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# Specificity of RNA Maturation Pathways: RNAs Transcribed by RNA Polymerase III Are Not Substrates for Splicing or Polyadenylation

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**To analyze the specificity of RNA processing reactions, we constructed hybrid genes containing RNA polymerase III promoters fused to sequences that are normally transcribed by polymerase II and assessed their transcripts following transfection into human 293 cells. Transcripts derived from these chimeric constructs were analyzed by using a combined RNase H and S1 nuclease assay to test whether RNAs containing consensus 5′ and 3′ splicing signals could be efficiently spliced in intact cells, even though they were transcribed by RNA polymerase III. We found that polymerase III-derived RNAs are not substrates for splicing. Similarly, we were not able to detect poly(A)$^+$ RNAs derived from genes that contained a polymerase III promoter linked to sequences that were necessary and sufficient to direct 3′-end cleavage and polyadenylation when transcribed by RNA polymerase II. Our findings are consistent with the view that in vivo splicing and polyadenylation pathways are obligatorily coupled to transcription by RNA polymerase II.**

Eucaryotic cell nuclei contain three types of RNA polymerase, each of which transcribes a distinct set of genes. The resultant RNA transcripts are processed through specific pathways to yield their respective mature RNA species. The maturation of mammalian cell mRNAs involves an extensive set of modifications of the primary transcript (2, 9, 42, 53). These include attachment of a modified guanine nucleotide at the 5′ end (44, 50) and methylation of this cap and of internal adenylate residues (7). Most mRNAs are also cleaved and then polyadenylated at a site ~20 to 30 nucleotides 3′ to a conserved AAUAAA sequence (15, 45, 46). Finally, most mRNA precursors undergo RNA splicing events to excise intervening sequences at precise intron-exon junctions (for reviews, see references 1, 9, 35, and 39). This splicing is directed by specific consensus sequences in the RNA and involves a number of the U-class small nuclear ribonucleoprotein particles (for reviews, see references 30, 39, and 52). Although the capping occurs cotranscriptionally, there is no obligatory temporal order of the other two processing events (58). Transcripts synthesized by RNA polymerase III, on the other hand, are neither capped nor polyadenylated. While some polymerase III-catalyzed transcripts do undergo splicing, it is of a different sort than that of mRNAs, inasmuch as it does not involve the same sequence determinants or the same U-class small nuclear ribonucleoprotein particles (18, 41). Transcripts catalyzed by RNA polymerase I utilize yet a third series of RNA maturation events (19, 33, 42).

Notwithstanding the recent major advances in our understanding of these complex RNA maturation events, it remains unclear whether these processes are, in fact, specific to RNAs transcribed by each polymerase. For mRNA maturation, two possibilities may be considered. Specificity in the in vivo processing reactions may depend on tight (perhaps obligatory) coupling of the RNA polymerase II transcription machinery to the additional activities responsible for capping, polyadenylation, and splicing. In this scenario, successful production of functional mRNAs would not be achieved if these transcripts were synthesized by RNA polymerase I or polymerase III transcription complexes. The second possibility is that specificity in RNA maturation is determined solely by the nucleotide sequences of the transcript. Here, maturation of RNA transcripts could occur independently of the identity of the polymerase complex that catalyzed transcription.

Until recently, few data were available to distinguish between these possibilities. However, Green et al. (17) demonstrated that in vitro-synthesized pre-mRNA templates containing capped 5′ termini are accurately spliced (albeit at a low efficiency of ~5%) following injection into *Xenopus* oocytes. This result indicates that accurate in vivo splicing can be uncoupled from the polymerase II transcription machinery, a conclusion that is also supported by successful splicing of purified pre-mRNA templates in vitro (23, 38). It is not clear, however, that this accurately reflects the in vivo processing specificity, which could involve compartmentalization, the presence of very large transcription and processing complexes, or other factors.

For RNAs transcribed by RNA polymerase I, transfection of hybrid genes has shown that RNA polymerase I-derived transcripts do not yield functional (translatable) mRNAs (27). Similarly, Smale and Tjian (55) found that chimeric RNAs transcribed by polymerase I do not become polyadenylated. These findings are consistent with the notion that transcription and processing reactions may be coupled in vivo, although they might also merely reflect a nucleolar compartmentalization of polymerase I-transcribed RNAs. On the other hand, Carlson and Ross (6) have documented in vivo splicing and polyadenylation of what is probably a polymerase III-catalyzed transcript of the β-globin gene, and Lewis and Manley (25) have recently concluded that chimeric RNAs containing an RNA polymerase III promoter (the adenovirus type 2 [Ad2] VA1 promoter) fused upstream of a protein-coding gene (the herpes simplex virus [HSV] thymidine kinase [*tk*] gene) were efficiently cleaved and polyadenylated in vivo. These data suggest that for polymerase III-derived transcripts the in vivo coupling between

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polymerase II transcription and processing pathways is not absolute.

To further analyze the specificity of processing of transcripts that are normally synthesized by RNA polymerase II, we constructed hybrid genes containing polymerase III promoters fused to sequences that are normally synthesized under the direction of polymerase II. In contrast to the conclusions of Lewis and Manley (25), transfection of these constructs into mouse L cells and into human 293 cells revealed that chimeric RNAs transcribed by RNA polymerase III cannot be polyadenylated, even though these molecules contain signals that are necessary and sufficient to direct 3'-end cleavage and polyadenylation when transcribed by RNA polymerase II. In addition, by using a combined RNase H and S1 nuclease assay, our results indicate that RNAs that contain consensus 5' and 3' splicing signals but that have been transcribed by RNA polymerase III are not substrates for splicing in vivo.

## MATERIALS AND METHODS

**Plasmid constructions. (i) p5SpA.** The construction of p5SpA, a plasmid that carries the 5S gene promoter linked to a polyadenylation signal sequence derived from the chicken β-globin gene, is shown in Fig. 1A. The 5S maxigene (4) was obtained from Donald Brown (Carnegie Institute, Baltimore, Md.). Plasmid pSDB4, which contains the chicken adult β-globin gene (10), was obtained from Gary Felsenfeld (National Institutes of Health, Bethesda, Md.). Plasmid pSDB4 was first digested with *Sac*I at a site that lies 64 nucleotides 3' to the polyadenylation site (at the junction between the β-globin DNA and the vector sequences of pSDB4), and was then treated with S1 nuclease to create flush ends. This DNA was next digested with *Sau*3A, which cleaves 92 nucleotides 5' to the polyadenylation site (15 nucleotides 3' to the translation termination codon), and the 152-base-pair (bp) *Sau*3A-*Sac*I fragment was isolated. It was ligated to the large (2,504-bp) *Pvu*II-*Bam*HI fragment from the 5S maxigene plasmid that contains 5S sequences from positions −48 to 115 (including the polymerase III promoter) plus essential vector sequences to produce p5SpA (Fig. 1A).

**(ii) pVATK and pLPTK.** The genes in which the HSV type 1 *tk* region was transcribed by the Ad2 major late promoter (pLPTK) or the Ad2 VA1 promoter (pVATK) are shown in Fig. 2A. The parental HSV-1 *tk* gene was from p5'Δ+1 (analogous to those described by McKnight et al. [31] and was obtained from Steven McKnight, Carnegie Institute, Baltimore, Md.), lacked the *tk* promoter and transcription initiation site, but retained all but the first nucleotide of the sequences contained on the *tk* mRNA as well as 294 bp downstream of the *tk* poly(A) addition site. The polymerase III and II promoters were isolated from plasmids pHK (containing the Ad2 VA1 gene) and pMLT (containing the Ad2 major late promoter), which were obtained from Gary Kettner (Department of Biology, The Johns Hopkins University) and Jeffrey Corden (School of Medicine, The Johns Hopkins University), respectively. Plasmid pVATK was assembled by ligating into the *Bam*HI site of p5'Δ+1 (at residue 1) a 1,781-bp *Bgl*II-*Bam*HI fragment from plasmid pHK that contained 76 bp of the Ad2 VA1-coding region (containing the VA1 promoter) and 1,705 bp of upstream sequences. Plasmid pLPTK was constructed by ligation into the *Bam*HI site of p5'Δ+1 a 292-bp fragment from plasmid pMLT that contained the Ad2 major late promoter, the transcription initiation site, and the first 33 bp of the 5'-untranslated region. These chimeric plasmids thus contained identical *tk* sequences but were transcriptionally dependent on either a polymerase III (pVATK) or a polymerase II (pLPTK) promoter.

**(iii) 5Sβ3.** 5S-promoted β-tubulin gene chimeras are diagrammed in Fig. 3A. The chicken β3 tubulin gene (pβG3) has been described previously (28). Plasmid p5Sβ3 was assembled by ligating a 255-bp *Sau*3A-*Ssp*I fragment from the chicken β3 tubulin gene that contained most of exons 2 and 3 and the entire second intron to the 2,504-bp *Pvu*II-*Bam*HI fragment from the 5S maxigene plasmid described above that contained the polymerase III promoter plus essential vector sequences. Plasmid pΔ5Sβ3, a promoterless construct, was identical to p5Sβ3 except that the 5S promoter sequences between residues 41 (a natural *Sau*3A site) and 115 (the *Bam*HI site of the 5S maxigene) were deleted. Because this construct did not contain the 5S internal control region that is necessary for polymerase III transcription (5, 48), it should not have directed any polymerase III initiation events. Plasmid pΔ5Scβ3 contained the identical 5S and vector sequences that were present in pΔ5Sβ3, but the corresponding β-tubulin fragment was isolated from a β3 cDNA clone (56). The plasmid was constructed by ligating a 181-bp *Sau*3A-*Ssp*I β3 cDNA fragment to a 5S maxigene fragment from which the 5S promoter sequences between residues 41 and 115 were deleted.

**Transient transfection assays and RNA isolation.** Transient DNA transfections were performed in cultured mouse LTK$^-$ cells by using the DEAE-dextran and dimethyl sulfoxide shock protocol (26). At 24 to 48 h posttransfection, cellular RNA was isolated by homogenization of cells in guanidine isothiocyanate and centrifugation through a 5.7 M cesium chloride cushion (8). Pelleted RNA was suspended, extracted with phenol-chloroform, and precipitated with ethanol. RNA was quantified by assuming an $A_{260}$ of 20 for 1 mg/ml.

For transient transfections into human 293 cells, calcium phosphate coprecipitation was used (16). At 24 h before transfection, $1.5 \times 10^6$ cells were plated in Dulbecco modified Eagle medium–10% fetal bovine serum. Calcium phosphate coprecipitates were then pipetted onto the cells and incubated at 37°C for 4.5 h, at which time the medium was replaced with fresh Dulbecco modified Eagle medium–10% fetal bovine serum. Total cellular RNA was isolated at 18 to 44 h posttransfection by homogenization in guanidine isothiocyanate and centrifugation through a 5.7 M cesium chloride cushion and was quantified as described above.

Cytoplasmic RNA was isolated by using a modification of the procedure described by Favaloro et al. (13). After washing once with 5 ml of ice-cold phosphate-buffered saline, 300 μl of lysis buffer (0.14 M NaCl, 1.5 mM $MgCl_2$, 10 mM Tris [pH 8.6], 0.5% Nonidet P-40, 10 mM vanadyl-ribonucleoside complexes) was added to the monolayer of cells. Cells were scraped, transferred to a 1.5-ml microfuge tube, and lysed by gentle vortexing. The cell extract was centrifuged for 3 min at 10,000 × *g* at 4°C to remove nuclei. The supernatant was removed and diluted with an equal volume of 2× proteinase K buffer (0.2 M Tris [pH 7.5], 25 mM EDTA, 0.3 M NaCl, 2% sodium dodecyl sulfate), and proteinase K was added to 200 μg/ml. After incubation at 37°C for 30 min, the lysate was extracted with phenol-chloroform and then with chloroform and was precipitated with ethanol. RNA was quantified as described above. Nuclear RNA was isolated following homogenization of the nuclear pellet in guanidine isothiocyanate and centrifugation through a 5.7 M cesium chloride cushion. Poly(A)$^+$ RNA was isolated following two cycles of chromatography of

either nuclear or cytoplasmic RNA on oligo(dT)-cellulose (type 3; Collaborative Research, Inc., Waltham, Mass.).

**In vitro transcription.** Plasmid p5Sβ3 was transcribed in mouse S-100 extract by using 500 μM of each of the four unlabeled ribonucleoside triphosphates and no α-amanitin, as described previously (33, 57). The RNA was isolated by treatment of the reaction with RNase-free DNase (Promega), phenol extraction, and ethanol precipitation following the addition of yeast tRNA carrier and was stored at −70°C.

**Probes for S1 nuclease analysis. (i) p5SpA.** To assess RNAs transcribed from p5SpA, two S1 nuclease probes were prepared by 5′ end labeling (Fig. 1A). The first of these (probe A) was prepared from the 5S maxigene by labeling at the *Bam*HI site that lies 118 nucleotides 3′ to the 5S transcription initiation site. The second probe (probe B) was prepared from p5SpA by labeling at the *Ava*I site that lies 31 bases 3′ to the site of polyadenylation. With this probe RNAs initiated at the 5S transcription start site protected a 235-base fragment. To detect RNAs that were derived from the authentic chicken β-globin gene, a probe from plasmid pSDB4 was 5′ end labeled at the *Nco*I site that lies 83 nucleotides downstream from the major β-globin start site (10).

**(ii) pVATK and pLPTK.** To detect RNAs that were derived from the Ad2 major late promoter transcription start site in pLPTK, a probe prepared from this plasmid was 5′ end labeled at the unique *Bgl*II site ~93 bp downstream of the late promoter initiation site. Ad2 VA1 *tk* chimeric RNAs were assayed with a probe that was 5′ end labeled at the *Bam*HI site that lies 76 bp downstream of the Ad2 VA1 initiation site (at the junction between Ad2 and *tk* sequences in plasmid pVATK).

**(iii) 5Sβ3.** Probes to assess RNAs initiated at the 5S transcription initiation site in p5Sβ3 were prepared from pΔ5Sβ3 and pΔ5Scβ3 DNAs that were 5′ end labeled at the unique *Ava*I site (69 nucleotides into exon 3 of the β3 tubulin sequence). The resultant probe was then cleaved with *Ssp*I in the upstream vector sequences before use.

**S1 nuclease analysis.** For hybridization and S1 nuclease analysis, 0.01 pmol of the end-labeled, double-stranded DNA probe was mixed with various amounts of the RNA sample and hybridized in a solution containing 80% formamide, 0.4 M NaCl, 40 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES; pH 6.4), and 1 mM EDTA for 12 to 16 h at the following temperatures: 59.5°C for the Δ5Sβ3 and Δ5Scβ3 probes, 54°C for the 5S and β-globin initiation site probes, and 50°C for the Ad2 VA1 and Ad2 major late promoter initiation site probes. Samples were then diluted 15-fold with ice-cold S1 nuclease buffer (3) and treated with 0.5 U of S1 nuclease per μl at 25°C for 60 min. The protected probe was visualized by autoradiography following electrophoresis on 4% acrylamide–9 M urea-containing gels.

**RNase H-directed cleavage assay.** To identify spliced chimeric RNAs which initiated at the normal polymerase III start site in p5Sβ3, we used a method in which RNase H was used in combination with S1 nuclease (see Fig. 5A; this procedure has been described in detail elsewhere [54]). Briefly, a purified DNA fragment corresponding to a portion of the intervening sequence was hybridized to the RNA sample, and resultant hybrids were treated with RNase H (which digests only RNA regions that are in DNA-RNA heteroduplexes [11]). Unspliced, intron-containing RNA molecules were thereby cleaved into two pieces, one 5′ to the intron and the other 3′ to the intron, while spliced RNAs were not affected by the RNase H treatment (54). The RNA sample was then hybridized to an intronless (cDNA) S1 nuclease probe that was end labeled downstream of the splice junction and that extended across this site and upstream of the initiation site. Following S1 nuclease digestion and electrophoresis under denaturing conditions, protected probe fragments were visualized by autoradiography. If the RNase H step was performed quantitatively and the S1 nuclease analysis was performed with excess probe, only spliced RNA molecules could protect the S1 nuclease probe from the labeled terminus through to the transcription initiation site.

## RESULTS

**Are RNAs transcribed by RNA polymerase III suitable substrates for polyadenylation?** To assess whether RNAs transcribed by polymerase III could undergo polyadenylation in vivo, we constructed plasmid p5SpA (Fig. 1A). This plasmid contained the RNA polymerase III promoter from the 5S maxigene plasmid (4) fused to a 152-bp segment of the adult chicken β-globin gene that carries the consensus AATAAA cleavage and polyadenylation signal, as well as a consensus YGTGTTYY sequence that is necessary for the efficient formation of processed 3′ termini (32). This β-globin sequence (containing sequences 92 bp upstream through 64 bp downstream of the β-globin polyadenylation addition site) was selected because it is devoid of T clusters, which can act to terminate polymerase III transcription (4). The *Xenopus borealis* somatic 5S gene promoter sequences were used for the following reasons. (i) This 5S gene has been extensively characterized with respect to the sequences (5, 48) and factors (24, 51) that specify transcription initiation by RNA polymerase III (for a review, see reference 22). (ii) Because the frog 5S gene is actively transcribed in mammalian extracts (24, 57), we expected that it would be active following transfection into mammalian cells. (iii) The frog 5S sequence is sufficiently diverged from the mammalian 5S RNA to avoid some problems that arise from trimolecular hybrids in S1 nuclease analyses (29).

To test whether RNAs derived from the chimeric gene carrying a polymerase III promoter and the β-globin polyadenylation sequences were capable of efficient cleavage and polyadenylation in vivo, p5SpA was transiently transfected into parallel dishes of mouse L cells; and total cellular RNA was isolated at 24, 36, or 48 h posttransfection. RNA that was isolated at each time point was selected for poly(A)$^+$ species by two cycles of chromatography on oligo(dT)-cellulose. Both the poly(A)$^+$ and poly(A)$^-$ fractions were then analyzed by S1 nuclease mapping with a probe that was 5′ end labeled 118 nucleotides downstream of the 5S initiation site (Fig. 1A, probe A). The results of this analysis demonstrate that although RNAs from transfected p5SpA are abundant in the poly(A)$^-$ fraction (Fig. 1B, lanes 2, 4, and 6), no transcripts that begin at the 5S transcription initiation site are detectable in the poly(A)$^+$ fraction (Fig. 1B, lanes 1, 3, and 5).

To assess whether the failure to observe polyadenylation could be due to the inability of the β-globin sequence to direct efficient polyadenylation of RNAs derived from transfected genes, we performed parallel transfection experiments using plasmid pSDB4. This plasmid carries the authentic chicken β-globin gene, including the region from which the p5SpA polyadenylation signal sequences were obtained. Plasmid p5SpA contains virtually the entire 3′-untranslated region plus all of the downstream β-globin sequences that are present in pSDB4 (as described above). When RNAs from pSDB4 were assayed for polyadenylation

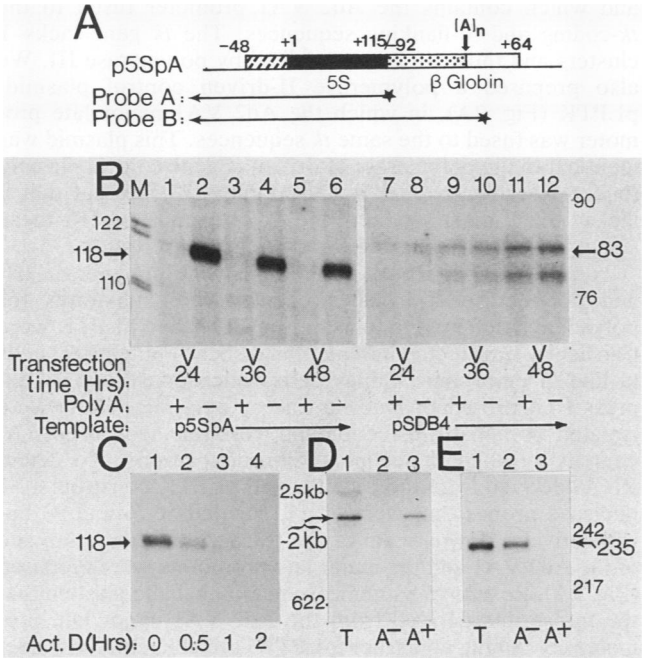


FIG. 1. Analysis of p5SpA, the polymerase III β-globin polyadenylation site containing plasmid. (A) The p5SpA gene. The chimeric gene in plasmid p5SpA consists of the 5S maxigene promoter (residues −48 to 115) followed by the polyadenylation site ($[A]_n$) of the chicken β-globin gene (from 92 nucleotides 5′ through 64 nucleotides 3′ of the poly(A) addition site; see text for details). Probes A and B used for the S1 nuclease analyses are shown below the gene. Stars denote sites of end labeling. (B) Analysis of polyadenylation of chimeric p5SpA RNAs in cells transfected with plasmid p5SpA. Triplicate dishes of L cells were transfected with plasmid p5SpA, and total cellular RNA was isolated at the indicated times and fractionated according to poly(A) content. RNAs were analyzed by S1 nuclease mapping with a probe that was 5′ end labeled 118 nucleotides downstream of the 5S transcription initiation site. Lanes 1, 3, and 5, S1 nuclease analysis of the poly(A)$^+$ fraction from 5 μg of total cell RNA; lanes 2, 4, and 6, analysis of 5 μg of poly(A)$^-$ RNA; a parallel set of transfections and RNA isolations was carried out with plasmid pSDB4, and resultant RNAs were analyzed by S1 nuclease mapping with a probe that was end labeled 83 nucleotides downstream of the β-globin transcription initiation site; lanes 7, 9, and 11, analysis of the poly(A)$^+$ RNA from 2.5 μg of total cell RNA; lanes, 8, 10, and 12, analysis of 2.5 μg of poly(A)$^-$ RNA. Times shown represent time from transfection to cell harvesting. M indicates size markers. (C) Stability of p5SpA RNAs. To examine the stability of p5SpA RNAs, replicate dishes of L cells were transfected with plasmid p5SpA, and at 24 h posttransfection the cells were treated with 20 μg of actinomycin D (Act. D) per ml. RNA was isolated at the indicated times after actinomycin D treatment and was assayed by S1 nuclease mapping with the 5S probe. (D) Analysis of p5SpA RNAs initiated at an upstream transcription initiation site. To examine whether RNAs initiated from a site(s) 5′ to the 5S initiation site in p5SpA were polyadenylated, RNA was prepared from L cells at 24 h posttransfection with p5SpA. The portion of the gel corresponding to the largest protected fragments are shown following S1 nuclease analysis with probe A (see panel A). Lane 1, 5 μg of total RNA; lane 2, 4 μg of poly(A)$^-$ RNA; lane 3, poly(A)$^+$ RNA recovered from passaging 4 μg of total RNA on an oligo(dT) column. kb, Kilobases. (E) Determination of whether polymerase III-derived RNAs from p5SpA extend 3′ to the site of polyadenylation. RNA prepared from L cells at 24 h posttransfection with p5SpA was analyzed by S1 nuclease analysis with probe B (5′ end labeled 31 bases 3′ to the usual site of polyadenylation; see panel A). Lane 1, 5 μg of total RNA; lane 2, 4 μg of poly(A)$^-$ RNA; lane 3, poly(A)$^+$ RNA recovered from passaging 4 μg of total RNA on an oligo(dT) column. In panels B to E, the numbers at the sides of the gels are the number of nucleotides.

(Fig. 1B, lanes 7 to 12), ~50% of the resultant transcripts that initiated at the β-globin transcription initiation site were found in the poly(A)$^+$ fraction at all time points. These results indicate that the polyadenylation signal sequences contained in p5SpA are sufficient to direct polyadenylation of authentic polymerase II transcription products.

Although one likely interpretation of the failure of p5SpA to yield 5S-initiated poly(A)$^+$ transcripts is that there was linkage of transcription and processing machineries, three alternative scenarios are also possible. In the first of these, p5SpA RNAs might be very unstable. If this were the case, then nascent RNAs might be degraded before they could be poly(A)$^+$. To directly examine the half-life of its transcripts, p5SpA was transiently transfected into L cells, and at 24 h posttransfection cells were treated with 20 μg of actinomycin D per ml, a level that is almost instantaneously sufficient to inhibit transcription by all three RNA polymerases (43, 47). RNA was isolated from the cells at 0, 0.5, 1, and 2 h after treatment with the drug and was assayed for remaining 5S-initiated transcripts. The results of this experiment (Fig. 1C) indicate that the p5SpA transcripts have a half-life of ~15 min. Because polyadenylation has been documented to occur very quickly (within 1 min) on normal polymerase II-derived RNAs (50), we conclude that transcript instability alone cannot be responsible for the failure of the p5SpA-derived RNAs to undergo polyadenylation.

A second explanation for the failure of p5SpA RNAs to be polyadenylated is that the conformation adopted by the 5S RNA after transcription inhibits subsequent polyadenylation independent of the promoter that is used. As shown in Fig. 1D, however, this cannot be the case since RNAs from the same p5SpA plasmid but which map to an upstream, fortuitous transcription initiation site (presumably transcribed by polymerase II) were efficiently polyadenylated, despite the juxtaposition of the same 5S RNA sequences adjacent to the site of polyadenylation.

A final possibility for the failure of polymerase III-derived RNAs from p5SpA to poly(A) is that the RNAs initiated by polymerase III may terminate before they reach the polyadenylation site. To test this, we prepared an S1 nuclease probe (Fig. 1A, probe B) that was 5′ end labeled at a site 31 bases downstream of the normal site of polyadenylation. When we analyzed RNA from cells transfected with p5SpA, an abundant 235-base protected fragment corresponding to RNAs that extended from the 5S transcription initiation site through to the site of labeling was observed (Fig. 1E). Thus, premature transcription termination cannot account for the failure of polymerase III-derived RNAs to be polyadenylated.

**Are other polymerase III-transcribed RNAs substrates for polyadenylation?** In light of our results described above, we were surprised by the results of a recent report by Lewis and Manley (25), in which it was concluded that polymerase III-transcribed RNAs are accurately 3′ cleaved and polyadenylated. These investigators used plasmid pVATK2, which contains the RNA polymerase III promoter from the Ad2 VA1 gene, which is fused to the polyadenylation region of the HSV *tk* gene. After transfection into human 293 cells, transcripts of this hybrid gene were stable and transported to the cytoplasm, and 10% of the polymerase III-derived RNA species was concluded to be poly(A)$^+$.

Since we detected no polyadenylation in our original experiments, we felt compelled to reexamine the apparent polyadenylation reported by Lewis and Manley (25). To this end, we constructed pVATK (Fig. 2A), which is identical to the plasmid pVATK2 described by Lewis and Manley (25)

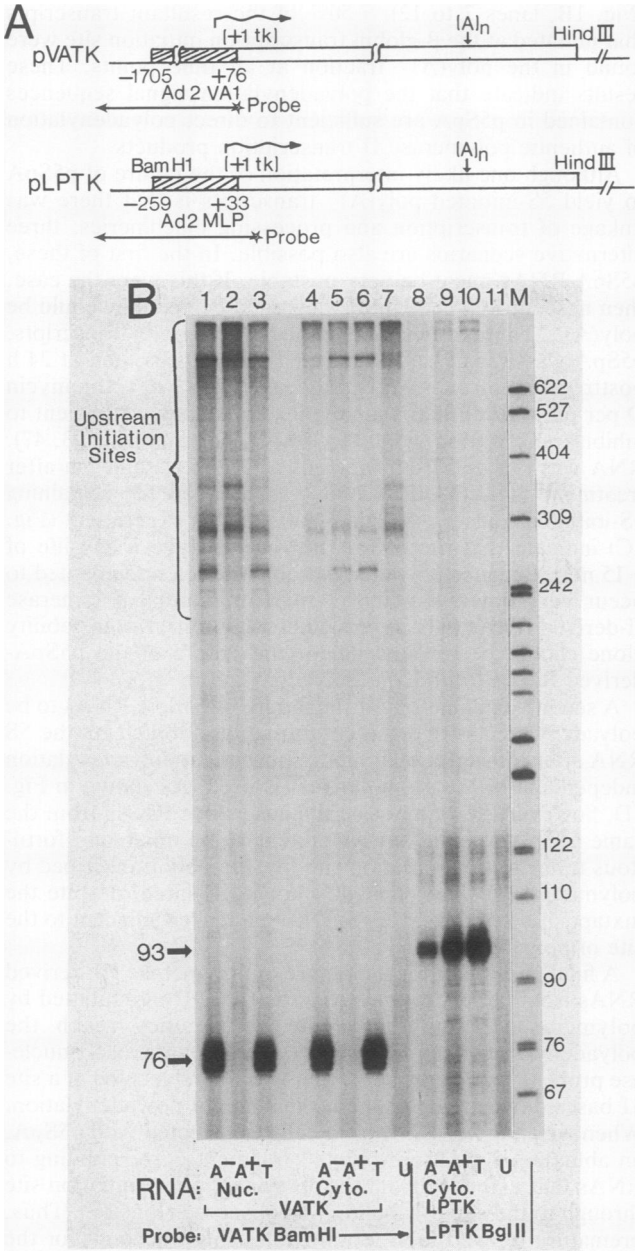


FIG. 2. Analysis of polyadenylation of chimeric Ad2 VA1 *tk* RNAs following transfection into human 293 cells. (A) Construction of chimeric Ad2 VA1 *tk* and major late promoter (MLP) *tk* plasmids. Plasmid pVATK contained a 1,781-bp *Bgl*II-*Bam*HI fragment of Ad2 DNA (residues −1705 to 76 relative to the Ad2 VA1 gene transcription initiation site) inserted at residue 2 of the *tk* gene in plasmid p5′Δ+1 [that retained all sequences that were present on the HSV type 1 *tk* messenger RNA and 294 bp downstream of the *tk* poly(A) addition site but that did not contain the promoter and transcription initiation site]. Plasmid pLPTK contained a 292-bp fragment of Ad2 DNA (residues −259 to 33 relative to the major late promoter) inserted into the *tk* gene plasmid, as described above. The transcription initiation site and polyadenylation sites are denoted by an arrow and $[A]_n$, respectively. Symbols: Hatched box, Ad2 DNA; open box, HSV type 1 *tk* DNA; single line, vector sequences. The S1 nuclease probes that were used to analyze transcripts from these genes are also shown. (B) Analysis of poly(A)$^+$ and poly(A)$^-$ RNAs produced from pVATK and pLPTK. Duplicate dishes of human 293 cells were transfected with the Ad2 VA1-driven construct pVATK or the major late promoter-driven construct pLPTK. At 44 h posttransfection nuclear (Nuc.) and cytoplasmic (Cyto.) RNAs were isolated and selected for poly(A) content. RNAs from pVATK were assayed with a probe that was 5′ end labeled at the *Bam*HI site 76 bp downstream of the known Ad2 VA1 transcription initiation site. RNAs from pLTPK were assayed with a probe that was 5′ end labeled at the *Bgl*II site approximately 93 bp downstream of the late promoter initiation site. Lanes 1 to 3 and 4 to 6, pVATK RNAs isolated from nuclear and cytoplasmic fractions, respectively; lanes 7 and 11, untransfected cell RNA; lanes 8 to 10, pLPTK RNAs isolated from the cytoplasmic fraction. T indicates total RNA, and M indicates size markers. Numbers at the sides of the gels are the number of nucleotides.

and which contains the Ad2 VA1 promoter fused to the *tk*-coding and 3′-flanking sequences. The *tk* gene lacks T clusters and thus can be transcribed by polymerase III. We also prepared a polymerase II-driven control plasmid, pLPTK (Fig. 2A), in which the Ad2 VA1 major late promoter was fused to the same *tk* sequences. This plasmid was identical to the polymerase II-driven *tk* gene control plasmid (pϕtk) described by Lewis and Manley (25), except that it lacked the 5′-most 146 bp of the Ad2 region of pϕTK; these sequences are dispensable for efficient transcription (36).

To test whether RNAs derived from the polymerase III- and polymerase II-driven *tk* genes were substrates for polyadenylation in vivo, plasmids pVATK and pLPTK were transiently transfected into parallel dishes of human 293 cells (a line of embryonic kidney cells which constitutively express E1A proteins). Nuclear and cytoplasmic RNAs were isolated 44 h posttransfection and were selected for poly(A) content by oligo(dT)-cellulose chromatography. To detect RNAs derived from the pVATK and pLPTK constructs, S1 nuclease probes that were 5′ end labeled at 76 and 93 bp, respectively, downstream of the transcription initiation site of the Ad2 VA1 and the major late promoters were prepared (Fig. 2A). Results of S1 nuclease mapping demonstrated that the transcripts derived from the Ad2 VA1 major late promoter containing construct (pLPTK) are efficiently polyadenylated(A)$^+$ (Fig. 2B, compare lanes 8 and 9). Thus, transcripts of transfected genes can be polyadenylated, and the polyadenylation signal sequences present on the pVATK construct are sufficient to direct polyadenylation of an authentic polymerase II-transcribed RNA. In contrast to the results with the polymerase II-driven construct, S1 nuclease mapping of RNA species that initiated from the Ad2 VA1 transcription initiation site in pVATK were not poly(A)$^+$. This same result was obtained regardless of whether the nuclear or cytoplasmic RNA fraction was analyzed (Fig. 2B, compare lanes 1 and 2 and 4 and 5).

Following transfection of pVATK there were abundant poly(A)$^+$ RNAs that initiated 5′ to the Ad2 VA1 transcription start site (Fig. 2B). These included the ~300-, ~350-, and ~800-base species in the nuclear fraction (lane 2) and the ~255-, ~300-, and ~800-base species in the cytoplasmic fraction (lane 5). Since the principal data on which Lewis and Manley (25) based their conclusion that polymerase III-derived RNAs were polyadenylated was a 3′ S1 nuclease analysis that could not determine the corresponding transcription initiation site, we propose that RNAs derived from initiation sites 5′ to the authentic VA1 initiation site (and which are almost certainly transcribed by RNA polymerase II) misled Lewis and Manley (25) into erroneously concluding that the pVATK RNAs could be polyadenylated. .

**Construction of plasmids to assess whether RNAs transcribed by RNA polymerase III are substrates for splicing.** To examine whether RNAs transcribed by RNA polymerase III were substrates for splicing in vivo, we constructed hybrid

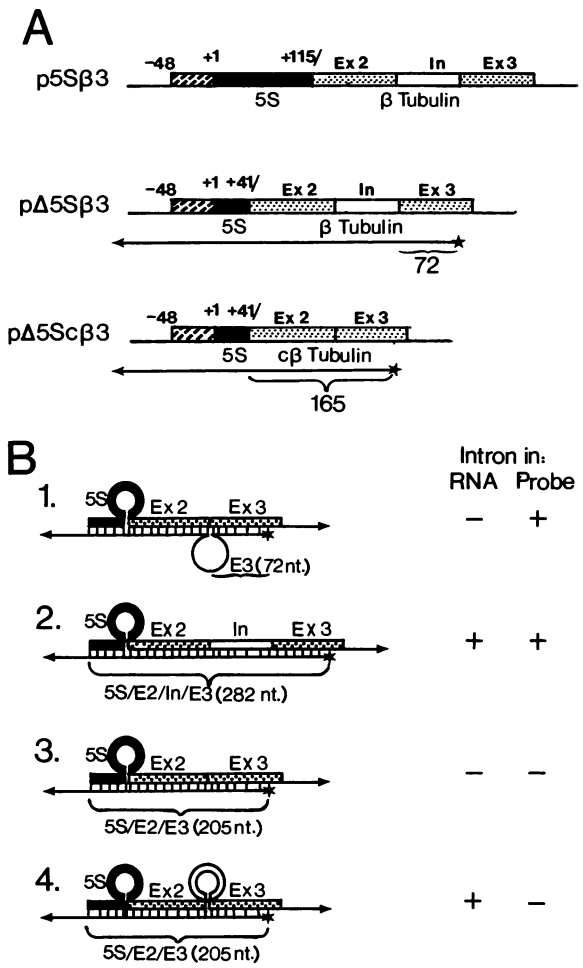


FIG. 3. The chimeric 5S-driven β-tubulin plasmids. (A) Plasmid constructs. A 255-bp segment of the chicken β3 tubulin gene containing the second intron and most of the second (Ex 2) and third (Ex 3) exons was fused to the 5S gene promoter to produce p5Sβ3. An analogous plasmid, pΔ5Sβ3, which lacked the internal control region of the 5S gene, was constructed by deletion of a *Sau*3A fragment that was contained between nucleotides 41 and 115 from the p5Sβ3 plasmid. Another analogous plasmid, pΔ5Scβ3, which lacked the intron (In) sequences, was constructed by using the corresponding 181-bp exon 2-exon 3 fragment from a cDNA clone of β3 tubulin. Symbols: Hatched boxes, 5S upstream sequences; solid boxes, 5S coding sequences; dotted boxes, β-tubulin exons; open boxes, β-tubulin intron. Relevant S1 nuclease probes are also shown. (B) Schematic drawings of hybridization and S1 nuclease analyses. The brackets indicate the probe DNA fragments predicted to be protected from S1 nuclease digestion when the probe (lower line of each duplex) was heteroduplexed with spliced or unspliced RNAs (upper line of each duplex). Spliced RNA was analyzed with the intron-containing probe (line 1) or the intronless probe (line 3). Intron-containing RNA was analyzed with the intron-containing probe (line 2) and the intronless probe (line 4). The S1 nuclease probes were as diagrammed in panel A. In line 4 the predicted protected radiolabeled probe fragment would be 72 or 205 nucleotides, depending on whether or not S1 nuclease was able to cleave the probe DNA opposite of the looped out intron on a hybridized but unspliced RNA. As we have shown previously (54) and verified here (Fig. 4), the probe was also resistant to S1 nuclease cleavage opposite the RNA loop (solid loop) formed by the use of the pΔ5Sβ3 or pΔ5Scβ3 probes. Abbreviations: Ex, exon; E2, exon 2; E3, exon 3; In, intron; nt, nucleotides. Other symbols were as defined above for panel A.

genes in which an intron-containing portion of a structural gene that is normally transcribed by RNA polymerase II was placed adjacent to the *Xenopus* 5S promoter. For this, we selected an intron-containing segment of the chicken β3 tubulin gene (beginning near the 5′ end of exon 2 and terminating near the 3′ end of exon 3). This region was advantageous because it is fairly short (facilitating S1 nuclease analysis) and it is devoid of T clusters, thereby permitting its transcription by RNA polymerase III. The resultant constructs are illustrated in Fig. 3A. Plasmid p5Sβ3 contains the promoter for 5S RNA transcription (residues −48 to +115) and the intron-containing β-tubulin segment. Plasmid pΔ5Sβ3 is a strictly analogous construct from which the internal polymerase III promoter sequences have been deleted; it contains the 5S sequences from residue −48 to +41. Finally, plasmid pΔ5Scβ3 is analogous to pΔ5Sβ3, except that it contains the cDNA (intronless) version of the β-tubulin region.

**Splicing in human 293 cells.** To demonstrate initially that normal polymerase II-catalyzed transcripts containing the chicken β3 tubulin intron were accurately and efficiently spliced after transfection of plasmid DNA, the authentic chicken β3 tubulin gene (contained on plasmid pβG3) was transiently transfected into human 293 cells. The resultant RNAs were analyzed by S1 nuclease mapping by using a probe prepared from the intron-containing plasmid pΔ5Sβ3 and labeled 69 nucleotides downstream of the acceptor splice site (Fig. 3A). Although the probe was labeled 69 nucleotides downstream of the acceptor splice site, a 72-base protected fragment was predicted because the trinucleotide CAG occurs immediately upstream of the acceptor splice site, as well as immediately upstream of the 5′ donor splice site. RNA transcribed from pβG3 protected only the expected 72-base fragment, which represents the distance from the site at which the probe was labeled to the 3′ splice junction (Fig. 4, lane 1). These data indicate that RNA transcribed from the authentic chicken β3 tubulin gene-containing plasmid is efficiently spliced after transfection into human 293 cells.

**RNAs transcribed from transfected chimeric 5S-tubulin genes are not spliced.** To determine whether the RNA derived from the chimeric 5S-β3 tubulin gene construct p5Sβ3 (Fig. 3A) could be spliced, we transfected p5Sβ3 plasmid DNA into human 293 cells. Total cellular RNA was recovered at 18 h posttransfection and assayed by S1 nuclease protection analysis. By using the intron-containing pΔ5Sβ3 probe (end labeled 69 bases 3′ to the β3 intron; Fig. 3A), some transcripts from cells transfected with p5Sβ3 were seen to be spliced, since an S1 nuclease-resistant fragment of 72 bases (extending from the labeled end of the probe to the 3′ side of the intron) was protected (Fig. 4, lane 2; see also Fig. 3B, line 1). However, there was also a prominent 282-base protected fragment which is indicative of RNAs that initiated at the authentic 5S start site and that were not spliced (Fig. 4, lane 2; see also Fig. 3B, line 2). Moreover, there was a 552-base fragment that represented unspliced RNAs that originated upstream of the 5S initiation site. In a parallel experiment, we also transfected plasmid pΔ5Sβ3, which lacks the sequences that are essential for promoting polymerase III transcription. By use of the intron-containing probe, analysis of RNAs derived from this transfection (Fig. 4, lane 3) revealed no 282-base protected fragment, which would have been diagnostic of RNA molecules which began at residue 1 of the 5S initiation site in pΔ5Sβ3. As expected, RNA from untransfected cells (Fig. 4, lane 4) also did not generate protected fragments.

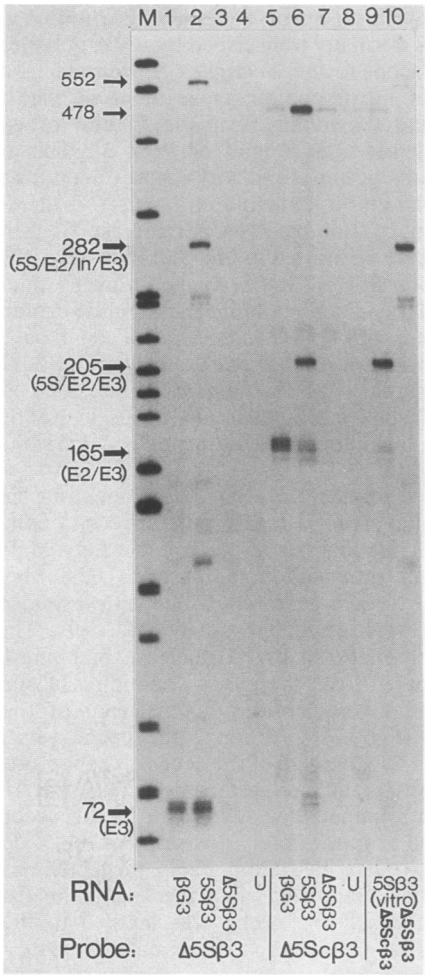


FIG. 4. S1 nuclease analysis of RNAs from transfected plasmids pβG3, p5Sβ3, and pΔ5Sβ3. Human 293 cells were transfected with the indicated plasmids, and RNA was isolated at 18 h posttransfection. The S1 nuclease probes from pΔ5Sβ3 or pΔ5Scβ3 (Fig. 3A) were 5′ end labeled at the unique *Ava*I site 69 nucleotides downstream of the β3 tubulin intron region. The autoradiogram represents the S1 nuclease-resistant fragments of hybrids between the pΔ5Sβ3 probe (lanes 1 to 4 and 10) or the pΔ5Scβ3 probe (lanes 5 to 9) and RNA from cells transfected with the indicated plasmid. U indicates untransfected cell RNA (lanes 4 and 8). Unspliced RNA transcribed in vitro from the p5Sβ3 template assayed with the pΔ5Scβ3 or the pΔ5Sβ3 probe is shown in lanes 9 and 10, respectively. Note that a bona fide unspliced RNA which is synthesized in vitro protects both spliced (lane 9) and unspliced (lane 10) S1 nuclease probes to the transcription initiation site, confirming our earlier observations (54) which documented that cDNA S1 nuclease probes that hybridize to unspliced RNAs are resistant to cleavage at the splice juncture. M indicates size markers. Numbers (in nucleotides) and arrows to the left denote positions of predicted S1-resistant fragments. The 552- and 478-base fragments observed in lanes 1 to 4 and 5 to 9, respectively, represent any residual undigested intron-containing probe molecules plus all upstream-initiated RNAs. Abbreviations: E2, exon 2; E3, exon 3; In, intron.

Unfortunately, in these experiments it could not be distinguished whether the spliced RNAs derived from p5Sβ3 (Fig. 4, lane 2) were initiated at the 5S initiation site (and presumably driven by polymerase III). This is because even though S1 nuclease analysis with an intron-containing probe (Fig. 4, lane 2; see also Fig. 3B, lines 1 and 2) can demonstrate that there is spliced RNA present, it cannot determine the site(s) at which the spliced transcripts are initiated. Since RNAs from fortuitous initiation sites (the 552-base species in Fig. 4, lane 2) would very likely be transcribed by RNA polymerase II (27), these RNAs would be expected to be spliced.

An alternate method of analyzing the splicing of the p5Sβ3 transcript would use an intronless S1 nuclease probe that is also complementary to the promoter region of the template (Fig. 4, lane 6). While this protocol should, in principle, allow the simultaneous detection of splicing and transcription initiation sites, in practice this cannot be done. Unspliced and spliced RNAs yielded a common 205-base protected fragment (Fig. 3B, lines 3 and 4; Fig. 4, lane 9). This effect, which we have recently documented in detail (54), occurs because cDNA S1 nuclease probes that are hybridized to unspliced RNA are resistant to cleavage at the splice junction (where only the intron RNA, but not the probe DNA, is looped out in a single-stranded conformation). Hence, one cannot determine whether the 205-base protected fragment (Fig. 4, lane 6) results from spliced or unspliced RNAs that initiated at the 5S start site.

To detect spliced RNAs and to map simultaneously their transcription initiation site(s), we developed an alternative approach in which an RNase H digestion step prior to a standard S1 nuclease analysis was used (54). The overall S1 nuclease and RNase H protocol is outlined in Fig. 5A. A DNA fragment that was homologous to the β3 intron sequence was hybridized with the RNA sample of interest. Following degradation of the RNA within the heteroduplexed region by treatment with RNase H, the remaining RNA was deproteinized and hybridized to an excess amount of the intronless S1 nuclease probe. If all the unspliced RNA molecules were cleaved in the RNase H step (Fig. 5A, unspliced RNA), the only RNA species that could protect the full-length cDNA probe in a bimolecular hybridization reaction were spliced molecules (Fig. 5A, spliced RNA). In the actual analysis, for the RNase H step we used an 83-bp fragment (generated by *Rsa*I digestion of p5Sβ3) that contained virtually the entire β3 intron plus nine nucleotides of the adjacent 3′ exon; this fragment only hybridized with the intron-containing RNA. For S1 nuclease analysis, we prepared the probe from pΔ5Scβ3 so that we could also assess any transcripts that may have been directed by the pΔ5Sβ3 template.

The results of a typical experiment with this RNase H and S1 nuclease method to evaluate unambiguously the splicing of transcripts driven from p5Sβ3 following transient transfection into human 293 cells are shown in Fig. 5B. p5Sβ3-derived RNAs isolated at 18 h posttransfection were analyzed (Fig. 5B, lanes 1 to 3). As expected, S1 nuclease analysis of RNAs that were not treated with RNase H or that were treated with RNase H in the absence of the intron oligonucleotide yielded the 205-base species representing both spliced and unspliced RNAs (Fig. 5B, lanes 1 and 2; see also Fig. 3B, lines 3 and 4). However, when S1 nuclease analysis was performed on RNA that was hybridized to the 83-base DNA fragment and that was treated with RNase H (Fig. 5B, lane 3), the 205-base fragment that is diagnostic of spliced RNA was quantitatively removed, and a new 65-base species was generated. This 65-base protected species was the expected product from S1 nuclease analysis of unspliced RNA following RNase H treatment (Fig. 5A). The absence of the 205-nucleotide band in RNA from cells transfected with pΔ5Sβ3 (Fig. 5B, lane 4) or from mock-transfected cells (Fig. 5B, lane 8) confirmed that molecules which mapped to the 5S initiation site in p5Sβ3 were authentic polymerase III

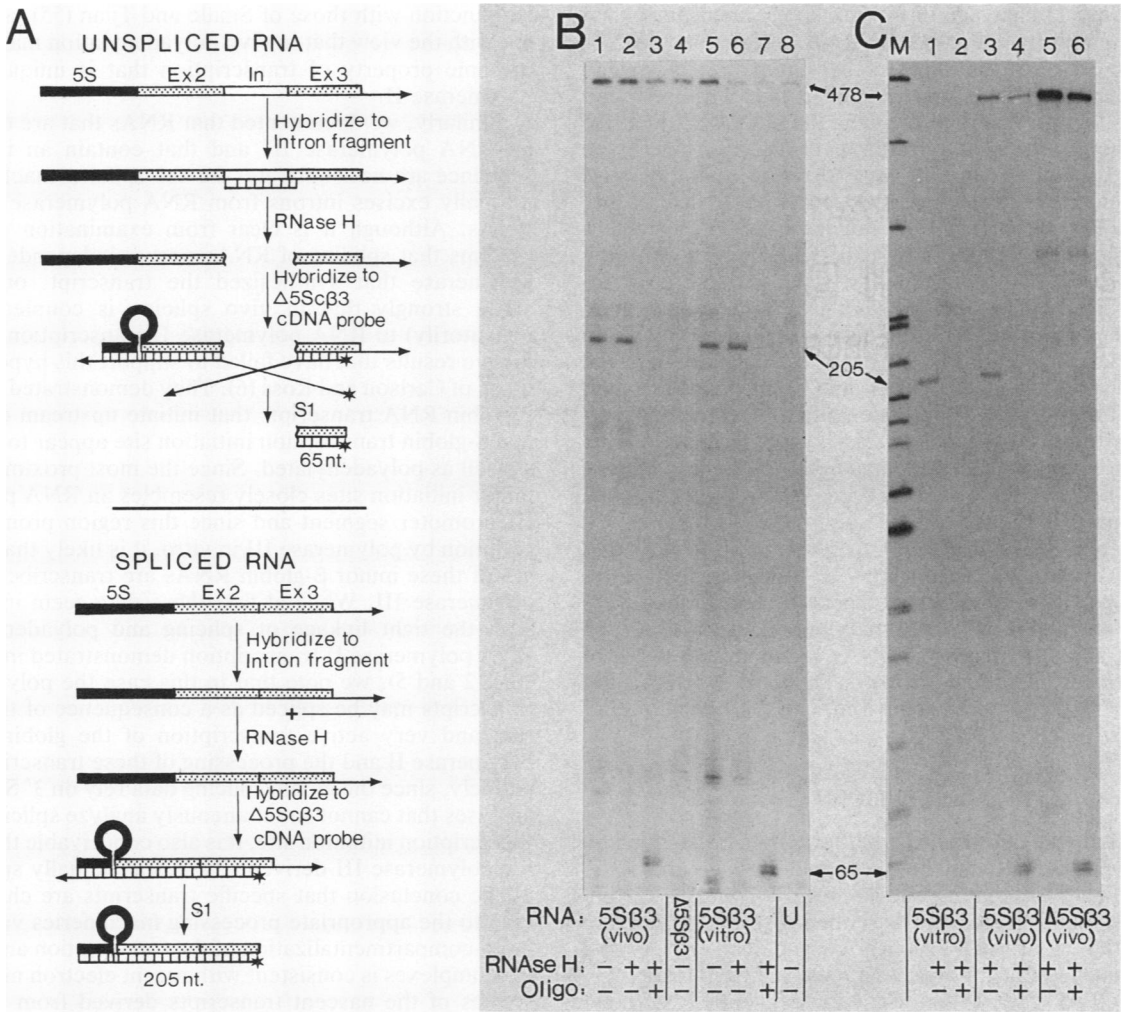


FIG. 5. Splicing of RNAs from p5Sβ3 by using the RNase H and S1 nuclease method. (A) Schematic of the RNase H and S1 nuclease method. Unspliced or spliced p5Sβ3 RNAs were hybridized to a DNA fragment that was homologous to the β3 intronic region and then were treated with RNase H. Only unspliced RNA molecules were affected. Subsequent conventional S1 nuclease analysis of the remaining RNAs by using an excess of intronless S1 nuclease probe from pΔ5Scβ3 yielded the protected species shown at the bottom of each diagram for unspliced and spliced RNA. nt, nucleotide. (B) Assessment of splicing of p5Sβ3 RNAs in transfected cells. Human 293 cells were transfected with the intron-containing plasmids p5Sβ3 (lanes 1 to 3) or pΔ5Sβ3 (lane 4), and total RNA was isolated at 18 h posttransfection. Fully unspliced RNA was also generated by in vitro transcription of p5Sβ3 (lanes 5 to 7). Untransfected cell RNA was analyzed in parallel (lane 8). For S1 nuclease analysis, the intronless pΔ5Scβ3 probe was used. Prior to S1 nuclease analysis RNAs were either hybridized (+) or not hybridized (−) to the intron-containing oligonucleotide and were either treated (+) or not treated (−) with RNase H, as indicated. (C) Analysis of splicing efficiency of p5Sβ3 RNAs at 44 h posttransfection. Duplicate dishes of human 293 cells were transfected with the chimeric intron-containing plasmid p5Sβ3 (lanes 3 and 4) or with the promoterless plasmid pΔ5Sβ3 (lanes 5 and 6), and RNA was isolated at 44 h posttransfection. Fully unspliced RNAs transcribed in vitro from the p5Sβ3 template were analyzed in parallel (lanes 1 and 2). RNAs were analyzed as described above for panel B. The 478-base protected fragment represents undigested probe molecules and probe protected following hybridization to upstream-initiated RNAs. M indicates molecular weight standards. Numbers between panels B and C are the number of nucleotides.

initiation events. As a final control, we verified that the RNase H-directed cleavage assay was performed quantitatively, by parallel analysis of bona fide unspliced p5Sβ3 RNAs synthesized in vitro (Fig. 5B, lanes 5 to 7). Quantitative RNase H cleavage was obtained (Fig. 5B, lane 7). We thus conclude that the majority of the polymerase III-initiated p5Sβ3 RNAs in transfected human 293 cells remain unspliced, despite the fact (described above; see also Fig. 4, lane 1) that the β3 intron was efficiently spliced when it was transcribed by RNA polymerase II.

**Spliced RNAs from p5Sβ3 do not accumulate at late times posttransfection.** Since the results of the experiment described above demonstrated that virtually all p5Sβ3 RNAs were unspliced at 18 h posttransfection, we reasoned that if spliced RNAs were more stable than unspliced ones, spliced RNAs would accumulate at later times. Consequently, p5Sβ3 and pΔ5Sβ3 were transfected into parallel dishes of human 293 cells, and RNAs were isolated at late times posttransfection (44 h). Each RNA sample was treated with RNase H, with and without initial hybridization to the 83-base intron fragment, and each was then hybridized to the intronless (pΔ5Scβ3) S1 nuclease probe. Autoradiograms of the resultant S1 nuclease-resistant fragments are shown in Fig. 5C. The control reactions showed (Fig. 5C, lanes 1 and

2) that RNase H cleavage of in vitro-transcribed, unspliced RNA is quantitative. For p5Sβ3 RNAs, the expected 205-base fragment corresponding to the summation of spliced and unspliced molecules that initiated at the 5S transcription initiation site was observed by using the cDNA S1 nuclease probe without prior RNase H treatment (Fig. 5C, lane 3; see also Fig. 3B, lines 3 and 4). Hybridization to the 83-base intron fragment followed by treatment with RNase H and analysis with the pΔ5Sβ3 S1 nuclease probe yielded a virtually quantitative loss of this 205-base species. On very long exposures (data not shown) a weak residual 205-base signal was observable, but it was not greater in intensity than that obtained from pΔ5Sβ3, the plasmid that was deleted in the essential 5S promoter sequences. We conclude that the very small amount of RNase H and S1 nuclease-resistant RNAs that map to the 5S initiation site are probably generated by fortuitous RNA polymerase II transcription events and that in vivo very few (perhaps none) of the polymerase III-derived p5Sβ3 RNAs are spliced either at early or late times posttransfection.

We also note that our inability to detect p5Sβ3 RNAs that were spliced cannot be due simply to transcript instability. After the addition of actinomycin D to levels that were sufficient to inhibit all three polymerases, we observed significant amounts of p5Sβ3 RNAs remaining after 2 h of drug treatment (data not shown). Thus, these RNAs are reasonably stable, at least compared with the typical rate of the splicing reaction (23).

## DISCUSSION

In this study we determined whether RNAs transcribed by RNA polymerase III can be processed through the RNA maturation pathway that is used by RNA polymerase II transcripts. We found that when transcribed by RNA polymerase III (from the 5S or Ad2 VA1 RNA promoters), RNAs that contain sequences that are necessary and sufficient to direct 3′ cleavage and polyadenylation when transcribed by RNA polymerase II are not substrates for this processing event.

The inability to detect poly(A)$^+$ RNAs transcribed in vivo by polymerase III from p5SpA (Fig. 1) or pVATK (Fig. 2) complements results of an earlier report by Smale and Tjian (55) involving RNA polymerase I-driven chimeric RNAs. In those experiments, plasmids containing the HSV *tk* gene fused to human rDNA promoter regions were transfected into COS cells, and transcripts that initiated at the normal rRNA start site were found not to be poly(A)$^+$. However, another class of *tk* RNA transcript, which probably initiated at a cryptic polymerase II promoter site within the rDNA, accumulated in the cytoplasm and was poly(A)$^+$, demonstrating that the plasmid contained the sequences sufficient to direct polyadenylation.

These in vivo results stand in contrast to results of several in vitro studies in which it has been documented that accurate cleavage and polyadenylation of purified precursor RNAs can be unlinked from polymerase II transcription in nuclear extracts (20, 34). Moreover, our results directly contradict those of Lewis and Manley (25), who concluded that transfection of a plasmid that is identical to pVATK yielded poly(A)$^+$ polymerase III-driven RNAs. Since the data of Lewis and Manley (25) relied heavily on 3′ S1 nuclease analyses that were inherently incapable of detecting the corresponding transcription initiation site, we believe that the poly(A)$^+$ species identified by Lewis and Manley (25) represent polymerase II-catalyzed RNAs that arose from fortuitous transcription initiation sites. Our results, in conjunction with those of Smale and Tjian (55), are consistent with the view that in vivo polyadenylation may be linked to some property of transcription that is unique to RNA polymerase II.

Similarly, we documented that RNAs that are transcribed by RNA polymerase III and that contain an intervening sequence are not substrates for the splicing machinery that normally excises introns from RNA polymerase II-derived RNAs. Although it is clear from examination of in vitro systems that splicing of RNAs occurs independently of the polymerase that synthesized the transcript, our findings argue strongly that in vivo splicing is coupled (perhaps obligatorily) to RNA polymerase II transcription. The only in vivo results that have failed to support this hypothesis are those of Carlson and Ross (6). They demonstrated that minor β-globin RNA transcripts that initiate upstream of the normal β-globin transcription initiation site appear to be spliced as well as polyadenylated. Since the most proximal of these minor initiation sites closely resembles an RNA polymerase III promoter segment and since this region promotes transcription by polymerase III in vitro, it is likely that much (or all) of these minor β-globin RNAs are transcribed by RNA polymerase III. While at first these data seem inconsistent with the tight linkage of splicing and polyadenylation to RNA polymerase II transcription demonstrated in this study (Fig. 2 and 5), we note that in this case the polymerase III transcripts may be spliced as a consequence of the concurrent and very active transcription of the globin locus by polymerase II and the processing of these transcripts. Alternatively, since the globin splicing data rely on 3′ S1 nuclease analyses that cannot simultaneously analyze splicing and the transcription initiation site, it is also conceivable that none of the polymerase III-derived RNAs was actually spliced.

The conclusion that specific transcripts are channeled in vivo to the appropriate processing machineries via coupling or co-compartmentalization of the transcription and processing complexes is consistent with recent electron microscopic studies of the nascent transcripts derived from two major *Drosophila melanogaster* chorion genes (37; A. Beyer, personal communication). In these studies distinct ribonucleoprotein particles at the splice junctions of the introns of these transcripts were observed. On the more mature transcripts, however, a single larger particle was observed. Analysis of the transcripts on a number of other *D. melanogaster* genes indicates that this large particle is probably a result of the coalescence of the two smaller particles just prior to intron excision. These data strongly suggest that at least some nascent polymerase II mRNAs are associated with processing complexes and are processed cotranscriptionally.

How is coupling of transcription by polymerase II and subsequent processing achieved? One obvious feature that distinguishes polymerase II- and polymerase III-derived RNAs is the presence of the 5′ cap on polymerase II RNAs. While 5′ caps have an important facilitating role in the initiation of translation (2, 14), they may also be involved in splicing. For instance, results of the studies of Patzelt et al. (40) indicate that the assembly of pre-mRNA splicing complexes (spliceosomes) is cap dependent in vitro. While Krainer et al. (23) have concluded that splicing in HeLa nuclear extracts does not require that the RNA substrate contain a 5′-terminal capped structure, Edery and Sonenberg (12) and Konarska et al. (21) have reported that only capped RNAs are efficiently spliced in HeLa nuclear or HeLa whole-cell extracts, respectively. In light of these data, we propose that one simple explanation for our inability to detect in vivo spliced or poly(A)$^+$ RNAs that are

transcribed by RNA polymerase III is that these transcripts are not capped. In particular, if 5'm$^7$G cap addition were obligatorily linked to polymerase II transcription (as it appears to be), the specificity of the remaining processing events could then be directed through cap recognition.

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