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# The Late Spliced 19S and 16S RNAs of Simian Virus 40 Can Be Synthesized from a Common Pool of Transcripts

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**The late transcripts from the simian virus 40 (SV40) are alternatively spliced into two classes of spliced RNAs, 19S and 16S in size. We are interested in understanding the precursor-product relationships that result in the excision of different intervening sequences (introns) from the late transcripts. SV40 mutants containing precise deletions of the introns for each of the spliced 19S and 16S RNA species, including a previously undetected doubly spliced 19S RNA species, were isolated. Analysis by S1 mapping and a modified primer extension technique of the viral RNAs made in monkey cells transfected with each of these mutants led to the following conclusions. (i) Spliced late 19S RNA is not an intermediate in the synthesis of the late 16S RNAs. (ii) The 3′ splice site used in the synthesis of the late 16S RNAs can join, albeit inefficiently, with alternative 5′ splice sites in the absence of the 5′ splice site normally used to synthesize 16S RNA. (iii) There is no obligatory order of excision of introns in the formation of the doubly spliced SV40 late 19S and 16S RNA species. A mutant was constructed by site-directed mutagenesis in which the 5′-proximal 3′ splice site used in the synthesis of the doubly spliced RNAs is inactive. Cells transfected with this mutant processed transcripts into 19S RNA which, in wild-type-transfected cells, would have become doubly spliced 16S RNA. Therefore, we conclude that some of the spliced late 19S and 16S RNA can be synthesized from a common pool of transcripts.**

The late transcripts of simian virus 40 (SV40) are alternatively spliced to give rise to a complex mixture of spliced RNA species. These species can be divided into three classes: precursor RNAs, spliced 19S RNAs, and 16S RNAs (for reviews, see references 20 and 43; see Fig. 1 for their structures). These classes of RNAs are functionally distinct; the spliced 19S RNAs are translated to synthesize the minor virion proteins VP2 and VP3, whereas the 16S RNAs are translated to synthesize the major virion protein, VP1, and LP1 (also referred to as the agnoprotein), a protein which facilitates virion assembly (P. J. Good, R. C. Welch, A. Barkan, M. B. Somasekhar, and J. E. Mertz, J. Virol., in press). Thus, the ratio of spliced 19S to 16S RNA determines the relative rates of synthesis of the different virion proteins.

The 5′ ends of these RNAs map to numerous locations within the late leader region from nucleotide residue (nt) 28 to 400, with the predominant 5′ end, referred to as the major cap site, mapping to nt 325 (13, 31, 39). Precursor RNAs are either unspliced or spliced within the leader region such that they lack the intron from nt 295 through 434 (RNA species A and B, respectively [Fig. 1]; 22, 38). Although a small percentage of these precursor RNAs can be isolated from the cytoplasm of SV40-infected cells, these RNAs are presumed to function predominantly as substrates for further RNA processing. All of the spliced 19S RNAs are synthesized by ligation of the 3′ splice site at nt 558 to one of the three 5′ splice sites situated at nt 294, 373, and 526 (RNA species C, D, and E, respectively [Fig. 1]). The 16S RNAs are normally synthesized by ligation of the 3′ splice site at nt 1463 to the 5′ splice site at nt 526 (RNA species G [Fig. 1]). Approximately 20% of the 16S RNA is additionally spliced either as in precursor species B to form doubly spliced 16S RNA or by ligation of the 5′ splice site at nt 526 to the 3′ splice site at nt 435 to form a duplication of part of the leader region (RNA species H and I, respectively [Fig. 1]; 34).

What are the pathways by which the spliced late 19S and 16S RNAs are synthesized? Kinetic experiments monitoring the fate of pulse-labeled RNA in SV40-infected cells lead to contradictory conclusions; whereas Aloni et al. (1, 2) and Chiu et al. (6) concluded that 19S RNA was a precursor to 16S RNA, Ford et al. (8) concluded that these RNAs were synthesized from distinct precursor RNA populations. Before the involvement of specific sequences surrounding splice sites in RNA processing was demonstrated, it was proposed that spliced 19S RNA species E could serve as precursor to the 16S RNAs (13, 44). The splice junction formed in this RNA species by excision of the intron from 527 through 557 regenerates a sequence which could theoretically function as a 5′ splice site in the synthesis of the 16S RNAs. Although our present knowledge of the mechanism of splicing makes this possibility unlikely, it has not been formally disproved. A similar model has been proposed to explain alternative splicing of other transcription units (37).

One plausible mechanism of generating alternatively spliced RNAs is the synthesis of distinct transcripts which are processed differently because of primary structural information in the RNA. Ghosh et al. (12) and Piatak et al. (32), noting a correlation between the 5′ end of an RNA and the splice sites used in its synthesis, hypothesized that the primary structure of the SV40 leader region, as determined by the 5′ end of the transcript, influences which splice sites are used in processing. At its extreme, this hypothesis proposes the existence of different precursor RNA pools which are defined by the 5′ ends of the primary transcripts.

We are interested in understanding the mechanisms which determine how an SV40 late transcript is processed. Here we asked which precursor RNAs can be used to synthesize the various spliced late 19S and 16S RNA species. To accomplish this, we constructed a set of mutants of SV40 with precise deletions of late region introns. Analysis of the structures of the viral RNAs from cells transfected with these mutants showed the following. (i) Spliced 19S RNAs are not precursors to the 16S RNAs. (ii) 16S-like RNAs can be synthesized, albeit inefficiently, with alternative 5′ splice

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sites. (iii) There is no obligatory order of excision of the introns present in the late transcripts of SV40. Analysis of the RNAs accumulated in cells transfected with a mutant in which the 3′ splice site used to synthesize doubly spliced RNA is inactive showed that RNA transcripts, which in wild-type (WT)-transfected cells would have become 16S RNA, can be processed alternatively into 19S RNA. Therefore, the spliced 19S and 16S RNAs can be synthesized from a common pool of transcripts.

## MATERIALS AND METHODS

**Cells and virus.** Plaque assays and virus infections were performed with CV-1P cells as previously described (28). A virus stock of SV40 strain WT830 was prepared from a single plaque isolate from viral DNA excised from the plasmid pSVS (9) by treatment with *Eco*RI. The origin of SV40 strain WT800 has been described previously (28); WT800 differs from WT830 in the precise size and endpoints of its duplicated enhancer region (39). The African green monkey kidney cell lines CV-1PD and BSC-1, obtained from C. Cole and the American Type Culture Collection (Rockville, Md.), respectively, were used interchangeably for transfections.

**Recombinant plasmids.** Standard recombinant DNA techniques were used in the construction of plasmids (23). DNA sequencing was performed either by the chemical degradation method (26) or by dideoxy sequencing of double-stranded plasmid DNA (5) with appropriate synthetic oligonucleotides as primers (Table 1). The plasmid pSD52, used for making S1 mapping probes, was constructed by insertion of the late region of SV40 from nt 1261 to 1782 (the *Eco*RI site) between the *Sal*I and *Eco*RI sites of pGEM2 (Promega Biotec). The plasmids pSVS (9) and pSV1-16SΔIVS (4) were gifts of M. Fromm and J. Sklar, respectively.

**Synthetic oligonucleotides.** All synthetic oligonucleotides used in this study were synthesized by the University of Wisconsin Biotechnology Center (Madison, Wis.) and are shown in Table 1. Deblocked oligonucleotides were purified with Sep-Pak C-18 columns (Waters Associates, Inc.) as previously described (30). Primers used for sequencing DNA, primer extension, and mutagenesis were gel purified as previously described (21). Primers were labeled with [$\gamma$-$^{32}$P]ATP and T4 polynucleotide kinase as previously described (21).

**Intron bypass cloning technique.** Plasmids pRWΔ294 and pRWΔ294Δ526 were obtained by the intron bypass cloning of cDNAs essentially as described by Steel et al. (40). The plasmid pRW101 was constructed by insertion of the *Hin*dIII C fragment of SV40 strain WT830 (nt 5171 to 1046) into the *Hin*dIII site of pUC9. The large *Kpn*I (nt 294)-to-*Eco*RV (nt 768) fragment of pRW101 was used both for priming cDNA synthesis from SV40-infected, cytoplasmic RNA and as a vector for cloning the cDNAs.

**Construction of cDNA plasmids with synthetic oligonucleotides.** The complementary strands for two restriction fragments with precise deletions of the introns for 19S RNA species D and E were synthesized; the sequences of the strands are presented in Table 1. Approximately 100 ng of each of the pair of complementary oligonucleotides was annealed in 4× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 65°C for 1 h, added to 100 ng of gel-purified, linearized pRW101 DNA, and ligated overnight at 14°C with T4 DNA ligase. For construction of plasmids corresponding to RNA species D and E, pRW101 was linearized with *Nae*I (nt 345) and *Nco*I (nt 560) or *Hpa*I (nt 501) and *Nco*I (nt 560), respectively. Afterwards, competent DH1 cells were transformed with the resulting plasmids and the DNA from single, ampicillin-resistant colonies was isolated and sequenced by dideoxy sequencing of double-stranded DNA. Plasmids pSDΔ373 and pSDΔ526 correspond to RNA species D and E, respectively.

**Site-directed mutagenesis.** The double primer method of Norris et al. (29) was used to generate a double point mutation at the nt 435 3′ splice site. First, an 18-mer oligonucleotide with two mismatches, one at nt 436 and the other at nt 433, was synthesized (443pmE [Table 1]). These changes eliminate the two AG dinucleotides in this region and introduce a *Sin*I restriction enzyme site. The second primer was the M13 sequencing primer from New England BioLabs, Inc. The template, obtained from M. Wickens, was a M13mp8-derived bacteriophage containing the late strand of the *Hin*dIII C fragment of SV40 (47). The resultant double-stranded fragment containing the mismatch was excised by cleavage with *Kpn*I (nt 294) and *Hin*dIII (nt 1046) and inserted into pUC18. Plasmids containing inserts were

TABLE 1. Sequences of oligonucleotides used

| Sequence name[a] | Sequence | Use |
|---|---|---|
| 373ΔL | 5′ G G C T G T C A C G C C A G G C C T C C G T T A A G G T C 3′ | Cloning[b] |
| 373ΔE | 3′ C C G A C A G T G C G G T C C G G A G G C A A T T C C A G G T A C 5′ | Cloning |
| 526ΔL | 5′ A A C T G A A A A A C C A G A A A G T T A A C T G G T C 3′ | Cloning |
| 526ΔE | 3′ T T G A C T T T T T G G T C T T T C A A T T G A C C A G G T A C 5′ | Cloning |
| 604E | 5′ C A G A T G C A A T T A G G T C C C C C 3′ | Primer |
| 682E | 5′ G C A C T T C A A T T G C A G C 3′ | Primer |
| 1543E | 5′ T G C C C C T G G A C A A C T T C C 3′ | Primer |
| 1562E | 5′ C C T T T G G T T T T T G G G A G C 3′ | Primer |
| 261L | 5′ C A C A T T C C A C A G C T G G T T C 3′ | Anti-*Pvu*II[c] |
| 1072L | 5′ G A C A T T C C T A G G C T C A C C 3′ | Anti-*Sty*I |
| 443pmE | 5′ G C A A A A A G G T C C A A A A C A A 3′ | Mutagenesis[d] |

[a] The letters E and L at the end of a sequence name refer to the oligonucleotide corresponding to either the early or late strand, respectively.

[b] Oligonucleotides 373ΔE and 373ΔL hybridize together to generate a *Nae*I (nt 345)-to-*Nco*I (nt 560) restriction fragment of a cDNA corresponding to RNA species D. Similarly, oligonucleotides 526ΔE and 526ΔL hybridize together to generate a *Hpa*I (nt 499)-to-*Nco*I (nt 560) restriction fragment of a cDNA corresponding to RNA species E.

[c] Oligonucleotides 261L and 1072L hybridize to cDNAs from late RNAs and bracket the recognition sequences, indicated by the underlines, of the restriction enzymes *Pvu*II (nt 272) and *Sty*I (nt 1080), respectively.

[d] The sequence of the oligonucleotide 443pmE differs from that of WT DNA by the two nucleotides indicated by asterisks; it was used to mutate specifically the 3′ splice site at nt 435. These changes also result in the generation of a *Sin*I restriction enzyme site, indicated by the underline.

screened by colony hybridization with end-labeled mismatch primer as the probe under stringent wash conditions (37°C for 10 min in 2× SSC). The resulting positive colonies were further screened by digestion with *Sin*I and sequenced by the dideoxy method.

**DNA transfections.** DNA transfections were performed by a modification of the DEAE-dextran protocol (36). Viral DNA was excised from the cloning vector by cleavage with *Eco*RI and ligated at 2.5 μg of DNA per ml into monomer circles. The DNA was concentrated by ultrafiltration with a Centricon-30 unit according to the manufacturer (Amicon Corp.), followed by ethanol precipitation. DNA was diluted into Dulbecco modified Eagle medium (DMEM) containing 500 μg of DEAE-dextran (molecular weight, $2 \times 10^6$; 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 1:4 the day before transfection, washed once with DMEM containing 20 mM HEPES (pH 7.1), and incubated with 1.0 ml of DNA solution per 100-mm-diameter dish for 1 h in a $CO_2$ incubator at 37°C. The DNA solution was removed and replaced with DMEM containing 20 mM HEPES (pH 7.1) and 100 μM chloroquine. After incubation for 1 h in 5% $CO_2$ at 37°C, the cells were washed twice with DMEM, fed with DMEM plus 2% fetal bovine serum, and incubated in 5% $CO_2$ at 37°C for the indicated length of time.

**RNA purification.** Whole-cell nucleic acids were purified as previously described (16). The DNA was degraded by suspension of the total nucleic acids in DNase I reaction buffer (100 μg of DNase I [DPRF grade; Organon Teknika] per ml, 600 U of RNasin [Promega Biotec] per ml, 5 mM $CaCl_2$, 10 mM $MgCl_2$, 5 mM dithiothreitol, 10 mM Tris hydrochloride [pH 7.4]) and incubation for 45 min at 37°C. The reaction was terminated by the addition of EDTA, EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid] and sodium dodecyl sulfate to final concentrations of 20 mM, 10 mM, and 0.2%, respectively, followed by extraction with phenol-chloroform-isoamyl alcohol (25:25:1). The samples were precipitated with ethanol and suspended in a solution containing 10 mM Tris (pH 7.6), 1 mM EDTA, and 0.1% sodium dodecyl sulfate. Poly(A)$^+$ RNA was prepared by a modification of the batch oligo(dT) cellulose procedure as described elsewhere (Good et al., in press).

**Modified primer extension technique.** cDNAs were prepared as previously described (27) from the indicated RNA samples using a 5'-end-labeled oligonucleotide primer (Table 1). After hydrolysis of the RNA with NaOH, the cDNAs were precipitated with ethanol and suspended in 10 μl of a solution containing 0.1 M NaCl, 10 mM Tris hydrochloride (pH 7.6), 1 mM EDTA, and 20 ng of a synthetic oligonucleotide specific to the restriction enzyme to be used for 3'-end cleavage (Table 1). The DNAs were incubated at 80°C for 5 min and at 60°C for 30 min and then were digested by the addition of an equal volume of 20 mM Tris hydrochloride (pH 7.6), 20 mM $MgCl_2$, 20 mM dithiothreitol plus 3 to 10 U of restriction enzyme and incubation at 37°C for 3 h. The resulting DNAs were precipitated with ethanol, suspended in a solution containing 80% formamide, 10 mM NaOH, and 1 mM EDTA, and electrophoresed in a gel containing the indicated percentage of polyacrylamide and 8 M urea (urea-PAG). The gel was dried and exposed to X-ray film with intensifying screens.

**S1 mapping.** The probe for quantifying the relative amounts of the late 16S and 19S SV40 RNAs was made by cleavage of the plasmid pSD52 with *Acc*I (nt 1630), incubation with calf alkaline intestinal phosphatase (Boehringer Mannheim), cleavage at the *Pvu*II site within the vector sequences, and agarose gel purification of the small fragment. This probe was labeled with [γ-$^{32}$P]ATP and T4 polynucleotide kinase as previously described (23). The procedure for S1 mapping was as previously described (4); the hybridization temperature used was 52°C.

## RESULTS

**Intron bypass technique for cloning plasmids with precise 19S RNA intron deletions.** In our initial attempts to generate cDNA plasmids of each of the late 19S RNA species, the intron bypass cloning technique of Steel et al. (40) was used. For this procedure, cDNA copies of the 19S RNAs were made by reverse transcription with a restriction fragment specific to the 19S RNAs as a primer. These cDNAs were annealed to plasmid DNA that had been cleaved to remove the intron sequences such that the cDNAs would join the ends of the plasmid. The annealed cDNA regenerated circular plasmid DNA, which was used to transform bacteria. Two different plasmids with intron deletions were obtained by this technique: one, pRWΔ294, corresponds to 19S RNA species C (Fig. 1); the other, pRWΔ294Δ526, corresponds to 19S RNA species F, a previously unidentified doubly spliced 19S RNA species.

**Detection of the doubly spliced 19S RNA species by a modification of the primer extension technique.** The doubly spliced 19S RNA species F lacks the same leader intron as the doubly spliced 16S RNA species H. The isolation of a plasmid corresponding to RNA species F from cDNAs of WT virus-infected cell RNAs strongly suggests that this RNA species exists in SV40-infected monkey cells. To confirm the existence and to quantify the amounts of the 19S RNA species F in WT-infected cells, we used a modification of the primer extension technique (38). cDNAs extended from primers hybridized to SV40 late RNAs are heterogeneous in length because of differences not only in the splice junctions used in the synthesis of these RNAs but also in the locations of their 3' ends (which correspond to the 5' ends of the RNAs). To eliminate the heterogeneity in the 3' ends of the cDNAs, the cDNAs are hybridized with a synthetic oligonucleotide that encodes the sequence surrounding a known restriction enzyme site and cleaved with this restriction enzyme. The ability to use a large molar excess of a synthetic oligonucleotide enables one to obtain quantitative cleavage at a known site on the cDNA (for examples, see Fig. 2 and 6). Possessing common 3' ends, the cDNAs generated by this technique can differ in size only because of deletions of different introns. The cDNAs that do not hybridize with the synthetic oligonucleotide, either because the intron the cDNAs lack encompasses the restriction site or because their 3' ends do not extend beyond the restriction site, will not change in size upon incubation with the restriction enzyme. A comparison of the sizes of the cDNAs before and after incubation with the restriction enzyme, along with knowledge of the splice junctions that can be present, allows for the identification and quantification of the spliced RNA species present in the RNA sample.

Analysis with this modification of the primer extension technique of the SV40 late 19S RNA species present in WT830-infected monkey cells is shown in Fig. 2A. A band 161 nt in length was seen after digestion of the cDNAs of cytoplasmic RNA with *Pvu*II (lane 6). This DNA was the size predicted for cDNAs made from 19S RNAs with 5' ends mapping upstream of the *Pvu*II site which are doubly spliced

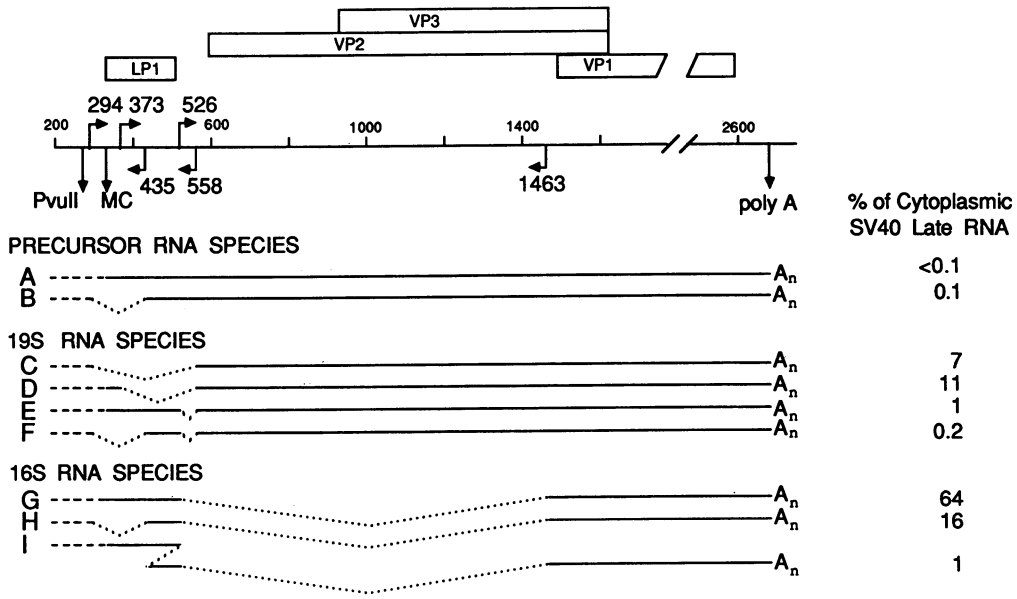


FIG. 1. Map of the late region of SV40. The numbered line at the top of the figure represents the late region of the SV40 genome with the nucleotide residues given in the SV numbering system (43). The rightward arrows indicate the locations of 5′ splice sites at nt 294, 373, and 526, and the leftward arrows indicate 3′ splice sites at nt 435, 558, and 1463. The downward arrows labeled *Pvu*II, MC, and poly A indicate the locations of the *Pvu*II site at nt 273, the major cap site at nt 325, and the cleavage-polyadenylation site at nt 2675, respectively. The lines labeled A through I show the structures of the RNAs, with the locations of the excised sequences indicated (·····). RNA species A, C, D, E, G, H, I (13, 34), and B (38) have been described previously; RNA species F is identified in this report. The heterogeneity of the 5′ ends of the RNAs is indicated (– – – –) at the left ends of the RNAs. The levels of the different RNA species in the cytoplasm of infected cells are listed at the right and were determined from Somasekhar and Mertz (39) and quantification of experiments similar to those presented in Fig. 2. The proteins encoded by these RNAs are indicated by the boxes labeled LP1, VP2, VP3, and VP1.

as in RNA species F; it comigrated with a size marker generated from the corresponding cDNA plasmid which was identical in sequence to the predicted cDNA (lane 2). Analysis of viral RNA isolated from cells infected with WT800, a different WT strain of SV40, showed clearly that this band was generated by *Pvu*II digestion of the cDNAs (Fig. 2B, lanes 9 and 10). Additional minor bands were present in this size range; since these bands were not sensitive to *Pvu*II digestion, they presumably represent minor RNA species with 5′ ends located between the major cap site and nt 273. cDNAs in this size range were not detected after analysis of the same amount of RNA isolated from mock-infected cells (Fig. 2, lanes 7 and 8). Another band, 192 nt in length, representing the previously identified spliced precursor RNA species B, was present in the cytoplasm in quantities approximately equal with the one representing RNA species F (lanes 6 and 10). Analysis of nuclear RNA (Fig. 2A, lanes 3 and 4) indicated that RNA species B accumulated preferentially in the nucleus, as does unspliced RNA. Quantitative S1 mapping with 5′-end-labeled, 19S RNA-specific probes confirmed that the doubly spliced RNA species F accounts for 0.5 to 1% of steady-state cytoplasmic 19S RNA (data not shown).

**Construction of SV40 mutants with precise deletions of intron(s) from the late region.** Plasmids corresponding to 19S RNA species D and E were not obtained by the intron bypass technique even after repeated attempts with different primers. Since the sequences of the splice junctions of these RNAs were already known, we chemically synthesized and cloned restriction fragments which lack precisely the corresponding introns. Two plasmids were obtained which correspond to the 19S RNA species D and E (Fig. 1). Restriction fragments containing the deletions of each of the 19S introns, plus a restriction fragment with a deletion of the 16S RNA intron, were recombined into cloned WT SV40 to generate a set of cloned SV40 mutants lacking precisely each of the introns excised during processing of the late RNAs (Table 2).

**Viability of SV40 mutants with precise intron deletions.** To test whether these mutants are defective in any viral function, we determined their viability after excision from their cloning vector by plaque assays on monolayers of CV-1P cells (Table 2). On the basis of previously published observations that leader region mutants are viable (3, 41) and intron deletion mutants are nonviable (15), we expected mutant SVΔ294 to be viable and the rest of the mutants to be nonviable. Surprisingly, mutants 19SΔ373 and 19SΔ526 were viable, although the mutant plaques arose much later than WT plaques. For both mutants, the plaques formed by them were uniform in size and arose within 1 to 2 days of each other (data not shown). In addition, the specific infectivities of the viral DNAs of mutants 19SΔ373 and 19SΔ526 were similar to those of WT DNA transfected in parallel. Therefore, it is highly unlikely that the plaques made by these two mutants were due to the appearance of revertants. These two mutants lack the 5′ splice site used to excise the normal 16S RNA intron. Perhaps, these two mutants are weakly viable because of inefficient synthesis of normal 16S RNAs or of novel 16S-like RNAs from the prespliced 19S RNA transcripts made from the mutant DNAs.

**Synthesis of novel 16S-like RNAs in cells transfected with SV40 mutants with precise intron deletions.** Can 16S-like RNAs be synthesized from transcripts that precisely lack 19S RNA introns? To answer this question, monkey cells were transfected with SV40 mutants that contain precise deletions of the various introns of the late region and the resultant RNA products synthesized from these transcripts

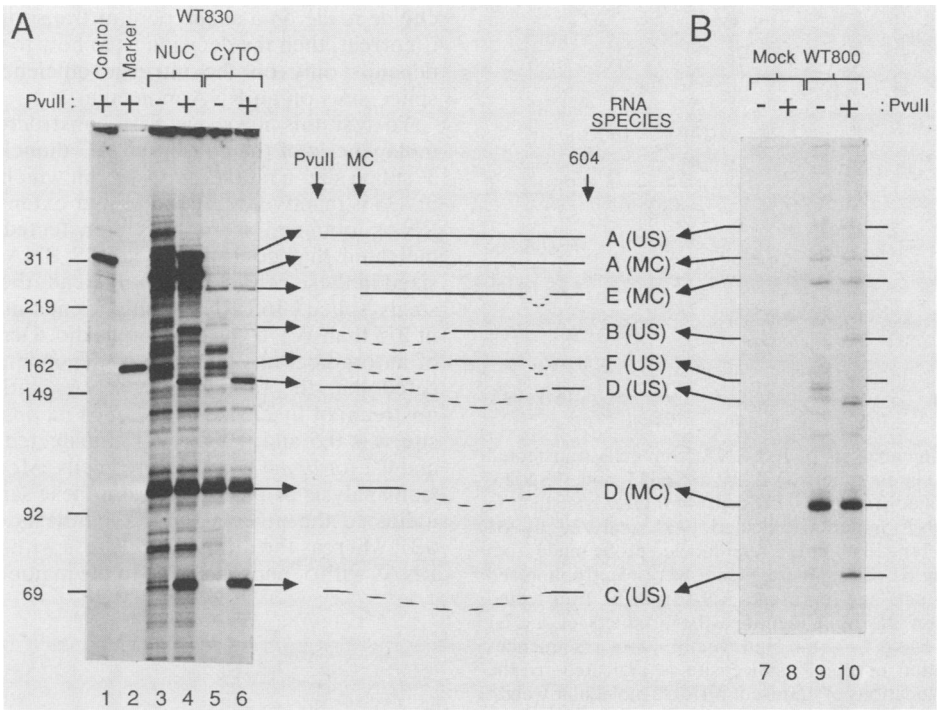


FIG. 2. Analysis by a modification of the primer extension technique of the SV40 late 19S RNAs. (A) cDNAs of cytoplasmic (CYTO) and nuclear (NUC) RNA, purified from $5 \times 10^5$ CV-1P cells infected for 40 h with SV40 strain WT830, were prepared with a 5′-end-labeled, 19S RNA-specific primer (604E of Table 1). The resulting cDNAs were cleaved with *Pvu*II as described in Materials and Methods and electrophoresed in an 8% urea-PAG. Lane 1, cDNAs cleaved with *Pvu*II of RNA made in vitro with SP6 RNA polymerase from a plasmid in which an SP6 promoter was located at SV40 nt 170; lane 2, a size marker made by annealing the 604E primer to the denatured plasmid p19SΔDS, extending with the Klenow fragment of DNA polymerase I, and cutting with *Pvu*II; lane 3, cDNAs from nuclear RNA; lane 4, cDNAs from nuclear RNA cleaved with *Pvu*II; lane 5, cDNAs from cytoplasmic RNA; lane 6, cDNAs of cytoplasmic RNA cleaved with *Pvu*II. The diagrams on the right indicate the structures of the corresponding bands as determined by their sizes before and after incubation with *Pvu*II. The RNA species are named as in the legend to Fig. 1; US and MC in parentheses indicate cDNAs with 3′ ends mapping upstream of nt 273 and to the major cap site, respectively. The locations of *Msp*I-cut pBR322 molecular weight markers are indicated on the left. (B) cDNAs of cytoplasmic 19S RNA isolated from WT800- and mock-infected cells were prepared as described above, except using the 682E primer, cleaved with *Pvu*II, and electrophoresed in a 5% urea-PAG. Lane 7, cDNAs from mock-infected cells; lane 8, cDNAs from mock-infected cells cleaved with *Pvu*II; lane 9, cDNAs from WT800-infected cells; lane 10, cDNAs from WT800-infected cells cleaved with *Pvu*II.

TABLE 2. Summary of SV40 mutants used

| Plasmid[a] | RNA species[b] | Nucleotide sequences deleted | Time (days) of first appearance of plaques[c] |
|---|---|---|---|
| pSVS(WT830) | | | 8 |
| pSV1761(SVΔ294) | B | 295 – 434 | 12 |
| pSV1762(19SΔ294) | C | 295 – 557 | NV[d] |
| pSV1763(19SΔ373) | D | 374 – 557 | 18 |
| pSV1764(19SΔ526) | E | 527 – 557 | 22 |
| pSV1765(19SΔDS) | F | 295 – 434, 527 – 557 | NV |
| pSV1766(16SΔ526) | G | 527 – 1462 | NV |
| pSV1767(16SΔDS) | H | 295 – 434, 527 – 1462 | NV |
| pSV1768(*dpm*435) | | 433 A→G; 436 G→C | 8 |

[a] The plasmids are identified by their official mutant numbers (1761 through 1768), along with descriptive names in parentheses. Throughout the paper, we shall use the descriptive names 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] Plaque assays were performed by transfection of monolayers of CV-1P cells with excised viral DNA.

[d] NV, Nonviable, i.e., plaques were still not visible 22 days after transfection.

were determined. An unavoidable assumption inherent in these and other of the experiments described below is that transcripts from mutants that lack an intron are subject to the same processing pathways as WT transcripts that have been processed initially by excision of the same intron.

The amount of RNA synthesized using the 3′ splice site at nt 1463 in cells transfected with the 19S cDNA mutants was quantified by S1 mapping. Whereas cells transfected with the nonviable 19S cDNA mutants did not accumulate 16S RNAs (data not shown), cells transfected with mutants 19SΔ373 and 19SΔ526 accumulated 16S RNAs, but to levels approximately 5- to 10-fold lower than that observed in WT-transfected cells (Fig. 3). As expected, mutant SVΔ294 synthesized near-WT levels of 16S RNA (Fig. 3). Thus, 16S RNAs can be synthesized, albeit inefficiently, from pre-spliced 19S RNAs.

Transcripts from mutant 19SΔ526 might be processed into 16S RNAs by using (i) a 5′ splice site generated by deletion of the intron, (ii) one of the alternative 5′ splice sites used to synthesize 19S RNAs, or (iii) a cryptic 5′ splice site. Transcripts from mutant 19SΔ373 might use the latter two of these possibilities. We examined the structures of the 16S RNAs made from these mutants to determine the 5′ splice sites that were used to synthesize these 16S RNAs. From

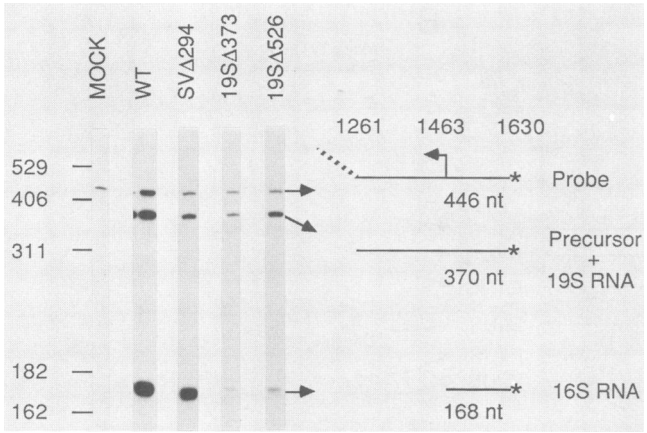


FIG. 3. S1 mapping analysis of 16S RNA from cells transfected with the viable SV40 late mutants SVΔ294, 19SΔ373, and 19SΔ526. Poly(A)$^+$ RNA, purified from CV-1PD cells 44 h after transfection with the viable cDNA mutants indicated, was analyzed by S1 mapping with an *Acc*I (nt 1630)-cut, 5′-end-labeled probe made from pSD52. The resulting S1-resistant fragments were electrophoresed in a 5% urea-PAG. Each lane represents RNA from $10^5$ transfected cells. The diagram on the right identifies the products of the S1 mapping, with the SV40 (———) and vector (▪▪▪) sequences indicated. The location of the 3′ splice site is indicated by the leftward arrow. The locations of *Msp*I-cut pBR322 molecular weight markers are indicated on the left.

cells transfected with these mutants, a cDNA product was detected which was consistent with an RNA species spliced from the nt 294 5′ splice site to the nt 1463 3′ splice site; a less abundant cDNA product was detected from cells transfected with mutant 19SΔ526 which was consistent with an RNA species spliced from the nt 373 5′ splice site to the nt 1463 3′ splice site (Fig. 4). The 482-nt cDNA product generated by cleavage with *Sty*I indicated that cells transfected with both mutants accumulated 19S RNA (Fig. 4). The ratios of the sum of the 16S cDNAs to the 19S cDNAs were similar to the ratios of 16S RNAs to 19S RNAs determined by S1 mapping (Fig. 3). Therefore, previously undetected splice site joinings can be used to synthesize 16S RNA-like RNAs in the absence of the nt 526 5′ splice site.

**Nonobligatory order of excision of late region introns.** The existence of both doubly spliced 16S and doubly spliced 19S RNA suggests that RNA species B may be an intermediate in a processing pathway used in the synthesis of both of these doubly spliced RNAs. To test whether the order of excision of the introns in the synthesis of these doubly spliced RNAs is obligatory, we determined the structures of the RNAs present in cells transfected with cDNA mutants in which the primary transcripts were prespliced for one or the other of these introns. Transcripts from both mutants SVΔ294 and 19SΔ526 (corresponding to RNA species B and E, respectively) were further processed to generate the doubly spliced 19S RNA species F (Fig. 5A). Similarly, transcripts from both mutants SVΔ294 and 16SΔ526 (corresponding to RNA species B and G, respectively) were further processed to generate the doubly spliced 16S RNA species H (Fig. 5B). Therefore, no obligatory order exists for the excision of introns from the late region of SV40.

**Synthesis of spliced 19S and 16S RNAs from a common precursor pool.** We have shown above that RNA species B can function as an intermediate for the synthesis of both spliced 19S and 16S RNAs. This observation implies that the decision to synthesize either 19S or 16S RNAs represents a choice made on a single pool of transcripts. If this pathway is correct, then the decision as to how to process a transcript depends only on the intrinsic efficiency of utilization of splice sites on an RNA molecule.

To test this idea, we first constructed, by site-specific mutagenesis of the conserved AG dinucleotide in the nt 435 3′ splice site, a mutant, *dpm*435, in which the 3′ splice site at nt 435 is inactive. Modified primer extension analysis of 16S RNAs obtained from cells transfected with this mutant indicated that doubly spliced 16S RNAs were not synthesized in these cells (Fig. 6A). Instead, these cells made more singly spliced 16S RNAs with 5′ ends mapping upstream of nt 273 than WT-transfected cells did. Densitometric analysis of autoradiograms from two independent experiments indicated that the ratio of 16S RNAs with 5′ ends mapping upstream of nt 273 versus 5′ ends mapping to the major cap site was 0.5 and 0.2 for WT-transfected cells, yet only 0.1 and 0.1 for *dpm*435-transfected cells. Modified primer extension analysis of 19S RNAs from these same transfected cells indicated the inverse for the synthesis of 19S RNAs (Fig. 6B); whereas the ratio of 19S RNAs with upstream 5′ ends to RNAs with 5′ ends mapping to the major cap site was 0.9 and

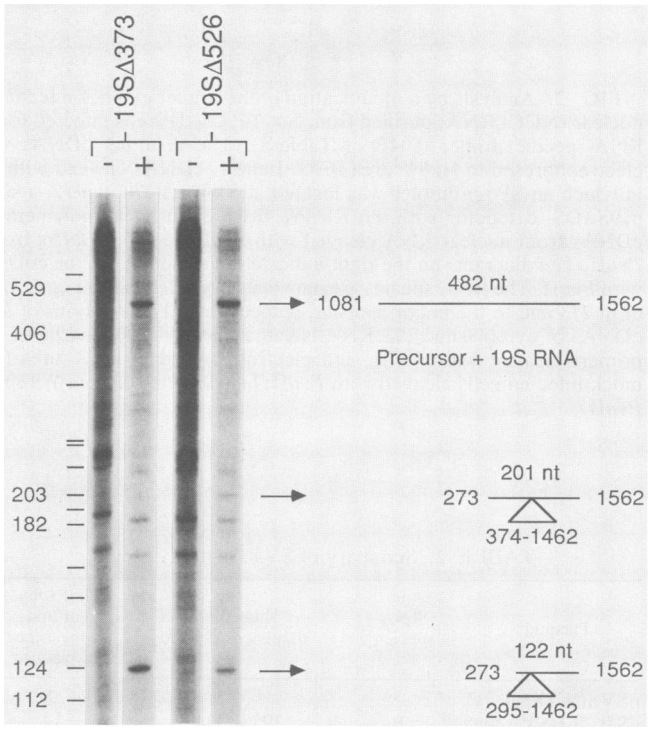


FIG. 4. Modified primer extension analysis of 16S RNA from cells transfected with the SV40 mutants 19SΔ373 and 19SΔ526. Poly(A)$^+$ RNA was purified from CV-1PD cells 44 h after transfection with the cDNA mutants indicated. cDNAs of RNA isolated from $5 \times 10^5$ cells were prepared with primer 1562E (Table 1). Each reaction was split into two portions; one portion was hybridized with the primers 1072L and 261L and incubated with *Sty*I and *Pvu*II (+), while the other portion was left intact (−). The resulting cDNAs were electrophoresed in a 5% urea-PAG. The diagrams at the right represent the predicted structures of the bands indicated. The bands not identified are bands also present in cDNAs synthesized from mock-transfected RNA (data not shown). Some loss in signal seen upon digestion with the restriction enzymes is probably due to contaminating phosphatase present in the restriction enzyme preparation. The locations of *Msp*I-cut pBR322 molecular weight markers (in nucleotide residues) are indicated at the left.

0.7 in WT-transfected cells, this ratio was 3.4 and 2.1 in *dpm*435-transfected cells (compare RNA species D(US) and C(US) to D(MC) in Fig. 6B). Thus, the mutation in the transcripts from *dpm*435 results in RNAs, which in WT-transfected cells would have become 16S RNAs, being processed to 19S RNAs.

## DISCUSSION

**Use of a common pool of transcripts for the synthesis of late 19S and 16S RNAs.** We have shown here that cells transfected with a mutant containing a point mutation somewhat removed from the 5′ ends of the late transcripts process to spliced 19S RNAs some transcripts that would have become 16S RNAs in cells transfected with WT SV40 (Fig. 6). Thus, the decision to process to 19S or 16S RNAs is not determined solely by the sequences at the 5′ end of a primary transcript. The validity of this conclusion depends upon several assumptions. First, the alterations in the point mutant must not affect the distribution of the 5′ ends of the late transcripts. This assumption is supported by the observation that the distribution of 5′ ends mapping upstream of nt 294 does not differ between WT and *dpm*435 (data not shown). Second, the mutations must not alter RNA sequences directly involved in the recognition by the splicing machinery of the cell of the 3′ splice sites at nt 558 and 1463. This assumption is probably valid since the mutation is a point mutation that maps a considerable distance from these sites. Last, the mutation must not affect the half-life of the RNAs. For the predominant 19S RNAs, the mutation maps within the intron and therefore cannot affect stability of the processed RNAs; the effect of the mutation on stability of the 16S RNAs, however, is unknown but would be expected to be minor, since deletions in this part of the leader region do not affect late RNA stability (32). Therefore, we conclude that the spliced 19S and 16S RNAs can be derived from a common precursor pool and the decision to process to one or the other of these RNAs represents a decision along a shared processing pathway.

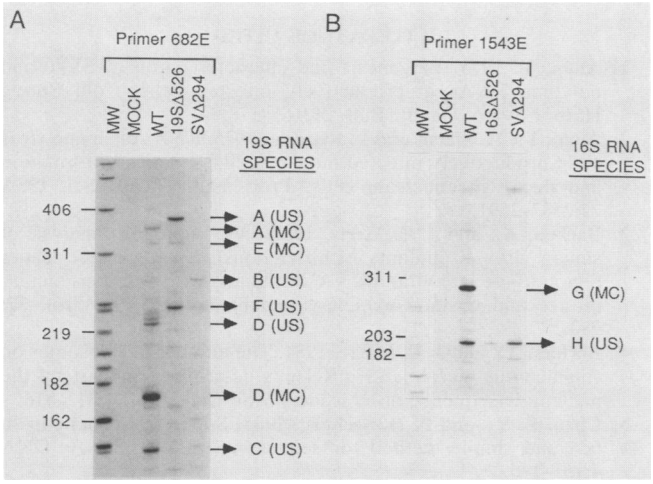


FIG. 5. Modified primer extension analysis of RNA from cells transfected with SV40 mutants that retain an excisable intervening sequence. Poly(A)$^+$ RNA was purified from CV-1PD cells 44 h after transfection with the SV40 mutants indicated. cDNAs of these RNAs were prepared with (A) the primer 682E and (B) the primer 1543E, cleaved with *Pvu*II, and electrophoresed in a 5% urea-PAG. The size markers (MW) are *Msp*I-cut pBR322 DNA. The identities of the bands are indicated as in the legend to Fig. 2.

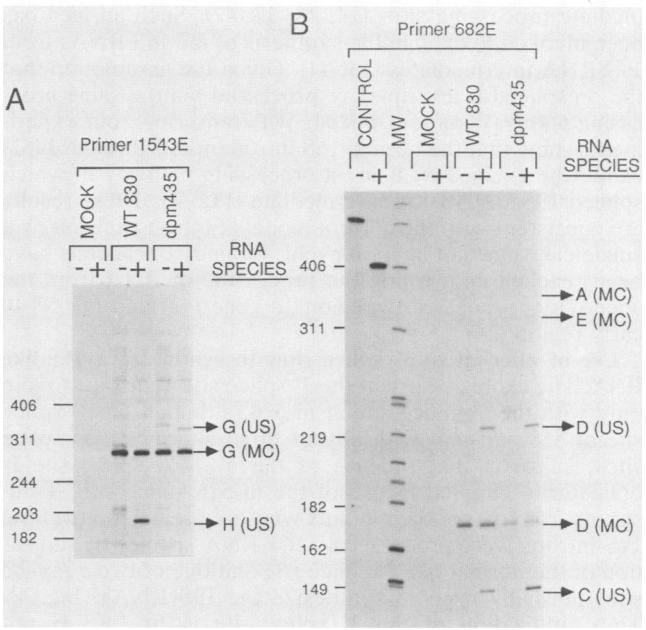


FIG. 6. Modified primer extension analysis of RNA from cells transfected with WT SV40 and mutant *dpm*435. Poly(A)$^+$ RNA was purified from CV-1PD cells 44 h after transfection. cDNAs were made with (A) primer 1543E and (B) primer 682E and analyzed as described in the legend to Fig. 5. Samples were undigested (−) or digested (+) with *Pvu*II. The control lanes are the same as in Fig. 2. The size markers (MW) are *Msp*I-cut pBR322 DNA.

**A nonobligatory, but preferred, order of excision of introns from the late region of SV40.** The results presented here show that there is no obligatory order of excision of the introns present in the late transcripts of SV40 (Fig. 5). Nevertheless, a strong preference exists for the formation of doubly spliced 16S RNA, rather than doubly spliced 19S RNA. Whereas approximately 0.2% of cytoplasmic SV40 RNA is doubly spliced 19S RNA, approximately 16% is doubly spliced 16S RNA (Fig. 2 and 6). This strong preference probably reflects the very low efficiency of excision of the intron from nt 527 through 557, as indicated by the fact that the 19S RNA species E and F represent only 1 to 2% of SV40 RNA found in WT SV40-infected cells (Fig. 2; also see reference 38). A likely reason for the low efficiency of excision of this intron is its size. This 31-nt intron is probably not excised from normal precursor RNAs. Rather, a 5274-nt intron is probably excised from a greater-than-full-length precursor RNA. These greater-than-full-length transcripts, while being common in polyomavirus-infected cells, are rare in SV40-infected cells (8).

The nonobligatory order of excision of two or more introns confirms similar conclusions reached with other multiply spliced transcripts. The excision of two introns from globin RNA, studied both in vivo (48) and in vitro (19), and from adenovirus type 2 transcripts (11, 17, 25) can occur in a nonobligatory order. In all cases, excision of the two introns occurs in a preferred order. These results are consistent with the hypothesis that introns are not excised in an obligatory order; however, introns have different intrinsic rates of excision which determine a preferential order of excision.

**Spliced 19S RNAs not intermediates in the synthesis of the 16S RNAs.** One model to explain the excision of introns involves discrete intermediate cleavage and ligation events using either known functional splice sites or cryptic inter-

mediate processing sites (14, 18, 37, 42). Such an idea has been invoked to explain the synthesis of the 16S RNAs from 19S RNA intermediates (13, 44). Given the assumption that the prespliced transcripts are processed via the same processing pathways as are spliced, WT transcripts, our experiments show that the normal 16S intron (nt 527 through 1462) cannot be excised by using a processing pathway in which spliced 19S RNA is an intermediate (Fig. 4). These results are consistent with the current model of intron excision by a single cleavage and ligation event. Similar conclusions have been reached concerning the processing of RNA from the adenovirus type 2 early region 1a gene (42) and the SV40 early region (24).

**Use of alternative 5′ splice sites to synthesize a 16S-like RNA.** The exclusive use of the 3′ splice site at nt 1463 to join only with the 5′ splice site at nt 526, although the 3′ splice site at 558 can join with any of three alternative 5′ splice sites, suggests that the use of the nt 1463 splice site is obligatorily coupled to use of the nt 526 splice site. Transcripts from two of the mutants with precise deletions of the 19S introns were processed to 16S RNA species by utilization of the normal 16S 3′ splice site and one of two 5′ splice sites normally used to synthesize the 19S RNAs (Fig. 4). Thus, utilization of the 3′ splice site at nt 1463 is not obligatorily coupled with the utilization of the 5′ splice site at nt 526. This result confirms the findings of Chu and Sharp (7) and Rautmann et al. (33) that heterologous and synthetic splice sites, respectively, are capable of joining together. It should be noted, however, that these alternative 16S RNA species did not accumulate in mutant-transfected cells to the levels of the 16S RNAs seen in WT-transfected cells (Fig. 3). This greatly reduced efficiency of synthesis was unexpected, and the reason for it is unknown.

**Function and origin of the doubly spliced 19S RNA species.** A plasmid with a deletion corresponding to a doubly spliced 19S RNA species was isolated, and the presence of this RNA species in the cytoplasm of SV40-infected monkey cells was confirmed (Fig. 2). The functions of this RNA species are unknown; a likely possibility is that this doubly spliced 19S RNA species serves no unique function (Good et al., in press).

Perhaps this RNA is merely a byproduct of the splicing pathways of the SV40 late RNAs. Transcripts initiated upstream of nt 294 are spliced preferentially with the nt 294 5′ splice site to form initially either RNA species B or species C (P. J. Good and J. E. Mertz, manuscript in preparation). RNA species B is further spliced to form 16S RNA species H. If this RNA species B is a greater-than-genomic-length transcript, however, the genomic-length intron from nt 526 through 557 can be excised to form 19S RNA species F. Evidence that RNA species B is an intermediate in processing of SV40 late transcripts includes the following. (i) Primary transcripts made from mutant SVΔ294, which correspond to RNA species B, were further processed to both the doubly spliced 19S and 16S RNA species (Fig. 5). (ii) RNA species B accumulated preferentially in the nucleus (Fig. 2). (iii) Mutations altering the accumulation of RNA species B also alter, in like fashion, accumulation of 16S RNA species H (38). (iv) RNA species B is found among the nascent SV40 late transcripts present in association with viral transcription complexes (22). As shown above, however, it is still possible for the excision of the intron from nt 295 through 434 to occur on RNA transcripts lacking either the 19S or the 16S RNA introns (Fig. 5). Therefore, these events can occur in either order.

**Regulation of selection of splice sites from the SV40 late region.** What determines the selection of splice sites used in the processing of the SV40 late transcripts? We adopt the hypothesis of Reed and Maniatis (35) and Fu and Manley (10) that selection of splice sites used during processing is governed by competition between different splice sites on a single RNA molecule for recognition and subsequent utilization. Two factors determine how an SV40 late transcript is spliced: the location of the 5′ end of the transcript and the relative intrinsic efficiencies of utilization of the various splice sites. The location of the 5′ end obviously determines which splice sites are encoded within the transcript. The relative intrinsic efficiency of utilization of a splice site determines what percent of a given population of identical transcripts will be spliced using a particular splice site.

This model is consistent with the altered ratios of spliced RNA species seen in cells infected with a variety of SV40 leader region mutants (12, 31, 32, 45, 46). The alteration in each of these mutants results in one or more of the following changes: removing competing splice sites, shifting the locations of the 5′ ends of the primary transcripts, altering the sizes of introns, and changing sequences surrounding splice sites. These changes affect the utilization of the different splice sites, thereby giving rise to altered ratios of the spliced RNA species. A complete understanding of SV40 late RNA processing will require a detailed biochemical knowledge of the mechanism of 5′ and 3′ splice site recognition, along with a mutational analysis of possible competing splice sites encoded within the SV40 late region.

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