise to a 330-nucleotide RNA of unknown function, but it is present in all species tested from insects to humans (28). The 4.5S$_I$ gene family is found only in rodents and encodes abundant 98- or 99-nucleotide RNAs of unknown function that are localized primarily but not exclusively in the nucleus (30). The B1 gene family is another short, highly repeated sequence found

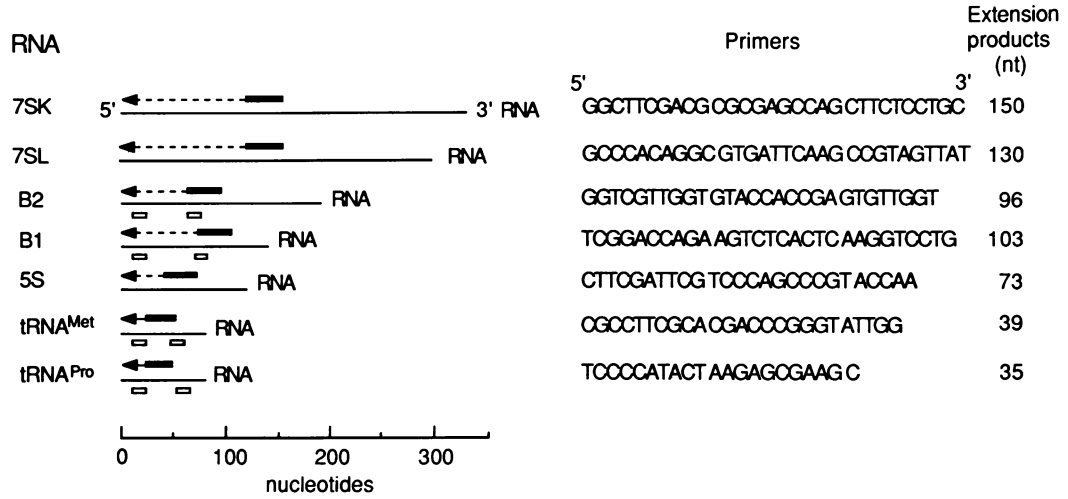

FIG. 3. Schematic representation of the reverse transcriptase primer extension procedure. The short bold segment is the ^{32}P-labeled primer hybridized to the indicated RNAs. The primer is elongated (dashed line) with reverse transcriptase and terminates at the 5′ end of the RNA; the arrow tips denote the 3′ end of the extension product. The open boxes below the B1, B2, and tRNAs are the 5′- and 3′-proximal conserved promoter elements designated the A- and B-boxes, respectively. The sequence of the primers and the sizes of the extension products are shown on the right. The primers are complementary to the published sequences of either the RNAs (7SK [28], 7SL [22]) or the cloned genes (B2 consensus [29], B1 consensus [17], 5S gene [8], tRNA^{Met} [13], and tRNA^{Pro} [15]). We detected only one significant homology of a primer to a different pol III-transcribed gene. The B1 primer was 82% homologous to nucleotides 75 to 92 of 4.5S RNA (distinct from 4.5S$_I$). The 4.5S genes are greater than 75% homologous to B1 and are considered members of the B1 superfamily (29). We did not attempt to distinguish between these two RNAs and therefore may be detecting both. nt, Nucleotides.

in rodent cells (18, 20). It is present at approximately 100,000 copies per genome and has an average size of 140 base pairs. Sequence analysis strongly suggests that B1 genes are derived from a 7SL gene (37). Our studies (see below) show that the B1 genes are also transcribed by pol III, but their function is unknown.

We employed primer extension analysis to measure the relative cytoplasmic levels of all the transcripts except 4.5S$_I$ RNA. The sequences of the primers and their complementary positions on their respective RNAs are shown in Fig. 3. Because there is considerable homology among several of the transcripts, we chose primers corresponding to unique regions in each RNA. In Fig. 4 we present the results on the expression of the 5S, B2, 7SL, and 7SK RNAs. We compared cytoplasmic RNAs from normal and transformed cells grown to five different cell densities, each representative of various stages of confluency. This permitted us to measure the effects of both growth and transformation on the amounts of each RNA species. Each set of RNAs was annealed to a mixture of four ^{32}P-labeled primers complementary to the 5S, B2, 7SK, and 7SL transcripts. Lanes 1 to 4 are controls in which the individual primers are annealed to RNA. The hybridized primers were then extended with reverse transcriptase, and the products were analyzed by polyacrylamide gel electrophoresis and autoradiography. The 7SL and 5S primers gave rise to extension products whose corresponding RNA levels were the same in the normal and transformed cells. In contrast, the B2 primer gave rise to extension products whose RNA levels were 20-fold enhanced in the transformed cells. In the normal BALB/c 3T3 cells, we also found a fourfold enhancement in B2 RNA levels in the low-density, actively growing cells compared with those in the high-density, growth-arrested cells (lanes 1 to 5).

Extension of the 7SK primer gave rise to two similarly sized products with transformed cell RNA. The RNA corresponding to the larger product was present at comparable levels in the normal cell. However, the RNA corresponding to the smaller extension product appeared to be present only in the transformed cell. Our sequence analysis of the two extension products revealed that their 5′ ends are the same. This suggests that they did not arise from differential initiation or processing of a single 7SK gene product but more likely were derived from two closely related 7SK genes. We looked to see whether the 7SK RNA corresponding to the smaller extension product is present in other normal or transformed mouse cell lines but were unable to detect it even in other cell lines transformed by SV40 (data not shown). This RNA species appears to be specific to SVT-2 cells. We have previously documented how various murine cell lines can show strikingly different expression patterns for certain genes (35); the 7SK gene family appears to be another example of this phenomenon.

We also measured the cytoplasmic levels of two different tRNA gene transcripts. The amounts of tRNA^{Met} were similar in the normal and transformed cells (Fig. 5), and we also found the same to be true for tRNA^{Pro} (data not shown).

When B1 gene expression was measured we obtained a very different result (Fig. 6). The B1 RNAs were present at 50- to 100-fold-lower levels than the B2 RNAs. However, like the B2 RNAs, B1 RNAs were increased 20-fold in the SVT-2 compared with the BALB/c cells. Moreover, the B1 RNAs were also increased fourfold in the growing BALB/c 3T3 cells compared with the confluent cells (lanes 1 to 5). It appears that the B1 and B2 families are coordinately affected by both the growth and transformation-inducible pol III expression system. Because the B1 transcripts are expressed so poorly, we did not show that the B1 induction like the B2 induction occurs mainly at the level of transcription, but we assume that this is the case.

To see whether other murine repeated-gene families transcribed by pol III are affected by SV40 transformation, we

measured the levels of $4.5S_I$ RNA. The moderately repeated $4.5S_I$ genes are present at 10^4 copies per genome (30), an order of magnitude less than the B1 and B2 gene families. We used a mouse genomic clone containing a $4.5S_I$ gene (30) as a probe in Northern analysis to show that the cytoplasmic levels of $4.5S_I$ transcripts are the same in the normal and transformed cells (Fig. 7). It appears therefore that only the very highly repeated pol III gene families are affected by the transformation-inducible transcription system.

Studies employing total genomic DNA as a template for in vitro transcription of B1 and B2 genes. We developed an assay for the study of B1 and B2 gene transcription in vitro.

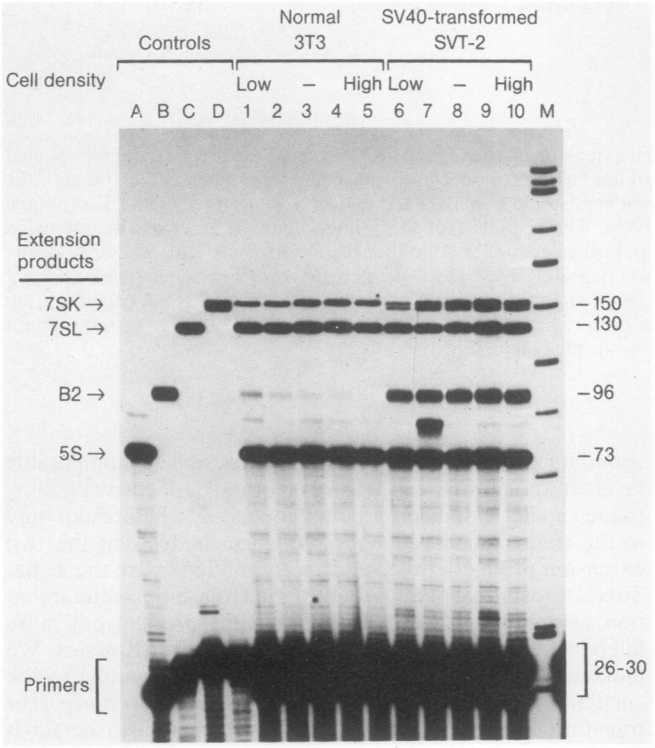

FIG. 4. Primer extension analysis of 5S, B2, 7SL, and 7SK RNAs in normal and transformed cells. The 20-μl hybridization mixtures contained 250 ng of total cytoplasmic RNA from BALB/c 3T3 or SVT-2 cells and ^{32}P-labeled primers complementary to 5S RNA (lane A), B2 RNA (lane B), 7SL RNA (lane C), 7SK RNA (lane D), or all four RNAs (lanes 1 to 10). The densities and approximate confluencies of the cell cultures were as follows: lane 1, 10^6 cells per 100-mm plate, 30 to 40% confluent; lane 2, 2.6×10^6, 80 to 90% confluent; lane 3, 3.0×10^6, 100 % confluent; lane 4, 2.9×10^6, 100% confluent for 1 day; lane 5, 2.5×10^6, 100% confluent for 3 days; lane 6, 5×10^6, 40 to 50% confluent; lane 7, 1.2×10^7, 80 to 90% confluent; lane 8, 1.8×10^7, 100% confluent; lanes 9 and A through D, 3.0×10^7, 100% confluent for 1 day; lane 10, 2.8×10^7, 100% confluent for 3 days. After 10 min at 85°C and 2.5 h at 55°C, the hybridized primers were extended by the addition of 80 μl of a reverse transcriptase synthesis mixture. The reactions were terminated after 1 h at 42°C. The products were fractionated by electrophoresis through polyacrylamide-urea gels and visualized by autoradiography. The DNA band between the 5S and B2 RNA extension products in lane 8 is an artifact of 5S RNA extension that we occasionally observe. It is represented in lesser amounts in lanes A, 1 to 7, and 9 and 10. We believe that it arises as a result of snapback synthesis from 5S RNA. The sizes of the extension products in nucleotides are indicated to the right of the autoradiograph. These were determined by electrophoresis alongside a sequencing ladder of known composition or alongside ^{32}P-labeled restriction fragments derived from a digest of pUC19 with *Hpa*II.

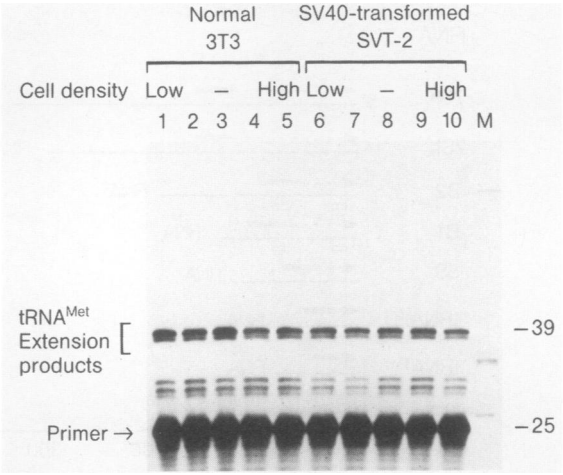

FIG. 5. Primer extension analysis of tRNA^{Met} in normal and transformed cells. Hybridization mixtures contained 250 ng of the indicated cytoplasmic RNAs and a ^{32}P-labeled primer homologous to tRNA^{Met}. The procedure was as described in the legend to Fig. 4. The DNA bands between the primer and the completely extended product represent positions at which reverse transcriptase extension was blocked. The strong secondary structure of tRNA prevented quantitative extension of the primers to the 5′ end of the RNA.

This assay employs deproteinized genomic DNA as a template in a cell-free transcription system. A similar assay has recently been devised (9). This approach has two chief advantages over the use of cloned templates: first, each member of the genomic B1 and B2 population is represented in a reaction and second, the physiological ratio is maintained between both B1 and B2 genes and other genes transcribed by pol III.

We developed the assay with a well-characterized transcription system. HeLa cell extracts have been chromatographically fractionated into two components designated transcription factors IIIB and IIIC which are necessary and sufficient for transcription of the tRNA and VA genes by pol III (33). We have shown previously that transcription of cloned B2 elements by pol III requires these two components (34). Transcription of genomic B2 elements also requires these components and is inhibited by the same levels of α-amanitin as are other pol III transcripts (Fig. 8, lanes 10 to 14). Furthermore, the 5′ ends of the RNAs aligned precisely with those observed in cytoplasmic RNA (Fig. 8, compare lanes 12 and 13 with lanes 15 to 18). This genomic assay therefore avoids the predicament observed in our previous study in which the 5′ ends of the RNAs transcribed from individual cloned B2 templates, although similar, were not identical to those found in vivo.

Using the genomic assay, we found that B1 transcription has the same factor requirements and α-amanitin sensitivity as the B2 genes (Fig. 8, lanes 1 to 5). Moreover, the 5′ ends of the B1 RNAs are 12 to 13 nucleotides upstream of the A-box in the B1 consensus sequence, a characteristic of all pol III transcripts except 5S RNA. Our studies, therefore, provide the first proof that the B1 RNAs are transcribed by pol III. We also observed cytoplasmic B1 transcripts whose 5′ ends are 6 nucleotides shorter than those observed in vitro; these are approximately 50% of the B1 RNA and may result from processing. The background bands in Fig. 8 are described in the figure legend.

Our genomic in vitro transcription studies also clarify why

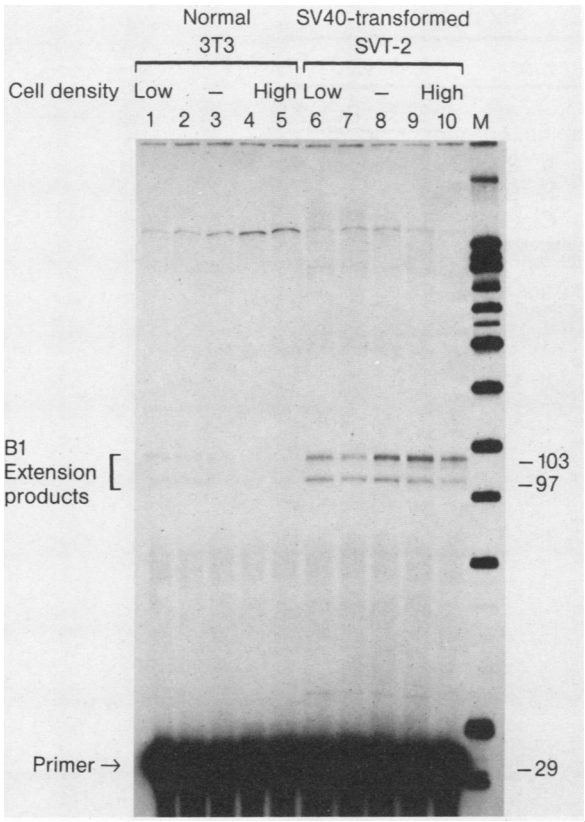

FIG. 6. Primer extension analysis of B1 RNAs in normal and transformed cells. Hybridization mixtures contained 2.5 μg of the indicated cytoplasmic RNAs and a ^{32}P-labeled primer homologous to the B1 consensus sequence. The procedure was as described in the legend to Fig. 4.

the cytoplasmic levels of B2 transcripts are approximately 50- to 100-fold greater than the levels of B1 transcripts, even though there are roughly equal numbers of both elements in the genome. We found that the ratio of B2 to B1 transcripts observed in vitro is almost equal (within a factor of 3) to that seen in cytoplasmic RNA, demonstrating that this ratio is a reflection of their nuclear transcription rates. One possibility is that B2 genes per se contain a much stronger promoter than the B1 genes and that this difference in promoter strength is maintained in our in vitro genomic transcription assay. Alternatively, there may be more transcriptionally competent B2 genes than B1 genes in the cell.

DISCUSSION

Analysis of transformation-mediated changes in cellular gene expression that result directly or indirectly from the action of a single viral gene product should provide insight into how such a gene brings about the large number of changes that accompany oncogenesis. We characterized a pol III expression system which is induced by SV40 transformation (34). Nuclear run-on transcription reactions demonstrated that a significant portion of this system is operating at the level of transcription. There was a 5- to 10-fold increase in the pol III transcription of B2 genes in SV40-transformed cells compared with that in the parental cells. Pulse-chase studies showed that this difference is not the result of differential stability of the B2 nuclear transcripts. However, this 5- to 10-fold increase in transcription is less than the 20-fold enhancement in the cytoplasmic levels of B2 RNAs in transformed cells. This difference may be the result of a technical artifact; it has been suggested that nuclear run-on assays are quantitatively less sensitive than techniques which measure the steady-state levels of RNA (11). Alternatively, posttranscriptional events such as cytoplasmic stability may also contribute to the enhanced expression of B2 genes in transformed cells.

In this context it is interesting that Edwards et al. (7) found no difference in B2 transcription between quiescent and serum-stimulated Swiss NIH 3T3 cells, even though they observed a 10-fold increase in the cytoplasmic levels of B2 RNAs in the serum-stimulated cells. The sensitivity of B2 cytoplasmic RNA levels to growth conditions, which we also observed, may be occurring posttranscriptionally. However, these workers may have missed a transcriptional component of this induction because they only studied total transcription (pol II and pol III) of B2 genes in the isolated nuclei. Our studies showed that in such nuclei the enhanced pol III transcription of B2 genes is in part masked by pol II transcription of B2-containing heterogeneous nuclear RNA (Fig. 1, lane 1 versus lane 5).

To study the specificity of this system, we measured the cytoplasmic levels of transcripts of a representative number of pol III genes in BALB/c 3T3 and SVT-2 cells. Transcripts of the highly repeated B1 and B2 gene families were specifically increased. In contrast, transcripts of less highly reiterated pol III genes such as 5S, 7SL, 7SK, $tRNA^{Met}$, $tRNA^{Pro}$, and $4.5S_I$ were not affected by this transformation-sensitive system. Our study was not an exhaustive one, and it is still possible that other pol III gene families are substrates for the system.

There are several models which could account for the specific induction of B1 and B2 gene transcription. We rule out the possibility of a serendipitous genetic change leading to increased B1 and B2 transcription because almost all transformed mouse cell lines show this induction (12, 21, 25, 32, 34) and cell lines harboring temperature-sensitive T antigen do not express augmented levels of the B2 transcripts at the restrictive temperature (32). Our models therefore focus on the *trans*-acting factors involved in the regulation of these pol III genes. Our preliminary observations are that the pol III transcription activities of extracts derived from SVT-2 cells are significantly greater than those pre-

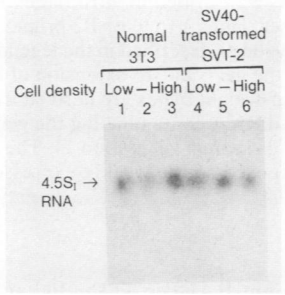

FIG. 7. Northern blot analysis of $4.5S_I$ cytoplasmic RNA in normal and transformed cells. The indicated cytoplasmic RNAs (10 μg of each) were analyzed by Northern blotting with a ^{32}P-labeled $4.5S_I$ DNA clone (30) as a hybridization probe. The cell densities of the cultures were as follows: lane 1, 10^6 cells per 100-mm plate, 30 to 40% confluent; lane 2, 3.0×10^6, 100% confluent for 1 day; lane 3, 2.6×10^6, 100% confluent for 3 days; lane 4, 5×10^6, 40 to 50% confluent; lane 5, 3×10^7, 100% confluent for 1 day; lane 6, 2.8×10^7, 100% confluent for 3 days. An autoradiograph of the blot is shown.

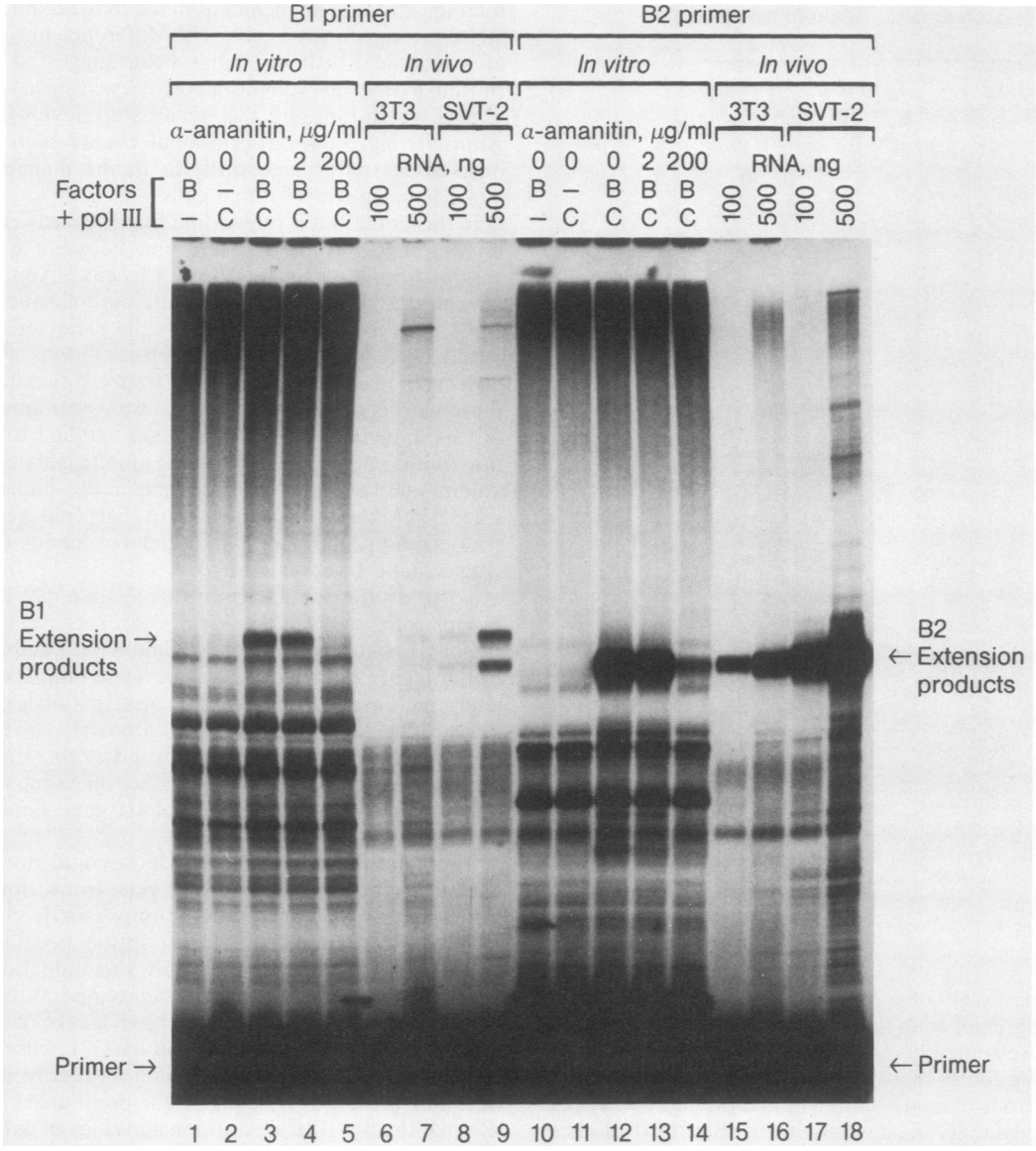

FIG. 8. In vitro transcription of genomic DNA. The 50-μl in vitro transcription mixtures contained the indicated components and genomic DNA as a template. After 1 h at 23°C, the products were isolated and analyzed by primer extension. The 20-μl hybridization mixtures contained the RNAs transcribed in vitro (lanes 1 to 5, 10 to 14) or the indicated cytoplasmic RNAs (lanes 6 to 9, 15 to 18) and 0.1 pmol of ^{32}P-labeled B1 primer (lanes 1 to 9) or B2 primer (lanes 10 to 18). The hybridized primers were extended with reverse transcriptase, and the products were analyzed as described in the legend to Fig. 4. An autoradiograph of the polyacrylamide gel is shown. The high-molecular-weight smear at the top of the gel is due to extension of B1 and B2 primers which annealed to their complementary sites in the genomic DNA. Some of the low-molecular-weight bands are derived from contaminants of the labeled primers; additional bands present only in the genomic assay result from degraded RNA contaminating the genomic DNA preparations. Genomic DNA incubated with the labeled B2 primer produces all the background bands seen in lanes 10 to 14. The background bands are considerably more pronounced than usual because the autoradiogram was heavily overexposed so the weakly expressed B1 gene family could be compared side by side with the B2 family.

pared from confluent BALB/c 3T3 cells, although this effect is not specific for B2 genes. One possibility is that an increase in general pol III transcription factors is responsible for enhanced B1 and B2 expression but that other pol III templates such as 5S, 7SL, and tRNAs are transcribed at maximal rates and are therefore unaffected by such changes. Alterations in chromatin structure might also contribute to this enhanced expression by exposing previously silent B1 and B2 genes to the factors. This is not unprecedented; changes in both chromatin structure and transcription factor IIIA concentration regulate the differential expression of *Xenopus* 5S RNA genes during development (3, 31).

An increase in general pol III transcription factors has been observed in other systems involving viral transforming genes. Exogenous pol III templates transfected into cells harboring the adenovirus E1A or the pseudorabies IE protein are transcribed at much greater efficiences than in control cells, whereas transcription of the endogenous 5S and tRNA genes is unaltered (10). This stimulation was attributed to a fivefold-greater activity of partially purified

transcription factor IIIC from the adenovirus-infected cell extracts compared with that prepared from uninfected cells (14).

Finally, we are interested in understanding the role of T antigen in the increased B1 and B2 gene transcription. We believe that this effect is indirect because purified T antigen has no effect on in vitro transcription of cloned or genomic B2 elements (unpublished observations). Further studies are needed to elucidate exactly how T antigen initiates a series of events that culminate in increased B1 and B2 transcription.

ACKNOWLEDGMENTS

We thank Corey Levinson for the oligonucleotide primers used in this study, Harris Busch and Ram Reddy for the plasmids containing the rat 5S RNA and $4.5S_I$ RNA genes, and R. Tjian for the plasmid containing the mouse rDNA.

This work was supported by Public Health Service grants CA 30490 to M.B. and GM 31657 to N.R.C. from the National Institutes of Health.

LITERATURE CITED

1. **Aaronson, S. A., and G. J. Todaro.** 1968. Development of 3T3-like lines for Balb/c mouse embryo cultures: transformation susceptibility to SV40. J. Cell. Physiol. **72**:141–148.
2. **Bennett, K. L., R. E. Hill, D. F. Pietras, M. Woodworth-Gutai, C. Kane-Haas, J. M. Houston, J. K. Heath, and N. D. Hastie.** 1984. Most highly repeated dispersed DNA families in the mouse genome. Mol. Cell. Biol. **4**:1561–1571.
3. **Brown, D. D., and M. S. Schlissel.** 1985. A positive transcription factor controls the differential expression of two 5S RNA genes. Cell **42**:759–767.
4. **Carey, M. F., S. Gerrard, and N. R. Cozzarelli.** 1986. Analysis of RNA polymerase III transcription complexes by gel filtration. J. Biol. Chem. **261**:4309–4317.
5. **Ciliberto, G., L. Castagnadli, and R. Cortese.** 1983. Transcription by RNA polymerase III. Curr. Top. Dev. Biol. **18**:59–88.
6. **Darnell, J. E., Jr.** 1982. Variety in the level of gene control in eukaryotic cells. Nature (London) **297**:365–371.
7. **Edwards, D. R., C. L. J. Parfelt, and D. T. Denhardt.** 1985. Transcriptional regulation of two serum-induced RNAs in mouse fibroblasts: equivalence of one species to B2 repetitive elements. Mol. Cell. Biol. **5**:3280–3288.
8. **Emerson, B., and R. Roeder.** 1984. DNA sequences and transcription factor interactions of active and inactive forms of mammalian 5S RNA genes. J. Biol. Chem. **12**:7926–7935.
9. **Endoh, M., and N. Okada.** 1986. Total DNA transcription in vitro: a procedure to detect highly repetitive and transcribable sequences with tRNA-like structures. Proc. Natl. Acad. Sci. USA **83**:251–255.
10. **Gaynor, R., L. Feldman, and A. Berk.** 1985. Transcription of class III genes activated by viral immediate early proteins. Science **230**:447–450.
11. **Greenberg, M. E., and E. B. Ziff.** 1984. Stimulation of 3T3 cells induces transcription of the c-fos proto-oncogene. Nature (London) **311**:433–438.
12. **Grigoryan, M. S., D. A. Kramerov, E. M. Tulchinsky, E. S. Revasova, and E. M. Lukanidin.** 1985. Activation of putative transposition intermediate formation in tumor cells. EMBO J. **4**:2209–2215.
13. **Han, J., R. Rooney, and J. Harding.** 1984. Structure and evolution of mammalian tRNA genes: sequence of a mouse $tRNA_{met}$ gene, the 5′ flanking region of which is homologous to a human gene. Gene **28**:249–255.
14. **Hoeffler, W., and R. Roeder.** 1985. Enhancement of RNA polymerase III transcription by the E1A gene product of adenovirus. Cell **41**:955–963.
15. **Hu, J., B. Cote, E. Lund, and J. Dahlberg.** 1983. Isolation and characterization of genomic mouse DNA clones containing sequences homologous to tRNAs and 5S RNA. Nucleic Acids Res. **11**:4809–4821.
16. **Jelinek, W. R., T. P. Toomey, L. Leinwand, C. Duncan, P. Biro, P. Choudary, S. Weissman, C. Rubin, C. Moauck, P. Deininger, and C. Schmid.** 1980. Ubiquitous, interspersed repeated sequences in mammalian genomes. Proc. Natl. Acad. Sci. USA **77**:1398–1402.
17. **Kalb, V., S. Glasser, D. King, and J. Lingrel.** 1983. A cluster of repetitive elements within a 700 base pair region in the mouse genome. Nucleic Acids Res. **11**:2177–2184.
18. **Kramerov, D., A. Grigoryan, A. Ryskov, and G. Georgiev.** 1979. Long double-stranded sequences (ds RNA-B) of nuclear pre-mRNA consist of a few highly abundant classes of sequences: evidence from DNA cloning experiments. Nucleic Acids Res. **6**:697–713.
19. **Kramerov, D. A., S. V. Tillib, I. V. Lekakh, A. P. Ryskov, and G. P. Georgiev.** 1985. Biosynthesis and cytoplasmic distribution of small poly(A)-containing B2 RNA. Biochim. Biophys. Acta **824**:85–98.
20. **Krayev, A., D. Kramerov, K. Skryabin, A. Ryskov, A. Bayev, and G. Georgiev.** 1980. The nucleotide sequence of the ubiquitous repetitive DNA sequence B1 complementary to the most abundant class of mouse fold-back RNA. Nucleic Acids Res. **8**:1201–1215.
21. **Krayev, A. S., T. V. Markusheva, D. A. Kramerov, A. P. Ryskov, K. G. Skryabin, A. A. Bayer, and G. P. Georgiev.** 1982. Ubiquitous transposon-like repeats B1 and B2 of the mouse genome: B2 sequencing. Nucleic Acids Res. **10**:7461–7475.
22. **Li, W.-Y., R. Reddy, D. Henning, P. Epstein, and H. Busch.** 1982. Nucleotide sequence of 7S RNA. J. Biol. Chem. **157**:5136–5142.
23. **Linial, M., N. Gunderson, and M. Groudine.** 1985. Enhanced transcription of c-*myc* in bursal lymphoma cells requires continuous protein synthesis. Science **230**:1126–1132.
24. **Lusky, M., and M. Botchan.** 1981. Inhibition of SV40 replication in simian cells by specific pBR322 DNA sequences. Nature (London) **293**:79–81.
25. **Majello, B., G. L. Mantia, A. Simeone, E. Boncinelli, and L. Lania.** 1985. Activation of major histocompatibility complex class I mRNA containing an Alu-like repeat in polyoma virus-transformed rat cells. Nature (London) **314**:457–459.
26. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning, a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
27. **Murphy, D., P. M. Brickell, D. S. Latchman, K. Willison, and P. W. Rigby.** 1983. Transcripts regulated during normal embryonic development and oncogenic transformation share a repetitive element. Cell **35**:865–871.
28. **Reddy, R., D. Henning, C. Subramanian, and H. Busch.** 1984. Primary and secondary structures of 7-3(K) RNA of Novikoff hepatoma. J. Biol. Chem. **259**:12265–12270.
29. **Roger, J. H.** 1985. The structure and evolution of retroposons. Int. Rev. Cytol. **93**:187–201.
30. **Saba, J., H. Busch, and R. Reddy.** 1985. A new moderately repetitive rat DNA sequence detected by a cloned $4.5S_I$ DNA. J. Biol. Chem. **260**:1354–1357.
31. **Schlissel, M. S., and D. D. Brown.** 1984. The transcriptional regulation of *Xenopus* 5S RNA genes in chromatin: the roles of active stable transcription complexes and histone H1. Cell **37**:903–913.
32. **Scott, M. R. D., K. Westphal, and P. W. J. Rigby.** 1983. Activation of mouse genes in transformed cells. Cell **34**: 557–567.
33. **Segall, J., T. Matsui, and R. Roeder.** 1980. Multiple factors are required for accurate transcription of purified genes by RNA polymerase III. J. Biol. Chem. **255**:11986–11991.
34. **Singh, K., M. Carey, S. Saragosti, and M. Botchan.** 1985. Expression of enhanced levels of small RNA polymerase III transcripts encoded by the B2 repeats in simian virus 40-transformed mouse cells. Nature (London) **314**:553–556.
35. **Singh, K., S. Saragosti, and M. Botchan.** 1985. Isolation of cellular genes differentially expressed in mouse NIH 3T3 cells and a simian virus 40-transformed derivative: growth-specific expression of VL30 genes. Mol. Cell. Biol. **5**:2590–2598.

36. **Thomas, C. A., K. J. Burns, Jr., and T. Kelly, Jr.** 1966. Isolation of high molecular weight rDNA from bacteria and cell nuclei, p. 535. *In* G. L. Cantoni and D. R. Davies (ed.), Procedures in nucleic acid research. Harper & Row, Publishers, Inc., New York.
37. **Ullu, E., and C. Tschudi.** 1984. Alu sequences are processed 7SL RNA genes. Nature (London) **312**:171–172.
38. **Vannice, J. L., J. M. Taylor, and G. M. Ringold.** 1984. Glucocorticoid-mediated induction of α1-acid glycoprotein: evidence for hormone-regulated RNA processing. Proc. Natl. Acad. Sci. USA **81**:4241–4245.
39. **Weinmann, R., and R. Roeder.** 1974. Role of DNA-dependent RNA polymerase III in the transcription of the tRNA and 5S RNA genes. Proc. Natl. Acad. Sci. USA **71**:1790–1794.