Splice site requirement for the efficient accumulation of polyoma virus late mRNAs

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ABSTRACT

Polyoma virus late nuclear primary transcripts are giant and heterogeneous, containing tandem repeats of the late strand of the circular viral genome. Late pre-mRNA processing involves the splicing of noncoding 'leader' exons to each other (removing genome-length introns), with the joining of the last leader to a coding 'body' exon. We have constructed a number of mutants blocked only in leader-leader splicing, or blocked in both leader-leader and leader-body splicing. We examined the accumulation of both nuclear and cytoplasmic late-strand RNAs in NIH3T3 cells. Consistent with our previous results, mutants lacking the 3′ splice site of the late leader (leader-leader splicing blocked) showed a 10–20 fold defect in late RNA accumulation. Mutants which lacked the leader 5′ splice site (leader-body splicing blocked) had a more profound defect, exhibiting virtually no late-strand cytoplasmic or nuclear RNA. This result was unexpected as a substantial proportion of wild type late cytoplasmic messages are unspliced. A mutant with no intron, but having functional 3′ and 5′ splice sites bordering the leader exon, is capable of producing large amounts of unspliced late mRNA. This demonstrates that an excisable intron is not a requirement for late mRNA accumulation. The accumulation of polyoma late mRNAs requires the presence of leader exons bordered by functional 3′ and 5′ splice sites, whether or not these sites are used during pre-mRNA processing.

INTRODUCTION

Our interest in polyoma virus late gene expression has led us to study the pathways by which late viral pre-mRNA molecules are processed. Polyoma virus late transcription termination and polyadenylation are both inefficient, leading to the production of a heterogeneous collection of primary transcripts, some of which are many times the size of the viral genome (1–5). Figure 1 illustrates the current model for how one such giant pre-mRNA molecule is processed into a mature cytoplasmic message for the major viral structural protein, VP1. As illustrated in the figure, the late leader, a noncoding exon which is flanked by consensus 3′ and 5′ splice sites, appears multiple times. During RNA processing, leaders are spliced to each other, removing genome-length introns. In addition, the final late leader exon can be alternatively spliced to either of two terminal coding exons. Thus, mature late cytoplasmic messages contain, at their 5′ ends, between one and 12 tandem copies of the late leader exon, followed by a single coding exon (6–9). Interestingly, one late message, that for VP2, contains no leader-body splice and represents 5–10% of late viral messages (10; Y.Luo and G.Carmichael, unpublished). About 30–40% of these VP2 messages contain single leaders at their 5′ ends and are thus completely unspliced (9).

We have been conducting extensive studies of the polyoma late leader in order to further define its role in late viral gene expression. Its position in late primary transcripts is unusual in that its presence generates molecules with 3′ splice sites at or near their 5′ ends. We have previously shown that the length but not the sequence of the late leader is important for virus viability (11, 12). Mutants with leaders of substituted sequence are phenotypically wild type in tissue culture, but those with leaders shorter than 33 nucleotides are nonviable and produce reduced levels of late messages. This accumulation defect was associated with inefficient leader-body splicing in these mutants. We have hypothesized that the defect in short leader mutants is due to the juxtaposition of the leader splice sites, one or both of which might be compromised as a result of steric hindrance (11).

We have shown that mutants with deletions or mutations in the late leader 3′ splice site also exhibited a deficiency in leader-body splicing (13) as well as a defect in accumulation of late-strand mRNA in both the cytoplasm and nucleus. These results indicated that accumulation of polyoma late RNA in both the nucleus and the cytoplasm is dramatically affected by mutations that interfere with leader-leader splicing, and led us to suggest that leader-leader splicing is required for efficient leader-body splicing (13).

Our laboratory is interested in understanding how alternative splice site choices are made in the polyoma system. In this report we have investigated the role of splicing and of late splice sites in the accumulation of late messages from this virus. Our approach has been to mutate specific late splice sites and then to determine the effects on late viral RNA processing and

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accumulation. After transfecting splice site mutants into NIH3T3 cells, the accumulation of both nuclear and cytoplasmic late RNAs was examined. Consistent with our previous results, mutants lacking the 3′ splice site of the late leader showed a defect in late RNA accumulation. However, mutants lacking the leader 5′ splice site had a more profound defect, with virtually no detectable accumulation of cytoplasmic or nuclear RNA. A mutant with both the leader 3′ and 5′ splice sites, but having no functional message body 3′ splice site and no excisable intron produced a wild type level of late cytoplasmic messages, a large fraction of which were unspliced. From these results we conclude that polyoma late pre-mRNA molecules with functional 3′ and 5′ splice sites bordering the late leader exon are a prerequisite for RNA accumulation in the nucleus and cytoplasm, whether or not these sites are used during pre-mRNA processing. Polyoma late RNA accumulation does not, however, appear to be intron-dependent.

MATERIALS AND METHODS

Materials

Restriction enzymes, DNA polymerase I Large Fragment (Klenow enzyme), T4 DNA ligase, T4 DNA polymerase, and T4 polynucleotide kinase were obtained from New England Biolabs, and used as suggested by the supplier. *E. coli* strains JM83, UT481, GM1634 (*dam*$^-$), and CJ236 (*dut*$^-$*ung*$^-$) were used to propagate plasmids by standard procedures (14, 15). RNase T2 and AMV reverse transcriptase were obtained from Bethesda Research Laboratories. Oligonucleotides were synthesized using a Milligen/Biosearch Cyclone DNA synthesizer. Mutagenesis was performed using the BioRad Labs Muta-gene® *in vitro* mutagenesis kit. Polymerase chain reaction analysis (PCR, ref. 16) was performed using the GeneAmp® kit from Perkin Elmer Cetus. Sequencing was performed by the dideoxy method using the Sequenase® kit from United States Biochemical Corp. [α-^{32}P]-labeled deoxynucleoside and ribonucleoside triphosphates and [γ-^{32}P]ATP were obtained from New England Nuclear. All polyoma constructs were made using the strain 59RA (17–19).

Construction of polyoma mutants

Bcl-BamΔ mutants. The polyoma truncated leader mutants, ALM and ALM11, and the wild-type plasmid pPK have been described previously (13). Each of these plasmids was grown in *dam*$^-$ *E. coli*, and purified two times by CsCl density gradient centrifugation. In order to create a large deletion which included both the late leader 5′ splice site and the VP3 3′ splice site, 200 μg of each plasmid was digested first with BclI and then with BamHI. Complete digestion liberated the polyoma sequences from the vector backbone, and also removed a 389 bp fragment representing the BclI to BamHI fragment from the polyoma late region (see Fig. 2). The digested plasmids were then recircularized under dilute ligation conditions as follows. DNAs (200 mg) were incubated in 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP with 400 U T4 DNA ligase for 16 hours at 16°C at a final DNA concentration of 0.01–0.025 mg/ml. After incubation, each mixture was extracted once with phenol:chloroform (1:1 v/v). The aqueous phase was removed to clean tubes, made 0.3 M with NaOAc, and the DNA precipitated with 2 vol ethanol. DNAs were pelleted, dried under vacuum, and resuspended in 2–5 ml TE (10 mM Tris-HCl, pH 7.5, 1 mM EDTA). The DNA solution was then passed through a 0.2μ filter, and reprecipitated. The final precipitate was resuspended in 1–5 ml ddH_2O and used in transfection experiments as described below.

Splice site deletion mutants (5′ssΔLL, 3′ssΔVP3/1). Oligonucleotide-directed mutagenesis was used to construct specific 6 or 9 bp in-frame deletions of the 5′ splice site of the late leader (LL), and the 3′ splice sites of the VP3 and VP1 exons (see Fig. 2). No exon sequences were removed. For mutant 5′ssΔLL the oligonucleotide used was 5′-GAAGCTGATCAAGAATTTT-CAAAA-3′. Mutagenesis removed 6 bp: the conserved GT dinucleotide and four additional base pairs downstream of the late leader exon. For mutant 3′ssΔVP3 the oligonucleotide used was 5′-CCTCTACTATTTGAATACAGCGGT-3′. Mutagenesis removed 6 bp: the conserved AG dinucleotide and four additional base pairs upstream of the VP3 splice acceptor site. For mutant 3′ssΔVP1 the oligonucleotide used was 5′-GGATGCTTCC-TTGGCTGTACGGTG-3′. Mutagenesis removed 9 bp: the conserved AG dinucleotide and four additional base pairs upstream of the VP1 splice acceptor site. The template used to

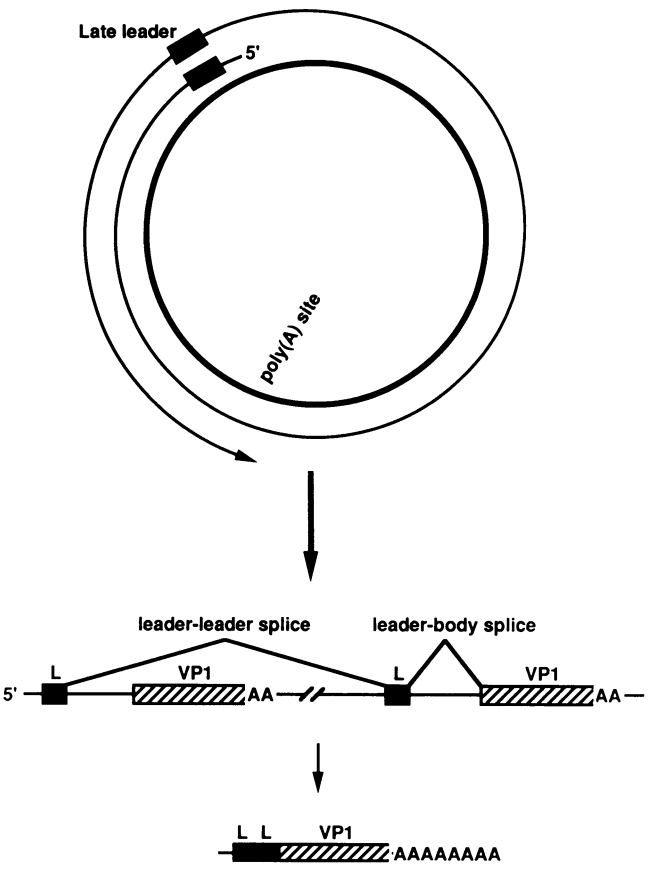

Figure 1. A model for the processing of polyoma virus late giant primary transcripts and the production of multiple, tandem non-translated late leader units on mature mRNA molecules. Due to inefficient termination by RNA polymerase II late viral transcripts are heterogeneous in length, with many representing multiple circuits of the viral genome by the polymerase. The example shown is for a transcript created by two passes through the viral late region. During pre-mRNA processing a leader-body splice joins the 3′-most non-coding late leader exon (L) to a message coding body [here mVP1, which accounts for 80–85% of late message (10, 39, Y. Luo and G. Carmichael, unpublished)]. In addition, leader-leader splicing removes a genome length intron, yielding a final mVP1 message with two tandem leader units at its 5′-end.

create 5'ssΔLL and 3'ssΔVP3 was M13BPorimp18 (BPori), which contains a 1169 bp BamHI-PstI origin fragment spanning the late promoter region of polyoma inserted into M13mp18. M13RPHD-12mp19 (RPHD12, ref. 9), was the template used to create 3'ssΔVP1. Single stranded DNA templates were made after growth of the M13 clones in the *dut⁻ung⁻* strain CJ236. After the annealing and synthesis steps, the resultant double strand templates were introduced into *E. coli* UT481, and mutants verified by dideoxy sequencing.

Fragments containing deletions were removed from the M13 clones and subcloned into the entire polyoma genome which had been inserted into the vector pBS⁺ (Stratagene) at the unique EcoRI site (pPyBS(RI)). To get the final 5'ssΔLL polyoma mutant required a two step subcloning. The first step consisted of removing the BamHI-NarI fragment containing the deletion and putting it into pHEoriBS to create p5'ssΔLL(HEoriBS). pHEoriBS was made by inserting the polyoma HindIII-EcoRI fragment spanning the polyoma replication origin into pBS⁺. The second step consisted of placing the EcoRV-NarI fragment from p5'ssΔLL(HEoriBS) into pPyBS(RI) creating the mutant 5'ssΔLL.

To obtain the final double deletion mutant, 3'ssΔVP3/1, required a three step subcloning. First, the BamHI-NarI fragment (containing 3'ssΔVP3) and the BamHI-HindIII fragment (3'ssΔVP1) were placed separately into pHEoriBS to create p3'ssΔVP3(HEoriBS) and p3'ssΔVP1(HEoriBS). Second, the BamHI-HindIII fragment from p3'ssΔ(HEoriBS) was subcloned into p3'ssΔVP3(HEoriBS) to create the double mutant p3'ssΔVP3/1(HEoriBS). Finally, the EcoRV-NarI fragment from p3'ssΔVP3/1(HEoriBS) was placed into pPyBS(RI) creating the mutant 3'ssΔVP3/1.

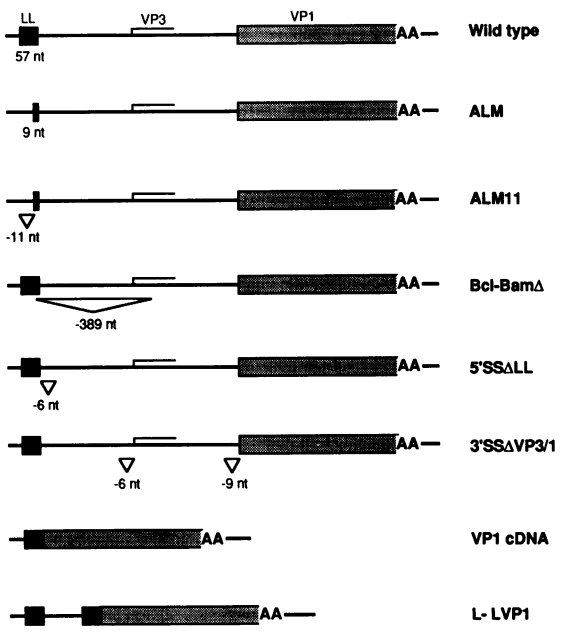

Figure 2. Constructs used in this study. Construction of mutants is described in Materials and Methods. The top line schematically illustrates the late transcription unit of wild type polyoma virus. The 57-base noncoding late leader exon (LL) is shown as a solid box, and the 3' splice site acceptors for the VP3 and VP1 splices are indicated. The VP1 coding exon is shown as a stippled box, with the late poly(A) site at its distal border. Mutant ALM (11, 12) contains a truncated, 9-base leader exon. Mutant ALM11 is ALM containing a further 11-base deletion of the 3' splice site upstream of the leader (13). The Bcl-BamΔ mutants lack the 389 nt fragment from the BclI site near the 3' end of the late leader, to the BamH1 site 70 nt downstream of the VP3 3' splice site. Mutant 5'ssΔLL lacks 6 nt (GTAAGT) at the 5' splice site bordering the late leader. Mutant 3'ssΔVP3/1 lacks 6 nt (TCCCTAG) at the VP3 3' splice site , and 9 nt (TAATTCTAG) at the VP1 3' splice site. Mutant VP1cDNA precisely lacks the mVP1 intron. Mutant L-LVP1 is VP1cDNA into which a 165 bp fragment containing the late leader exon and flanking sequences has been inserted.

VP1cDNA and L-LVP1 mutants. To study the effects of exon splice site removal and intron retention on splicing, a VP1 cDNA and a second late leader cDNA mutant were made. The VP1cDNA mutant was made by annealing two complementary synthetic oligonucleotide adaptors to create a double strand fragment with a EcoRV blunt end and a BclI 5' overhang. The oligonucleotides used were 5'-ATCACCGTACAGCCTT-3' and 5'-GATCAAGGCTGTACGGTGAT-3'. The two oligonucleotides were mixed in equimolar amounts in TE, boiled 2 min, and slow cooled to 25°C. This fragment was then mixed in a 5:1 ratio with BclI-cut PyBS(RI) and ligated at 16°C overnight. Ligated plasmids were introduced into JM83, and correct candidates were sequence-verified. pVP1cDNA was made *dam⁻* by growth in *E. coli* strain GM1634 for the next cloning.

The mutant L-LVP1, which consists of a second complete late leader exon and part of the VP1 intron, was made by removing a 165 bp BclI fragment from the double leader mutant pYL-5 (Y. Luo and G. Carmichael, submitted), and inserting it into pVP1cDNA. pYL-5 was made by inserting the EcoNI to PvuII fragment (rendered blunt-ended with Klenow enzyme) containing the late leader into the filled in EcoNI site immediately downstream of the late leader into pT7-1 (Boehringer Mannheim).

Cell culture techniques, transfections, and nuclei and RNA isolations

Mouse NIH3T3 cells were propagated, and transfections performed using a modification of the Chen and Okayama procedure (20) as described elsewhere (19, 21). At 40 to 48 hr after transfection total RNA, nuclear RNA, cytoplasmic RNA, and nuclei for run-on transcription were isolated using modifications of procedures as previously described by our laboratory (19, 22).

Preparation of probes and RNA analysis

Total, cytoplasmic and nuclear RNAs were analyzed by RNase T_2 protection experiments as previously described (13). The riboprobes used are as follows: analysis of the VP1 3' splice site was done using a 714-bp BamHI-HindIII late fragment cloned into pBS⁺ (11). As a control, early splicing was analyzed using a 359-bp AvaI early fragment cloned into pBS⁺ (11). Late 3' end processing was analyzed using pXP1, which consists of the XbaI-PstI late fragment cloned into pBS⁺.

PCR assay

The polymerase chain reaction was performed as described (9). Briefly, oligonucleotides 275 (5'-TATCACCGTACAGCCTTG) and 276 (5'-TGAAAATTCACTTACTTG) were made complementary to the late mRNAs for VP1 and VP2, respectively, and span the junction site of the late leader to these exons. Oligonucleotide 278 (5'-CCTGACATTTT-CTATTTTAAG) binds to a reverse transcript of the immediate upstream region of the late leader mRNA. Cytoplasmic RNAs were isolated from transfected cells, and copied with AMV reverse transcriptase, using oligonucleotide 275 or 276 as primers, at 45°C for 1 hr under Taq polymerase reaction

conditions. Taq polymerase and oligonucleotide 278 were then added and the mixtures cycled as follows. Samples were incubated at 94°C for 2 min, followed by 30 cycles of incubation at 94°C for 2 min (melting), 45°C for 1 min (annealing), and 72°C for 1.5 min (extension). Samples were then incubated at 72°C for 10 min, and brought to 4°C. Leaders were resolved by electrophoresis on 6% polyacrylamide/7M urea sequencing gels.

RESULTS

In previous work we have shown that mutations that interfere with leader-leader splicing result in inefficient accumulation of polyoma virus late RNA molecules (11, 13). In order to better understand the role of splicing in polyoma late mRNA accumulation, we first constructed and analyzed a series of mutants lacking a 389 bp fragment of late DNA which spans from the late leader 5′ splice site through the VP3 3′ splice site (Bcl-BamΔ, Fig. 2). Mutants with this deletion would be expected to be unable to undergo any late splicing events, as the 5′ splice site of the late leader exon is involved in all late splicing. This deletion was introduced into our wild type polyoma genome, into mutant ALM, which has a short, 9 base leader exon but which retains both the leader 5′ and 3′ splice sites (Fig. 2, ref. 11), and into mutant ALM11, which has an 11 bp deletion of the ALM leader 3′ splice site (Fig. 2, ref. 13). Mutants ALM and ALM11 have been previously shown to produce only 5−10% wild type levels of late mRNAs (13). Each mutant genome was transfected into NIH3T3 cells and both cytoplasmic RNA (for RNAse protection assays) and nuclei (for nuclear run-on assays) were harvested. Results of RNase protection assays to measure early and late RNA levels are shown in Fig. 3. Analysis of early-strand messages served as an internal control for transfection efficiencies. The wild type, ALM and ALM11 late RNAs gave the same results as seen in earlier work (12, 13), namely, efficient accumulation and VP1 splicing in wild type, and decreased late RNA accumulation and splicing in mutants ALM and ALM11. In contrast, the Bcl-BamΔ mutants produced no detectable late RNA, even though early RNAs were present at the same levels as wild type (Fig. 3). Nuclear run-on experiments confirmed that in these mutants promoter strength was not significantly different from wild type (data not shown), indicating that failure to accumulate late transcripts in the nucleus is not due to a failure of transcription initiation. These results suggest that blocking both leader-leader and leader-body splicing leads to a more severe defect than blocking only leader-leader splicing by removal of the leader 3′ splice site.

In order to examine further the role of splicing and the requirement for the leader 5′ splice site in late viral RNA accumulation, two mutants lacking the leader 5′ splice site were made. Mutant 5′ssΔLL (Fig. 2), was constructed by oligonucleotide directed mutagenesis and contained a 6 bp deletion of the 5′ splice site immediately downstream of the late leader exon (GTAAGT). Mutant VP1cDNA can only produce prespliced mVP1 molecules, since it precisely lacks the VP1 intron. RNase protection experiments of nuclear and cytoplasmic RNAs obtained after transfection using these constructs are shown in Fig. 4. As can be seen, both mutants produced early-strand

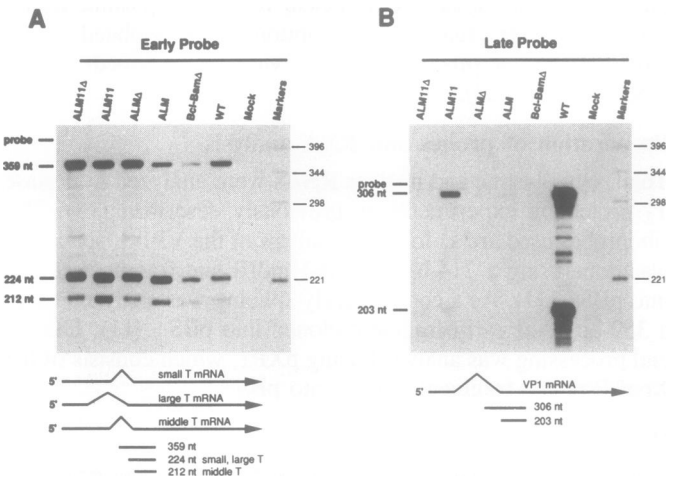

Figure 3. RNase protection analysis of Bcl-BamΔ mutants. Viral DNAs were prepared and transfected into NIH3T3 cells as described in Materials and Methods and total cellular RNA harvested 48 hrs post transfection and subjected to RNase protection analysis using riboprobes specific for early or late viral transcripts (13). **A.** Analysis of early RNAs. Since all constructs contained identical early viral transcription units, this analysis served as a control for transfection efficiencies. At the bottom is shown a schematic diagram of the structures of the three early messages, and the sizes of the protected species predicted. **B.** Analysis of late RNAs. The same amounts of RNA were used as for panel A, but using a late-specific riboprobe. Molecules with the VP1 splice generate a protected band of 203 nt, and other late RNAs lead to the 306 nt band.

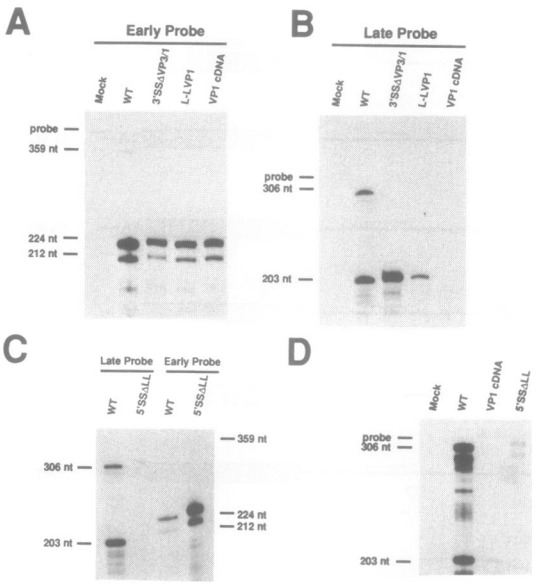

Figure 4. RNase protection analysis of splice site mutants. Transfections and RNA analyses were as in Figure 3. **A.** Early probe analysis of cytoplasmic RNA harvested from cells mock transfected, or transfected with wild type, or with mutant 3′ssΔVP3/1, L-LVP1 or VP1cDNA. **B.** Late probe analysis of the constructs shown in panel A. Assays with the late probe used the same amounts of RNA as those using the early probe. The late-specific 306 nt protected band does not appear in the lanes for 3′ssΔVP3/1, L-LVP1 or VP1cDNA because these mutants are discontinuous with the probe at the VP1 3′ splice site. **C.** Early and late probe analysis of mutant 5′ssΔLL. The assays were as in panel A. **D.** Analysis of nuclear RNA samples from WT and mutants VP1cDNA and 5′ssΔLL. Nuclear RNA was harvested from transfections as described in Materials and Methods. Equal amounts of RNA were subjected to RNase protection analysis using the late-specific riboprobe. The 203 nt band in the VP1cDNA lane reflects the total late RNA signal for this mutant. The 306 nt band in the 5′ssΔLL lane reflects the total late RNA signal for this mutant. In control experiments (not shown), WT and VP1cDNA showed approximately the same intensity of signals using the early probe, while mutant 5′ssΔLL showed about 5 times higher early signal.

RNA at normal levels. However, these two mutants failed to accumulate detectable late RNA in both the cytoplasm and nucleus. These results demonstrate that blocking both leader-leader and leader-body splicing leads to a more dramatic defect in late RNA accumulation than does blocking only leader-leader splicing. In other experiments, derivatives of mutant VP1cDNA were made which lacked the leader 3′ splice site and thus contained no splice sites at all. These constructs also exhibited no detectable late RNA accumulation in the nucleus or cytoplasm (data not shown).

The above results pointed to the critical roles played by the splice sites flanking the late leader exon in late RNA accumulation. We next asked whether the functional splice sites flanking the leader exon, or the presence of a functional intron, was the key determinant of late RNA accumulation. Two additional mutants were constructed to answer these questions. Mutant L-LVP1 consists of the VP1cDNA mutant into which a 165 bp fragment containing the late leader exon and flanking sequences has been inserted. This mutant contains a functional intron just upstream of the VP1cDNA sequences. Mutant 3′ssΔVP3/1 is a double mutant of the wild type genome where 6 bp at the VP3 3′ splice site sequence (TCCCTAG) and 9 bp at the VP1 3′ splice site (TAATTCTAG) are removed. The deletions introduced were designed to eliminate any possible 3′ splice site consensus sequence. This mutant should be incapable of VP1 or VP3 body splicing as it retains functional leader 3′ and 5′ sites. However, giant late primary transcripts from this mutant should be capable of undergoing leader-leader splicing. These constructs are illustrated schematically in Fig. 2. Both constructs were transfected into NIH3T3 cells, and cytoplasmic RNA harvested and again analyzed by RNase protection experiments. Results are shown in Fig. 4. In contrast to the 5′ splice site mutants (5′ssΔLL and VP1cDNA), both of these mutants demonstrated efficient accumulation of late RNA, at essentially wild type levels (Table 1).

Table 1. Relative Accumulation of Late Cytoplasmic mRNAs.

Wild Type	1
3′ssΔVP3/1	3.2
L-LVP1	0.45
VP1cDNA	0
5′ssΔLL	0.009

The intensities of bands from the RNAse protection experiment shown in Figure 4 were determined using a Betagen Betascope 603 Blot Analyzer. Early RNA levels were used to normalize transfection efficiencies.

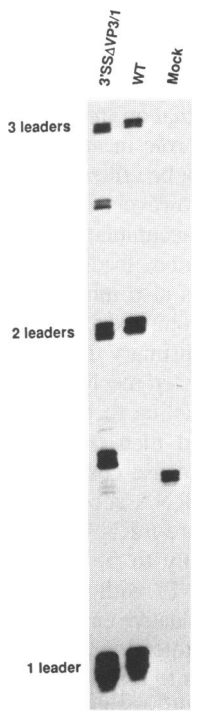

Figure 5. PCR analysis of late cytoplasmic RNA from mock, wild type and mutant 3′ssΔVP3/1-transfected cells. Cytoplasmic RNA was prepared and subjected to reverse transcription and PCR analysis as described in Materials and Methods. For mock and WT lanes, primer 275 (specific for mVP1) was used, while for 3′ssΔVP3/1, primer 276 (specific for mVP2) was used. The bands seen in the mock lane and between the positions of leader repeats in the mutant 3′ssΔVP3/1 sample are artifacts of this PCR reaction and were not seen in other experiments.

Finally, an RNA PCR analysis was performed to determine the distribution of late leaders at the 5′ ends of late messages from mutant 3′ssΔVP3/1. Work to be presented elsewhere (Y. Luo and G. Carmichael, submitted) shows that L-LVP1 produces abundant late messages with multiple leader structures at their 5′ ends. The PCR results for wild type (WT) and 3′ssΔVP3/1 are shown in Figure 5. This assay was performed as previously described from our laboratory, and has been shown to accurately reflect the distribution of late leader exons on polyoma late mRNA molecules (9). Mutant 3′ssΔVP3/1 has about the same levels of 1, 2, and 3 leader messages as wild type (determined by quantitation using a Betascope Blot Analyzer). Therefore, not only are giant transcripts produced in this mutant, but leader-leader splicing occurs at about the same efficiency as in wild type. In addition, the levels of single leader transcripts observed indicate that RNAs that contain a viable leader 5′ splice site do not need to be spliced in order to accumulate in the cytoplasm. The key observation here is that in mutant 3′ssΔVP3/1, 30−40% of all of its cytoplasmic late viral RNA appears to be unspliced, although the precursors to such messages contain no functional introns. In order to rule out the possibility that cryptic 3′ splice sites were used in this mutant, we performed RNase protection analysis using a probe which spanned much of the late region. We were unable to detect any evidence whatsoever for cryptic splice site usage (data not shown). These results lead us to conclude that functional leader splice sites, but not a functional intron, are important for polyoma late RNA accumulation.

DISCUSSION

In eukaryotic cells, most pre-mRNAs contain introns which are removed by splicing to produce mature mRNA. The need to have excisable intervening sequences present in order to get efficient mRNA accumulation has been demonstrated in many systems, such as SV40 late genes (23−26), rabbit β-globin (27), mouse dihydrofolate reductase (27, 28), rat growth hormone (29), maize alcohol dehydrogenase-1 (30) and immunoglobin heavy chain μ (31). However, splicing is not always necessary for mRNA accumulation. Cloned chicken thymidine kinase cDNA, which contains no introns, can produce stable mRNA after transfection into mouse cells (32). Likewise, the genes for histones (33), α-interferon (34) and c-*jun* (35) lack introns. A mutant of polyoma virus early middle T antigen lacking introns can also accumulate mRNA after transfection of mouse cells (36).

How do introns or splice sites affect polyoma virus late mRNA accumulation? Polyoma late pre-mRNA processing is complex, involving both leader-leader splicing in giant and heterogeneous RNAs, and alternative usage of coding exon splice sites. An interesting feature of polyoma late primary transcripts is that a 3′ splice site appears at their 5′ ends. Noncoding late leader exons can splice to each other in giant primary transcripts, thus removing genome-length introns. The terminal leader exon can be joined to two alternative body exons, which encode the viral structural proteins VP1 and VP3. Our laboratory is interested in understanding how alternative splice site choices are made in this system. In this report we have investigated the role of late splice sites and splicing in the accumulation of late messages from this virus. Our approach has been to mutate specific late splice sites and then determine the effects on late viral RNA processing and accumulation.

We have previously studied two types of mutants which exhibit defects in leader-leader splicing, and which also show the accumulation of reduced levels of late mRNAs. The first consists of mutants with short leaders. We have previously shown that the length but not the sequence of the late leader is important for virus viability (11, 12). Mutants with leaders of substituted sequence are phenotypically wild type in tissue culture, but those with leaders shorter than 33 nucleotides are nonviable and produce reduced levels of late messages (5−10% WT). In these mutants the accumulation defect was also associated with inefficient leader-message body splicing. This can be seen here, in Figure 3 (mutant ALM). Although the reason for late RNA instability in ALM and other short leader mutants remains unclear, we have hypothesized that the defect is due to the juxtaposition of the two efficient leader splice sites, one or both of which might be compromised as a result of steric hindrance (11).

The second type of mutants defective in leader-leader splicing consists of those with mutations in the 3′ splice site just upstream of the leader exon. In other work we have shown that mutants with deletions or mutations in the late leader 3′ splice site exhibited the same phenotype as the short leader mutants, namely, a deficiency in leader-body splicing (13) as well as a 10−20 fold defect in accumulation of late mRNA in both the cytoplasm and nucleus. These results indicated that accumulation of polyoma late RNA in both the nucleus and the cytoplasm is dramatically affected by mutations that interfere with leader-leader splicing, and led us to suggest that leader-leader splicing is required for efficient leader-body splicing (13). This can be seen here, in Figure 3 (mutant ALM11). We do not yet know why leader-body splicing is inefficient in the absence of leader-leader splicing. However, recent work from our laboratory has suggested that this may be important to the virus in its temporal regulation of late-strand gene expression (9, 22).

Here we report the construction and study of different mutants that are specifically blocked in their ability to undergo leader-body splicing, but which exhibit very different effects on late mRNA accumulation. The first type lacks a functional leader 5′ splice site, and cannot undergo any late splicing. These are the Bcl-BamΔ mutants, and mutants VP1cDNA and 5′ssΔLL. Each of these is capable of producing wild type levels of early-strand RNAs, but each accumulates little or no detectable late-strand RNA in the cytoplasm (Figs. 3, 4 and Table 1). The defect is not one of mRNA transport from the nucleus, because nuclear RNA is also greatly diminished (Fig. 4D). It is important to note that in these mutants the accumulation defect is more profound than the defect in mutants only blocked in leader-leader splicing. As these leader-body splicing mutants all lack functional introns, the results obtained could indicate that polyoma virus late RNAs are members of the class of RNA molecules that require the presence of excisable introns for their accumulation. Consistent with this view, addition of a functional intron to the VP1cDNA construct (mutant L-LVP1), resulted in almost wild type levels of late message accumulation.

A requirement for an excisable intron is inconsistent, however, with results seen with the second type of mutant blocked in leader-body splicing, 3′ssΔVP3/1. This mutant lacks both late message body splice acceptor sites, but can still undergo leader-leader splicing in giant primary transcripts. This mutant produces wild type levels of late cytoplasmic mRNA (Fig. 4 and Table 1), demonstrating that the VP3 and VP1 body 3′ splice sites themselves are not critical for late RNA accumulation. RNA PCR analysis (Fig. 5) reveals that this mutant produces a large fraction of late messages with single leader units at their 5′ ends. These single leader mRNAs are significant because they do not contain any excisable introns and therefore indicate that polyoma late RNAs do not require an intron in order to accumulate. However, these mRNAs contain both 3′ and a 5′ splice sites, namely, those bordering the late leader exon. As we can find no evidence for usage of cryptic splice sites in these molecules, we conclude that late leader splice sites are important for the accumulation of polyoma late messages, whether or not they are used during pre-mRNA processing. It is important to note that the RNA PCR method used to detect single leader species on 3′ssΔVP3/1 mRNA molecules was confirmed in separate experiments to accurately reflect the fraction of messages with particular leader structures at their 5′ ends (9).

How could the presence of splice sites themselves stabilize polyoma late pre-mRNA molecules in the nucleus? Our results with mutant 3′ssΔVP3/1 suggest that a pair of functional splice sites is necessary, but that an intron is not. The leader 5′ splice site can clearly play a role in nuclear RNA stabilization, even in cases where it cannot be utilized for splicing. Our data do not allow us to conclude, however, that the leader 3′ splice site plays a direct role in RNA accumulation. This is because whenever this splice site is mutated, leader-to-body splicing (use of the leader 5′ splice site) is also inhibited. It is quite possible that splicing factors, when complexed with RNA molecules, can either stabilize or destabilize primary transcripts. Destabilization could occur by a snRNP-directed mechanism, such as proteins binding to 3′ splice sites and failing to find or interact with a 5′ splice site. This event would then trigger RNA degradation. This scenario is unlikely because constructs lacking any splice sites show little or no late RNA accumulation. On the other hand, it is more likely that pre-mRNAs are stabilized by snRNPs or proteins binding directly to 5′ splice sites. For example, the interaction of U1 snRNPs with 5′ splice sites might prevent or delay the action of degradative enzymes in the nucleus. This could occur by masking or protecting the pre-mRNA backbone, or by targeting the molecules to a subcellular location removed from ribonucleases. Also in this scenario, leader 3′ splice site mutants such as ALM11 might be less stable because the 5′ splice site is more efficiently recognized when paired with an upstream 3′ splice site, as postulated in the 'exon definition' model (40). In light of our results it is also interesting to speculate that in intron-independent transcription units, there may exist 5′ splice sites which are not used in splicing and therefore have not been detected. If this were true, then intron-dependent and intron-

independent gene expression would both reflect an underlying requirement for splice sites for nuclear pre-mRNA stability.

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