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Efficient transcription of a protein-coding gene from the RNA polymerase I promoter in transfected cells

(RNA polymerase I promoter/transfection/gene expression/chloramphenicol acetyltransferase gene)

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ABSTRACT **The activity of the mouse ribosomal promoter was examined after fusion to the gene coding for chloramphenicol acetyltransferase (CAT) and transfection into mouse cells. Very little CAT enzyme but high levels of CAT-specific RNA correctly initiated at the ribosomal DNA start site were synthesized. The amount of specific transcripts was neither influenced by long stretches of upstream spacer sequences nor by the insertion of the Moloney murine sarcoma virus enhancer. The deletion mutant pMrΔ-39, which has been shown to be fully active *in vitro*, exhibited a 90% decrease in template activity *in vivo*. A mutant in which 22 base pairs of ribosomal DNA (between positions −35 and −14) were substituted by foreign DNA sequences proved transcriptionally inactive. The fusion genes were only transcribed in mouse cells, indicating that species-specific transcription factors are involved in ribosomal promoter recognition.**

Previously we have defined some of the sequence elements that are required for correct transcription initiation on cloned mouse ribosomal DNA (rDNA). Using a cell-free transcription system, we have shown that there are at least two functional domains that constitute the rDNA promoter. The proximal promoter element is located within a short region in front of the transcription start site (between positions −39 and −14) and may be part of a larger functional domain that extends some nucleotides into the transcribed region (1, 2). Mutations within this region can alter both the efficiency and specificity of transcription. The upstream element is not well defined yet. It is located between positions −169 and −40, is not required for faithful transcription, but plays a role in modulating the efficiency of transcription (1). rDNA control elements of *Drosophila* (3), *Xenopus laevis* (4, 5), and human ribosomal genes (6) are found at positions similar to those in mouse. All these previous studies have been performed by testing the template activity of normal and mutant rDNA gene fragments either in crude cellular extracts or after microinjection into frog oocytes. However, as it has been shown that *in vitro* and *in vivo* assays delineate different boundaries for the RNA polymerase II promoter, the functional analysis of the RNA polymerase I promoter requires the study of the expression of wild-type and mutant copies of rDNA introduced into somatic cells. Therefore, we fused 5′ terminal rDNA fragments containing various lengths of spacer sequences to the gene for chloramphenicol acetyltransferase (CAT) and monitored the expression of these hybrid genes in mouse 3T6 cells. The results show that the rDNA–*CAT* gene fusions are effectively and faithfully transcribed *in vivo* but that the hybrid mRNA molecules are inefficiently translated into active CAT protein.

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MATERIALS AND METHODS

Recombinant DNA Constructions. The rDNA–*CAT* hybrid genes were constructed by blunt-end ligation of the *Hin*dIII/*Bam*HI fragment derived from pSV2-CAT (7, 8) into the *Sma* I site present in the external transcribed spacer (ETS) of mouse rDNA at position +155 (see Fig. 1). pMrCAT-1 contained a 324-base-pair (bp) *Sal* I–*Sma* I fragment (from positions −169 to +155), and pMrCAT-2 contained a 477-bp *Pvu* II–*Sma* I fragment (from −322 to +155) in front of the CAT structural gene. pMrCAT-3 was generated by cleaving the 11.35 kilobase (kb) rDNA clone pMr974 (9) with *Sal* I, followed by insertion of the 1.75-kb nontranscribed spacer fragment D into the *Sal* I site of pMrCAT-1. For the construction of pMSV-MrCAT, a 200-bp *Xba* I/*Sau*3A fragment from pM15 (10) was inserted into the *Bam*HI site of pMrCAT-2 by blunt-end ligation. In pMSV-A10CAT, the murine sarcoma virus (MSV) fragment was ligated into the *Sal* I site of pA10-CAT (11). The mutant rDNA clones pMrΔ-39 and pMrΔ-14-35 have been described before (1, 2). They were linearized with *Sma* I, and the *Hin*dIII/*Bam*HI fragment from pSV2-CAT was inserted to generate the clones pMrΔ-39CAT and pMrΔ-14-35CAT, respectively.

Assay for CAT Activity. Subconfluent cultures of 3T6 cells were transfected with 20 μg of supercoiled plasmid DNA by the calcium phosphate technique (7, 8, 12). The cells were harvested 44 hr after transfection and assayed for CAT activity by the method of Gorman *et al.* (8).

RNA Preparations. Cells from one to three plates of transfected cells were lysed in 2.5 ml of 4 M guanidinium isothiocyanate/10 mM EDTA/50 mM Tris·HCl, pH 7.6/2% Sarcosyl/0.14 M 2-mercaptoethanol. One gram of CsCl was added, and the solution was layered over a 1.2-ml cushion of 5.7 M CsCl/0.1 M EDTA. RNA was pelleted by centrifugation at 35,000 rpm in a Beckman rotor SW 55 for 16 hr. The RNA pellet was dissolved in 10 mM Tris·HCl (pH 7.4)/5 mM EDTA/1% $NaDodSO_4$ and extracted with chloroform/butanol, 4:1 (vol/vol), before precipitation with ethanol. The RNA recovered was dissolved in sterile water at 2 mg/ml for dot-blot analysis and primer extension.

Primer Extension Analysis. A 102-bp *Pvu* II–*Eco*RI fragment from the CAT coding region was labeled by T4 DNA polymerase and [^{32}P]dNTPs. Approximately 100,000 cpm of the primer was mixed with 40–50 μg of total cellular RNA from transfected cells, precipitated with ethanol, and dissolved in 25 μl of hybridization buffer (40 mM Pipes, pH 6.4/0.4 M NaCl/1 mM EDTA/0.2% $NaDodSO_4$). After incubation for 5 min at 70°C, the primer was annealed to the RNA by incubating for 3 hr at 42°C. Hybrids recovered by

Abbreviations: rDNA, ribosomal DNA; CAT, chloramphenicol acetyltransferase; ETS, external transcribed spacer; MSV, murine sarcoma virus; SV40, simian virus 40; Mo-MSV, Moloney MSV; bp, base pair(s); kb, kilobase(s).

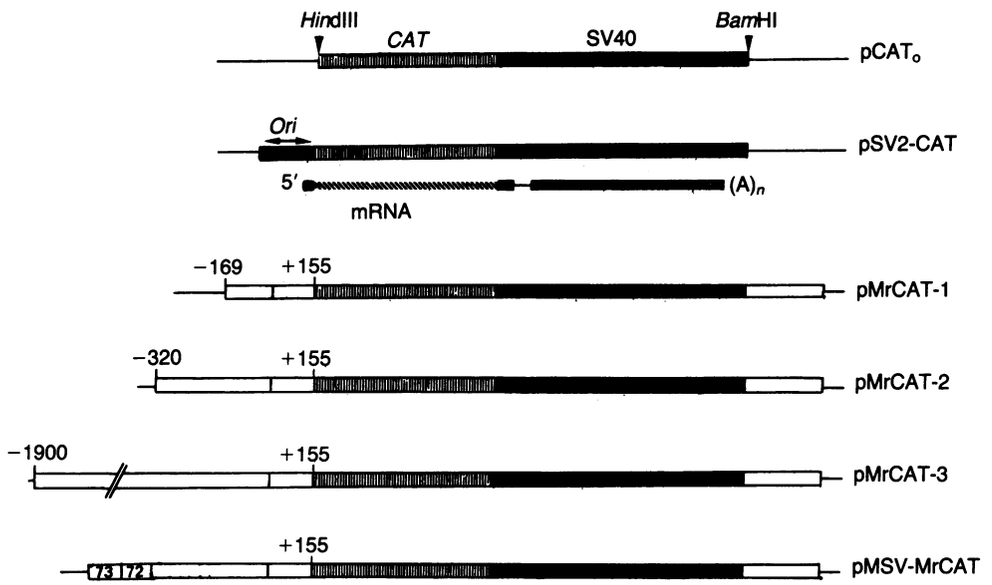


FIG. 1. Structure of the recombinant *CAT* constructs used. The plasmids pCAT$_o$ and pSV2-CAT have been described before (7). They contain the CAT coding region (hatched areas) followed by SV40 sequences including the small t tumor antigen intron and the SV40 early polyadenylylation signal (solid bars). In the pMrCAT plasmids, the *Hin*dIII–*Bam*HI fragment from pCAT$_o$ or pSV2-CAT was ligated into the *Sma* I site (position +155) of the rDNA clones pMrSP or pMr600 (1, 16), yielding pMrCAT-1 and pMrCAT-2, respectively. The ribosomal DNA sequences are represented by open bars. In pMrCAT-3, another 1.75 kb of spacer sequences are inserted into pMrCAT-1. The plasmid pMSV-MrCAT contains the 73/72-bp repeat sequences from the Mo-MSV adjacent to the rDNA of pMrCAT-2.

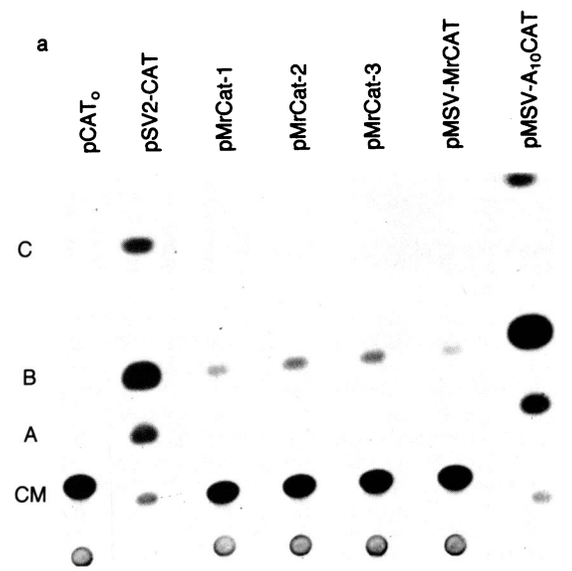
precipitation with ethanol were extended with reverse transcriptase as described by Hernandez and Keller (13).

RESULTS

Detection of CAT Activity in 3T6 Cells Transfected with Different rDNA–*CAT* Gene Fusions. Studies on the *in vivo* activities of cloned rDNA promoter fragments have to take into account that species-specific factors are involved in the transcription initiation process (3, 14, 15). Therefore, it is necessary to carry out transfection experiments in homologous cell systems. To identify the level of transcription from foreign DNA fragments above the background of endogenous cellular pre-rRNA transcripts, we fused rDNA promoter fragments to the bacterial gene coding for CAT. The *CAT* fragment present in the recombinant plasmids contained the CAT coding region and both the simian virus 40 (SV40) small tumor (t) antigen intron and SV40 polyadenylylation signal downstream of the CAT gene sequence. Since it has been reported that spacer sequences affect the transcription efficiency of *Xenopus laevis* rDNA after injection into frog oocytes (21, 22), we used three rDNA fragments that contain the same amount of rDNA coding sequences (from +1 to +155) but vary in the length of spacer sequences upstream of the start site (Fig. 1). The spacer DNA in the rDNA–*CAT* hybrid genes was 169 bp in pMrCAT-1, 300 bp in pMrCAT-2, and ≈1900 bp in pMrCAT-3.

3T6 cells were transfected with the different *CAT* fusion plasmids, and the transient expression of CAT was monitored after 40–44 hr. Fig. 2 shows the result of one typical experiment (Fig. 2*a*) and the average data from six different experiments (Fig. 2*b*). All three rDNA–*CAT* gene fusions directed *CAT* expression at a low but significant level. When the rDNA promoter fragment was inserted in the opposite orientation relative to the CAT structural gene no enzyme activity could be detected (not shown).

For a more precise quantitation of the rDNA-promoted *CAT* expression, kinetic experiments were performed. Fig. 3 shows the time course of CAT activity in extracts from cells transfected with pSV2-CAT, pCAT$_o$ or pMrCAT-1. There was a linear increase in the amount of chloramphenicol acetylation for at least 2 hr in extracts from cells transfected with pMrCAT-1, which led to conversion of ≈8% of the chloramphenicol into its acetylated forms. Within this period, only 1.4% chloramphenicol was acetylated in the negative control pCAT$_o$. Comparison of the linear parts of the curves showed that the pSV2-CAT plasmid yielded 20–50 times the level of CAT activity promoted by pMrCAT DNA in 3T6 cells. Interestingly, the level of CAT in extracts of cells transfected with any one of the three rDNA–*CAT* constructs was the



b

Plasmid	CM-acetylation, %
pCat$_o$	1.1
pMrCat-1	7.3
pMrCat-2	7.5
pMrCat-3	7.5
pMSV-MrCat	7.3

FIG. 2. CAT activity in 3T6 cells transfected with pSV2-CAT and different rDNA–*CAT* gene fusions. The enzyme reaction was performed with 30 μl of extract at 37°C for 90 min. The [^{14}C]chloramphenicol (CM) was separated from the monoacetylated forms (A and B) and the diacetylated form (C) by TLC. (*a*) Autoradiograph of one typical experiment. (*b*) Average conversion rates of chloramphenicol into its acetylated forms after incubation for 90 min with 30 μl of extract. The data represent mean values of at least six separate experiments.

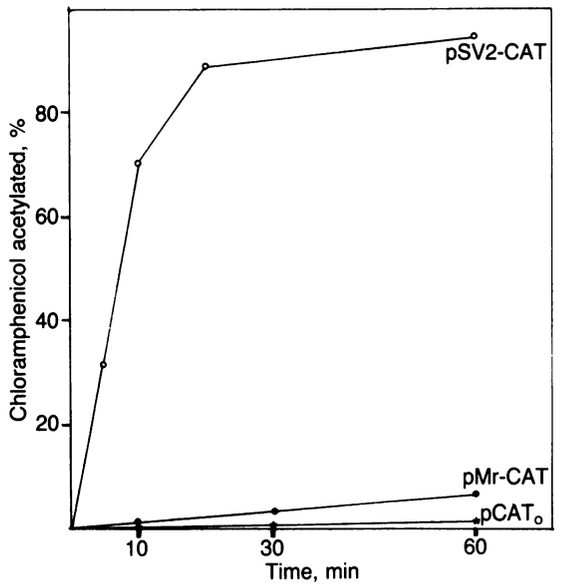


FIG. 3. Time course of CAT enzyme activity in extracts from transfected cells.

same. This means (*i*) that sequences from −169 to +155 of the ribosomal transcription unit are sufficient for promoter activity *in vivo* and (*ii*) that spacer sequences that include the 135-bp repetitive elements of mouse rDNA (9, 17) do not augment CAT expression from the rDNA promoter.

The Mo-MSV Enhancer Does Not Affect Expression of the rDNA-*CAT* Constructs. Viral enhancers have been shown to increase transcription from a variety of polymerase II promoters (for review, see ref. 18). To investigate whether enhancers also exert an effect on RNA polymerase I genes, we inserted the 73/72-bp tandem repeats from Moloney MSV (Mo-MSV) in front of the ribosomal sequences of pMrCAT-2 (see Fig. 1). This 73/72-bp tandem repeat unit from the Mo-MSV long terminal repeat has been shown to enhance transcription from a variety of RNA polymerase II promoters (18, 19). The CAT activity in extracts from cells transfected with pMrCAT fusions with or without the MSV enhancer was equally low (Fig. 2). As a control, the plasmid MSV-A10-CAT was used. In this construct, the 72-bp enhancer element present in pSV2-CAT was replaced by the 73/72-bp Mo-MSV fragment. As already reported by others, the Mo-MSV enhancer functionally replaced the SV40 enhancer in 3T6 cells promoting CAT levels comparable to pSV2-CAT (Fig. 2). This finding indicates that the MSV repeats that activate RNA polymerase II genes do not affect rDNA-promoted CAT expression. However, it remains to be seen whether or not RNA polymerase I is generally nonresponsive to such enhancer elements.

Analysis of CAT-Specific RNA by Primer-Extension. To prove that the CAT-specific mRNAs were transcripts from the authentic rDNA initiation site, the 5′ ends of the hybrid transcripts were mapped. For this, a 102-bp *Pvu* II–*Eco*RI fragment derived from pSV2-CAT was hybridized to total cellular RNA and used as primer for a reverse transcriptase reaction. As a control we used authentic CAT mRNA isolated from chloramphenicol-resistant *E. coli* harboring the plasmid pBR325. Elongation of the bacterial mRNA produced the predicted 250-nucleotide-long cDNA (Fig. 4, lane 3). RNA from cells transfected with pSV2-CAT yielded three bands characteristic for transcripts that were initiated at the SV40 early cap sites (lane 2). When RNA from cells transfected with the pMrCAT hybrid genes was analyzed by primer extension, a 405-nucleotide product was obtained (lane 1). This length corresponds to the distance from the rDNA initiation site to the 3′ end of the primer. In spite of the fact that very little CAT protein was produced in cells transfected with rDNA–*CAT* gene fusions, the amount of CAT mRNA that had been transcribed from the ribosomal promoter was almost as high as that transcribed from the SV40 promoter. Obviously, the rDNA promoter directed the synthesis of high levels of RNA, but the hybrid mRNA was poorly translated into protein. A more precise determination of the relative amounts of RNA produced has been performed by dot-blot analysis (not shown) and primer extension analysis of RNA transcribed from different rDNA–*CAT* constructs. In lanes 4 and 5 of Fig. 4, the transcripts synthesized from pMrCAT and pMSV-MrCAT are compared. There is no significant quantitative or qualitative change of CAT-specific

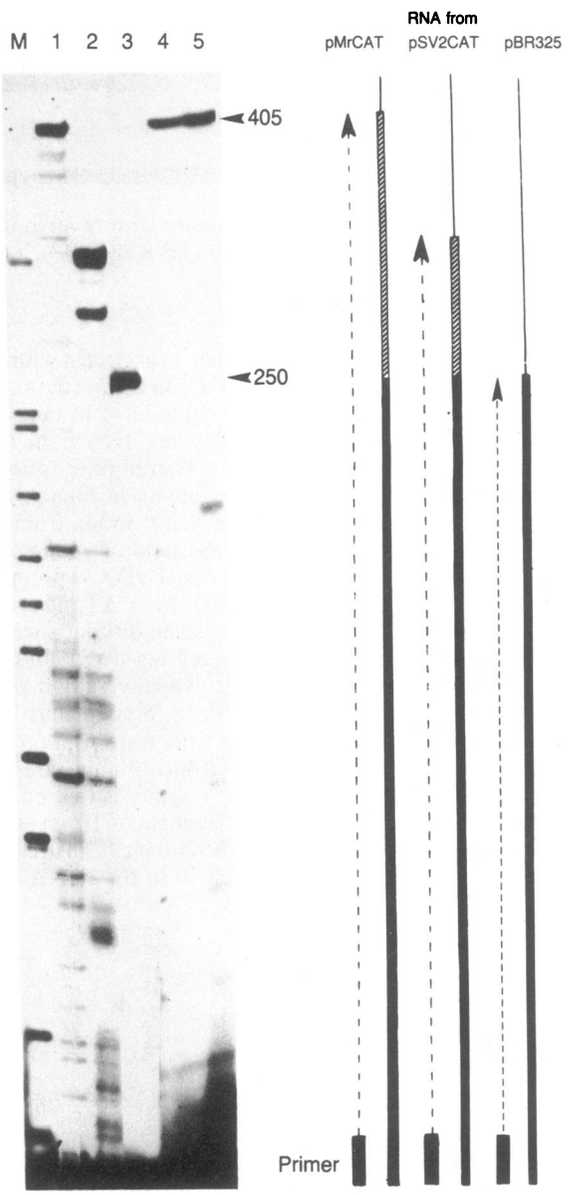


FIG. 4. Primer extension analysis of CAT-specific RNA. Cellular RNA (50 μg) was hybridized to a labeled 102-bp *Pvu* II/*Eco*RI fragment derived from the CAT gene, and the hybrids were transcribed by reverse transcriptase. The [^{32}P]cDNAs were electrophoresed on a 6% denaturing polyacrylamide gel along with [^{32}P]DNA markers (lane M) from a *Hpa* II digest of pBR322 DNA. The cDNA transcripts of RNA from cells transfected with pMrCAT-2 (lane 1), pSV2-CAT (lane 2), and from *E. coli* harboring the plasmid pBR325 (lane 3) are shown. Lanes 4 and 5 are from a different experiment showing the reverse transcripts from cells transfected with pMrCAT-1 (lane 4) and pMSV-MrCAT (lane 5).

FIG. 5. Primer extension analysis of RNA from cells transfected with wild-type and mutant pMrCAT gene fusions. The autoradiograph shows the position of the reverse transcripts of RNA obtained from cells transfected with pMrCAT-1 (lane 1), pMrΔ-39CAT (lane 2), and pMrΔ-14-35CAT (lane 3) along with [^{32}P]DNA markers (lane M) from a *Hpa* II digest of pBR322.

RNA in the presence or absence of the MSV enhancer. Similarly, no significant differences were found when the rDNA constructs with different spacer lengths were compared (not shown). This indicates that neither spacer sequences nor the Mo-MSV enhancer affect transcription from the ribosomal promoter.

Assay of Hybrid Genes Containing Mutations in the rDNA Control Region. Recently we performed a detailed mutational analysis of the mouse rDNA promoter by assaying the template activity of several deletion mutants, deletion-substitution mutants, and point mutants in a cell-free transcription system (1, 2). In order to study whether the sequences that are essential for promoter activity *in vitro* are required for specific transcription *in vivo*, we replaced the ribosomal sequences in the pMrCAT constructs by two mutant rDNA clones. pMrΔ-39CAT is a deletion mutant containing only 39 nucleotides of the 5′ flanking sequences. The template activity of this deletion mutant was identical to the wild type in the *in vitro* transcription system (1). In pMrΔ-14-35CAT, the 22 bp between positions −14 and −35 of the rDNA region

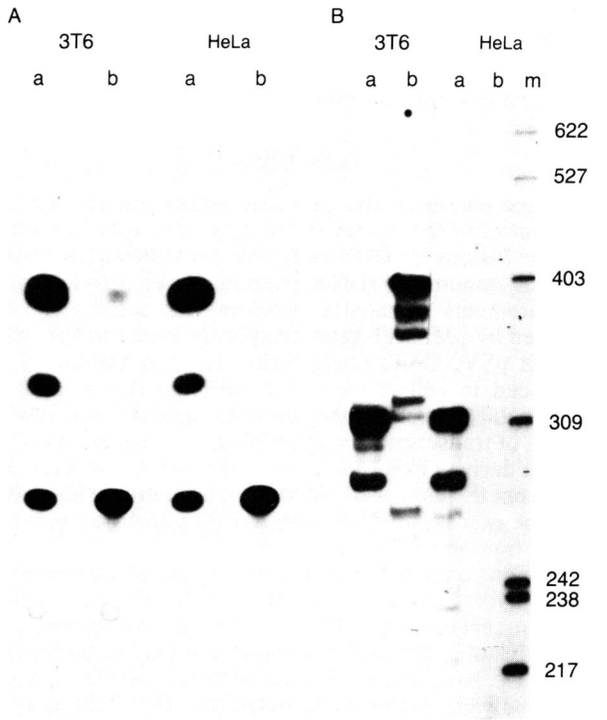


FIG. 6. Species-specific expression of rDNA-*CAT* constructs. (*A*) CAT activity in 3T6 and HeLa cells transfected with pSV2-CAT (lanes a) or pMrCAT-2 (lanes b). The autoradiograph shows the acetylation of chloramphenicol in 30 μl of extract incubated for 15 min (for pSV2-CAT-transfected cells) or 90 min (for pMrCAT-2-transfected cells) at 37°C. (*B*) Primer extension analysis of RNA derived from 3T6 or HeLa cells transfected with pSV2-CAT (lanes a) or pMrCAT-2 (lanes b). Lane m shows labeled pBR322/*Hpa* II marker fragments.

have been deleted and substituted by vector sequences, which resulted in an absolute loss of template activity *in vitro* (2). Fig. 5 shows a primer extension analysis of the CAT-specific RNAs derived from 3T6 cells transfected with the wild-type or the two mutant rDNA clones. The clone pMrΔ-39CAT showed an approximate decrease to 1/5th to 1/10th in the amount of transcripts (lane 2) compared to the wild-type pMrCAT-2 (lane 1), while pMrΔ-14-35CAT proved to be transcriptionally absolutely inactive (lane 3). The inability of the mutant pMrΔ-14-35CAT to direct transcription supports our previous *in vitro* data, which have demonstrated that an essential promoter element is contained within the region −14 to −35.

Species-Specific Expression of rDNA-*CAT* Gene Fusions. Previous experiments in cell-free transcription systems have shown that species-specific factors are required for accurate transcription initiation by RNA polymerase I (14). To investigate whether this involvement of species-specific factors is not restricted to the *in vitro* systems, we transfected in parallel mouse 3T6 and human HeLa cells with pSV2-CAT and pMrCAT-1, and assayed for both CAT activity and CAT-specific RNA. The CAT enzyme levels were about the same in pSV2-CAT-transfected 3T6 or HeLa cells (Fig. 6*A*, lanes a). In contrast, the low but significant expression of pMrCAT was detected only in 3T6 and not in HeLa cells (lanes b). The complete absence of CAT activity in pMrCAT-transfected HeLa cells indicates that the low CAT levels in 3T6 cells is not due to abortive initiations by RNA polymerase II. Once again, the differences in CAT expression are much more pronounced at the RNA level. The SV40-specific bands are present in approximately the same intensity in both mouse or human cells (Fig. 6*B*, lanes a).

However, the rDNA-specific 405-base reverse transcript is present only in 3T6 and not in HeLa cells (lanes b), which suggests that species-specific factors are part of the RNA polymerase I transcription machinery.

DISCUSSION

We have measured the transient expression of *CAT* under the control of the mouse rDNA promoter after introduction of gene fusions into 3T6 cells. We could detect low but significant amounts of rDNA-promoted CAT activity. Kinetic measurements revealed a lower enzyme activity (≈1/50th) directed by pMrCAT gene fusions compared to the positive control pSV2-CAT. Surprisingly, the low amount of CAT produced in cells transfected with the rDNA–*CAT* constructs did not reflect the promoter activity—i.e., the efficiency of transcription of the CAT gene. The amount of transcripts derived from the ribosomal promoter was as high as that from the enhanced SV40 early promoter. This indicates that the expression of the rDNA–*CAT* constructs is impaired at the translational level.

Obvious explanations for the low translational efficiency of the hybrid RNA are: (*i*) The rDNA–*CAT* gene transcripts are not capped or (*ii*) the transcripts are not properly polyadenylylated. The latter possibility seems to be less likely since we have found that the rDNA promoter transcripts were polyadenylylated, provided that the SV40 early polyadenylylation site was present in the recombinant plasmids (unpublished data). This finding suggests that the enzymes responsible for cutting the primary transcript and poly(A) addition are not associated with the RNA polymerase II transcription machinery.

On the contrary, cap formation appears to be coupled with initiation of transcription, and it has been suggested that the mechanism of this coupling involves a close association between RNA polymerase II and the capping enzymes (19). Jove and Manley (20) have shown that addition of *S*-adenosylhomocysteine to a soluble whole-cell extract inhibited transcription initiation by RNA polymerase II but had no effect on transcription by RNA polymerase III. This suggests that 5′-modification enzymes are components of the RNA polymerase II transcription initiation complex. Cap structures, on the other hand, have been shown to increase the ability of mRNAs to be translated and, thus, to play an important role in the function and utilization of mRNAs. We also have some preliminary evidence that the transport of the pMrCAT transcripts from the nucleus into the cytoplasm may be impaired. After fractionation of cells into nuclei and cytoplasm, we consistently observed a higher percentage of CAT RNA in the nucleus than in the cytoplasm of cells transfected with pMrCAT plasmids, whereas more CAT RNA was found in the cytoplasm in pSV2-CAT-transfected cells (data not shown).

We also investigated whether spacer sequences could affect the strength of the polymerase I. In fact, in the case of *X. laevis*, the upstream spacer sequences seem to influence the promoter strength. When two rDNA templates are coinjected into oocytes, those with larger spacer regions are transcribed in preference to those with smaller ones (21). This effect has been attributed to the duplicated 42-bp promoter element located between −73 and −114, which is present as a repetitive sequence within the nontranscribed spacer of *X. laevis*. The number of these 42-bp elements has been shown to enhance the frequency with which the rDNA promoter becomes active (22). In mouse, a 135-bp repetitious region is located 5′ to the origin of the rRNA initiation site (9, 17). When tested in the transfection assay, the constructs without the repetitive spacer region were transcribed equally well. This means that the repeated nontranscribed spacer elements in mouse do not seem to affect the promoter strength. However, we cannot exclude that these sequences serve a function in the regulation of rRNA synthesis *in vivo*.

A mutational analysis of the rDNA promoter confirmed our previous suggestion that an important promoter element is located within positions −14 and −35 (2). In contrast, the deletion mutant pMrΔ-39, which proved to be transcriptionally fully active under noncompetitive assay conditions *in vitro*, showed a reduction of template activity to ≈1/10th *in vivo*. This finding is reminiscent of the different sequence requirements for transcription of class II RNA polymerase genes *in vitro* and *in vivo*. Furthermore, it was demonstrated that the rDNA promoter was only active in mouse cells, which is in accord with our *in vitro* data showing that initiation by RNA polymerase I requires species-specific factors (14). In view of the fact that the rDNA promoter by itself was as active as the enhanced SV40 early promoter, it is not too surprising that we did not observe an effect of the Mo-MSV enhancer on the polymerase I promoter. Electron microscopic studies have shown that ribosomal gene transcription is an all-or-none process (22). Once a given polymerase I promoter is activated, it loads RNA polymerase I with maximal density. Therefore, the dense polymerase packing seems to be a property of the rDNA promoter itself. A more or less efficient transcription is probably brought about by factors that switch the promoter into an active or inactive conformation.

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