

A Suboptimal 5' Splice Site Is a *cis*-Acting Determinant of Nuclear Export of Polyomavirus Late mRNAs

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Mouse polyomavirus has been used as a model system to study nucleocytoplasmic transport of mRNA. Three late mRNAs encoding the viral capsid proteins are generated by alternative splicing from common pre-mRNA molecules. mRNAs encoding the virion protein VP2 (mVP2) harbor an unused 5' splice site, and more than half of them remain fully unspliced yet are able to enter the cytoplasm for translation. Examination of the intracellular distribution of late viral mRNAs revealed, however, that mVP2 molecules are exported less efficiently than are mVP1 and mVP3, in which the 5' splice site has been removed by splicing. Point mutations and deletion analyses demonstrated that the efficiency of mVP2 export is inversely correlated with the strength of the 5' splice site and that unused 3' splice sites present in the mRNA have little or no effect on export. These results suggest that the unused 5' splice site is a key player in mVP2 export. Interestingly, mRNAs carrying large deletions but retaining the 5' splice site exhibited a wild-type mVP2 export phenotype, suggesting that there are no other constitutive *cis*-acting sequences involved in mVP2 export. RNA stability measurements confirmed that the subcellular distribution differences between these mRNAs were not due to differential half-lives between the two cellular compartments. We therefore conclude that the nuclear export of mVP2 is strongly influenced by a suboptimal 5' splice site. Furthermore, results comparing spliced and unspliced forms of mVP2 molecules indicated that the process of splicing does not enhance nuclear export. Since mVP2 and some of its mutant forms can accumulate in the cytoplasm in the absence of splicing, we propose that splicing is not a prerequisite for mRNA export in the polyomavirus system; rather, removal of splicing machinery from mRNAs may be required. The possibility that export of other viral mRNAs can be influenced by suboptimal splicing signals is also discussed.

The mechanism of nuclear export of mRNAs is not well understood. Recently, it has become increasingly clear that migration of mRNAs from the nucleus to the cytoplasm is regulated not only by recognition of specific *cis*-acting signals on the substrate mRNAs but also by *trans*-acting factors that interact with them (23, 27, 39, 47). For intron-containing genes, mRNA export usually does not occur unless introns are removed by splicing. Many intronless transcripts encoded by cDNAs of intron-containing genes are not exported but are degraded in the nucleus (9, 17, 18, 41). It has therefore been hypothesized that splicing and export may be coupled processes and that splicing may be a prerequisite for export. In a study performed with *Saccharomyces cerevisiae* (28), a synthetic yeast intron sequence was inserted into a bacterial β -galactosidase gene and export of unspliced mRNAs was monitored by measuring β -galactosidase activity. Results showed that both *cis*- and *trans*-acting mutations that reduced the efficiency of splicing enhanced β -galactosidase activity, consistent with increased cytoplasmic accumulation (or translation) of unspliced mRNAs. On the basis of these observations, a second, alternative model, in which unspliced transcripts may be trapped in the nucleus because of association of spliceosomes, was proposed (28). The two models described above are not mutually exclusive.

Many animal viruses generate multiple mRNA products from a single pre-mRNA by alternative splicing. Consequently, some of the resulting mRNA products retain splicing signals yet are transported to the cytoplasm (3, 7, 25, 34, 37). In

retroviruses, many full-length pre-mRNAs are delivered to the cytoplasm to serve as genomic mRNAs for progeny virions and as mRNAs for proteins. Mechanisms by which unspliced or partially spliced viral mRNAs enter the cytoplasm differ. In the retrovirus human immunodeficiency virus type 1 (HIV-1), cytoplasmic accumulation of unspliced or partially spliced viral mRNAs is mediated by the interaction between the virus-encoded Rev protein and a specific *cis* element (the Rev responsive element [RRE]) present in the viral pre-mRNAs (11, 12). In the case of the Mason-Pfizer monkey retrovirus, cytoplasmic accumulation of unspliced and partially spliced viral RNAs does not appear to depend on the expression of viral proteins. Instead, an interaction between a particular sequence in the viral mRNAs and an as yet unknown cellular factor(s) is required (8). For some other viruses, such as the avian sarcoma virus and the murine polyomavirus, the mechanism by which unspliced and partially spliced viral RNAs reach the cytoplasm remains unknown.

In the present study, we used polyomavirus as a model system to study nucleocytoplasmic transport of mRNA. Polyomavirus has a double-stranded DNA genome of about 5.3 kb. Late-strand gene expression from this virus resembles that of retroviruses in that the viral pre-mRNA undergoes alternative splicing and a fraction of the pre-mRNA remains fully unspliced yet is exported to the cytoplasm. More importantly, this virus relies entirely on the cellular machinery for the synthesis, processing, and export of RNA. Therefore, it provides us with a valuable model system to study general mechanisms of how cells and viruses can regulate mRNA export.

Figure 1 illustrates splicing of primary transcripts synthesized from the late strand of the polyomavirus genome. The late transcription unit includes a single 5' splice site and three

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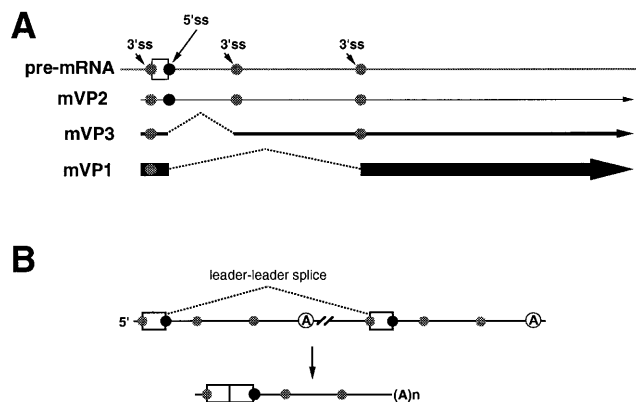


FIG. 1. Splicing of polyomavirus late pre-mRNA. (A) Leader-body splicing. The stippled line at the top represents polyomavirus late pre-mRNA, with the noncoding late leader exon indicated by the open box. The solid and stippled circles mark the late 5' splice site and three late 3' splice sites, respectively. The dotted lines denote leader-body splices. The relative amounts of late mRNAs are indicated by the thicknesses of the lines. (B) Leader-leader splicing. Late transcription termination is inefficient, and many primary transcripts are multigenomic in length. Processing includes the splicing of noncoding late leader exons and the removal of genome-length introns. The A within the open circle depicts the late polyadenylation signal. (A)_n indicates the poly(A) sequence. Dotted lines indicate leader-leader splicing.

alternative 3' splice sites (Fig. 1A). The promoter-proximal 3' splice site and the 5' splice site define the noncoding "late-leader" exon. Splicing between the 5' splice site and either of the downstream two alternative 3' splice sites (leader-body splicing) generates mRNAs for VP1 and VP3, which encode two of the viral capsid proteins and consist of ~80 and ~15% of the total late mRNAs, respectively (7). mRNA for VP2, the third capsid protein, lacks a leader-body splice and accounts for ~5% of the total late mRNA. Curiously, at late times in infection, termination of transcription from the late strand is inefficient. This leads to the production of multigenomic primary transcripts, which are subsequently resolved by splicing the leader exons (leader-leader splicing) to remove genome-length introns (Fig. 1B) (22, 46). While most late pre-mRNAs undergo leader-leader splicing prior to leader-body splicing (resulting in multiple tandem repeats of the late leader exons at the 5' ends of mRNAs), a majority of mVP2 molecules remain fully unspliced and possess a single leader exon.

How unspliced mVP2 is transported to the cytoplasm is the focus of this study. The questions we wanted to address were as follows. (i) Is there a constitutive transport element responsible for the export of unspliced mVP2, as is found in HIV-1 and Mason-Pfizer monkey virus? (ii) Does the process of splicing enhance export? (iii) Do unused splicing signals retard export? To approach these issues, we systematically altered the strength of the 5' splice site and demonstrated that the efficiency of export of mVP2 is inversely correlated with the strength of the 5' splice site. Deletion of all the 3' splice sites had little or no effect on export. Moreover, mRNAs with large deletions but containing the 5' splice site exhibited a similar export phenotype to that of wild-type mVP2, suggesting that no constitutive transport element or other negative *cis*-elements were involved in export. We therefore conclude that the suboptimal 5' splice site plays a crucial role in influencing mVP2 export. Finally, results have shown that leader-leader splicing does not improve the export of mVP2 (spliced and unspliced forms of mVP2 are exported to the cytoplasm with the same efficiency) and that a substantial fraction of mVP2 and some of its mutant forms can reach the cytoplasm in the

absence of splicing. This leads us to propose that the process of splicing in the polyomavirus system is not necessary for mRNA export and does not make export more efficient. Instead, removal of splicing machinery from mRNAs may be required for export.

MATERIALS AND METHODS

Materials. Restriction enzymes, T4 DNA ligase, and DNA polymerase I large fragment were from New England BioLabs and were used as suggested by the manufacturer. RNase T₁/T₂ was prepared as previously described (29). [α -³²P]UTP was from New England Nuclear. Actinomycin D was from Sigma. RQ1 DNase I and T3 and T7 RNA polymerases were from Promega. A PolyATtract mRNA isolation system kit was purchased from Promega and was used as recommended by the supplier. Mutagenesis was performed with a Bio-Rad Mutagen *in vitro* mutagenesis kit. *Escherichia coli* JM83, GM1634 (*dam*), and CJ236 (*dut ung*) were used to propagate plasmids by standard procedures (4, 35).

Cell culture and transfection. Mouse NIH 3T3 cells were maintained in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum, L-glutamine, penicillin, and streptomycin at 37°C in 5% CO₂. Transfections were carried out by a modified CaPO₄ DNA coprecipitation method (10). Briefly, approximately 6 h prior to transfection, cells were diluted twofold and replated in 150-mm plates. The total amount of DNA used per plate for transfection was 40 μ g. Before transfection, recombinant plasmids were digested with *Eco*RI and dilute-ligated with T4 DNA ligase as described previously (1).

Constructs. The salient features of some of the constructs used in this study are illustrated in Fig. 3A and 4A. For each construct, a polyomavirus genome was inserted into the unique *Eco*RI site of pBlueScribe (2). Before transfections, genomes were released from plasmid backbones with *Eco*RI and recircularized by dilute ligation.

The wild-type polyomavirus construct (5) and constructs Py5-1 and Py5-5 (Fig. 3A) (6) have been described previously. For construct Py-G, site-directed oligonucleotide mutagenesis (5) was used to alter base -1 of the late-leader 5' splice site to the indicated base (Fig. 3A) to generate a consensus 5' splice site. In Fig. 4A, site-directed mutagenesis was used to remove 11 nucleotides (nt) upstream of and including the leader 3' splice site, 6 nt upstream of and including the VP3 3' splice site, and 9 nt upstream of and including the VP1 3' splice site, creating mutant 3 \times 3'ss Δ . Mutants Mini and 3' Δ mini were built by deleting large sequences by site-directed mutagenesis. In Mini, sequences between immediately downstream of the VP3 3' splice site and 50 nt upstream of the AAUAAA late polyadenylation site were removed. 3' Δ mini is identical to Mini except that an 11-nt sequence upstream of and including the VP3 3' splice site has been deleted.

RNA preparation. Nuclear and cytoplasmic RNAs were isolated 48 h after transfection. For the preparation of cytoplasmic RNA, cells were rinsed free of media with ice-cold phosphate-buffered saline and were then disrupted with a modified Nonidet P-40 lysis buffer (10 mM *N*-2-hydroxyethylpiperazine-*N*'-2-ethylsulfonic acid [HEPES; pH 7.6], 10 mM NaCl, 3 mM CaCl₂, and 0.5% NP-40) on ice for 30 s. Cytoplasmic lysates were collected in new tubes, and appropriate amounts of guanidinium thiocyanate crystals were added to give a final concentration of 4 M. Cytoplasmic RNA was then purified through cesium chloride step gradients. For nuclear RNA, the above intact cell nuclei which were still attached to the plates were rinsed with ice-cold Nonidet P-40 buffer twice and then lysed in 4 M guanidinium isothiocyanate-20 mM sodium acetate (pH 5.2)-0.1 mM dithiothreitol-0.5% *N*-laurylsarcosine. The RNA was then pelleted through cesium chloride as described above. The ratio of the total yield of cytoplasmic RNA and nuclear RNA was consistently about 1:1. The PolyATtract mRNA isolation system kit was used to treat the purified nuclear RNAs to select poly(A)⁺ mRNAs.

RNase protection assays. Internally labeled RNA probes were made by *in vitro* transcription with T3 or T7 RNA polymerase in the presence of [α -³²P]UTP. DNA templates were removed by RQ1 DNase digestion followed by phenol-chloroform extraction. The labeled riboprobes were hybridized to target RNAs at 60°C overnight, as described previously (2). The hybridization products were digested with an RNase T₁-T₂ mixture at 37°C for 1.5 h, and the resulting samples were resolved on 6% denaturing polyacrylamide gels (2). The protected bands were quantitated with a Packard Instant Imager. Background was subtracted by using regions of identical size located immediately above each of the experimental bands. Routinely, 25% (about 40 μ g of RNA) of the total nuclear and cytoplasmic RNA fractions were used in each RNase protection assay. This amount of RNA used is approximately equal to the amount of RNA obtained from 10⁶ cells.

For actinomycin D time course analysis, nuclear and cytoplasmic RNAs were extracted 0, 3, and 6 h after treatment with a 5- μ g/ml final concentration of actinomycin D and were analyzed by quantitative RNase protection assays as described above.

RESULTS

mVP2, which contains an unused 5' splice site, is exported inefficiently from the nucleus. To examine the export of poly-

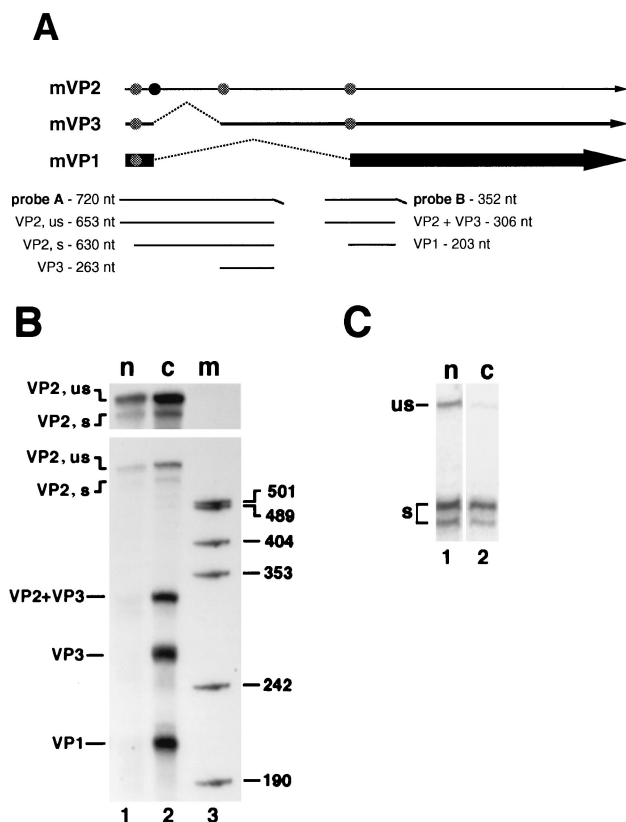


FIG. 2. Nucleocytoplasmic transport of wild-type polyomavirus late mRNAs. (A) Schematic representation of polyomavirus late mRNAs and the predicted protection bands with the indicated riboprobes. The diagonal lines represent nonhomologous sequences within the probes that derive from their plasmid vectors. VP2, us, unspliced mVP2; VP2, s, leader-leader spliced mVP2; VP1, mVP1; VP3, mVP3. (B) RNase protection results. Wild-type (WT) polyomavirus genomic DNA plasmid was transfected into mouse NIH 3T3 cells. Nuclear and cytoplasmic RNAs were isolated 48 h post transfection. Cytoplasmic RNA and poly(A)⁺ nuclear mRNA were used in the RNase protection assays. Lanes: n, nuclear poly(A)⁺ mRNA; c, cytoplasmic RNA; m, molecular size markers labeled in nucleotides. Shown on top is a longer exposure of the gel, highlighting only the mVP2 protection bands. (C) RNase protection of polyomavirus early mRNAs. In this RNase protection assay, total nuclear RNA, instead of poly(A)⁺ nuclear mRNA, was used. us, unspliced early mRNA (357-nt band); s, spliced early mRNAs (224- and 212-nt bands).

omavirus late mRNAs, mouse NIH 3T3 cells were transiently transfected with wild-type polyomavirus genomic DNA excised and recircularized from a recombinant plasmid vector. Nuclear and cytoplasmic RNAs were isolated 48 h after transfection (late in the viral life cycle), and levels of late-strand mRNA between the two cellular compartments were analyzed by RNase protection assays with two internally labeled, nonoverlapping riboprobes (Fig. 2A, probe A and probe B). As mentioned above, inefficient termination of late-strand transcription at late times in infection leads to the production of multigenomic pre-mRNAs. Since these giant pre-mRNAs (as well as splicing intermediate products) would also hybridize to the probes used, and since this might complicate interpretations of the data obtained, we used only polyadenylated nuclear mRNAs in the RNase protection assays, except for the internal control experiments (see below). To select poly(A)⁺ mRNAs, we treated nuclear RNAs with the PolyATtract mRNA isolation system kit as described in Materials and Methods.

As shown in Fig. 2B, lanes 1 and 2, mVP1 and mVP3 are

localized almost exclusively in the cytoplasm, with only about 8% of the total cellular RNA being retained in the nucleus (data are also summarized in Fig. 7, lanes 1 and 2), indicating efficient export. Importantly, although these mRNAs are efficiently exported, they possess either one or two unused 3' splice sites. This suggests that the unused viral late 3' splice sites most probably do not by themselves act as nuclear retention signals. In the unspliced mVP2, however, a much larger proportion of the RNA (about 37% [Fig. 2B, lanes 1 and 2; also see Fig. 7, lanes 3 versus lanes 1 and 2]) accumulated in the nuclear compartment, indicating the inefficient export. Interestingly, the leader-leader spliced mVP2 exhibited a similar transport phenotype to the fully unspliced mVP2 (Fig. 2B, lanes 1 and 2; also see Fig. 7, lanes 3). This suggests that the process of splicing does not improve mVP2 export.

To monitor that relatively clean nuclear and cytoplasmic fractionations had been achieved, polyomavirus early-strand gene expression was always used as an internal control in parallel experiments. In mouse NIH 3T3 cells, unspliced polyomavirus early-strand RNAs are confined to the nuclear compartment while the spliced early-strand RNAs are distributed almost equally between the two compartments (Fig. 2C). Such a distribution pattern of early-strand RNAs serves as a useful marker for efficient subcellular fractionations and as a control for sample loading.

The late 5' splice site influences mVP2 export. The above results revealed that export of mVP2 is inefficient compared with that of mVP1 and mVP3. We next asked which sequence(s) on the RNA is responsible for this phenotype. One of the structural differences between mVP2 and the other two lane mRNAs is that in mVP2, the 5' splice site is not removed. The strength of a 5' splice site correlates well with its ability to form base-paired interaction with U1 small nuclear RNA (U1 snRNA) (48). A perfect match would be a strong consensus 5' splice site. Base changes can alter strength of the splice site, with some positions being more important than others. For example, positions -1, +1, and +5 are especially important for the strength of a 5' splice site (31, 48). We postulated that the single-base-pair mismatch of the polyomavirus late 5' splice site with U1 snRNA may render polyomavirus late pre-mRNAs unfavorable for spliceosome assembly, thus allowing their export to the cytoplasm. To test this hypothesis, three mutant clones were created by introducing point mutations into the late 5' splice site (Fig. 3A). In Py-G, an A residue at position -1 was replaced with a G so that the 5' splice site sequence was perfectly complementary to that of U1 snRNA. For Py5-1 and Py5-5, G residues at positions +1 and +5 were converted to U and C, respectively, to disrupt the base pairing with U1 snRNA. These mutant constructs were each introduced into NIH 3T3 cells, and RNase protection assays were performed with the same probes shown in Fig. 2A. Since there is only a single-base-pair mismatch between probe A and the mutant transcripts, the nucleases used did not cleave at the mismatched site under the RNase protection conditions used. Results shown in Fig. 3B revealed that a single-base-pair substitution in the 5' splice site led to significant changes in intracellular distributions of VP2 mRNAs as well as alternations in splicing patterns. In Py-G, a consensus 5' splice site mutation resulted in a nearly 1.5-fold increase in nuclear accumulation of mVP2 (Fig. 3B, compare lanes 1 and 2 with lanes 3 and 4 also see Fig. 7, lanes 3 versus lanes 4). The mutation also changes the ratio between mVP1 and mVP3 (Fig. 3B, compare lane 4 with lane 2), with mVP1 now apparently favored even more. In Py5-5 and Py5-1, on the other hand, significantly improved mVP2 nuclear export was accompanied by the abolition of splicing, indicated by the absence of the splicing prod-

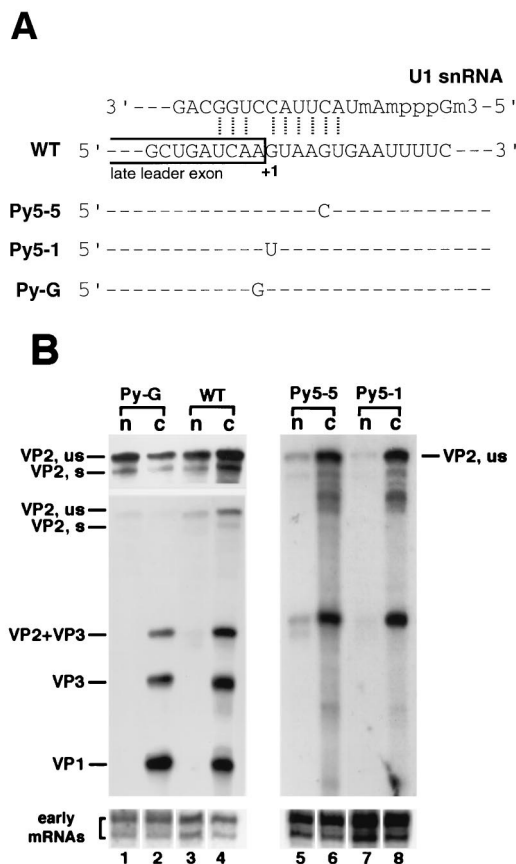


FIG. 3. Nuclear export of polyomavirus late mRNAs with 5' splice site mutations. (A) At the top is shown the wild-type (WT) polyomavirus late 5' splice site and its complementarity to the 5' end of U1 snRNA. A portion of the 57-nt late leader exon is boxed, and position +1 of the 5' splice site is marked. The three mutant polyomavirus constructs, each with the mutated bases indicated, are shown below. Dashes denote bases not altered by mutagenesis. (B) RNase protection results for mutant and wild-type (WT) polyomavirus late mRNAs. Mutant and wild-type polyomavirus plasmids were individually transfected into mouse NIH 3T3 cells, and RNase protection assays of nuclear and cytoplasmic mRNAs were performed with the same riboprobes shown in Fig. 2A. Shown at the bottom are RNase protection results for spliced early mRNAs as an internal control for subcellular fractionations.

ucts (Fig. 3B, compare lanes 5 to 8 with lanes 3 and 4). The nuclear accumulations of mVP2 are 18 and 12% of total cellular RNA for Py5-5 and Py5-1, respectively (see Fig. 7, lanes 5 and 6 versus lanes 3). The simplest interpretation of these observations is that a "weak" 5' splice site gives rise to "weak" association of spliceosomes and allows more rapid export of unspliced mRNAs. Alternatively, a "strong" 5' splice site leads to "strong" association of spliceosomes, hence acting to retard the export of unspliced mRNAs. These data therefore suggest that the 5' splice site is an important *cis*-acting determinant of mVP2 export.

Differences in subcellular distributions of VP2 mRNAs are not due to differential stabilities. To exclude the possibility that differences in the intracellular distribution of VP2 mRNAs result from different half-lives of these mRNAs, RNase protection assays following actinomycin D treatment of the transfected cells were carried out. Nuclear and cytoplasmic RNAs were extracted from cells transfected with the indicated constructs. As shown in Fig. 4, none of the mVP2 mRNAs shows half-life differences between the two cellular compartments. Thus, for the wild type, the half-life for mVP2 is greater than

12 h in both compartments (Fig. 4 and data not shown), for Py-G, the half-life is 3 h in both compartments, and for Py5-5, the half-life is 6 h in both compartments. The half-lives of mVP2 for Py5-1 in both compartments were similar to those for Py5-5 (data not shown). Although actinomycin D can lead to artifacts in the interpretation of RNA half-lives, these results are consistent with the conclusion that changes in RNA turnover rates do not contribute significantly to the different subcellular distribution patterns among the VP2 mRNAs. The same data also show that conversion from mVP2 to its splicing products in the wild type and Py-G occurs extremely slowly, if at all, during the experimental period. This again supports the idea that the differential subcellular distributions of VP2 mRNAs indeed result from the different export kinetics of these mRNAs.

Unused 3' splice sites have little effect on mVP2 export. To determine whether the apparent effects on export of the 5' splice site require cooperation with 3' splice sites, we carried out the following experiments. The mutant construct $3 \times 3' \text{ss}\Delta$, in which all three late 3' splice sites were deleted, was used (Fig. 5A). An RNase protection assay with the indicated probe (probe C) revealed that the mutant construct expressed stable and only fully unspliced mRNA (Fig. 5B, lanes 1 and 2). This result is consistent with previously published observations showing no late splicing for this construct (6). Like fully unspliced wild-type mVP2, transcripts from $3 \times 3' \text{ss}\Delta$ are not spliced but are exported. Surprisingly, this unspliced mRNA, indicated by the 361-nt protection band, was not exported as efficiently as were mVP1 and mVP3. Instead, the export phenotype closely resembles that of wild-type mVP2 (compare Fig. 5B, lanes 1 and 2, with Fig. 2B, lanes 1 and 2; also see Fig. 7, lanes 7 versus lanes 3). Taken together with the finding that the 3' splice sites retained in mVP1 and mVP3 did not interfere with export (Fig. 2B), this result leads us to conclude that the late 3' splice sites do not contribute to mVP2 export and further supports the hypothesis that the 5' splice site is a key determinant of mVP2 export.

It is noteworthy that mRNAs from $3 \times 3' \text{ss}\Delta$ are not spliced and therefore are not precursors for mVP1 or mVP3. Similarly, transcripts from Py5-1 and Py5-5 cannot be converted to mVP1 or mVP3. Since mRNAs from Py5-1 and Py5-5 are exported more efficiently than those from $3 \times 3' \text{ss}\Delta$, this result is again consistent with the notion that the unused 5' splice site plays a crucial role in mVP2 export. To further evaluate the role played by spliceosomes in mRNA export, we examined

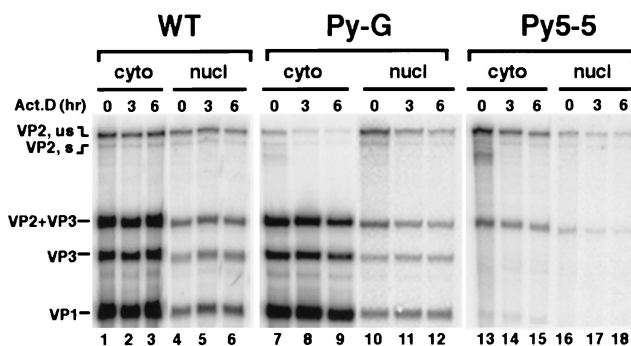


FIG. 4. Nuclear and cytoplasmic half-lives of VP2 mRNAs from wild-type (WT) and mutant constructs. Cells were individually transfected with each of the indicated constructs. Nuclear poly(A)⁺ (nucl) and cytoplasmic (cyto) RNAs were extracted 0, 3, and 6 h after treatment with a 5- μ g/ml final concentration of actinomycin D (Act.D) and were analyzed by RNase protection assays as described in Materials and Methods.

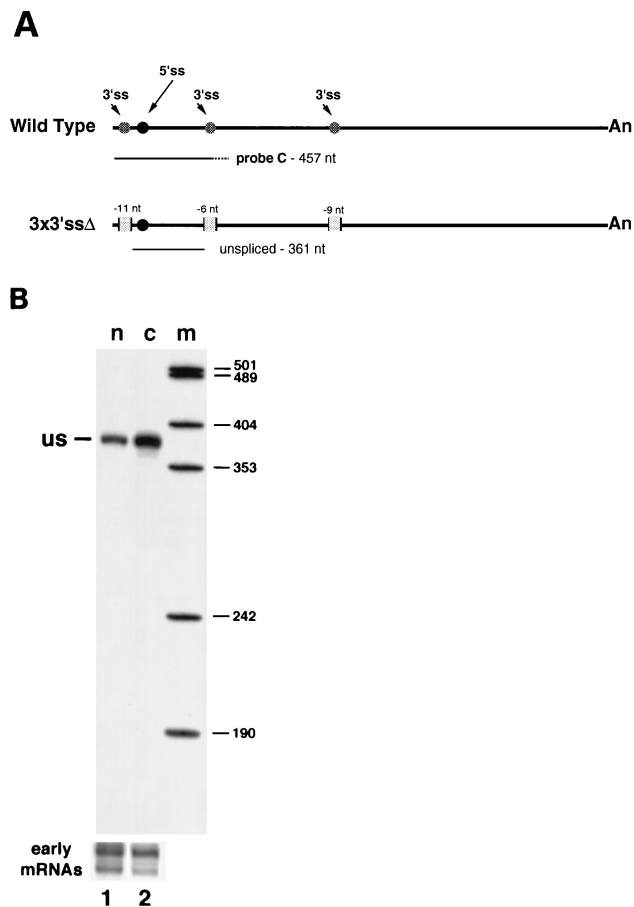


FIG. 5. Effect of 3' splice sites on mVP2 export. (A) Wild-type and $3 \times 3'ssd\Delta$ constructs. The shaded boxes represent deleted sequences, with the number of deleted nucleotides indicated. The riboprobe used in RNase protection assays and the predicted protection bands are also shown. (B) Nuclear and cytoplasmic distributions of $3 \times 3'ssd\Delta$ mRNAs. us, unspliced mRNA, indicated by the 361-nt protected band.

expression from a mutant lacking all late splice sites. Expression from this construct was extremely low (too low for meaningful interpretation of RNA transport and stability measurements [data not shown]).

Roles of other *cis* elements in mVP2 export. To investigate whether other *cis* elements may be involved in controlling mVP2 export, we made large deletions in the polyomavirus late region to eliminate most of the other possible *cis*-acting elements that might influence export. Previous work from this laboratory has shown that the sequence of the late leader exon is not important for late RNA synthesis or processing (1). In the Mini construct (Fig. 6A), a 1.75-kb fragment including sequences from immediately downstream of the mVP3 3' splice site to 50 nt upstream of the AAUAAA polyadenylation site was removed. This construct expresses the late leader exon, the downstream 310-nt mVP3 intron up to and including the mVP3 3' splice site, and the late polyadenylation site. Construct $3'\Delta$ mini is identical to Mini except that in this construct the mVP3 3' splice site and upstream 11 bases were also removed. The two mutant constructs were each transfected into NIH 3T3 cells, and nuclear and cytoplasmic RNAs were extracted. The intracellular distributions of polyadenylated mRNAs were examined with the indicated probe (probe C) shown in Fig. 6A. As shown in Fig. 6B, lanes 1 and 2, two RNA

species are produced from the Mini construct. The 57-nt protection band labeled with s represents the spliced leader exon (leader-leader and leader-body spliced mRNAs), a majority of which are found in the cytoplasmic fraction, suggesting efficient export. In repeated experiments, this small protected band always appeared broad, owing both to its small size and to the nature of digestion with our RNase T_1/T_2 preparation. The 367-nt protection band labeled with us is the leader-leader-splice-only mRNAs (mRNAs with no leader-body splice but having a leader-leader splice). The us mRNAs contain an unused 5' splice site and two unused 3' splice sites (Fig. 6A). Interestingly, these mRNAs resemble wild-type mVP2 in that nearly one-third of the mRNAs accumulated in the nuclear compartment (compare Fig. 6B, lanes 1 and 2, with Fig. 2B, lanes 1 and 2; also see Fig. 7, lanes 8 versus lanes 3). This suggests that sequences defined by the deletion have little effect on mVP2 export.

In contrast to Mini, $3'\Delta$ mini expresses only the leader-leader-splice-only RNAs, indicated by the 356-nt protection band

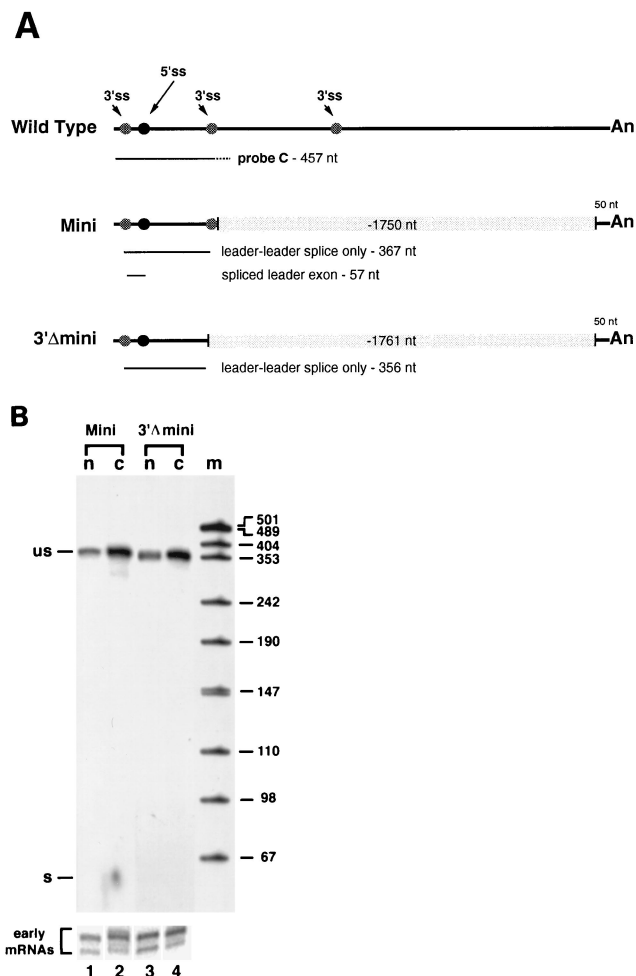


FIG. 6. Nucleocytoplasmic transport of polyomavirus late mRNAs with deletion mutations. (A) Schematic representations of wild-type and deletion constructs. The stippled boxes in Mini and $3'\Delta$ mini represent the deleted sequences. Predicted protection bands by probe C are indicated under each of the constructs. (B) RNase protection results of mRNAs generated from the indicated mutant constructs. us (lanes 1 and 2), leader-leader-splice-only Mini mRNA, indicated by the 367-nt protected band; us (lanes 3 and 4), leader-leader-splice-only $3'\Delta$ mini mRNA, indicated by the 356-nt protected band; s (lanes 1 and 2), spliced leader exon from Mini.

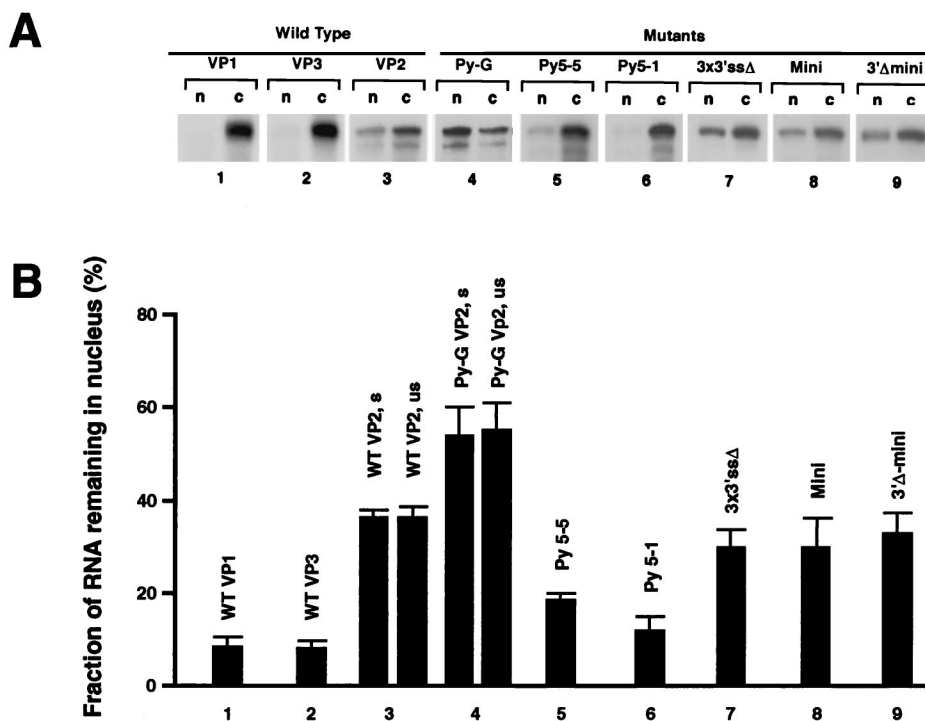


FIG. 7. Summary of the data. (A) Summary of mVP2 transport data. RNase protection bands of nuclear and cytoplasmic mRNAs from wild-type and the mutant constructs used in this study are shown. (B) Bar graph quantitation of the data shown in panel A. Each of the quantitation results was based on at least three independent experiments. WT, wild type.

(Fig. 6B, lanes 3 and 4). These RNAs contain an unused 5' splice site and an unused 3' splice site (Fig. 6A). Like the Mini leader-leader-splice-only RNAs, these RNAs showed a wild-type intracellular distribution pattern (compare Fig. 6B, lanes 3 and 4, with Fig. 2B, lanes 1 and 2; also see Fig. 7, lanes 9 versus lanes 3). Since the leader-leader spliced forms of 3'Δmini RNAs were not exported as efficiently as were those of mVP1 and mVP3 (Fig. 2B), the process of splicing, per se, again cannot enhance mRNA export. These constructs do not express RNAs with different half-lives in different subcellular compartments (data not shown). These data, together with those presented above (summarized in Fig. 7), make it unlikely that other *cis*-acting elements are involved in mediating mVP2 export. The only portions of the late region not ruled out by these studies are the 50 nt immediately upstream of the late polyadenylation signal and about 300 nt immediately downstream of the late leader exon. In other work, removal of 200 nt within the mVP3 intron had no effect on RNA distribution (data not shown). The observations that mRNAs lacking leader-body splices from both the Mini and 3'Δmini mutants have a wild-type mVP2 export phenotype and that these mRNAs contain an unused 5' splice site are consistent with our conclusion that the 5' splice site is a key determinant of mVP2 export.

The relatively high level of the 367-nt band with respect to the 57-nt band in Fig. 6b, lanes 1 and 2, indicates that in the Mini construct, the efficiency of leader-body splicing is low compared with the wild type (compare Fig. 2B, lanes 1 and 2, with Fig. 6B, lanes 1 and 2). One possible explanation for this is that the short distance (50 nt) between the 3' splice site and the polyadenylation site resulting from deletion interferes with the binding of splicing factors to the 3' splice site as a result of a steric hindrance with the polyadenylation machinery; this is consistent with previous results from this laboratory (7).

DISCUSSION

The aim of this study was to use polyomavirus as a model system to evaluate the relative importance of some mechanisms by which the nuclear export of mRNAs is influenced or regulated. We focused on how unspliced polyomavirus late mRNA, mVP2, can be exported to the cytoplasm. Results revealed that mVP2, which harbors an unused 5' splice site, is exported less efficiently than mRNAs in which the 5' splice site has been removed by splicing (Fig. 7, compare lanes 1 and 2 with lanes 3). We next systematically mutated the 5' splice site, and the results demonstrated that the strength of the 5' splice site correlates positively with splicing efficiency and inversely with the efficiency of mVP2 export (Fig. 3B and Fig. 7, compare lanes 4 with lanes 3). Furthermore, 3' splice sites retained in mature late mRNAs did not interfere with the export of these mRNAs, and deletion of all the three late 3' splice sites did not affect mVP2 export (Fig. 2B and 4B; Fig. 7, compare lane 7 with lanes 3). Finally mRNAs with large deletions and retaining the 5' splice site exhibited similar export phenotypes to wild-type mVP2 (Fig. 7, compare lanes 8 and 9 with lane 3). We therefore conclude that (i) the polyomavirus late 5' splice site plays a crucial role in the efficiency of mVP2 export; (ii) the late 3' splice sites have little, if any, effects on export; and (iii) it is unlikely that other positive or negative *cis* elements are involved.

How does the 5' splice site influence the export of mVP2?

Pre-mRNA splicing occurs in large complexes (spliceosomes) which include pre-mRNA, splicing components, and other regulatory protein factors (16, 36, 43). The formation of a spliceosome is a dynamic process and follows an ordered pathway. In mammalian cells, the *cis*-acting elements required in splicing include the splice donor site (5' splice site, consensus, AG/G UAAGU), the splice accept site (3' splice site, consensus

CAG/A), and a weakly conserved branch point sequence located 20 to 50 nt upstream of the 3' splice site. The first step involves the recognition of the 5' splice site by U1 snRNP (15, 42). Subsequent steps include recognition of the branch point sequence by U2 snRNP and other splicing factors, followed by assembly of active spliceosomes by recruitment of U4-U5-U6 snRNPs and other factors (15). The efficiency of splicing can be regulated by both *cis*-acting elements and *trans*-acting factors (reviewed in references 15 and 44).

The wild-type polyomavirus late 5' splice site is not perfectly consensus; rather, it can base pair to the 5' end of U1 snRNA at 8 of 9 positions (Fig. 3A). It is possible that the mismatch at position -1 makes this pre-mRNA a somewhat unfavorable target for spliceosome assembly, thus rendering it more accessible to the export machinery. Evidence supporting this derives from several results. First, in the mutational analysis, when the late 5' splice site was mutated to a consensus one (Py-G), about a 1.5-fold increase in the nuclear accumulation of mVP2 was observed (Fig. 7, compare lanes 4 with lanes 3). On the other hand, two other point mutations (Py5-5 and Py5-1), which gave rise to 5' splice sites that were further from consensus, led to the abolition of splicing and to lower levels of nuclear accumulation of mVP2 (Fig. 7, compare lanes 5 and 6 with lanes 3). These phenomena are recapitulated by the observations that the unspliced mRNAs from Py5-1 and Py5-5 are exported more efficiently than are those from 3×3'ssΔ (Fig. 7, compare lanes 5 and 6 with lanes 7). These results suggest direct links between the strength of the 5' splice site and mVP2 export. Thus, it appears that a "weak" 5' splice site cannot retain pre-mRNAs in the nucleus as well as a "strong" 5' splice site can. Second, in 3×3'ssΔ, in which only fully unspliced RNAs were expressed, the nuclear accumulation of these RNAs was about 30%, similar to that of wild-type mVP2 (Fig. 7, compare lanes 7 with lanes 3). Similarly, the large deletion mutant 3'Δmini produced only RNAs containing leader-leader splices. These RNAs also showed a wild-type mVP2-like export phenotype (Fig. 7, compare lanes 9 with lanes 3). In mRNAs from Py5-1, Py5-5, 3×3'ssΔ, and 3'Δmini, leader-body splices failed to occur and the 5' splice sites were not removed. These mRNAs, however, were exported to the cytoplasm, although not as efficiently as were mVP1 and mVP3. One possible explanation for these observations is that the intrinsically weak 5' splice site and possibly the branch point sequences still present in these mutant transcripts are not efficiently recognized and bound by splicing factors. As a result, some unspliced RNAs are able to escape from the splicing machinery and are exported to the cytoplasm.

Our data suggest that the process of splicing is not necessary for mRNA export and does not improve export. The fact that transcripts generated from 3×3'ssΔ did not undergo splicing yet could be exported to the cytoplasm indicated that splicing is not a prerequisite for mRNA export. Consistent with this, other unspliced mRNAs, such as the fully unspliced wild-type mVP2 and those generated from Py5-1 and Py5-5, can be transported to the cytoplasm, although not as efficiently as mVP1 and mVP3 (Fig. 7, compare lanes 3 to 7 with lanes 1 and 2). The process of splicing does not improve mRNA export. This is supported by the observations that leader-leader unspliced and leader-leader spliced VP2 mRNAs from wild-type Py-G, Mini, and 3'Δmini exhibit similar export phenotypes (Fig. 7, lanes 3, 4, 8, and 9).

Our results suggest that there may exist a competition between the process of RNA export and the binding of splicing factors to pre-mRNA. In this model, export efficiency would depend on the relative kinetics of these two processes. For example, when interactions between splicing factors and

mRNAs are strong, the splicing process would be favored and fewer unspliced RNAs would be available for export. Alternatively, when the interactions are weak, more unspliced RNAs would be available for export. In polyomavirus late-strand gene expression, just such a competition can account for the observed export properties of mVP2 molecules. Since only a relatively small proportion of viral late mRNAs need to remain unspliced to produce sufficient VP2 for capsid assembly, such an effect is sufficient in the absence of any other *cis*-acting transport elements. Further, the efficiency of late splicing in this system also appears to be governed largely by the properties of the splice sites and the relative distances between them, and no other *cis*-acting elements or virus-encoded proteins appear to be involved (7, 32).

Export of unspliced mRNAs in other viral systems. Regulation of export by suboptimal *cis*-acting processing signals may also apply to the avian sarcoma virus. Katz, and Skalka have reported that splicing in avian sarcoma virus may be controlled by a suboptimal 3' splice site and sequences within the downstream exon (25). On the basis of these results and data from Legrain and Rosbash (28), Katz and Skalka suggested that nucleocytoplasmic transport of unspliced avian sarcoma virus mRNAs may be regulated by similar if not the same *cis*-acting elements. However, it is quite possible that in retroviral systems, other positive *cis* elements also play a role in regulating the export of unspliced and partially spliced RNAs. This might be necessitated by the requirement for the cytoplasmic expression of rather large amounts of unspliced mRNAs, unlike the rather small fraction of unspliced mRNAs that are required to accumulate in the cytoplasm in the polyomavirus system.

We do not yet know the reason why export of polyomavirus late unspliced mRNA is strongly influenced only by the 5' splice site whereas that of avian sarcoma virus appears to be affected mainly by 3' splice sites. It is possible that other *cis*-acting viral sequences help to determine the roles played by individual splicing signals. Alternatively, the context of the splice sites or their location within the RNA transcript might be critical. In this regard, it is noteworthy that if the polyomavirus late 5' splice site is moved downstream of its normal location within about 100 nt of transcription start sites, late-gene expression is severely affected (6).

In HIV-1, cytoplasmic accumulation of unspliced and partially spliced viral mRNAs is dependent on the expression of the virus-encoded Rev protein, which specifically interacts with the RRE present on the viral RNA (11, 12). Two non-mutually exclusive models have been proposed regarding the regulation of mRNA nuclear export by Rev: (i) Rev promotes the export of unspliced and partially spliced mRNA by displacing spliceosomes from the viral pre-mRNAs (11, 26, 31); (ii) Rev facilitates export through a pathway independent of that used by normal cellular mRNAs (13, 14, 24, 33, 38, 40, 45). Like polyomavirus late pre-mRNA, HIV-1 possesses splice signals which are not efficiently used during splicing. In the human lymphocytes, unspliced HIV-1 mRNAs are not exported to the cytoplasm and are rapidly degraded in the nucleus in the absence of Rev (33). This suggests that in these cells, a mechanism may have been evolved to quickly eliminate the viral transcripts that are not spliced and/or not exported in the absence of Rev. However, a recent study has shown that in the absence of Rev, a small fraction of unspliced mRNAs transcribed from an HIV-1-based construct was able to enter the cytoplasm of COS7 cells transiently transfected with the plasmid (20). This observation is consistent with the data reported here in that a small proportion of unspliced mVP2 can be transported to the cell cytoplasm. Hence, it is plausible to hypothesize that the suboptimal splicing signals in the HIV-1 transcripts may also

play roles in influencing export of the viral pre-mRNAs and that these regulation effects are superimposed by that of the Rev/RRE.

Export of the unspliced mRNAs and intronless gene transcripts. Accumulated data have suggested that export of intronless transcripts requires specific positive *cis*-acting RNA elements. A *cis*-acting RNA element (PPE) necessary for high efficiency of RNA processing and export has been identified in the herpes simplex virus thymidine kinase gene (30). PPE interacts specifically with the heterogeneous nuclear ribonucleoprotein particle (hnRNP) L protein (one of the most abundant nuclear proteins) and functions independently of splicing. Not surprisingly, another *cis*-acting element (PRE), present in an intronless hepatitis B virus transcripts, is also required for the cytoplasmic expression of the viral mRNAs (19, 21). Like PPE, PRE acts in the absence of splicing signals. Both PPE and PRE can activate the cytoplasmic accumulation of a prespliced β -globin transcript. PRE has also been reported to be able to functionally replace the HIV-1 Rev/RRE (21). Our studies have shown that unspliced mVP2 can most probably be exported to the cytoplasm in the absence of positive *cis*-acting elements.

Is the export of mVP2 molecules different from that of intronless mRNAs? Perhaps mRNAs with inefficient 5' splice sites must associate with spliceosomes to enter the export pathway. Once associated, dissociation can lead to entry into the export pathway, even if splicing does not occur. In this model, mRNAs with no splicing signals could not associate with spliceosomes and therefore would be excluded from the pathway. Consistent with this, prespliced mRNAs have been shown to have a transport block (20a, 41).

Thus, unspliced mVP2 may be exported through a pathway different from that used by some intronless mRNAs, and this may be a general phenomenon for the export of some intron-containing cellular and other viral mRNAs. Since deletion of splice sites in many intron-containing gene transcripts often abolishes accumulation of RNAs in both cellular compartments (6, 9, 17, 18, 31, 41), we suggest that for intron-containing transcripts, splice sites may serve not only for splicing but also to stabilize mRNAs in the nucleus. This model has been supported directly in the polyomavirus system (6).

In summary, by examining nuclear export of polyomavirus mRNAs, we have come to the following conclusions: (i) a suboptimal 5' splice site serves as a *cis*-acting determinant of the efficiency of export of polyomavirus late unspliced mRNA; (ii) for intron-containing gene transcripts, the process of splicing may not be necessary for mRNA export and does not improve export; rather, removal of functional splicing signals seems to be required; and (iii) for intron-containing genes, unspliced mRNAs may be exported by a pathway different from that used by the intronless gene transcripts.

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