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Simian Virus 40 Late Transcripts Lacking Excisable Intervening Sequences Are Defective in Both Stability in the Nucleus and Transport to the Cytoplasm

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Little or no simian virus 40 (SV40) late mRNA accumulates in the cytoplasm when the primary transcript lacks an excisable intervening sequence. To begin to understand why, we analyzed the synthesis, processing, transport, and stability of SV40 late transcripts accumulated in the nucleus and cytoplasm of monkey cells cotransfected with the DNAs of wild-type and mutants of SV40 lacking precisely various introns. The data from these experiments indicated that (i) the presence of excisable intervening sequences in SV40 late transcripts is necessary for efficient accumulation in the cytoplasm of any of the SV40 late RNA species and (ii) SV40 late transcripts lacking excisable intervening sequences are defective in both stability in the nucleus and transport to the cytoplasm but not in stability in the cytoplasm. We hypothesize that SV40 late transcripts need to be processed via a pathway that couples stabilization of the primary transcript within the nucleus, excision of intervening sequences, proper 5′- and 3′-end formation, and transport to the cytoplasm.

Most protein-coding genes in higher eucaryotes contain intervening sequences which are removed from primary transcripts by RNA splicing. The use of cell-free splicing systems has led to rapid advancement in our knowledge concerning the mechanism of splicing of pre-mRNAs (for reviews, see references 32, 38, and 40). However, little is known concerning the role(s) played by RNA splicing in mRNA biogenesis other than that of the precise excision of intervening sequences from primary transcripts. Furthermore, the issue of whether splicing is necessary for efficient accumulation in the cytoplasm of mRNAs of intron-containing genes remains unresolved.

Early work with simian virus 40 (SV40) recombinants indicated that splicing plays an essential role in the biogenesis of at least some mRNAs. Cells infected with SV40 mutants encoding various combinations of splice sites from SV40 and the mouse β-globin gene accumulate stable hybrid mRNAs only if at least one excisable intervening sequence is present in the transcript (22). Similarly, cells infected with an SV40 mutant lacking the intron excised in synthesis of the major late 16S mRNA species fail to accumulate viral late mRNA; however, viral late mRNA accumulates when a heterologous intron is inserted into the mutant genome (20, 21). The need for excisable intervening sequences for efficient mRNA accumulation has been demonstrated for several cellular genes as well, including those encoding rabbit β-globin (7), mouse dihydrofolate reductase (7, 13), rat growth hormone (6), immunoglobulin μ (37), and maize alcohol dehydrogenase-1 (8).

On the other hand, accumulation in the cytoplasm without splicing of RNAs transcribed from genes that normally contain introns has also been reported. For example, some unspliced SV40 late 19S RNA is detectable in the cytoplasm of cells infected with either wild-type SV40 (14) or an SV40 late leader region deletion mutant (15). Cells infected with an adenovirus early region E1a 3′ splice site mutant accumulate unspliced E1a region mRNA in the cytoplasm (9). A polyomavirus mutant lacking the intron excised during synthesis of the mRNA species that encodes middle T antigen transforms rat cells, albeit inefficiently (44). Recently, stable mRNA encoding thymidine kinase was shown to exist in the cytoplasm of mouse L cells that had been stably transformed with cloned cDNA of chicken thymidine kinase mRNA (19). These findings indicate that splicing is not always essential for at least some accumulation in the cytoplasm of mRNAs of genes that normally contain introns.

The previously reported studies with SV40 recombinants showed that the defect of transcripts lacking excisable intervening sequences is posttranscriptional in nature but did not determine at which of the numerous posttranscriptional steps in mRNA biogenesis the defect occurs (21, 22). To answer this question, we analyzed the synthesis, processing, transport, and stability of SV40 late transcripts made in monkey cells transfected with SV40 mutants which lack precisely the sequences excised in the synthesis of each of the numerous spliced late mRNA species. We show here that (i) SV40 late mRNAs accumulate efficiently in the cytoplasm of transfected cells only if their precursors contain excisable intervening sequences, and (ii) the presence of excisable intervening sequences in SV40 late transcripts is necessary both for initial stabilization of the transcripts in the nucleus and for their transport to the cytoplasm.

MATERIALS AND METHODS

Cells, viral DNAs, and transfection. African green monkey kidney cell lines CV-1P, CV-1PD, and BSC-1 were grown in Dulbecco modified Eagle medium (DMEM) supplemented with 5% fetal bovine serum as described previously (35) and were used interchangeably for transfections. Mutant viral DNAs grown as recombinant plasmids in *Escherichia coli* DH1 cells were prepared by excision from their cloning vector by cleavage with *Eco*RI restriction endonuclease, ligation with T4 DNA ligase at 2.5 μg of DNA per ml to form monomer circles, concentration by ultrafiltration with Centricon-30 units as recommended by the manufacturer (Amicon Corp.), extraction with phenol-chloroform, and precip-

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TABLE 1. Summary of SV40 mutants used

Plasmid[a]	RNA species[b]	Nucleotide sequence(s) deleted (inserted)	Reference
pSVS(WT830)	SVS		12
pSVSM	SVSM (marked)	1629–1635, (770[CGGATCCG]771)[c]	This paper
pSV1761	SVΔ294	295–434	17
pSV1762	19SΔ294	295–557	17
pSV1763	19SΔ373	374–557	17
pSV1764	19SΔ526	527–557	17
pSV1765	19SΔDS	295–434, 527–557	17
pSV1766	16SΔ526	527–1462	17
pSV1767	16SΔDS	295–434, 527–1462	17
pSV1769	16SΔDSM (marked)	295–434, 527–1462, 1627–1636	This paper

[a] Each plasmid is identified by its official mutant number (1761–1769). Throughout this report, we use the names of RNA species when referring to the mutants.
[b] Mutants 1761 through 1767 lack precisely the intron(s) excised in the synthesis of the RNA species depicted in Fig. 1.
[c] Mutant pSVSM contains an insertion of the indicated *Bam*HI linker DNA between SV40 nt 770 and 771, as well as the indicated deletion of SV40 nt 1629 to 1635, inclusive.

itation with ethanol. DNA transfections were performed by a modification of the DEAE-dextran protocol of McCutchan and Pagano (33) essentially as described previously (17). Briefly, viral DNA was diluted into DMEM containing 500 μg of DEAE-dextran (molecular weight, 2×10^6; Pharmacia) per ml and 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.1) to a final concentration of 1.0 μg/ml. Confluent monolayers of cells were subcultured 1:5 1 day before transfection, washed twice with DMEM containing 20 mM HEPES (pH 7.1), and incubated with 1.0 ml of DNA solution per 100-mm-diameter dish for 45 min in a CO_2 incubator at 37°C. CV-1P cells were incubated with DEAE-dextran for 15 min, since incubation for longer periods is toxic to these cells. The DNA solution was removed and replaced with DMEM containing 20 mM HEPES (pH 7.1) and 100 μM chloroquine, and the cells were incubated for 2 to 4 h in an incubator containing 5% CO_2 at 37°C. Afterward, the cells were washed twice with DMEM, fed with DMEM plus 2% fetal bovine serum, and incubated at 37°C in a CO_2 incubator until harvesting.

Recombinant plasmids. Standard recombinant DNA techniques were used in the construction of plasmids (31). The starting plasmid used in the construction of all viral mutants was either pSVS, a plasmid in which the DNA of the SV40 wild-type (WT) strain 830 was inserted via its *Eco*RI site into a pBR322-derived vector (12), or pSVS(ΔA), a derivative of pSVS which lacks the *Acc*I site within the vector sequences (17). Construction of plasmids pSV1761 through pSV1767 (i.e, the cDNA mutants) has been described previously (17). The sequences they lack are summarized in Table 1. Plasmid pSVSM (i.e., marked WT), obtained from P. Good, contains two alterations from pSVS: (i) a 7-base-pair (bp) deletion of SV40 nucleotides (nt) 1629 to 1635, inclusive, to allow the RNA made from this marked-WT DNA to be readily distinguishable by S1 mapping from RNA made from the cDNA mutants and (ii) an insertion of *Bam*HI linker DNA (CGGATCCG) between SV40 nt 770 and 771 to enable us to distinguish readily, by digestion with *Bam*HI, replicated marked-WT DNA from replicated cDNA mutant DNA. The first of these mutations was introduced by cleavage with *Acc*I, treatment with S1 nuclease, and ligation with T4 ligase. The *Bam*HI insertion was subsequently introduced by cleavage with *Eco*RV and ligation in the presence of *Bam*HI linker DNA. Plasmid pSV1769(16SΔDSM) was constructed by digestion of pSV1767(16SΔDS) with *Acc*I, treatment with S1 nuclease, incubation with T4 DNA polymerase in the presence of all four deoxynucleoside triphosphates, and ligation with T4 DNA ligase. This plasmid differs from pSV1767(16SΔDS) by lack of SV40 nt 1627 to 1636, inclusive.

Plasmid pLZ103, obtained from P. Good and used for making S1 mapping probes, contains the late region of SV40 from nt 1261 through 1786 (an *Eco*RI site) between the *Sal*I and *Eco*RI sites of pUC18. Plasmid p14Tβ17 (39), containing the 14-kilobase *Eco*RI fragment of the human β-actin gene cloned into the *Eco*RI site of pBR322, was obtained from L. Kedes. Plasmid pP7 (41), containing LINE-1 DNA (i.e., DNA of the major primate family of highly repeated, long interspersed sequences), was obtained from M. Singer.

RNA purification. Nuclear and cytoplasmic RNAs were fractionated from transfected monkey cells by disruption of the cells in Tris-buffered saline containing 0.5% Nonidet P-40 (Sigma) and 5 mM vanadyl ribonucleoside complex (Bethesda Research Laboratories, Inc.). After incubation on ice for 5 min, the nuclei were pelleted by centrifugation and the supernatants (i.e., cytoplasm) were transferred to new tubes. To minimize contamination of nuclear RNA with cytoplasmic RNA, the nuclear pellets were washed with Tris-buffered saline containing 0.5% Nonidet P-40. Afterward, both nuclear and cytoplasmic fractions were incubated for 45 min at 37°C with proteinase K (500 μg/ml), extracted twice with phenol-chloroform-isoamyl alcohol, precipitated twice with ethanol, and treated with RNase-free DNase I (DPRF grade; Organon Teknika) as described previously (25) before mapping with S1 nuclease. Contamination of cytoplasmic RNA by nuclear RNA was determined by either of two assays. In the first, Southern blot analysis was performed as described previously (24) to quantify the percentage of the replicated (i.e., *Dpn*I-resistant) SV40 DNA present in cytoplasmic fractions before DNase I treatment. In the second assay, RNA slot blot analysis was performed with the 320-bp *Bgl*II-to-*Taq*I fragment of pP7 (i.e., LINE-1 DNA) (41) as the probe. Skowronski and Singer (41) have shown that LINE-1 RNA, made by RNA polymerase II, is localized almost exclusively to the nuclei of mammalian cells (41). These two assays yielded essentially identical results (data not shown).

S1 nuclease mapping. S1 nuclease protection experiments were performed as described previously (3). The probe for quantifying the relative amounts of late viral RNAs was made by cleavage of plasmid pLZ103 with *Eco*RI (SV40 nt 1786), incubation with calf alkaline intestinal phosphatase (Boehringer Mannheim Biochemicals), cleavage at the *Nde*I site within the vector sequences, and agarose gel purification of the smaller fragment. The probe was 5′ end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase (31). The hybrids

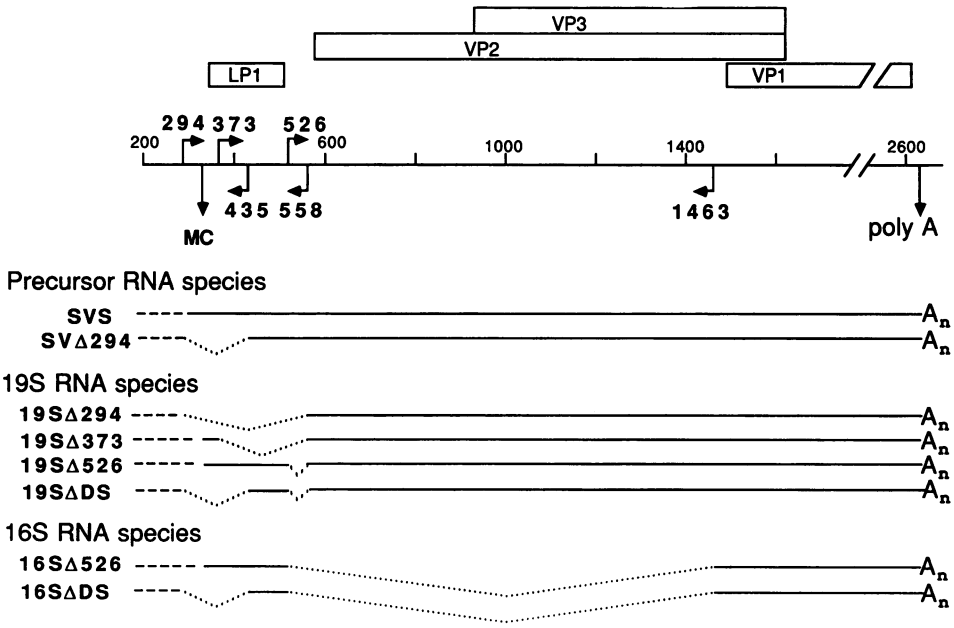

FIG. 1. Structures of the SV40 late RNA species. The late region of SV40 is shown at the top, and the nucleotide residues are given in the SV numbering system (43). The open reading frames that encode the late proteins LP1, VP1, VP2, and VP3 are indicated by boxes. The dashed lines indicate the heterogeneity in the locations of the 5′ ends of the RNAs. MC indicates the location of the major cap site at nt 325 to which 70 to 80% of the 5′ ends of the WT late strand viral mRNA molecules map. The right- and leftward arrows indicate the locations of the three 5′ splice sites at nt 294, 373, and 526 and the three 3′ splice sites at nt 435, 558, and 1463, respectively, encoded by the late transcripts. These transcripts are processed by using various combinations of these splice sites, resulting in the synthesis of two classes of viral mRNAs 19S and 16S in size. RNA species SVΔ294 is classified as a precursor, since it can be processed further by excision of nt 527 to 557 or 527 to 1462 (17). The intervening sequences excised during synthesis of each species are indicated by dotted lines. A_n, polyadenylated 3′ end.

were treated with S1 nuclease at 37°C for 1 h in 200 mM NaCl–30 mM sodium acetate (pH 5.5)–1 mM $ZnSO_4$–10 μg of denatured calf thymus DNA per ml. The resulting protected DNA fragments were fractionated by electrophoresis through 8 M urea–5% polyacrylamide gels.

Nuclear run-on transcription assays. Nuclear run-on transcription assays were performed essentially as described by Linial et al. (30) and Greenberg and Ziff (18). At 42 h after transfection, nuclei were prepared from BSC-1 cells transfected with each of the mutants indicated, suspended at a concentration of 2×10^8/ml in nuclear freezing buffer (40% glycerol, 50 mM Tris hydrochloride [pH 8.3], 5 mM $MgCl_2$, 0.1 mM EDTA), and incubated for 30 min at 30°C with [α-^{32}P]UTP (1.5 mCi/ml, 3,000 Ci/mmol). Afterward, each nuclear RNA sample was purified, partially degraded by treatment with 0.2 N NaOH for 10 min at 4°C, and incubated under hybridization conditions with nitrocellulose filters containing 5 μg of denatured, immobilized DNAs of pBR322, pSVS (i.e., SV40), and p14Tβ17 (i.e., human β-actin) per slot. Since early strand transcription at late times in the lytic cycle of infection by SV40 accounts for only 5% of total viral transcription both in nuclear run-on assays (16) and in vivo (29), a double-stranded, rather than SV40 early strand-specific DNA was used for convenience to make filters. Each filter was hybridized with the radiolabeled run-on product at equal counts per minute. After hybridization, each filter was washed for 1 h in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 65°C, incubated at 37°C in 2× SSC with RNase A (10 μg/ml) for 30 min, washed in 2× SSC at 37°C for 1 h, and subjected to autoradiography.

RESULTS

SV40 late mRNAs accumulate efficiently in the cytoplasm only if their precursors contain excisable intervening sequences. Gruss et al. (21) showed that cells transfected with a mutant of SV40 lacking the sequences excised in the synthesis of the major late 16S mRNA of SV40 (RNA species 16SΔ526 in Fig. 1) fail to accumulate this RNA species in the cytoplasm because of a posttranscriptional defect in mRNA biogenesis. Plausible reasons for this finding include the following: (i) the sequences deleted in the 16S cDNA mutant encode elements needed for stabilization of SV40 late transcripts, (ii) excision of an intervening sequence is a prerequisite for transport of the RNA from the nucleus to the cytoplasm, and (iii) excision of an intervening sequence is required for stabilization of the RNA in the cytoplasm. The studies presented below were designed to test these and other possibilities.

SV40 late strand primary transcripts encode three 3′ splice sites at nt 435, 558, and 1463 and two 5′ splice sites at nt 373 and 526. When transcripts initiate upstream of nt 294, they encode an additional 5′ splice site at nt 294 as well (Fig. 1). These transcripts are alternatively processed not only by splicing out of nt 527 to 1462 to yield the major late 16S RNA species 16SΔ526 but also by splicing out of other intervening sequences to yield a variety of other spliced RNA species (see Fig. 1 for a summary and reference 43 for a review). We have previously described the isolation of a set of SV40 mutants lacking precisely the sequences excised in the synthesis of each of the spliced SV40 late RNA species (17; see Fig. 1 and Table 1 for a summary of their structures).

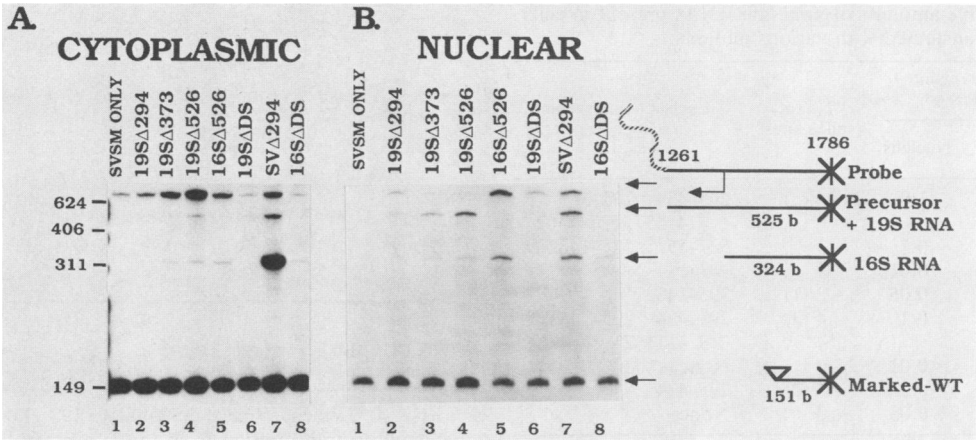

FIG. 2. S1 mapping analysis of the nuclear and cytoplasmic RNAs accumulated in monkey cells cotransfected at a 2:1 molar ratio with the indicated SV40 late cDNA mutants and pSVSM (i.e, marked WT), respectively. The sequences deleted in each mutant are indicated in Table 1. Nuclear and cytoplasmic RNAs purified from CV-1PD cells 40 h after transfection were analyzed by S1 mapping with an *Eco*RI (nt 1786)-cut, 5′-end-labeled probe made from pLZ103. Hybridization was performed at 50°C. Afterward, the hybrids were treated with S1 nuclease at 37°C for 1 h and the resulting protected fragments were electrophoresed in a 5% polyacrylamide gel containing 8 M urea. Each lane represents the analysis of (A) cytoplasmic RNA from 1×10^5 cells or (B) nuclear RNA from 4×10^5 cells. The diagrams on the right identify the S1 nuclease-protected fragments produced from each RNA species. The location of the nt 1463 3′ splice site used in the synthesis of 16S RNA is indicated on the probe by the leftward arrow. The pUC18 sequences at the 3′ end of the probe are indicated by the broken squiggly line. The location of the 7-bp deletion present in the marked WT is indicated by a small triangle. The size markers were *Msp*I-cut pBR322 DNA; the locations of some of them are indicated on the left by their molecular sizes in bases (b).

Primary transcripts synthesized from cells transfected with these cDNA mutants are identical in sequence to the corresponding spliced mRNAs from which the mutants were constructed.

The first question we asked was whether the previously reported failure of cells transfected with cDNA mutant 16SΔ526 (i.e., a mutant identical in sequence to *dl*2350 of reference 21) to accumulate viral late RNA in the cytoplasm is a general attribute of transcripts synthesized from SV40 late cDNA mutants or peculiar to the fact that transcripts synthesized from this mutant lack the 936 bases of the viral sequence from nt 527 through nt 1462, inclusive. To answer this question, monkey cells were cotransfected at a 2:1 molar ratio with each of the cDNA mutants and SVSM, a marked-WT SV40 which contains an additional *Bam*HI site between nt 770 and 771 and lacks nt 1629 to 1635 (Table 1). The marked WT served as an internal control, and its alterations from the WT enabled us to distinguish its DNA and late transcripts readily from those of the cDNA mutants. After incubation at 37°C for 42 h, the cells were harvested. Nuclear and cytoplasmic RNAs and plasmid DNA were purified from the cotransfected cells and analyzed by S1 nuclease mapping (Fig. 2) and Southern blotting (Fig. 3), respectively.

In confirmation of the previous studies (21), late RNA made from cDNA mutant 16SΔ526 accumulated in the cytoplasm to only 1/100 of the level of late RNA made from the marked WT (Fig. 2A, lane 5). Except for mutant SVΔ294, all of the other cDNA mutants were also very defective in accumulation of late RNA in the cytoplasm (Fig. 2A), even though their primary transcripts are identical in sequence to one of the viral RNA species that accumulate in monkey cells transfected with WT SV40. Especially noteworthy is the fact that mutant 19SΔ526 was also very defective. The 31 bp missing from this mutant encode only the 5′ splice site needed to synthesize the 16S RNAs and the 3′ splice site and adjacent pyrimidine-rich stretch needed to synthesize the spliced 19S RNAs. Therefore, the absence of sequences that can function as splice sites is solely responsible for the defect of the cDNA mutants in accumulation of late RNA in the cytoplasm. Only mutant SVΔ294 did not have this defect (Fig. 2A, lane 7). However, the primary transcripts made from this late mutant are identical in sequence to a spliced precursor (RNA species SVΔ294 in Fig. 1) and have been shown previously (17) to be spliced to produce both the doubly spliced 19S and doubly spliced 16S RNA species (i.e., RNA species 19SΔDS and 16SΔDS, respectively, in Fig. 1). Therefore, the presence of excisable intervening sequences or their excision is necessary for each

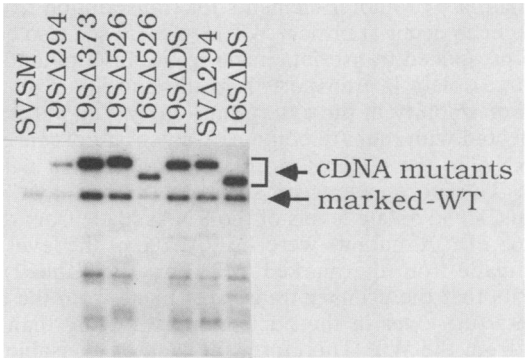

FIG. 3. Evidence that the SV40 late cDNA mutants are not defective in viral DNA replication. CV-1PD cells were cotransfected at a 2:1 molar ratio with each of the cDNA mutants and pSVSM. Replicated SV40 DNAs present at 40 h after transfection were purified and incubated with (i) *Dpn*I to degrade unreplicated DNA and (ii) *Bam*HI, which cleaves DNA of the cDNA mutants once but that of pSVSM twice. Southern blot analysis was performed as described previously (24). Each lane was loaded with DNA from 1/10 of a 100-mm-diameter dish of cells. This experiment was done in parallel with the experiment shown in Fig. 2. The bands representing the replicated DNAs of the cDNA mutants and the marked WT are indicated on the right.

TABLE 2. Relative amounts of viral late RNAs present in cells transfected with various mutants

Mutant	Relative amt. of RNA[a]		No. of splice sites[b]	Excisable intervening sequence(s)
	Cytoplasm	Nucleus		
SVSM	1.0	1.0	6 (5)	295–434[c]; 295–557[c]; 374–557; 527–1462
SVΔ294	0.3	0.3	3	527–557[d]; 527–1462
19SΔ294	<0.01	0.01	1	None
19SΔ373	0.01	0.06	2 (1)	295–1462[c,e]
19SΔ526	0.03	0.1	4 (3)	295–434[c]; 295–1462[c,e]; 374–1462[e]
19SΔDS	<0.01	<0.01	0	None
16SΔ526	0.01	0.2	3 (2)	295–434[c]
16SΔDS	<0.01	0.03	0	None

[a] The amounts of nuclear and cytoplasmic viral late RNAs made from the cDNA mutants indicated relative to that from the cotransfected marked WT. The data were obtained by quantitative densitometry of the autoradiograms shown in Fig. 2, with normalization to the relative amounts of DNA transfected into the cells. The ratio of the amount of nuclear-to-cytoplasmic viral late RNAs in cells transfected with pSVSM was 1:5 (data not shown).

[b] Number of splice sites encoded within transcripts initiated upstream (or downstream) of nt 294.

[c] Excisable only from transcripts initiated upstream of nt 294.

[d] The intervening sequence from nt 527 to 557 has been shown previously to be excised (17), although its size is below the minimum necessary for excision. This intervening sequence is probably excised only from multigenomic-length transcripts (P. J. Good, R. C. Welch, and J. E. Mertz, manuscript in preparation). The intervening sequence from nt 374 to 434 is not excised from transcripts of any of the mutants because it is below the minimal size necessary for excision in mammalian cells (Good et al., manuscript in preparation).

[e] These intervening sequences are excised at low efficiency and only in the absence of other intervening sequences (17).

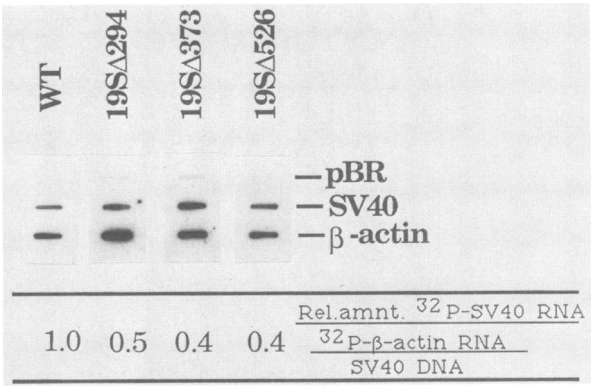

FIG. 4. Evidence that the SV40 late 19S cDNA mutants are not defective in transcription from the late promoter. Nuclear run-on transcription assays of SV40 late 19S cDNA mutants were performed as described in Materials and Methods. Shown here are autoradiograms of the resulting filters. Each band was cut out and counted by scintillation spectroscopy. SV40-specific counts (i.e., the counts from the SV40 band minus the counts from the pBR band) were normalized both to β-actin-specific counts (to correct for variability in the nuclear run-on assay) and to the SV40 DNA present in the samples as determined by Southern blot analysis (to correct for variability in transfection efficiencies). The relative transcription rates determined by this procedure are presented at the bottom of each lane. Rel. amnt, Relative amount.

of the SV40 late mRNAs to accumulate efficiently in the cytoplasm of monkey cells.

The presence of excisable intervening sequences also facilitates stabilization of SV40 late transcripts in the nucleus. Most of the cDNA mutants used in the above-described experiments retain all known elements of the late promoter. Even those whose deletion impinges upon the late promoter retain sufficient late promoter elements for transcription from this promoter to occur at or near WT levels (25; see also below). If the prespliced transcripts made from these mutants were defective solely in transport from the nucleus to the cytoplasm or stability in the cytoplasm, one would expect cells transfected with them to contain as much, if not more, viral late RNA in the nucleus as do cells transfected with WT SV40. Therefore, we were surprised to find that in the nucleus, steady-state levels of the RNAs made from the 19S and 16S cDNA mutants were 1/5 to 1/100 of the level of the RNA made from the marked WT (Fig. 2B; Table 2). This suggests that the primary transcripts made from the cDNA mutants turn over in the nucleus more rapidly than those made from the WT. Therefore, the lack of excisable intervening sequences or their excision results either in rapid degradation of the primary transcripts within the nucleus or faster-than-usual transport to the cytoplasm, followed by rapid degradation in the cytoplasm.

Also noteworthy is the finding that very little viral late RNA was present in the nuclei of cells transfected with cDNA mutants 19SΔ294, 19SΔDS, and 16SΔDS, which lack most of the known splice sites and encode no excisable intervening sequences, whereas moderate amounts of viral late RNA were present in the nuclei of cells transfected with the cDNA mutants which retain some splice sites and at least one excisable intervening sequence (Table 2). In other words, a correlation exists between the number of splice sites or excisable intervening sequences encoded by an SV40 late transcript and the amount of nuclear viral RNA. This finding suggests that the presence of sequences that encode splice sites or excisable intervening sequences plays a role in the initial stabilization of SV40 late transcripts in the nucleus.

The defects in the cDNA mutants are posttranscriptional. Although unlikely, alternative explanations for the above-described finding are that the cDNA mutants are primarily defective either in viral DNA replication or in transcription from the late promoter. To eliminate the first of these possibilities, we determined by Southern blot analysis the amount of mutant DNA relative to marked-WT DNA present in cells cotransfected in parallel with the cells used in the RNA analyses presented in Fig. 2. To distinguish readily the mutant DNAs from marked-WT DNA, each DNA sample was incubated not only with *Dpn*I to degrade unreplicated DNA but also with *Bam*HI. The latter enzyme cleaves the cDNA mutant DNAs to unit length linear DNA but cleaves SVSM, the marked-WT DNA, to two smaller fragments. The results of this experiment (Fig. 3) indicate that all of the cDNA mutant DNAs replicated as efficiently as marked-WT DNA in cotransfected cells.

To eliminate the possibility that the cDNA mutants are defective in transcription from the late promoter, we performed nuclear run-on transcription assays with nuclei isolated from cells transfected with the cDNA mutants. To correct for possible variations both in transfection efficiencies and in labeling and recovery of RNA, the amounts of radiolabeled SV40 RNA obtained from each nuclear run-on transcription assay were normalized both to the amount of SV40 DNA present in the isolated nuclei and to the amount of radiolabeled β-actin mRNA hybridized to the filter. For all of the mutants tested here (Fig. 4) and elsewhere (*dl*2350, the same as 16SΔ526; see references 21 and

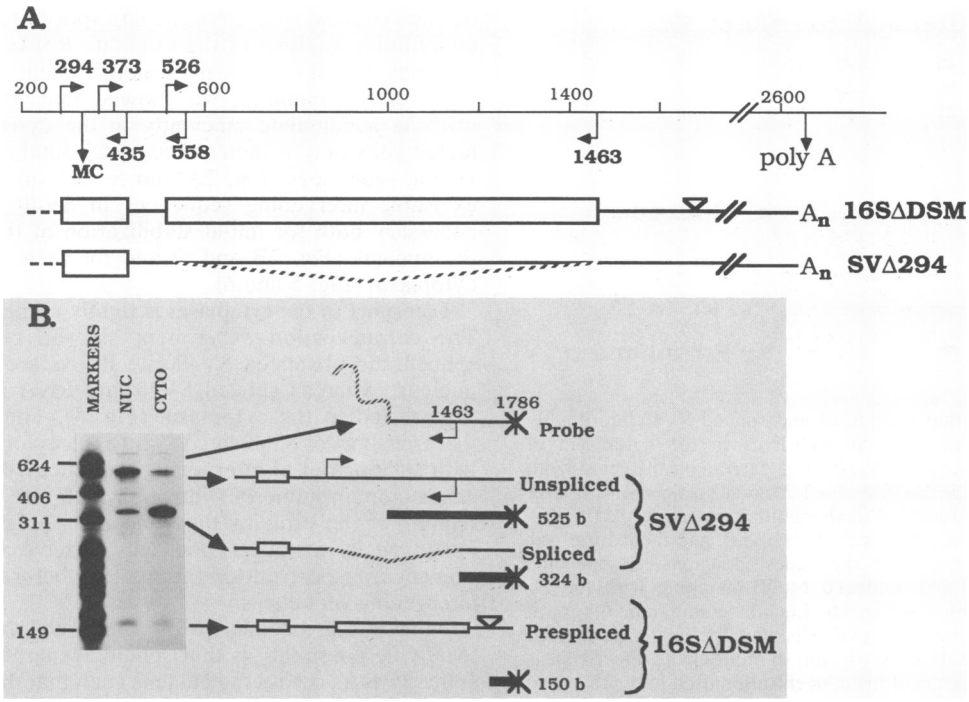

FIG. 5. S1 mapping analysis of the nuclear and cytoplasmic RNAs accumulated in cells cotransfected with mutants SVΔ294 and 16SΔDSM. (A) Structures of mutants SVΔ294 and 16SΔDSM. The top line represents the SV40 late region with the symbols and abbreviations used in Fig. 1. The locations of the intervening sequence(s) missing in the transcripts made from each cDNA mutant are indicated by open boxes. The small triangle on 16SΔDSM RNA indicates the location of the 10-base deletion present at the *Acc*I site (nt 1630) which allows the RNAs made from the two mutants to be distinguished by S1 mapping. The dashed line indicates the location of the excisable intervening sequence present in RNAs made from SVΔ294. (B) Analysis by S1 mapping of nuclear (NUC) and cytoplasmic (CYTO) RNAs harvested 42 h after cotransfection of CV1-PD cells with equal molar amounts of the DNAs of mutants SVΔ294 and 16SΔDSM. The *Eco*RI-cut, 5′-end-labeled probe is the same as the one shown in Fig. 2. The diagrams on the right identify the structures of the RNAs and the S1 nuclease-protected fragments generated from each. The size markers were *Msp*I-cut pBR322 DNA; the numbers on the left indicate molecular sizes in bases of some of them.

27), the average rate of transcription initiation measured by this assay was within threefold of that of WT SV40. Any differences, if significant, are not large enough to account for the greatly reduced amounts of viral late RNA observed in the nuclei and cytoplasm of cells transfected with these cDNA mutants.

In summary, since the cDNA mutants are not defective either in viral DNA replication or in transcription from the late promoter, we conclude that their defects in late RNA accumulation are posttranscriptional in nature.

Cotransfection experiments confirm the requirement of splicing for efficient RNA accumulation in the cytoplasm. Interpretation of the data presented in Fig. 2 and Table 2 is complicated by the facts that (i) some of the cDNA mutants lack some of the elements of the late promoter (2 and references therein), and (ii) several different spliced RNA species, which may differ in their half-lives, are produced from these mutants. To eliminate these complications, we also analyzed by S1 mapping the steady-state levels of SV40 late RNA present in the nuclei and cytoplasm of monkey cells cotransfected at a 1:1 molar ratio with mutants SVΔ294 and 16SΔDSM, a marked version of mutant 16SΔDS (Fig. 5). These two mutants are identical in sequence throughout the region that encodes late promoter elements (Fig. 5A). In addition, except for a 10-bp deletion which was introduced into mutant 16SΔDSM so that the RNAs made from the two mutants could be distinguished by S1 mapping, the spliced RNA produced from mutant SVΔ294 is identical in sequence to the prespliced RNA produced from mutant 16SΔDSM. Furthermore, a control experiment involving cotransfection of cells with mutants 16SΔDS and 16SΔDSM showed that this 10-bp deletion does not affect the stability of this RNA (data not shown).

As described above, the SV40 late RNA that underwent splicing (i.e., the spliced RNA from SVΔ294) accumulated predominantly in the cytoplasm, whereas the prespliced RNA (i.e., the RNA from 16SΔDSM) was present predominantly in the nucleus (Fig. 5B). This preferential accumulation of the spliced RNA in the cytoplasm was qualitatively reproducible (data not shown). These data confirm the conclusion that the presence of excisable intervening sequences in SV40 late transcripts is necessary for efficient accumulation in the cytoplasm.

Prespliced RNAs are defective in transport but not in stability in the cytoplasm. Our findings suggest that RNAs that are processed via a pathway that involves the splicing out of intervening sequences are either (i) transported to the cytoplasm more efficiently than RNAs that bypass this pathway or (ii) modified in some way (e.g., by methylation) that results in their possessing longer half-lives than RNAs that bypass this pathway. The first hypothesis predicts that the small percentage of prespliced RNA that manages to get transported to the cytoplasm has a half-life similar to that of RNA that was synthesized via splicing. On the other hand, the second hypothesis predicts that this RNA turns over in the cytoplasm with a shorter half-life.

To distinguish between these two hypotheses, monkey cells were cotransfected with mutants 16SΔDSM and

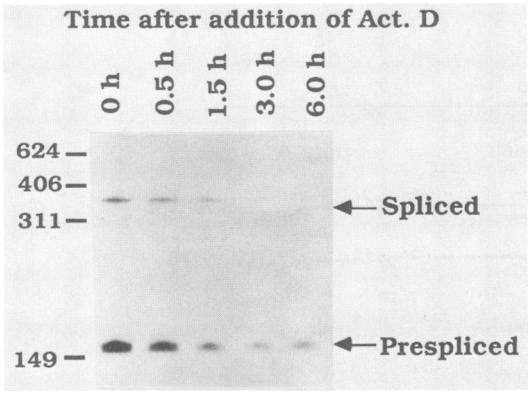

FIG. 6. Evidence that prespliced and spliced SV40 late doubly spliced 16S RNAs have similar half-lives in the cytoplasm of transfected monkey cells. CV-1P cells were cotransfected with mutants SVΔ294 and 16SΔDSM at a 1:25 molar ratio. At 42 h after transfection, the cells received fresh medium containing dactinomycin (Act. D; Calbiochem-Behring) at 10 μg/ml, and incubation was continued until harvesting at the times indicated. Cytoplasmic RNAs were purified and analyzed by S1 mapping with the 5′-end-labeled probe shown in Fig. 5B. Each lane contained 6.4 μg of cytoplasmic RNA. Other dishes of cells were metabolically labeled in parallel with [^{3}H]uridine (10 μCi/ml) to monitor the effectiveness of the chase; dactinomycin at the concentration used here prevented incorporation of [^{3}H]uridine into RNA within 15 min of addition (data not shown). The size markers were *Msp*I-cut pBR322 DNA; the locations of some of them are indicated on the left by their molecular sizes in bases.

SVΔ294 (molar ratio, 25:1) as described above. At 42 h later, dactinomycin (10 μg/ml) was added to the culture medium, and cytoplasmic RNA was isolated at various times thereafter. Analysis by S1 mapping of the SV40 late cytoplasmic RNAs present in these samples indicated that the half-lives of the prespliced and spliced SV40 late 16S RNAs were similar, i.e., approximately 1.0 h (Fig. 6). Southern blot and RNA slot blot analyses of the nuclear and cytoplasmic nucleic acid samples obtained in this experiment indicated that less than 4% of either nuclear SV40 DNA or LINE-1 RNA (i.e., RNA, localized almost exclusively to the nucleus, which is synthesized by RNA polymerase II transcription of LINE-1 DNA [41]) had contaminated any of the cytoplasmic samples (data not shown). Therefore, the half-life measured here for cytoplasmic prespliced RNA was truly that of cytoplasmic RNA and not that of nuclear prespliced RNA that inadvertently contaminated the cytoplasmic samples. Thus, these data rule out the hypothesis that the presence of excisable intervening sequences in primary transcripts or their being spliced out increases the stability of the resulting RNA in the cytoplasm. Therefore, we conclude that SV40 late transcripts that are processed via a splicing pathway are transported to the cytoplasm more efficiently than RNAs that bypass this pathway.

DISCUSSION

Since most protein-coding genes in higher eucaryotes contain introns, it is of interest to understand how the presence of excisable intervening sequences in primary transcripts or their being spliced out during processing affects RNA accumulation in the cytoplasm. In these experiments, we sought to elicit the functional roles that splicing plays in efficient accumulation of RNA in the cytoplasm. We used a set of SV40 mutants lacking the intron(s) excised during synthesis of each of the late spliced RNA species. By quantitative analysis of the viral late RNAs accumulated in the nucleus and cytoplasm of monkey cells transfected with these cDNA mutants, we showed that (i) the SV40 late mRNAs accumulate efficiently in the cytoplasm of transfected cells only if their precursors contain excisable intervening sequences (Fig. 2A and 5) and (ii) the presence of excisable intervening sequences in SV40 late mRNAs is necessary both for initial stabilization of the transcripts in the nucleus (Fig. 2B and 5) and for their transport to the cytoplasm (Fig. 5 and 6).

Transport to the cytoplasm is tightly regulated by splicing. The cotransfection experiment showed clearly that prespliced and unspliced SV40 late RNAs are retained in the nucleus, whereas spliced SV40 late RNA is preferentially transported to the cytoplasm (Fig. 5). These data suggest that transport of SV40 late RNA to the cytoplasm is coupled with splicing out of intervening sequences. Most eucaryotic genes contain numerous introns which interrupt the coding regions of the proteins they encode. Therefore, it is obviously crucial for eucaryotic cells to have a mechanism to prevent transport out of the nucleus before removal of all intervening sequences.

What is the mechanism by which cells accomplish this feat? One possibility is that splicing generates a gradient of spliced RNAs across the nucleus such that the spliced RNAs are highly localized near nuclear pores. Another possibility, which we favor, is that pre-mRNAs are tightly associated with the splicing machinery through interactions between intron-encoded sequences and small nuclear ribonucleoprotein (RNP) particles (32, 38, 40); the removal of all intervening sequences is, therefore, necessary for release of the RNA from the splicing machinery and subsequent transport of the RNA to the cytoplasm. This latter model is consistent with the findings that (i) only completely spliced products are released from splicing complexes in a cell-free splicing reaction (5, 28) and (ii) the major spliced SV40 late 19S RNA (i.e., RNA species 19SΔ373 of Fig. 1), containing the 3′ splice site at nt 1463 and adjacent 16S sequences, fails to be transported efficiently unless these sequences are deleted (J.-H. Roe, J.-S. Park, and J. E. Mertz, manuscript in preparation).

In spite of tight regulation of nuclear transport by the process of splicing, a small percentage of the prespliced SV40 late mRNA was found in the cytoplasm. The amount of this RNA varied not only from one experiment to the next but also in repeats on different days of a given experiment. For example, whereas the amount of prespliced SV40 late RNA in the cytoplasm was as low as approximately 1% of that of the WT in the experiment shown in Fig. 2A, it was as high as 20% in the experiment shown in Fig. 6. Similarly, the amounts of this prespliced RNA in the cytoplasm in four repeats of the experiment shown in Fig. 5 were 3 (Fig. 5B), 3, 15, and 20%, respectively (data not shown). The fact that less than 4% of SV40 DNA or LINE-1 RNA was found in the cytoplasmic fractions (data not shown) indicated that most of the cytoplasmic SV40 late RNA observed in these latter cases could not have resulted from leakage from nuclei during fractionation. Furthermore, S1 mapping analyses performed with probes homologous to the 5′ quarter of these prespliced cytoplasmic RNAs indicated that they were probably not spliced via utilization of cryptic splice sites (data not shown). Thus, it appears that some SV40 late RNA can make it to the cytoplasm without being spliced. Unfortunately, the experimental variables responsible for the inconsistency observed in the amount of this RNA remains

unknown. However, it may be responsible for the discrepancies that exist in the literature as to whether excisable introns are necessary for production of functional amounts of a variety of mRNAs.

Posttranscriptional nuclear defect of some prespliced transcripts. In addition to the defect in transport, the SV40 late prespliced RNAs also fail to accumulate to WT levels in the nucleus (Fig. 2B), and a correlation exists between the number of splice sites or excisable intervening sequences and the amount of nuclear viral RNA (Table 2). We did not directly show instability of the prespliced RNA in the nucleus by, for example, pulse-chase experiments designed to measure the half-life of prespliced nuclear RNA. We interpret these data (Fig. 2B) as indicating that the half-life in the nucleus of the SV40 late prespliced RNAs is much shorter than that of WT transcripts, because (i) the cDNA mutants are not defective in either DNA replication (Fig. 3) or transcription (Fig. 4), and (ii) the half-life of the small percentage of prespliced RNA that makes it to the cytoplasm is similar to that of spliced RNA in the cytoplasm (Fig. 6). The instability of prespliced SV40 late RNA in the nucleus is consistent with a recently published finding on polyomavirus late leader region deletion mutants in which a defect in splicing was correlated with a defect in nuclear stability (1). Prespliced RNAs made from cDNA versions of the rabbit (7) and human (W.-S.R. and J.E.M., manuscript in preparation) β-globin genes also accumulate in the nucleus and in the cytoplasm to much lower levels than RNAs made from the corresponding intron-containing genes.

Why are these prespliced transcripts rapidly degraded? The intron-exon junction sequences present in pre-mRNAs have been shown to associate strongly with both small nuclear RNP particles (32, 38, 40) and heterogeneous nuclear RNP particles (42; for a review, see reference 11) in cell-free splicing extracts. However, prespliced mRNAs and mutant pre-mRNAs lacking the *cis*-acting sequences required for binding of small nuclear RNP particles fail to form spliceosome complexes in cell-free splicing reactions (28). Furthermore, it has been found that pre-mRNAs containing excisable intervening sequences associate during transcription or immediately thereafter with RNP particles (4). Therefore, we hypothesize that SV40 late prespliced RNAs which do not contain excisable intervening sequences fail to associate properly with the machinery of their processing pathway; consequently, they are readily accessible to nucleases and degrade rapidly. Since SV40 nt 527 to 557 are missing from all of the defective cDNA mutants, including mutant 19SΔ526 which lacks only these 31 bp (Table 1), we speculate that a *cis*-acting sequence element mapping within this region of the SV40 genome is involved in directing SV40 late transcripts to their proper RNA-processing pathway.

Separate pathways for processing of different classes of RNA polymerase II transcripts. The β-globin and SV40 late pre-mRNAs define a class of transcripts in which the presence of excisable intervening sequences is necessary both for stabilization of the primary transcript within the nucleus and for efficient transport of mRNA to the cytoplasm. To explain the multitude of defects exhibited by prespliced versions of these mRNAs, we hypothesize that these transcripts need to be processed via a pathway that couples stabilization of the primary transcript within the nucleus, splicing out of all intervening sequences, proper 5′- and 3′-end formation, and transport to the cytoplasm.

On the other hand, some viral and cellular genes lack introns, yet they are expressed at functional levels (e.g., the genes for herpes simplex virus type 1 thymidine kinase [34], herpesvirus saimiri dihydrofolate reductase [45], histone [26], α-interferon [36], and c-*jun* [23]). Likewise, cDNA versions of some viral and cellular genes that normally contain introns are also expressed at functional levels (e.g., polyomavirus middle T antigen [44]). The efficiencies with which transcripts from these latter two classes of genes are processed into stable cytoplasmic mRNAs is not clear. However, we have found that insertion of human β-globin intron 1 into the 5′ leader region of the herpes simplex virus gene for thymidine kinase does not increase *tk* mRNA accumulation in the cytoplasm (W.-S.R. and J.E.M., manuscript in preparation). In addition, data in the literature indicate that at least some other prespliced transcripts also accumulate in the cytoplasm much more efficiently than those of the prespliced β-globin and SV40 late RNAs (9, 44). Therefore, the presence of excisable intervening sequences in transcripts is not required for expression of all mammalian genes. The genes encoding U1 RNA also lack introns, yet they are transcribed by RNA polymerase II to produce large quantities of RNA that are transported efficiently to the cytoplasm before returning to the nucleus (for a review, see reference 10). Taken together, these findings suggest that eucaryotic cells probably contain several pathways for the synthesis, processing, and transport of RNA polymerase II transcripts. It remains to be determined whether the signals that dictate which pathway a transcript takes are encoded in sequence elements within the transcript (7; W.-S.R. and J.E.M., manuscript in preparation), in sequence elements within the promoter (37; W.-S.R. and J.E.M., manuscript in preparation), by the location of the gene within the nucleus of the cell, or by the particular form of RNA polymerase II used to synthesize the transcript.

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