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RNA polymerase specificity of mRNA production and enhancer action

(RNA processing/enhancer selectivity/transient expression/RNA polymerase I promoter/chloramphenicol acetyltransferase gene)

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ABSTRACT **To examine the RNA polymerase (EC 2.7.7.6) specificity of RNA maturation/utilization and transcriptional enhancement, we constructed a chimeric plasmid (pPolI-CAT) in which a promoter for mouse rRNA gene transcription was placed adjacent the coding sequences for chloramphenicol acetyltransferase (CAT; EC 2.3.1.28). A number of other constructs, including plasmids also containing a murine sarcoma virus enhancer or lacking any natural eukaryotic promoter sequences, were also prepared. In apparent agreement with earlier conclusions that an RNA polymerase I transcript can act as a messenger RNA, transient transfection of mouse L cells with pPolI-CAT yielded both high levels of transcription from the RNA polymerase I promoter and enzymatically active CAT protein. However, further examination revealed that CAT protein is not translated from RNA that begins at the normal rRNA transcription initiation site. Polysomal RNA is devoid of such RNA and instead consists of CAT-encoding transcripts that begin elsewhere in the mouse ribosomal DNA (rDNA) region. Since transcription of these aberrant RNAs is stimulated by the addition of a murine sarcoma virus enhancer segment, they are probably transcribed by RNA polymerase II. Transcripts that map to the authentic rRNA start site are not similarly enhanced. Moreover, unlike the RNAs deriving from the rRNA initiation site, these aberrant RNAs are more stable and the level of translatable CAT transcripts is suppressed by inclusion of larger segments of the rDNA promoter regions. Fortuitously initiated mRNAs are also formed in the absence of any natural eukaryotic promoter sequence. From these data we conclude (*i*) that there is no evidence that normal RNA polymerase I transcription yields functional mRNA and (*ii*) that transcriptional enhancement appears to be RNA polymerase specific.**

Precisely how nuclear transcripts whose synthesis is catalyzed by RNA polymerase I, II, or III (DNA-directed RNA polymerase, EC 2.7.7.6) are directed through the appropriate processing/transport/utilization pathway remains largely unresolved. *A priori*, the choice of the maturation pathway could be specified solely by the primary sequence of the transcript. Alternatively, it could be dependent upon the polymerase that catalyzes the transcription either through subnuclear compartmentalization or cotranscriptional attachment of processing complexes. Certain aspects of this general question have been approached by Green *et al.* (1), who followed the fate of RNA synthesized *in vitro* that was injected into *Xenopus* oocytes. These authors showed that the injected RNA was spliced accurately, albeit quite inefficiently. This finding, along with the development of *in vitro* systems that support splicing (2–4), polyadenylylation (5), and 3′-end cleavage (6, 7) of purified RNAs normally transcribed by RNA polymerase II, has demonstrated that there is not an obligatory coupling of processing of mRNA precursors to concomitant synthesis by a polymerase II transcription complex.

Other studies have begun to address the companion questions of whether ribonucleoprotein particles that arise from transcription by RNA polymerase I or III are appropriate substrates for mRNA processing and transport pathways, and moreover, whether such RNAs are competent to direct protein synthesis. Carlson and Ross (8, 9) have reported that alternative transcripts of the human and mouse β-globin genes, evidently produced by RNA polymerase III, appear to be spliced normally. These unusual globin transcripts are either polyadenylylated (8, 9) or "transspliced" onto normal globin RNA. Moreover, from microinjection and transfection studies, Grummt and co-workers have reported that the promoter of the mouse rRNA gene can direct transcription of a juxtaposed simian virus 40 (SV40) tumor (T) antigen (10) or chloramphenicol acetyltransferase (CAT; EC 2.3.1.28) (11) coding region and that these RNAs are subsequently translated into the corresponding polypeptides. Since a substantial proportion of the transcripts do initiate at the normal rRNA initiation site, Grummt and coworkers concluded that normal polymerase I transcription can yield a functional translatable mRNA (11).

We have further investigated whether functional polypeptides can be encoded by a hybrid gene transcribed by RNA polymerase I. To this end, we have cloned the mouse RNA promoter region juxtaposed to the coding region of the CAT gene (12). After expression in transiently transfected mammalian tissue culture cells, the resultant CAT mRNA-containing transcripts were characterized by S1 nuclease and primer extension analyses, and production of functional CAT protein was determined by enzymatic assays. We find that synthesis of CAT protein is indeed directed by these constructs, as originally reported by Grummt and Skinner (11). However, none of the polysomal RNAs begins at the normal rRNA initiation site. Instead, the 5′ terminus of this translatable RNA maps to another site within the rRNA sequence. Inasmuch as the levels of this aberrant RNA and its translation product are increased by the presence in *cis* of a murine sarcoma virus (MSV) enhancer sequence, we suggest that the translatable CAT RNA may arise from a fortuitous polymerase II start in the rRNA initiation region. RNAs deriving from the authentic mouse rRNA start site are indeed detected, but they are not found on polysomes, are not increased in amount by the presence of an MSV enhancer, and are very short lived.

MATERIALS AND METHODS

Plasmids. The constructs (Fig. 1) were formed from pSV2-CAT (12), kindly provided by George Khoury. For pSV2′-CAT, the *Pvu* II site at the upstream boundary of the SV40

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Abbreviations: SV40, simian virus 40; T antigen, tumor antigen; CAT, chloramphenicol transacetylase; MSV, murine sarcoma virus; rDNA, DNA encoding rRNA and the flanking spacer regions; bp, base pair(s); kb, kilobase(s).

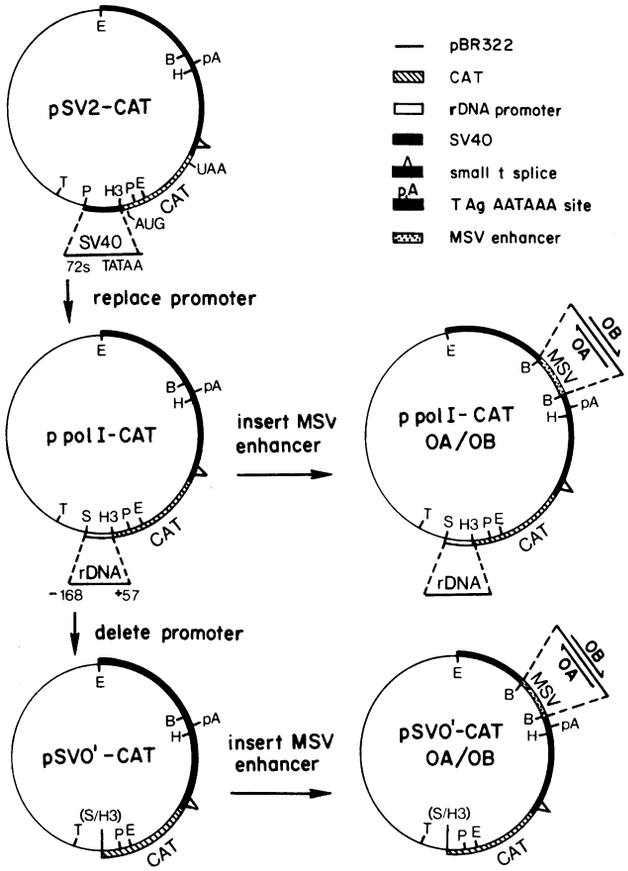

FIG. 1. Construction of hybrid CAT gene plasmids. As detailed in *Materials and Methods*, the SV40 promoter/enhancer region of pSV2-CAT (12) was replaced with the promoter region for mouse rRNA gene transcription [nucleotides −168 to +57 (13)] to yield pPolI-CAT, or the SV40 control region was deleted entirely to yield pSV0'-CAT. The translation initiation signals for the CAT gene are 3' to the *Hin*dIII site and are retained in all constructs. An MSV enhancer region was also inserted in both orientations (OA and OB) in pPolI-CAT and pSV0'-CAT. Restriction endonuclease sites: P, *Pvu* II; S, *Sal* I; H3, *Hin*dIII; E, *Eco*RI; B, *Bam*HI; T, *Tth* III 1; H, *Hpa* I. Small t, SV40 small tumor antigen; 72s, SV40 72-bp repeats; rDNA, DNA encoding rRNA and the flanking spacer regions.

promoter/enhancer region was converted to a *Sal* I site by partial *Pvu* II digestion and ligation to *Sal* I linkers. This plasmid was used for subsequent constructs and as a "divergence probe" for S1 nuclease analysis of pSV2-CAT RNA. To form pPolI-CAT, the mouse rDNA plasmid p5'Sal-Pvu (14) was cleaved sequentially with *Sal* I (rDNA position −168) and *Taq* I (rDNA position +57). Next, pSV2'-CAT was cleaved with *Sal* I and *Hin*dIII to remove the entire SV40 promoter/enhancer region. The 235-base-pair (bp) rDNA promoter fragment (*Sal–Taq* fragment) and the CAT/vector (*Sal–Hin*dIII fragment) were isolated and joined by using *Hin*dIII–*Taq* converters to form pPolI-CAT. The CAT/vector fragment was also circularized by itself after treatment with the Klenow fragment of DNA polymerase to blunt the ends, forming the promoterless pSV0'-CAT. (Note that pSV0'-CAT is strictly homologous to pSV2-CAT and pPolI-CAT, while the standardly used pSV0-CAT (12) has different pBR322 sequences abutting the CAT region.) A 398-bp fragment of the MSV long terminal repeat (LTR) containing the 72/73-bp MSV enhancer repeats (ref. 15, kindly provided by G. Khoury) was inserted into the unique *Bam*HI site of pPolI-CAT and pSV0'-CAT in both orientations, termed OA (natural orientation) and OB (reverse orientation). Finally, to form p-2kbPolI-CAT a 1.8-kilobase (kb) *Sal* I fragment of mouse rDNA extending from position ≈−2 kb to −168 (16) was inserted at its natural position relative to the rDNA promoter at the unique *Sal* I site in pPolI-CAT.

Transfections and CAT Assay. Mouse L cells and Chinese hamster ovary (CHO) cells were transfected as described by using DEAE-dextran coupled with a dimethyl sulfoxide shock (17). Extracts were prepared 24 hr after transfection and assayed for CAT activity as described (12, 17).

RNA Analyses. RNA was prepared 24 hr after transfection by using the guanidinium isothiocyanate/cesium chloride method (18). Alternatively, polysomes were prepared (19) prior to RNA isolation. For S1 nuclease analysis, single-stranded DNA probes were prepared as described (20) and end labeled at the *Pvu* II site within the CAT gene. Probe (0.2 pmol) was hybridized to 10 μg of cellular RNA in 0.3 M NaCl/2 mM EDTA/100 mM Tris·HCl, pH 7.6, at 65°C for 12 hr, and single-stranded regions were digested with S1 nuclease (20). Alternatively, a primer (the 5'-end-labeled coding strand of the 27-bp *Hin*dIII–*Dde* I fragment at the 5' end of the CAT coding region) was hybridized to the RNA and extended by using reverse transcriptase as described (21). The resultant radioactive fragments were denatured, fractionated on 6% polyacrylamide sequencing gels, and visualized by autoradiography (20).

RESULTS

To examine whether RNA whose synthesis is catalyzed by RNA polymerase I can direct translation of a functional protein, we have utilized the hybrid plasmids displayed in Fig. 1. These constructs are based on pSV2-CAT of Gorman *et al.* (12), in which the bacterial CAT gene is inserted between the SV40 early transcription initiation segment and the SV40 small tumor antigen splice and polyadenylylation regions. To form a CAT gene transcribed under direction of a polymerase I promoter, we have replaced the entire SV40 promoter/enhancer region of pSV2-CAT with sequences from the mouse rRNA promoter region. The resultant pPolI-CAT contains rDNA sequence −168 to +57 (where +1 is the transcription start site) that includes the ≈150-bp polymerase I promoter defined *in vitro* (13). A second plasmid, pSV0'-CAT, which is strictly analogous to pPolI-CAT and pSV2-CAT but lacks all natural eukaryotic promoter regions, was constructed from pPolI-CAT by excision of the rDNA sequences. Derivatives of pPolI-CAT and pSV0'CAT into which an MSV enhancer segment was inserted in the natural (OA) or reverse (OB) orientation 3' to the CAT gene were also formed (Fig. 1), yielding pPolI-CAT-MSV(OA/OB) and pSV0'-CAT-MSV(OA/OB). A final plasmid (p-2kbPolI-CAT) containing an additional 1.8 kb of upstream rDNA sequence (residues ≈−2kb to +57) was also constructed from pPolI-CAT (not shown).

These plasmids were introduced into mouse L cells by transient transfection using DEAE-dextran and a dimethyl sulfoxide shock (17). Extracts prepared from the cells 24 hr after transfection were assayed for CAT activity (12) (Fig. 2). Consistent with an earlier report involving a similar construct (11), pPolI-CAT directs production of CAT enzymatic activity (Fig. 2, lane 4). Indeed, the CAT activity from pPolI-CAT is substantially greater than from the analogous "promoterless" construct, pSV0'-CAT, which yields a small but detectable level of CAT activity (lane 7). Strikingly, the presence of the MSV enhancer in pPolI-CAT results in markedly increased CAT activity (lanes 5 and 6), demonstrating that translatable RNAs from pPolI-CAT are elevated by this polymerase II enhancer segment. The level of CAT expression from pPolI-CAT-MSV(OA/OB) is comparable to that from pSV2-CAT (lane 10). However, the presence of the MSV enhancer also substantially raises the level of CAT expression from the promoterless constructs pSV0'-CAT-MSV(OA/OB) (lanes 8 and 9).

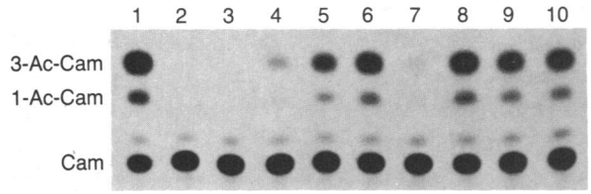

FIG. 2. Assay of CAT enzymatic activity from transfected L cells. L cells were transfected with the various plasmids and 40% of the extract prepared from a 60-mm dish was assessed for CAT activity. Unreacted chloramphenicol (Cam) and its 1- and 3-acetate derivatives were separated chromatographically. Lane 1, 10^{-2} unit of CAT enzyme (P-L Biochemicals); lane 2, extract from untransfected L cells; lanes 3–10, RNA from L cells transfected with p-2kbPolI-CAT (lane 3), pPolI-CAT (lane 4), pPolI-CAT-MSV(OA) (lane 5), pPolI-CAT-MSV(OB) (lane 6), pSV0′-CAT (lane 7), pSV0′-CAT-MSV(OA) (lane 8), pSV0′-CAT-MSV(OB) (lane 9), or pSV2-CAT (lane 10).

To directly assess expression of these plasmids in transfected L cells, we sought to analyze their transcripts. Unfortunately, S1 nuclease analysis cannot be used to characterize the transcripts of pPolI-CAT in mouse cells (20). This is because the primary rRNA transcript endogenous in the mouse L cells forms an S1-resistant trimolecular hybrid with pPolI-CAT probe DNA and pPolI-CAT-derived RNA, preventing detection of the actual initiation site of the pPolI-CAT RNA. Consequently, we have analyzed RNAs from in the transfected L cells by primer extension. A 27-nucleotide end-labeled primer complementary to the 5′ end of the CAT sequence (extending from 62 to 88 nucleotides 3′ to the natural rRNA start site of pPolI-CAT) was prepared and annealed with the RNA. After extension by reverse transcriptase, the products were analyzed by electrophoresis under denaturing conditions (Fig. 3). The major extension product obtained with RNA from cells transfected with pPolI-CAT (lane 3) co-migrates with the 88-nucleotide extension product obtained with RNA faithfully initiated on this plasmid in an *in vitro* transcription reaction (lane 2). Thus, transcription initiates at position +1 of the transfected pPolI-CAT, and the amount of this RNA compares favorably

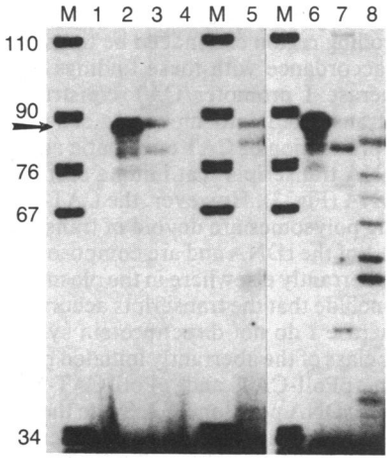

FIG. 3. Primer extension analysis of RNAs from transfected L cells. A 27-nucleotide primer from the 5′ end of the CAT gene was hybridized to 20 μg of transfected RNA cell and the hybrids were extended by using reverse transcriptase. Lane 1, untransfected L cell RNA; lane 2, RNA transcribed *in vitro* from pPolI-CAT; lanes 3–5, RNA from L cells transfected with pPolI-CAT (lane 3), pSV0′-CAT (lane 4), or pSV2-CAT (lane 5); lane 6, RNA transcribed *in vitro* from pPolI-CAT-MSV(OB); lanes 7 and 8, RNA transcribed from L cells transfected with pPolI-CAT-MSV(OB) (lane 7) or pSV0′-CAT-MSV(OB) (lane 8). M, markers, with numbers of nucleotides indicated on the left.

to that obtained from cells transfected with pSV2-CAT (lane 5). However, since extension products of many other lengths are also observed in lane 3, it appears that transcripts in cells transfected with pPolI-CAT also originate from other than the rDNA initiation site.

RNA isolated from cells transfected with pSV0′-CAT directs production of only a relatively low level of extension product (Fig. 3, lane 4). The amount of this RNA is, however, increased by the presence of the MSV enhancer on the transfected pSV0′-CAT (lane 8), consistent with the CAT enzymatic assays (Fig. 2). This confirms that fortuitous initiation sites are present in pSV0′-CAT plasmid sequences and suggests that these direct synthesis of a limited amount of RNA that in turn is translated into CAT protein. Moreover, this fortuitously initiated translatable RNA is elevated by the presence of the MSV enhancer even when it is ≈2 kb away from the CAT gene.

In contrast, the MSV enhancer does not increase the level of RNA initiated accurately at rDNA residue +1 of pPolI-CAT (lanes 3 and 7, Fig. 3). Since the enhancer-containing plasmid pPolI-CAT-MSV(OA/OB) directed synthesis of considerably more CAT enzymatic activity than did pPolI-CAT (see Fig. 2), this suggests that polymerase I-derived transcripts do not yield translatable RNAs. However, since the MSV enhancer resulted in elevated levels of RNAs that initiated at sites other than rDNA position +1 in pPolI-CAT-MSV(OB) (Fig. 3, lane 7), it appears that RNA from pPolI-CAT that directs CAT protein synthesis does not initiate at the natural rDNA start site.

To further investigate which RNA species does direct CAT protein synthesis, polysomal RNA was isolated from cells transfected with pPolI-CAT, pPolI-CAT-MSV(OB), or pSV2-CAT, and the 5′ ends of the resultant RNAs were identified. [Since polysomes do not contain RNA bearing the 5′ end of the primary transcript of the cellular rRNA genes, polysomal RNA can be analyzed by S1 nuclease mapping without the complication of trimolecular hybrids (ref. 20; see above).] Fig. 4*A* shows an S1 nuclease analysis of this RNA. As expected, the 5′ end of polysomal RNA from cells transfected with pSV2-CAT (lane 3) maps to the series of start sites known to be utilized by the SV40 early promoter region (22). Control RNA transcribed *in vitro* by polymerase I from pPolI-CAT in an S-100 transcription reaction similarly begins at the site expected for correct rRNA initiation (lane 4) and yields an ≈207-nucleotide protected fragment. (Since the SV40 and the rDNA promoters contribute approximately equal lengths of transcribed regions to pSV2-CAT and pPolI-CAT, respectively, their transcripts yield similar lengths of S1 nuclease or primer extension products.) Strikingly, however, no polysomal RNA from transfected pPolI-CAT (lane 1) or pPolI-CAT-MSV-OB (lane 2) begins at this normal start site. Instead, there are two other classes of polysomal pPolI-CAT-derived transcripts, both more prevalent in cells transfected with pPolI-CAT-MSV(OB) (lane 2) than in cells transfected with pPolI-CAT (lane 1). The 5′ terminus of one of these RNAs maps to approximately position +32 of the rRNA gene; it corresponds to a relatively minor band of ≈55 nucleotides in the primer extension analysis of whole cell RNA (Fig. 3). The other class of RNA, detected as a 375-nucleotide protected "divergence band" (Fig. 4*A*, lanes 1 and 2), is transcribed through the rDNA promoter region from an initiation site or sites 5′ to the rDNA sequences. We thus conclude that CAT enzymatic activity resulting from transfection of L cells with pPolI-CAT and pPolI-CAT-MSV does not derive from correct initiation by polymerase I at rDNA position +1, but rather it derives from aberrantly initiated RNAs.

To extend this result, we transfected CHO cells with the various CAT plasmids. Fig. 5 displays the resultant CAT enzymatic activity. CAT activity is indeed obtained with

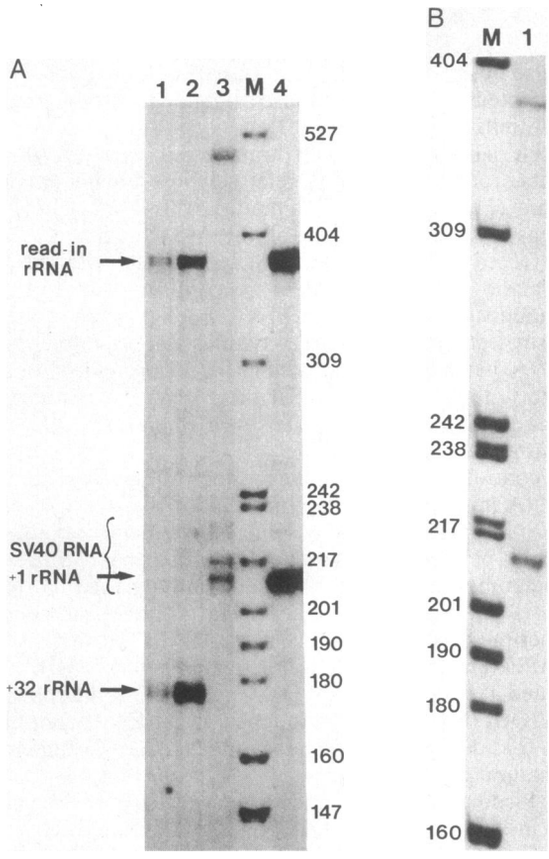

FIG. 4. S1 nuclease protection analysis of RNAs derived from transfected cells. (*A*) Analysis of polysomal RNA from L cells. Polysomal RNA was isolated from L cells transfected with pPolI-CAT (lane 1), pPolI-CAT-MSV(OB) (lane 2), or pSV2-CAT (lane 3). Lane 4, RNA transcribed from pPolI-CAT *in vitro* was analyzed. The probe for the pPolI-CAT RNAs (lanes 1, 2, 4) contains an insertion 5′ to the RNA polymerase I promoter region (375 nucleotides upstream of the labeled terminus). Correctly initiated RNA will thus yield a 207-nucleotide band protected from S1 nuclease, while any RNA that reads into this promoter region from upstream will yield a discrete 375-nucleotide band. The pSV2-CAT RNA was hybridized to an analogous "divergence" probe from pSV2′-CAT; correctly initiated RNAs will yield a series of bands of ≈205–225 nucleotides, while upstream initiated RNA will yield an ≈500-nucleotide "divergence band." M, markers, with numbers of nucleotides indicated on the right. (*B*) Analysis of RNA from CHO cells. RNA from CHO cells transfected with p-2kbPolI-CAT was mapped by S1 nuclease analysis as in *A*. RNA initiating at the correct rRNA transcription start site will yield a 207-nucleotide protected fragment, whereas RNAs that initiate 5′ to the rDNA sequences and transcribe into the rDNA sequences are visualized in a "divergence" band of 375 nucleotides.

pSV2-CAT (lane 10), demonstrating that CHO cells can be successfully transfected by this protocol. In contrast to the results found with the L cells in Fig. 2, however, no CAT activity is detectable in cells transfected with pPolI-CAT (lane 4) or its MSV enhancer-containing derivatives, pPolI-CAT-MSV(OA/OB) (lanes 5 and 6). Nor was CAT activity observed in pSV0′-CAT (lane 7) or pSV0′-CAT-MSV(OA/OB) (lanes 8 and 9) transfectants.

To test whether the lack of CAT activity in CHO cells transfected with rDNA-promoted CAT genes was due to an inability of the mouse RNA polymerase I promoter to function in hamster cells, we examined the RNA obtained from these transfected cells by S1 nuclease mapping. As seen in Fig. 4*B*, the mouse polymerase I promoter indeed directs synthesis of transcripts that initiate at position +1 of the rDNA promoter region. These results provide additional support for the conclusion that the translatable RNAs directed by pPolI-CAT do not derive from accurate initiation by RNA polymerase I.

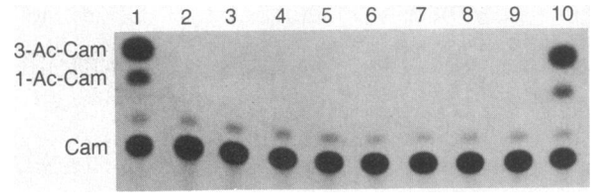

FIG. 5. Assay of CAT enzymatic activity from transfected CHO cells. CHO cells were transfected with the plasmids indicated below and the extract prepared from a 60-mm dish was assessed for CAT activity (see Fig. 2). Lane 1, 10^{-2} unit of CAT enzyme (P-L Biochemicals); lane 2, RNA from untransfected CHO cells; lanes 3–10, extract from CHO cells transfected with p-2kbPolI-CAT (lane 3), pPolI-CAT (lane 4), pPolI-CAT-MSV(OA) (lane 5), pPolI-CAT-MSV(OB) (lane 6), pSV0′-CAT (lane 7), pSV0′-CAT-MSV(OA) (lane 8), pSV0′-CAT-MSV(OB) (lane 9), or pSV2-CAT (lane 10).

DISCUSSION

The experiments described in this paper explore whether the rDNA promoter can direct the transcription of a gene that is normally transcribed by RNA polymerase II, and if so, whether the resultant RNA is a suitable substrate for the maturation pathway that leads to translatable RNAs characteristic of polymerase II-directed transcripts. Interest in these questions derives principally from two sources. First, an answer would serve to define the requisite specificity of the mRNA maturation pathway: is splicing/polyadenylylation/translation linked *in vivo* to an RNA polymerase II transcription complex, or alternatively, are RNAs segregated into appropriate processing and utilization pathways solely on the basis of a property inherent to the RNA transcript itself? Second, since the rRNA promoter is among the most active known transcriptional initiators it could potentially serve as a very useful promoter for active expression of a desired gene after transfection into growing cells.

The question of whether RNA polymerase I-directed transcripts can yield translatable RNAs has been addressed in two previous reports (10, 11), which concluded that RNAs that initiate at the rRNA start site and read into a T-antigen- or CAT-encoding region can indeed be translated into active protein. In accordance with these findings, using an analogous polymerase I promoter/CAT construct (pPolI-CAT) transiently transfected into mouse L cells, we do indeed observe the production of CAT enzymatic activity (Fig. 2) as well as of RNA transcripts that initiate correctly at position +1 of the rDNA (Fig. 3). However, the CAT-encoding RNAs on translating polysomes are devoid of transcripts that begin at position +1 of the rDNA and are composed only of species that initiate aberrantly elsewhere in the plasmid (Fig. 4*A*). We therefore conclude that the transcripts accurately initiated by RNA polymerase I do not direct protein synthesis.

The major class of the aberrantly initiated polysomal RNAs derived from pPolI-CAT and pPolI-CAT-MSV begins at approximately rDNA position +32. Since the normal ATG of the CAT sequence is the first ATG encountered in this RNA, it is likely that it is translated into CAT protein. Synthesis of a second class of polysomal RNAs initiated upstream of the rDNA region is also directed by pPolI-CAT, but these RNAs appear less likely to encode enzymatically active CAT protein since there are at least three ATGs (at rDNA positions −125, −100, and −45) prior to the ATG at the start of the CAT-encoding region.

The conclusion that RNA transcripts that initiate at the normal rRNA initiation site do not yield translatable mRNA is further strengthened by results with p-2kbPolI-CAT, a construct that contains the entire rDNA promoter sequence

from −2 kb to +57 but does not direct synthesis of translatable CAT RNA (Fig. 2, lane 3). The production of aberrantly initiated RNAs in cells transfected with plasmids bearing small rDNA promoter regions is also consistent with data of Smale and Tjian (23), who transfected COS cells with hybrid genes containing human rDNA promoter regions that had undergone 5′ deletions. These authors found that genes with large upstream rDNA segments (≥150 bp) initiate principally at +1 of the rDNA sequence, while genes carrying upstream domains with deletions direct production of increasing amounts of aberrantly initiated RNA (in the human case beginning at rDNA position −15 and −20), which are proposed to derive from transcription by RNA polymerase II.

The analogy between the present mouse data and the human data (23) suggests that the RNA initiated at +32 of the mouse rDNA might also be transcribed by RNA polymerase II rather than polymerase I. Several additional lines of evidence support this hypothesis. First, this RNA does not appear to be made in an S-100 extract of mouse cells that initiates transcription by polymerase I and III but not by polymerase II (B.S.-W. and K. Miller, unpublished observations). Second, the level of this species is increased when an MSV enhancer is located in *cis* (Fig. 4*A*, lanes 1 and 2), while RNA initiated correctly at position +1 is not (Fig. 3, lanes 3 and 7). Third, the +32 start occurs at a site 3′ to a TATAA-like sequence. Finally, as we have previously shown (20), in pPolI-CAT-transfected cells treated with actinomycin D for 1 hr the +32 species remains while the +1 species is lost. This difference in stability is most likely due to the presence of a 5′ cap structure on the +32 species, since in previous studies the lack of a 5′ cap structure has been shown to cause dramatically lower RNA stability (1).

Transfection into CHO cells shows that neither pPolI-CAT, pSV0′-CAT, nor their enhancer-containing derivatives direct synthesis of functional CAT mRNAs that produce measurable CAT enzymatic activity (Fig. 5). Moreover, in these cells pPolI-CAT-MSV yields detectable transcripts initiating only at position +1. Thus, our data mandate the unexpected conclusion that gene expression in the CHO cells is considerably more stringent than in the L cells, since sequences that are fortuitously utilized in L cells to direct mRNA production are not utilized in CHO cells.

The mouse rDNA promoter is active in CHO cells as well as in mouse cells (Fig. 4*B*). Thus, the species-specific interactions that discriminate mouse from human rDNA templates (24–26) do not do so between mouse and CHO rDNA. This result [which is consistent with recent data of Dahr *et al.* (27)] reinforces the growing body of evidence suggesting that speciation is not causally coupled to changes in rRNA promoter specificity.

Since the RNA polymerase I-transcribed hybrid RNAs are not able to direct protein synthesis, what then is the fate of these transcripts? Are these RNAs recognized by the enzyme systems that polyadenylylate and splice pre-mRNAs and transport them to the cytoplasm? Unfortunately, strong conclusions concerning these questions cannot be drawn from the present data. As the result of the short-lived nature of the +1-initiated pPolI-CAT RNA, we have not yet demonstrated spliced transcripts initiating at +1. Such problems are exacerbated in mouse cells by the presence of other CAT transcripts, which likely derive from synthesis by polymerase II (see above). We have also failed to detect rRNA initiated at +1 that is polyadenylylated or localized in the cytoplasm (M.A.L., unpublished data), but this may simply reflect the instability of the polymerase I-directed transcripts rather than their unsuitability for the polymerase II processing/transport pathway.

Finally, our experiments address the mechanism of action of RNA polymerase II enhancer sequences. The presence of MSV enhancer sequence on the CAT plasmid that lacks any normal eukaryotic promoters (pSV0′-CAT) markedly raised the level of CAT RNAs that initiated fortuitously (presumably under direction of polymerase II). Similarly, the MSV enhancer also raised the level of RNAs that initiated aberrantly within the rRNA region of pPolI-CAT. In contrast, the level of RNA that was correctly initiated at residue +1 was not increased by an MSV enhancer located in *cis* (Fig. 3, lane 7). Thus, transcription by polymerase I is not stimulated by this polymerase II enhancer segment. Complementary studies (J. Windle and B.S.-W., unpublished observations) have shown that an RNA polymerase II promoter placed in *cis* with a frog polymerase I enhancer segment also fails to enhance the level of transcription. These data therefore suggest that polymerase II and I enhancers act in polymerase-specific manners.

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