MOLECULAR AND CELLULAR BIOLOGY, Feb. 1985, p. 352–362
0270-7306/85/020352-11$02.00/0

Vol. 5, No. 2

Transcription of Herpes Simplex Virus *tk* Sequences Under the Control of Wild-Type and Mutant Human RNA Polymerase I Promoters

STEPHEN T. SMALE AND ROBERT TJIAN*

Department of Biochemistry, University of California, Berkeley, California 94720

Received 6 August 1984/Accepted 2 November 1984

We studied RNA polymerase I transcription in cells transfected with a plasmid, prHuTK, containing the herpes simplex virus *tk* gene fused to a human rRNA promoter. Primer extension analysis of *tk* RNA isolated from COS cells transfected with prHuTK reveals that transcription from the RNA polymerase I promoter is highly efficient and initiates at the same position used for the synthesis of endogenous rRNA in HeLa cells. The RNA products derived from prHuTK are distinguishable from normal RNA polymerase II transcripts of *tk* in that they are not polyadenylated, are extremely unstable, and are found predominantly in the nucleus. Moreover, the transcription observed is resistant to 300 μg of α-amanitin per ml. These results strongly suggest that prHuTK transcription is under the control of the human rRNA promoter and RNA polymerase I. To further characterize the activity of the human rDNA promoter in vivo, a series of 5′ and 3′ deletion mutants was tested in this transfection assay. The deletion analysis indicates that a core region of ca. 40 base pairs overlapping the initiation site is critical for transcription. In addition, a region between nucleotides −234 and −131 upstream from the core sequence serves to modulate the efficiency of transcription. Insertion into prHuTK of additional ribosomal nontranscribed spacer DNA or the simian virus 40 enhancer element has no apparent effect on the promoter activity. Surprisingly, RNA polymerase II transcripts synthesized at low levels from two start sites within the core control element of the wild-type RNA polymerase I promoter are activated upon deletion of upstream RNA polymerase I promoter sequences. However, these RNA polymerase II transcripts are not expressed from the endogenous rRNA promoter.

The current information about the promoter specificity of RNA polymerase I transcription in mammalian cells has been derived almost exclusively from in vitro experiments with cell-free transcription systems that faithfully initiate rRNA synthesis (9, 18, 27, 29, 30). The rRNA promoter sequences from both mice and humans have been defined extensively by analyzing the behavior of various mutant templates in the cell-free system (10, 19, 48, 54). More recently, these in vitro studies have allowed progress to be made in the purification and characterization of a specific transcription factor required to confer promoter recognition to RNA polymerase I (28, 31; R. M. Learned and R. Tjian, unpublished data). Although these in vitro experiments have been an invaluable approach to studying rRNA synthesis, in vivo studies are also needed for a complete understanding of the molecular events that regulate the initiation of transcription.

In the case of RNA polymerase I, the unique structural and functional characteristics associated with rRNA and ribosomal DNA (rDNA) within the cell may have a profound influence on the mechanism of transcriptional control. One characteristic of rRNA transcription is its localization within the nucleolus (24, 46). Although it is not known whether transcription by RNA polymerases II and III is also sequestered within specialized sites in the nucleus, the synthesis of neither tRNA nor mRNA is associated with a structure as obvious and distinct as the nucleolus. This densely packed structure contains all of the transcriptionally active rDNA repeats, as well as the proteins necessary to transcribe the rRNA and assemble the RNA products into ribosome precursors (40). The structure and function of the nucleolus are therefore important aspects of RNA polymerase I transcription that will need to be understood before a complete picture of rRNA synthesis and regulation is obtained.

Another characteristic associated with rRNA synthesis that may be important to transcriptional regulation is the structure of the rDNA (20). In mammalian cells, the rRNA genes are arranged as tandem repeats on several different chromosomes. Each repeat contains 35 to 40 kilobases (kb) of nontranscribed spacer (NTS) sequences, followed by the sequences encoding the 45S precursor transcript. Although a much shorter NTS in amphibians has been found to affect the efficiency of transcription (5, 33), no role for the mammalian NTS has yet been demonstrated.

The fate of the 45S ribosomal transcripts is also different from that of RNA polymerase II transcripts (36, 40, 47). Unlike mRNA, the RNA derived from ribosomal transcription units is neither capped nor polyadenylated [poly $(A)^+$]. Instead, these transcripts are rapidly processed through multiple cleavage steps into the mature 18S, 5.8S, and 28S rRNA subunits. The mature subunits are then packaged into precursor ribosomes which serve to protect them from degradation.

This paper describes the use of a transient assay procedure which was adapted for the study of RNA polymerase I transcription in mammalian cells. For this method, a fragment of DNA containing a human RNA polymerase I promoter was fused to the coding sequence from the herpes simplex virus (HSV) *tk* gene. This hybrid transcription unit was inserted into a vector containing a simian virus 40 (SV40) origin of replication, allowing plasmid replication in the COS-7 cell line. The HSV *tk* gene was used because the

* Corresponding author.

lack of homology to endogenous genes simplified the detection of specific transcripts. In addition, we wanted to explore the properties of an RNA polymerase II gene when it is transcribed under the direction of RNA polymerase I. After transfection of the hybrid template DNA into the COS-7 monkey cell line, specific RNA transcripts derived from the fused genes were isolated and analyzed for three different aspects of polymerase I transcription. First, the ability of this plasmid to direct specific transcription catalyzed by RNA polymerase I was determined. Next, the fate of the transcripts derived from the ribosomal promoter region was analyzed and compared with that of RNA molecules transcribed by RNA polymerase II. And finally, by mutating the wild-type promoter, the sequences required for transcription by RNA polymerase I were mapped in vivo and compared with in vitro results.

MATERIALS AND METHODS

Plasmid constructions. Restriction enzymes, nuclease Bal 31, and *Escherichia coli* DNA polymerase I Klenow fragment were purchased from Bethesda Research Laboratories or New England Biolabs. T4 DNA ligase was a gift from D. Rio. The plasmids pSVT2 and pSVTK3 were constructed by D. Rio, and the construction of the former was described previously (44; see Fig. 1). The plasmid pSVTK3 was constructed by inserting the purified 2.8-kb *Bgl*II-*Bam*HI fragment from pTK-2 (13) into the *Bgl*II-*Bam*HI sites in the pSVT2 polylinker. The plasmid prHuTK, constructed by A. Bagg, contains the *Cla*I-*Bst*EII rDNA promoter fragment from prHu4b (18) and the 950-base pair (bp) *Eco*RI-*Sal*I putative rDNA termination fragment from pDES (6) inserted into pSVTK3. The *Bst*EII site on the promoter fragment was extended with Klenow fragment and deoxynucleotide triphosphates to fill in the single-stranded tail for blunt-end ligation. The fragment was then inserted into the pSVTK3 *Cla*I-*Xba*I (filled-in) sites in the polylinker. The *Eco*RI-*Sal*I termination fragment was treated with nuclease Bal 31 to produce blunt ends and inserted into the HSV *tk* *Pvu*II site. The *Bst*EII site in the rDNA promoter fragment was recreated in this construction. A second *Bst*EII site in the HSV *tk* sequences was removed from prHuTK by partial digestion, treatment with Klenow fragment, and recircularization with T4 ligase. The plasmid pMLRIIGHSVTK (pMLTK) was a gift from M. Lusky and contains the entire 3.6-kb HSV *tk* *Bam*HI fragment inserted into the pMLRIIG vector (21, 22).

A series of deletion mutants constructed by successively removing increasing amounts of the human rDNA promoter region from the 5′ end (19) was inserted into the prHuTK vector by digesting the mutants with *Acc*I (filled in with Klenow) and *Bst*EII and ligated into *Cla*I (filled in)-*Bst*EII-digested prHuTK. Deletion mutants with sequences removed from the 3′ end of the rDNA promoter (19) were cleaved with *Acc*I-*Eco*RI and inserted into *Sal*I-*Bst*EII-digested prHuTK, after all four ends were filled in with Klenow fragment. The plasmid prHuTKNTS contains the 5.1-kb *Bam*HI-*Bst*EII rDNA fragment (18), consisting of 5 kb of NTS DNA in addition to the promoter, inserted into the *Bam*HI (partial digestion)-*Bst*EII-digested prHuTK. The plasmid prHuTK Enh. contains the SV40 enhancer (nucleotides 114 to 273) bounded by *Bam*HI linkers (52; from H. Weiher) and inserted upstream from the ribosomal promoter at a prHuTK *Bam*HI site. The pseudo-wild-type plasmid was constructed by inserting a 35-bp pBR322 fragment into the prHuTK *Bgl*II site.

Cells and DNA transfections. COS-7 monkey cells (8) were grown at 37°C on plastic dishes in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum and the antibiotics penicillin and streptomycin. DNA transfection (23) for RNA analysis was typically done on 100-mm dishes with cells at ca. 50 to 70% confluency. The cells were washed with phosphate-buffered saline and Tris-buffered saline and incubated with 1 μg of plasmid DNA (1.6 μg for prHuTKNTS) and 500 μg of DEAE-Dextran (Pharmacia Fine Chemicals) per ml in 500 μl of Tris-buffered saline at 33°C for 30 min. After being washed with Tris-buffered saline and phosphate -buffered saline, the cells were incubated at 37°C for 8 h with 7 ml Dulbecco modified Eagle medium with 10% fetal calf serum and 100 μM chloroquine. The cells were finally washed with phosphate-buffered saline and layered with 10 ml of Dulbecco modified Eagle medium plus 10% fetal calf serum. HSV *tk* protein from pMLTK was detected by immunofluorescence in >20% of the cells transfected by this procedure. Transfections for replication experiments were done by the above procedure, except scaled down for 60-mm dishes and with only 150 ng of plasmid DNA.

Preparation of RNA. COS cells were harvested 40 to 50 h after transfection, and the cytoplasmic and nuclear RNAs were isolated by a procedure similar to that of Berk and Sharp (2). The cells were lysed on ice by isotonic Nonidet P-40 buffer at 0.5 ml per plate (0.65% Nonidet P-40 [Sigma Chemical Co.], 150 mM NaCl, 10 mM Tris [pH 7.8]), and the nuclei were pelleted by centrifugation at 20,000 × g for 10 min. The supernatant (cytoplasmic fraction) was transferred to another tube, and the nuclei were resuspended in the same volume of Nonidet P-40 buffer. An equal volume of a denaturing urea-sodium dodecyl sulfate buffer (7 M urea, 350 mM NaCl, 10 mM Tris [pH 7.6], 10 mM EDTA, 1% sodium dodecyl sulfate [Bio-Rad Laboratories]) (15) was added to both cytoplasmic and nuclear fractions. The high-molecular-weight DNA from the lysed nuclei was sheared with a 20-gauge syringe, and both fractions were extracted with phenol, chloroform-isoamyl alcohol, and ether. After an ethanol precipitation, the RNA was further purified on a 5.7 M CsCl step gradient (53). Poly(A)$^+$ and poly(A)$^-$ RNAs were separated on oligodeoxythymidylate [oligo(dT)]-cellulose (type II; Collaborative Research, Inc.) (1). Purified RNA samples were stored in ethanol at −20°C.

Plasmid replication experiments. Recombinant plasmid DNA was isolated from transfected COS-7 cells by the method of Hirt (14). The purified DNA was digested with *Mbo*I restriction endonuclease to cleave plasmids produced by replication in the monkey cells (37). Southern analysis (50) was then performed as described previously (35), with nick-translated pBR322 as the probe.

RNA analysis. The 24 nucleotide *tk* and rDNA synthetic primers were gifts of J. Merryweather and Chiron Corp. Relative to the RNA polymerase I transcription initiation site in prHuTK, the rDNA and *tk* primers contain sequences from +73 to +50 and from +116 to +93 nucleotides, respectively. The single-stranded primers were 5′ end labeled with T4 polynucleotide kinase (New England Biolabs). Primer extension analysis of purified cytoplasmic or nuclear RNA was performed as previously described (26) with minor modifications. The hybridization reaction was done for 60 min at 60°C with the *tk* primer and at 68°C with the rDNA primer. After the extension reaction, the samples were extracted with phenol, precipitated with ethanol, and subjected to electrophoresis on 8% denaturing polyacrylamide gels. Autoradiography and densitrometric scanning were performed as described previously (44).

RNA blot analysis was performed on oligo(dT)-selected

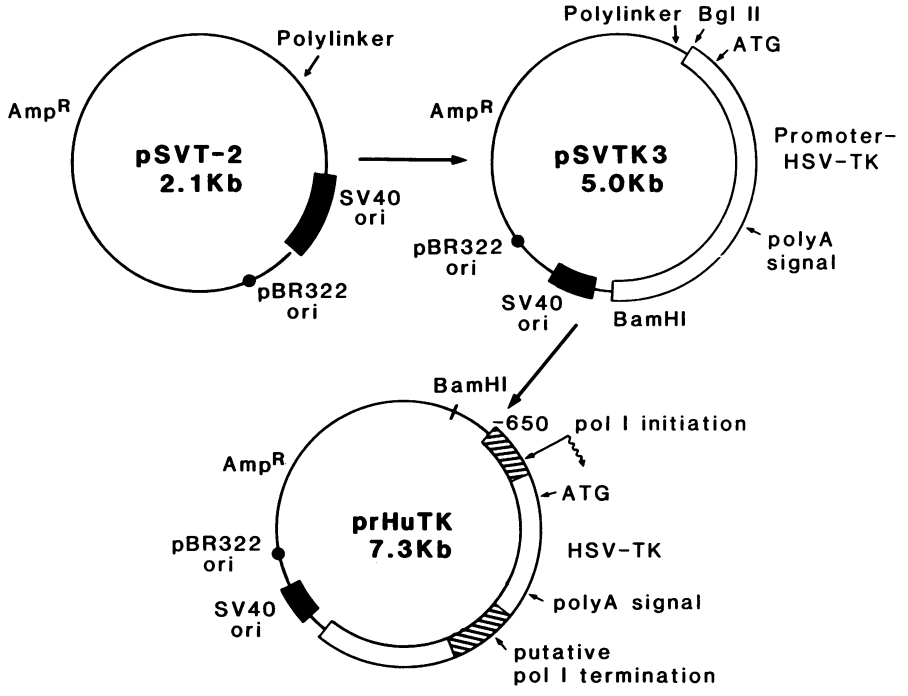


FIG. 1. Construction of the polymerase I-*tk* fusion plasmid. The original vector, pSVT2, contains the pBR322 Amp^r gene (solid line), ColE1 origin of replication (solid circle), and the SV40 origin (solid bar). pSVTK3 was formed by inserting the HSV *tk* gene lacking its own promoter (open bar). The final plasmid, prHuTK, was constructed by insertion of the human rDNA fragments containing the promoter and putative terminator sequences (hatched bars). The direction of transcription from the RNA polymerase I initiation site is depicted, as are the *tk* translation initiation codon and polyadenylation sequences. Details of the construction are given in the text.

cytoplasmic RNA as described (44). The nitrocellulose filters (Schleicher & Schuell, Inc.) were hybridized to nick-translated pSVTK3.

RESULTS

Construction of prHuTK. The shuttle vector (pSVT-2) (44) used to construct prHuTK contains all of the components necessary both for replication in bacteria and for use in a COS-7 transient assay (Fig. 1). These components include a bacterial origin of replication, an ampicillin resistance marker, an SV40 origin of replication, and deletion of pBR322 sequences that inhibit plasmid replication in mammalian cells (22, 35). This vector also contains a polylinker into which the HSV *tk* coding sequence was inserted, generating the plasmid pSVTK3. The inserted HSV *tk* *Bgl*II-*Bam*HI fragment contains no RNA polymerase II promoter, but includes all of the *tk* protein-coding sequences and the polyadenylation signal.

To place the transcription of *tk* under the control of RNA polymerase I, a cloned fragment of human rDNA was abutted to the 5′ end of the HSV *tk*-coding sequences. This promoter fragment extends from nucleotide −650 to nucleotide +78 relative to the transcription initiation site and contains all of the sequences necessary for efficient rRNA synthesis in vitro (18). Although rRNA transcription appears to be species specific (11), in vitro experiments suggest that a human rDNA promoter should be active in monkey cells (18). An attempt was also made to allow termination of RNA polymerase I transcription within this plasmid. Based on R-loop mapping (6) and S1 nuclease analysis (data not shown), the 3′ end of the 28S sequence was found to lie within a 950-bp *Eco*RI-*Sal*I restriction fragment. The termination point for 45S rRNA transcription has been proposed to lie at the 3′ end of the 28S sequence (6). Therefore, this *Eco*RI-*Sal*I putative termination fragment was inserted into the HSV *tk* *Pvu*II site, transcriptionally downstream from the polyadenylation signal. Thus, the completed plasmid, prHuTK, contains sequences that allow replication in COS-7 cells and transcription of *tk* RNA by RNA polymerase I. Moreover, depending on the behavior of cellular proteins involved in RNA termination and polyadenylation, the construction makes possible a discrete 3′ end for the *tk* transcripts. The transcripts could be either cleaved and poly(A)$^+$ at the appropriate site or terminated within the rDNA termination fragment.

Replication in COS-7 cells. Because the plasmid copy number may be related to the amount of RNA produced, we determined the relative amounts of template DNA after its replication in COS-7 monkey cells (8). This cell line contains integrated copies of SV40 DNA and expresses large T antigen, thus allowing efficient replication of plasmid DNAs that contain an SV40 origin. Low-molecular-weight DNA was isolated by Hirt extraction at 0 or 48 h after transfection into COS-7 cells. The DNA was then digested with *Mbo*I restriction endonuclease. This enzyme is unable to cleave plasmid DNA containing methylated adenine residues. Because the input plasmid DNA was prepared from *dam*$^+$ bacteria that methylate adenines, this DNA will not be cleaved by *Mbo*I. By contrast, plasmid DNA that has undergone replication in the mammalian cells will have unmethylated adenine residues and will be cleaved by *Mbo*I. Thus, the cleavage method (37) allows DNA replicated in the tissue culture cells to be distinguished from input DNA. After the *Mbo*I digestion, Southern blot analysis DNA was carried out as described previously (35).

Although the Southern analysis in Fig. 2 reveals several *Mbo*I cleavage products, the 800-bp band is common to every plasmid vector that we have used and therefore is a

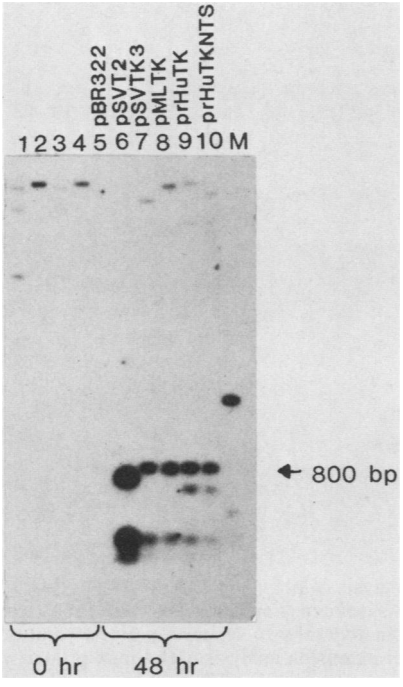


FIG. 2. Replication of plasmids in COS-7 cells. COS-7 cells were transfected with the various plasmids, and low-molecular-weight DNA was isolated after 0 or 48 h. The DNA was digested with *Mbo*I restriction endonuclease and analyzed by Southern blot hybridization (see the text). The 0-h time points include uncut and *Mbo*I-digested pBR322 (lanes 1 and 3) and prHuTK (lanes 2 and 4). Forty-eight-hour time points are shown for pBR322 (lane 5), pSVT-2 (lane 6), pSVTK3 (lane 7), pMLTK (lane 8), prHuTK (lane 9), and prHuTKNTS (lane 10). The marker DNA was *Hin*fI-digested pBR322. The 800-bp fragment used for comparison of levels of replication is indicated. The smaller fragments cannot be used for comparison because they are from different regions of each plasmid that do not hybridize equally to the pBR322 probe.

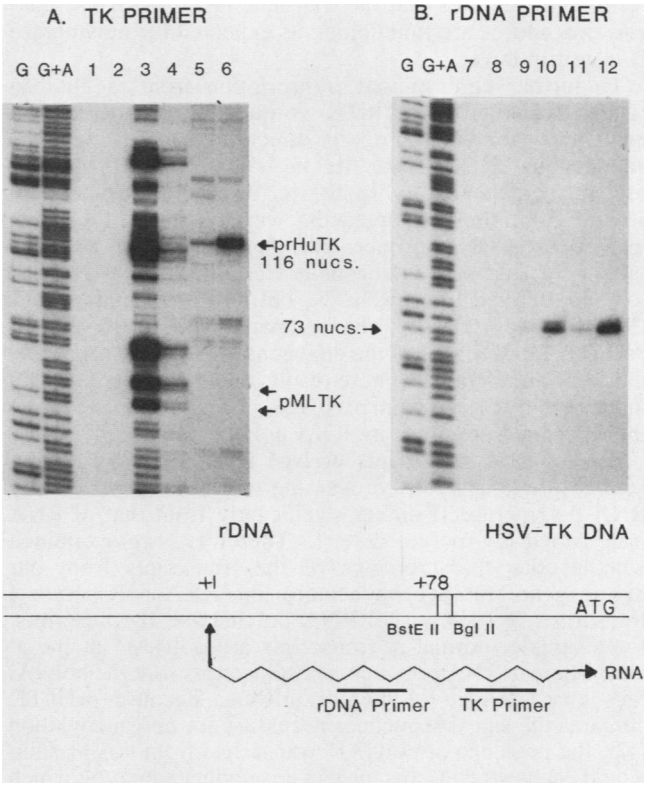


FIG. 3. Primer extension analysis of RNA isolated from transfected COS-7 cells. Primer extension analysis was performed on total cytoplasmic or nuclear RNA isolated from ca. 3×10^6 COS-7 cells (ca. 6×10^5 transfected cells) 48 h after transfection. Cytoplasmic RNA was used for odd-numbered lanes, and nuclear RNA was used for even-numbered lanes. Markers are from Maxam-Gilbert sequencing (25) of the *Bst*EII (5′ end labeled)-*Sal*I fragment of prHuTK. (A) The 24-nucleotide *tk* primer depicted was hybridized with RNA from COS cells transfected with pSVTK3 (lanes 1 and 2), pMLTK (lanes 3 and 4), or prHuTK (lanes 5 and 6). (B) The 24-nucleotide rDNA primer was hybridized with RNA from COS cells transfected with either pSVTK3 (lanes 7 and 8) or prHuTK (lanes 9 and 10). HeLa RNA (lanes 11 and 12) was isolated from 3×10^5 cells.

good measure of replication taking place in the COS-7 cells. As expected, no replication was observed in any of the 0-h time points. Also, no replicated DNA was observed at the 48-h time point after transfection of pBR322, which lacks SV40 *ori* sequences. Efficient replication was found, however, 48 h after transfection of five other plasmids, all of which contain an SV40 origin. Plasmid DNAs that contain the HSV *tk* sequences (*Bgl*II-*Bam*HI fragment) replicated 10-fold less efficiently than did parental vectors such as pSVT-2. However, the presence of different promoter sequences fused to the *tk* gene did not significantly alter the level of replication. Thus, plasmids containing no promoter (pSVTK3), the HSV *tk* polymerase II promoter (pMLTK), the rRNA promoter (prHuTK), or the ribosomal promoter with 5 kb of NTS (prHuTKNTS) were replicated with similar efficiencies.

Transcription initiation from prHuTK. The question that we must first address is whether specific initiation of *tk* RNA synthesis by RNA polymerase I can be observed in the transient assay. To assay transcription initiation, we used a primer extension assay to accurately map the 5′ end of transcripts from the plasmids transfected into COS-7 cells. The ability of the rRNA promoter in prHuTK to direct *tk* RNA synthesis was tested and compared to the *tk* transcription from two control plasmids, one containing no promoter and the other containing an RNA polymerase II promoter.

Forty-eight hours after transfection of plasmid DNA into COS-7 cells, both cytoplasmic and nuclear RNAs were purified. Primer extension analysis (see above) was then performed by using a 5′-end-labeled DNA primer of 24 nucleotides that is complementary to the HSV *tk* sequences just upstream from the translation initiation site (*tk* primer). The results from this analysis are shown in Fig. 3. The analysis of RNA after transfection with prHuTK, which contains the human RNA polymerase I promoter, reveals that transcription starts primarily at the nucleotide expected for RNA polymerase I initiation. Analysis of RNA from cells transfected with pSVTK3, which lacks a promoter sequence, reveals a lack of detectable RNA complementary to the primer. Therefore, a promoter is required to drive specifc *tk* transcription, and the *tk* primer does not hybridize to any endogenous COS RNA. When the transcription of HSV *tk* sequences was directed by its own polymerase II promoter (pMLTK), the initiation of RNA synthesis was found at the specific polymerase II start sites, as well as at upstream start sites. These strong, upstream start sites within the *tk* promoter are commonly found in mammalian cells during transient expression experiments before HSV infection (38; S. McKnight, personal communication). This

result demonstrates that the transient assay and RNA analysis procedures are functioning as expected for polymerase II transcription.

To further confirm that transcription from the human rDNA promoter in prHuTK is initiating at the correct nucleotide, the start site was directly compared with the endogenous rRNA start site in human cells. A second primer, complementary to transcribed rRNA located upstream from the *tk* sequences, was synthesized for this experiment (rDNA primer; see diagram in Fig. 3). This primer hybridizes to endogenous HeLa (human) rRNA and to RNA derived from prHuTK, but not to the endogenous COS (monkey) rRNA. The comparison of the 5′ end of prHuTK RNA to that of the endogenous HeLa rRNA shows that they are identical. These results taken together strongly indicate that *tk* RNA from prHuTK is being transcribed from the ribosomal promoter by RNA polymerase I.

Fate of RNA transcripts derived from prHuTK. As described previously, the processing of RNA transcribed by RNA polymerase II differs significantly from that of RNA transcribed by polymerase I. Therefore, we examined whether the characteristics of the transcripts from our hybrid gene would behave more like RNA polymerase I transcripts or like typical RNA polymerase II transcripts. For example, normal *tk* transcripts are poly(A)$^+$ at the 3′ end, whereas RNA polymerase I transcripts lack the poly(A) tails characteristic of mature mRNA. Because prHuTK contains the signal sequences necessary for polyadenylation (42), the presence of poly(A)$^+$ transcripts from this plasmid would suggest that this process is independent of which RNA polymerase is acting on the gene. Alternatively, the absence of poly(A) tails would suggest either that polyadenylation is linked to the process of transcription by RNA polymerase II or that the polymerase I transcripts are unavailable to the polyadenylation enzymes because of a different location within the nucleus. In addition to polyadenylation, we have characterized the intracellular localization, termination, and stability of the RNA products because these properties are also different for normal RNA polymerase I and RNA polymerase II transcripts.

The localization of the transcripts was determined after fractionation of the RNA into nuclear and cytoplasmic pools (Fig. 3). The major *tk* transcripts from prHuTK were found predominantly in the nucleus, whereas the RNA polymerase II transcripts from pMLTK were found in the cytoplasm. The presence or absence of poly(A) tails was determined by selection of the isolated RNA on oligo(dT)-cellulose, followed by primer extension analysis. The results (Fig. 4) reveal that practically all of the RNA with the correct RNA polymerase I initiation site from prHuTK is not poly(A)$^+$. The RNA polymerase II transcripts from pMLTK, on the other hand, bound with ca. 50% efficiency to the oligo(dT). Northern analysis (see Fig. 8) and 3′ S1 analysis were also employed to study the 3′ end of the RNA transcripts and their possible polyadenylation (data not shown). Using these assays, we were unable to detect a common 3′ end for the prHuTK transcripts.

To assay the stability of the transcripts, we took advantage of the characteristics of the transcription inhibitor actinomycin D (43). When added to COS-7 cells at a concentration of 10 μg/ml, this compound can enter the cells and completely inhibit transcription by all three RNA polymerases (data not shown). Actinomycin D was added to the cells 48 h after transfection, and the RNA was isolated after incubations for 0, 1, or 5 h. The decrease in the level of RNA is indicative of the rate of degradation of the RNA transcripts. The results from Fig. 5A show that whereas the total pMLTK RNA level remained constant over 5 h as expected (12), the prHuTK RNA level greatly decreases even 1 h after the addition of actinomycin D. Thus, the major transcripts from prHuTK are poly(A)$^-$, are extremely unstable, and are found in the nucleus, whereas RNA polymerase II transcripts from pMLTK are poly(A)$^+$, stable, and found in the cytoplasm.

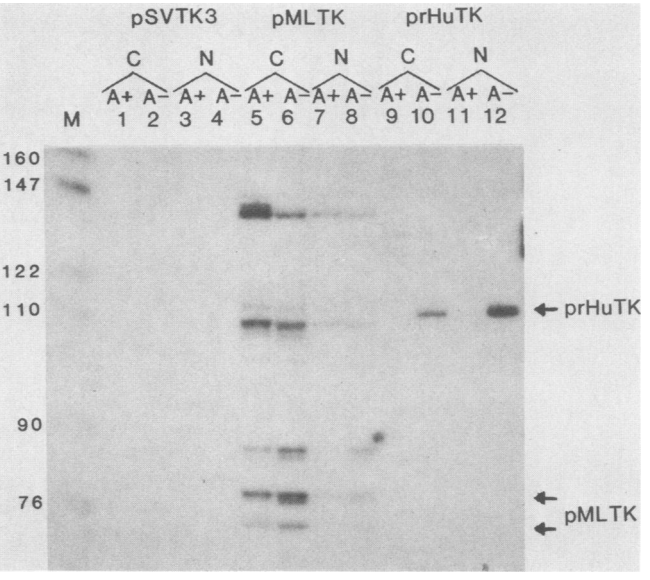
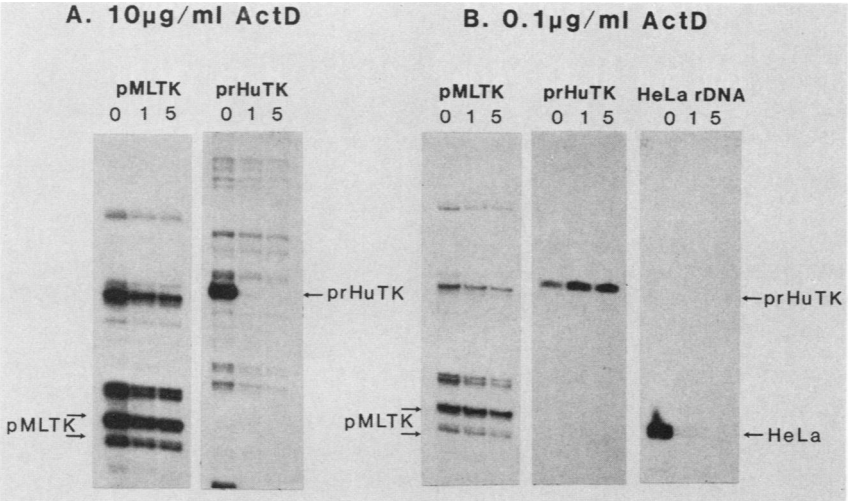


FIG. 4. Analysis of poly(A)$^+$ and poly(A)$^-$ RNA from transfected cells. RNAs isolated from COS cells were subjected to oligo(dT)-cellulose chromatography to separate poly(A)$^+$ and poly(A)$^-$ transcripts. Primer extension analysis with the *tk* primer was then used to locate the *tk* transcripts. The RNA which bound to oligo(dT) (A$^+$) and that which did not bind (A$^-$) were assayed from both cytoplasmic (C) and nuclear (N) fractions. Markers are *Hpa*II-digested pBR322.

Effect of α-amanitin and 0.1 μg of actinomycin D per ml on prHuTK transcription. A common method for distinguishing between transcription by RNA polymerases I, II, or III is through their differential sensitivities to the drug α-amanitin (3). Transcription by RNA polymerase II and III has been found to be inhibited by α-amanitin at 2 and 200 μg/ml, respectively, whereas transcription by RNA polymerase I is resistant at these concentrations. As with other cell lines (16), however, the drug cannot enter COS-7 cells at sufficiently high concentrations to inhibit transcription by both RNA polymerases II and III (data not shown). The sensitivity of prHuTK transcription to α-amanitin was therefore determined by measuring transcription in permeabilized cells (51). Forty-eight hours after transfection of COS-7 cells with either pMLTK or prHuTK DNA, the cells were treated with digitonin to facilitate the entry of α-amanitin (300 μg/ml) and [α-^{32}P]UTP into the nucleus. After the cells were incubated for 15 min to allow elongation of preinitiated transcripts, the ^{32}P-labeled RNA was isolated and hybridized to HSV *tk* DNA immobilized on nitrocellulose dots. The results shown in Fig. 5C indicate that whereas all of the pMLTK transcription is sensitive to 300 μg of α-amanitin per ml, ca. 35% of the transcription from prHuTK is resistant. Since true RNA polymerase I transcription is almost completely resistant to α-amanitin, we believe that the transcription inhibited by α-amanitin in prHuTK-transfected cells derives from the accumulation of minor polymer-



C. α-amanitin

	pML	prHuTK
No inhibitor	2447	1484
300μg/mL α-amanitin	15	512
α-amanitin +10μg/mL Actino.D	60	136

FIG. 5. Analysis of prHuTK RNA in the presence of transcription inhibitors. (A and B) HeLa cells or pMLTK- or prHuTK-transfected COS cells were incubated at 37°C 48 h after transfection with medium containing the indicated concentration of actinomycin D (Act D). After 0, 1, or 5 h, the total RNA was isolated and analyzed by primer extension analysis with the *tk* or rDNA (HeLa) primer. (C) The digitonin treatment and RNA labeling of COS cells transfected with pMLTK or prHuTK was performed as previously described (51). The background hybridization of 255 cpm was substracted from all the numbers listed.

ase II start sites found within prHuTK (see Fig. 3 and 8). However, the remaining α-amanitin resistance strongly suggests that RNA polymerase I transcription is taking place in prHuTK.

In addition to its resistance to α-amanitin, rRNA transcription can also be identified by its unique sensitivity to low concentrations of actinomycin D (39, 41, 43, 45). Although it is well established that 0.1 μg of Actinomycin D per ml inhibits transcription of rRNA but not transcription by RNA polymerases II and III, the reason for the inhibition is unknown. A reasonable explanation for this enhanced sensitivity is that the high guanine-cytosine content of rDNA results in preferential binding of actinomycin D, thereby inhibiting transcription (43). The results in Fig. 5B reveal that although 0.1 μg of actinomycin D per ml abolishes HeLa rDNA transcription and has no effect of pMLTK transcription in COS-7 cells, the transcription from prHuTK is stimulated ca. threefold. One possible explanation for this stimulation is that by abolishing transcription from the endogenous guanine-cytosine-rich rDNA, more RNA polymerase I transcriptional machinery is available for the transcription of prHuTK.

Deletion analysis. The results presented in this study suggest that the human ribosomal promoter fragment between nucleotides −650 and +78 is sufficient to direct transcription by monkey RNA polymerase I in a transient assay. To more accurately define the rRNA promoter sequences in vivo, a series of 5′ and 3′ deletion mutants was tested in this transcription system. Ten 5′ mutants and six 3′ mutants were inserted into prHuTK in place of the wild-type promoter as described above. All of the plasmids were found to replicate at the same level in COS-7 cells as did the wild-type prHuTK (data not shown). The results for the RNA analysis of the 3′ mutants are shown in Fig. 6. Each transfection included both the mutant to be tested and the Δ3′+22 mutant, which was used as a standard for the amount of total RNA used in each assay. The extended products vary in size due to the deletion of sequences within the transcribed region. The primer extension analysis with the *tk* primer revealed that deletions Δ3′+71, +22, +18, and +16 are transcribed at wild-type levels. The efficiency of transcription, however, was reduced slightly upon deletion to nucleotide +7. The final 3′ deletion, Δ3′−8, was incapable of directing RNA synthesis at a detectable level. Also, by using an additional primer which hybridizes closer to the initiation site, it was found that the observed transcription begins at the correct nucleotide (data not shown). To assay the 5′ deletion mutants, a pseudo-wild-type plasmid was used as an internal standard. This control plasmid contains a 35-bp pBR322 fragment inserted at the HSV *tk* *Bgl*II site, resulting in a transcript that will be 35 nucleotides longer than the wild-type RNA. The primer extension analysis (Fig. 6) indicates that several regions of the promoter strongly influence the RNA polymerase I transcription. A small effect was observed when the sequences between nucleotides −650 and −234 were deleted from the promoter. Further deletion of sequences to nucleotide −167 diminished the level of transcription ca. fivefold. When sequences between nucleotides −167 and −131 were removed, transcription decreased even further (ca. five fold). This reduced level of transcription was approximately maintained in deletions Δ5′−109, −83, −55, and −52, whereas transcription from mutant Δ5′−26 was barely detectable. No specific transcription was observed from deletions Δ5′−17 or +10. As with the 3′ deletions, the transcription initiation site appears to remain the same through all of these deletions.

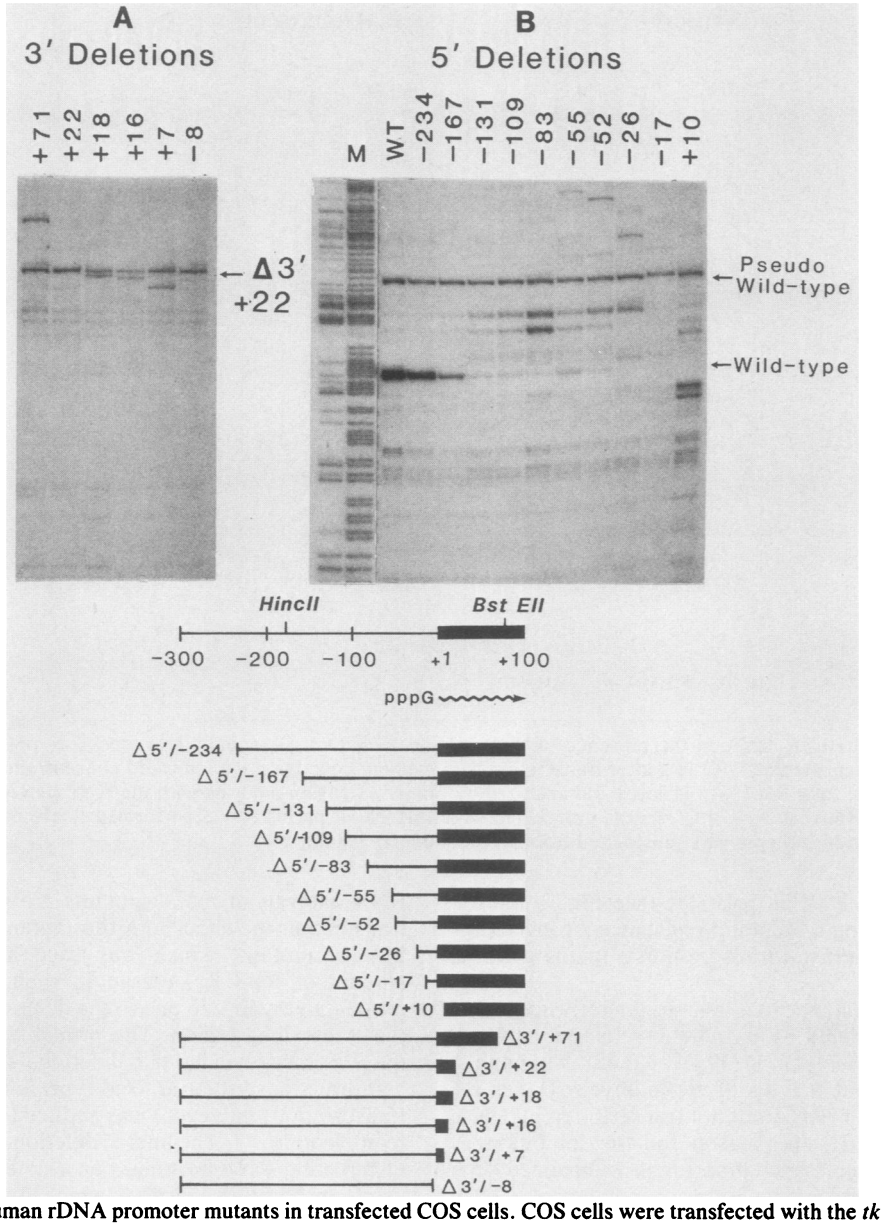


FIG. 6. Analysis of human rDNA promoter mutants in transfected COS cells. COS cells were transfected with the *tk* vector containing the 5′ and 3′ deleted ribosomal promoters depicted (see the text for construction). Isolated nuclear RNA was assayed by primer extension analysis with the *tk* primer. The longest extension products (for Δ3′+71 and for the pseudo wild type) exhibit somewhat reduced signals. Although we do not know the cause of this reduction, the efficiency of extension by reverse transcriptase may be influenced by the length of the extension products resulting from our CsCl-purified RNA. (A) The 3′ deletions were cotransfected with Δ3′+22 as an internal standard. The promoters were deleted from the 3′ side to the nucleotide indicated above the lane. The extended products are longer than usual due to the plasmid construct used. (B) The 5′ mutants were deleted from the 5′ side to the nucleotide indicated and were cotransfected with the pseudo-wild-type construction as an internal standard. Markers are the same as described in the legend to Fig. 3.

Effect of the ribosomal NTS and the SV40 enhancer. Although the ribosomal promoter in prHuTK directs efficient RNA polymerase I transcription, a stimulation of this transcription by additional NTS sequences or the SV40 enhancer element is possible. In the prHuTK construction we have used thus far, the RNA polymerase I promoter sequences extended to about nucleotide −650. To determine the effect of sequences further upstream in the NTS, we constructed a plasmid that includes an additional 4.5 kb of NTS sequence. The level of transcription from this new plasmid, prHuTKNTS, in transfected COS-7 cells was not stimulated and may be somewhat diminished (Fig. 7). Similarly, a plasmid containing the SV40 enhancer element directly upstream from the polymerase I promoter had no significant effect on polymerase I transcription in the transient assay.

Activated RNA polymerase II transcripts. An interesting observation from the 5′ deletion analysis is that transcription from two initiation sites is stimulated as the upstream sequences are deleted. These start sites are at nucleotides −15 and −20 and reach a maximum when the RNA polymerase I promoter is deleted from the 5′ end to nucleotide −83. Analysis of these two transcripts suggests that they are transcribed by RNA polymerase II rather than by RNA

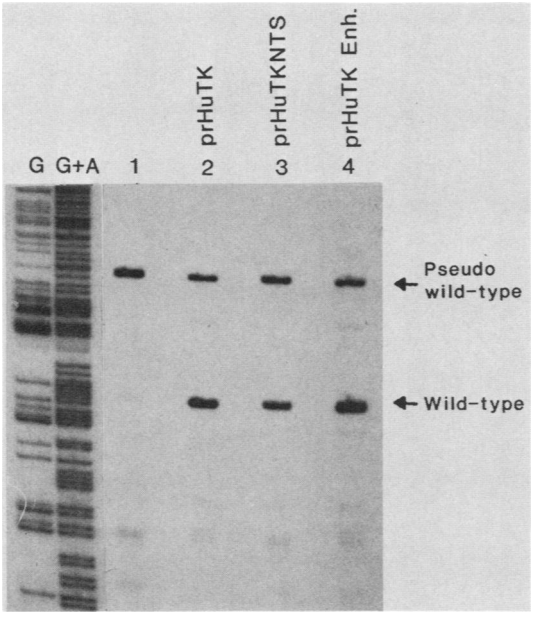


FIG. 7. Effect of ribosomal NTS and SV40 enhancer on prHuTK transcription. Primer extension analysis was performed on nuclear RNA from ca. 3×10^6 COS cells transfected with pseudo-wild-type plasmid (lane 1) or cotransfected with equimolar amounts pseudo-wild-type plasmid and prHuTK (lane 2), prHuTKNTS (lane 3), or prHuTK Enh. SV40 (lane 4). Markers are as described in the legend to Fig. 3.

polymerase I. They are found predominantly in the cytoplasm, they bind to oligo(dT) (Fig. 8), and they are stable after addition to 10 μg of actinomycin D per ml (data not shown). Furthermore, unlike RNA polymerase I transcripts from prHuTK, these two RNA polymerase II transcripts from Δ5′−83 produce a discrete band when analyzed by Northern blot hybridization (Fig. 8). Taken together, these data suggest that there are specific initiation sites for RNA polymerase II contained within the core region of the ribosomal promoter element and that transcription arising from these sites is activated by debilitating the polymerase I promoter.

DISCUSSION

To study RNA polymerase I transcription in vivo, we fused human rRNA transcriptional control sequences to HSV *tk*-coding sequences and used this vector in a COS-7 transfection system. Through the analysis of transcription initiation, the human rRNA promoter elements were mapped, and the characteristics of *tk* transcripts synthesized by RNA polymerase I were determined. Transcription of prHuTK is resistant to α-amanitin, and the *tk* transcripts are unstable, poly(A)$^-$, and localized in the nucleus. Although we primarily used COS-7 cells because they support the replication of transfected plasmids and produce higher RNA levels, similar results were also obtained in cell lines that do not permit plasmid replication, such as CV1, HeLa, and 293 cell lines (data not shown). The only cell line we tested in which initiation of transcription from prHuTK was not observed was the mouse 3T6 cell line. However, a possible explanation for this difference with 3T6 cells is that in vitro transcription experiments have found that rRNA transcription is species specific, with the human rDNA promoter active in monkey extracts but not in mouse extracts (11, 18). Indeed, specific transcripts were observed in mouse 3T6 cells and not in COS-7 cells from a plasmid containing a mouse ribosomal promoter fused to the HSV *tk* gene (unpublished data).

Our findings strongly suggest that RNA polymerase I is responsible for transcription of *tk* sequences from prHuTK. However, these data do not reveal the location or the efficiency of prHuTK transcription. Although endogenous rRNA transcription appears to take place exclusively within the nucleolus (24, 46), in situ hybridization experiments are needed to determine whether prHuTK transcription is also limited to the nucleolus. Alternatively, RNA polymerase I and all of the auxiliary components needed for specific rRNA transcription may be present and active outside of the nucleolus. In addition to the location of transcription, the efficiency of prHuTK transcription also cannot be determined from our results. Even though the prHuTK RNA level was shown to be comparable to the endogenous HeLa 45S rRNA level, many more vector DNA copies than endogenous rRNA genes are present in the cells 48 h after transfection. If most of the plasmids are transcriptionally active, then RNA synthesis from prHuTK may be inefficient. However, a possible requirement of nucleolar localization for plasmid transcription may allow efficient transcription of only a small number of plasmids.

An important application of the transient assay is that it allowed the mammalian rDNA promoter elements to be mapped in vivo. The results presented suggest that the human ribosomal promoter consists of at least two elements that control rRNA transcription in monkey cells. The core element is required for basal-level RNA polymerase I transcription. This region contains ca. 40 bp and possibly overlaps the transcription initiation site. In addition to being required for transcription, the core appears to be responsible for limiting transcription initiation to the correct nucleotide position. The second control element, located at least partially between nucleotides −234 and −131, serves to stimulate polymerase I transcription. This upstream element appears to be an isolated control element because removal of sequences between nucleotides −130 and −52 has little effect on the efficiency of transcription. Indeed, in vitro linker-scanning mutagenesis studies (M. Haltiner and R. Tjian, unpublished data) confirm the idea of an upstream control element that is ca. 100 bp removed from the core. Also, a deletion analysis performed in vitro (19) with the human ribosomal promoter defined both upstream and core elements similar to those found in this study. The only major difference between the in vivo and in vitro studies is that Δ5′−167 directs wild-type levels of transcription in vitro (R. M. Learned and S. T. Smale, unpublished data), whereas the transcription of this mutant is ca. fivefold reduced in the transfection assay. It is possible that control sequences exist near nucleotide −167 that cannot be detected in vitro or, alternatively, that the pBR322 sequences abutted to nucleotide −167 in this mutant inhibit the stimulatory activity of the nearby −130 control element only in the in vivo assay.

In sharp contrast to results obtained by microinjection of *Xenopus* rDNA into frog oocytes, there was no effect on the level of RNA polymerase I transcription after the insertion of an additional 4.5 kb of NTS DNA into prHuTK. In the oocytes, the efficiency of ribosomal transcription was found to be stimulated by the *Xenopus* ribosomal NTS (5, 33). This stimulation is possibly due to an effect of sequences homologous to the *Xenopus* rDNA promoter that are present in multiple copies within the NTS (34, 49). The mammalian NTS does not appear to contain reiterated promoter se-

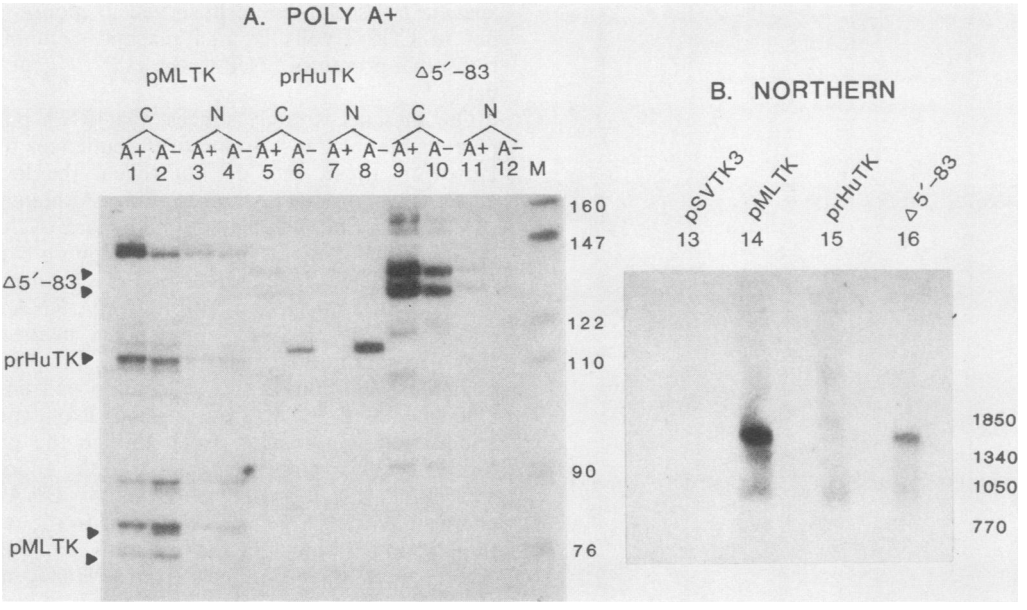


FIG. 8. Analysis of RNA polymerase II transcripts initiating at cryptic sites. (A) Poly(A)$^+$ and poly(A)$^-$ RNA isolated from COS cells transfected with pMLTK, prHuTK, or Δ5′−83 were analyzed by primer extension with the *tk* primer. Cytoplasmic (C) and nuclear (N) RNAs were analyzed for transcripts which bound (A$^+$) or did not bind (A$^-$) to oligo-(dT) cellulose. Size markers are from pBR322 digested with *Hpa*II. (B) Northern analysis (see the text) was used to analyze cytoplasmic poly(A)$^+$ transcripts from cells transfected with pSVTK3 (lane 13), pMLTK (lane 14), prHuTK (lane 15), and Δ5′ - 83 (lane 16).

quences. However, an effect on ribosomal transcription of the mammalian NTS cannot be ruled out. The presence of more than 4.5 kb of mammalian NTS or integration of the DNA into the chromosome may be necessary for a transcriptional effect.

The lack of poly(A)$^+$ *tk* transcripts from prHuTK is a noteworthy result because it may shed some light on the process of polyadenylation. A recently developed cell-free polyadenylation system (32) suggests that this process does not require active transcription in vitro, but that it is linked to transcription. Our results are consistent with the view that polyadenylation may be linked to some property of transcription unique to RNA polymerase II. An alternative explanation, however, is that transcripts synthesized in the nucleolus are not accessible to the polyadenylation enzymes. The polyadenylation results obtained with prHuTK are, however, not due to the presence of the 28S sequences 3′ to the *tk* gene. A precursor to prHuTK, which lacks the 28S sequences, behaves like prHuTK in the localization, polyadenylation, and stability experiments (data not shown). Furthermore, the RNA polymerase II transcripts originating from the Δ5′−83 mutant promoter are poly(A)$^+$, even though the mutant plasmid contains the 28S sequences.

Polyadenylation and 5′ capping, both characteristic of mRNA, are thought to stabilize RNA polymerase II transcripts (47, 55). Because the prHuTK transcripts are not poly(A)$^+$ and probably not capped, it is not surprising that they appear to be relatively unstable. Also, the nuclear localization of the transcripts may be influenced by this instability in that a rapid degradation rate could serve to prevent the accumulation of prHuTK RNA in the cytoplasm (55). The instability and nuclear localization of the prHuTK transcripts are similar to the characteristics found for endogenous precursor rRNA transcripts. Although mature rRNA transcripts are extremely stable, their stability is likely due to the immediate association with ribosomal proteins. In contrast, the first 4 kb of the 45S precursor transcripts do not associate with ribosomal proteins, and their stability and localization were shown to be similar to those of the prHuTK transcripts.

The discovery of two activated RNA polymerase II transcripts initiating at sequences within the rRNA promoter was an unexpected outcome of the analysis of the 5′ deletion mutants. Although several minor RNA polymerase II start sites were found in prHuTK, only the transcripts initiating at nucleotides −15 and −20 were stimulated by the deletion of RNA promoter sequences. We do not know whether there is any physiological significance to these transcripts. They may simply reflect the presence of a TATA box (TATAT) at nucleotide −40 that is more accessible in the absence of the polymerase I upstream element (4). However, transcripts initiating at these sites are not detectable from endogenous ribosomal genes in HeLa cells grown under a variety of conditions (unpublished data). Therefore, RNA polymerase II transcription from within the ribosomal promoter may require plasmid DNA or, alternatively, DNA that is present outside of the nucleolus.

A recent report (7) suggests that when the SV40 T antigen gene is fused to a mouse rRNA promoter, large T antigen can be detected by indirect immunofluorescence of microinjected cells. The discovery of a low level of RNA polymerase II transcription initiating in the polymerase I promoter complicates our ability to determine whether *tk* RNA transcribed by RNA polymerase I is also translated into *tk* protein. When cytoplasmic RNA is assayed from cells transfected with prHuTK containing the complete RNA polymerase I promoter, more transcripts are found that have initiated at the various RNA polymerase II sites than at the authentic RNA polymerase I site. Nevertheless, *tk* immunofluorescence experiments (unpublished data) were attempted with the plasmids prHuTK and Δ5′−83. In addition, the wild-type and −83 mutant promoters, lacking a cryptic ATG

codon at nucleotide +50 in the rDNA that could potentially interfere with efficient expression of *tk* (17), were fused to a neomycin resistance gene and selected in HeLa cells (A. Levinson, personal communication). Low-level expression of protein was observed from the −83 mutant, but not from the wild-type promoter. Thus, RNA transcribed under the direction of RNA polymerase I is incapable of producing enough protein to be detected by these assays. By contrast, when the cryptic RNA polymerase II transcription is stimulated, expression of protein can be detected. In light of these findings, we find it difficult to determine whether relatively small amounts of protein such as that observed in the rDNA-T antigen fusion studies (7) are actually expressed as a result of RNA polymerase I or RNA polymerase II transcription.

ACKNOWLEDGMENTS

We thank D. Rio for helpful comments and J. Rine, M. Learned, K. Jones, W. Dynan, and M. Haltiner for critical reading of the manuscript. We are also grateful to K. Erdley for preparation of the manuscript and to R. Smale for assistance in preparing the figures.

This work was funded by Public Health Service grants from the National Institutes of Health. S.T.S. was the recipient of a predoctoral fellowship from the National Science Foundation.

LITERATURE CITED

1. **Aviv, H., and P. Leder.** 1972. Purification of biologically active globin messenger RNA by chromatography on oligo-thymidylic acid-cellulose. Proc. Natl. Acad. Sci. U.S.A. **69**:1408–1412.
2. **Berk, A. J., and P. A. Sharp.** 1977. Sizing and mapping of early adenovirus mRNAs by gel electrophoresis of S1 endonuclease-digested hybrids. Cell **12**:721–732.
3. **Blatti, S., C. Ingles, J. Lindell, P. Morris, R. Weaver, F. Weinberg, and W. Rutter.** 1970. Structure and regulatory properties of eukaryotic RNA polymerase. Cold Spring Harbor Symp. Quant. Biol. **35**:649–658.
4. **Breathnach, R., and P. Chambon.** 1981. Organization and expression of eucaryotic split genes coding for proteins. Annu. Rev. Biochem. **50**:349–383.
5. **Busby, S., and R. H. Reeder.** 1983. Spacer sequences regulate transcription of ribosomal gene plasmids injected into Xenopus embryos. Cell **34**:989–996.
6. **Erickson, J. M., C. L. Rushford, D. J. Dorney, G. N. Wilson, and R. D. Schmickel.** 1981. Structure and variation of human ribosomal DNA: molecular analysis of cloned fragments. Gene **16**:1–9.
7. **Fleischer, S., and I Grummt.** 1983. Expression of an mRNA coding gene under the control of an RNA polymerase I promoter. EMBO J. **2**:2319–2322.
8. **Gluzman, Y.** 1981. SV40-transformed simian cells support the replication of early SV40 mutants. Cell **23**:175–182.
9. **Grummt, I.** 1981. Mapping of a mouse ribosomal RNA promoter by *in vitro* transcription. Nucleic Acids Res. **9**:6093–6102.
10. **Grummt, I.** 1982. Nucleotide sequence requirements for specific initiation of transcription by RNA polymerase I. Proc. Natl. Acad. Sci. U.S.A. **79**:6908–6911.
11. **Grummt, I., E. Roth, and M. R. Paule.** 1982. RNA transcription *in vitro* is species specific. Nature (London) **296**:173–174.
12. **Guyette, W. A., R. J. Matusik, and J. M. Rasen.** 1979. Prolactin-mediated transcriptional and post-transcriptional control of casein gene expression. Cell **17**:1013–1023.
13. **Hanahan, D., D. Lane, L. Lipsich, M. Wigler, and M. Botchan.** 1980. Characteristics of an SV40-plasmid recombinant and its movement into and out of the genome of a murine cell. Cell **21**:127–139.
14. **Hirt, B.** 1967. Selective extraction of polyoma DNA from infected mouse cell culture. J. Mol. Biol. **26**:365–369.
15. **Holmes, D. S., and J. Bonner.** 1973. Preparation, molecular weight, base composition, and secondary structure of giant nuclear ribonucleic acid. Biochemistry **12**:2330–2334.
16. **Kedinger, C., and R. Simard.** 1974. The action of α-amanitin on RNA synthesis in chinese hamster ovary cells. J. Cell Biol. **63**:831–842.
17. **Kozak, M.** 1978. How do eukaryotic ribosomes select initiation regions in messenger RNA? Cell **15**:1109–1123.
18. **Learned, R. M., and R. Tjian.** 1982. *In vitro* transcription of human ribosomal RNA genes by RNA polymerase I. J. Mol. Appl. Genet. **1**:575–584.
19. **Learned, R. M., S. T. Smale, M. M. Haltiner, and R. Tjian.** 1983. Regulation of human ribosomal RNA transcription. Proc. Natl. Acad. Sci. U.S.A. **80**:3558–3562.
20. **Long, E. O., and I. B. Dawid.** 1980. Repeated genes in eukaryotes. Annu. Rev. Biochem. **49**:727–764.
21. **Lusky, M., L. Berg, H. Weiher, and M. Botchan.** 1983. Bovine papilloma virus contains an activator of gene expression at the distal end of the early transcription unit. Mol. Cell. Biol. **3**:1108–1122.
22. **Lusky, M., and M. Botchan.** 1981. Inhibition of simian virus 40 replication in simian cells by specific pBR322 sequences. Nature (London) **293**:79–81.
23. **Luthman, H., and G. Magnusson.** 1983. High efficiency polyoma DNA transfection of chloroquine treated cells. Nucleic Acids Res. **11**:1295–1308.
24. **Maul, G. G., and T. H. Hamilton.** 1967. The intranuclear localization of two DNA-dependent RNA polymerase activities. Proc. Natl. Acad. Sci. U.S.A. **57**:1371–1378.
25. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-labeled DNA with base-specific cleavages. Methods Enzymol. **65**: 499–560.
26. **McKnight, S. L., and R. Kingsbury.** 1982. Transcriptional control signals of a eukaryotic protein-coding gene. Science **217**:316–324.
27. **Miesfeld, R., and N. Arnheim.** 1982. Identification of the *in vivo* and *in vitro* origin of transcription in human rDNA. Nucleic Acids Res. **10**:3933–3949.
28. **Miesfeld, R., and N. Arnheim.** 1984. Species-specific rDNA transcription is due to promoter-specific binding factors. Mol. Cell. Biol. **4**:221–227.
29. **Miller, K. G., and B. Sollner-Webb.** 1981. Transcription of mouse rRNA genes by RNA polymerase I: *in vitro* and *in vivo* initiation and processing sites. Cell **27**:165–174.
30. **Mishima, Y., O. Yamamoto, R. Kominami, and M. Muramatsu.** 1981. *In vitro* transcription of a cloned mouse ribosomal RNA gene. Nucleic Acids Res. **9**:6773–6785.
31. **Mishima, Y., I. Finanesek, R. Kominami, and M. Muramatsu.** 1982. Fractionation and reconstitution of factors required for accurate transcription of mammalian ribosomal RNA genes: identification of a species-dependent initiation factor. Nucleic Acids Res. **10**:6659–6670.
32. **Moore, C. L., and P. A. Sharp.** 1984. Site-specific polyadenylation in a cell-free reaction. Cell **36**:581–591.
33. **Moss, T.** 1983. A transcriptional function for the repetitive ribosomal spacer in *Xenopus laevis*. Nature (London) **302**: 223–228.
34. **Moss, T., and M. L. Birnstiel.** 1979. The putative promoter of a *Xenopus laevis* ribosomal gene is reduplicated. Nucleic Acids Res. **6**:3733–3743.
35. **Myers, R. M., and R. Tjian.** 1980. Construction and analysis of simian virus 40 origins defective in tumor antigen binding and DNA replication. Proc. Natl. Acad. Sci. U.S.A. **77**:6491–6495.
36. **Nevins, J. R.** 1983. The pathway of eukaryotic mRNA formation. Annu. Rev. Biochem. **52**:441–466.
37. **Peden, K. W. C., J. M. Pipas, S. Pearson-White, and D. Nathans.** 1980. Isolation of mutants of an animal virus in bacteria. Science **209**:1392–1396.
38. **Pelham, H. R. B.** 1982. A regulatory upstream promoter element in the Drosophila hsp70 heat-shock gene. Cell **30**:517–528.
39. **Perry, R. P.** 1962. The cellular sites of synthesis of ribosomal and 4S RNA. Proc. Natl. Acad. Sci. U.S.A. **48**:2179–2181.
40. **Perry, R. P.** 1976. Processing of RNA. Annu. Rev. Biochem. **45**:605–630.
41. **Perry, R. P., and D. E. Kelley.** 1970. Inhibition of RNA synthesis by actinomycin D: characteristic dose-response of

different RNA species. J. Cell. Physiol. **76**:127–140.
42. **Proudfoot, N. J., and G. G. Brownlee.** 1976. 3′ Non-coding region sequences in eukaryotic messenger RNA. Nature (London) **263**:211–214.
43. **Reich, E., and I. H. Goldberg.** 1964. Actinomycin and nucleic acid function. Prog. Nucleic Acid Res. Mol. Biol. **3**:184–230.
44. **Rio, D. C., and R. Tjian.** 1983. SV40 T antigen binding site mutations that affect autoregulation. Cell **32**:1227–1240.
45. **Roberts, W. K., and J. F. E. Newman.** 1966. Use of low concentrations of actinomycin D in the study of RNA synthesis in Ehrlich Ascites cells. J. Mol. Biol. **20**:63–73.
46. **Roeder, R. G., and W. J. Rutter.** 1970. Specific nucleolar and nucleoplasmic RNA polymerases. Proc. Natl. Acad. Sci. U.S.A. **65**:675–682.
47. **Shatkin, A. J.** 1976. Capping of eukaryotic mRNAs. Cell **9**:645–653.
48. **Skinner, J. A., A. Ohrlein, and I. Grummt.** 1984. *In vitro* mutagenesis and transcriptional analysis of a mouse ribosomal promoter element. Proc. Natl. Acad. Sci. U.S.A. **81**:2137–2141.
49. **Sollner-Webb, B., and R. H. Reeder.** 1979. The nucleotide sequence of the initiation and termination sites for ribosomal RNA transcription in *X. laevis*. Cell **18**:485–499.
50. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
51. **Ucker, D., and K. Yamamoto.** 1984. Early events in the stimulation of mammary tumor virus RNA synthesis by glucocorticoids. J. Biol. Chem. **259**:7416–7420.
52. **Weiher, H., and M. R. Botchan.** 1984. An enhancer sequence from bovine papilloma virus DNA consists of two essential regions. Nucleic Acids Res. **12**:2901–2912.
53. **Weingartner, B., and W. Keller.** 1981. Transcription and processing of adenoviral RNA by extracts from HeLa cells. Proc. Natl. Acad. Sci. U.S.A. **78**:4092–4096.
54. **Yamamoto, O., N. Jakakusa, Y. Mishima, R. Kominami, and M. Muramatsu.** 1984. Determination of the promoter region of mouse ribosomal RNA gene by an *in vitro* transcription system. Proc. Natl. Acad. Sci. U.S.A. **81**:299–303.
55. **Zeevi, M., J. R. Nevins, and J. E. Darnell, Jr.** 1982. Newly formed mRNA lacking polyadenylic acid enters the cytoplasm and the polyribosomes but has a shorter half-life in the absence of polyadenylic acid. Mol. Cell. Biol. **2**:517–525.