JOURNAL OF VIROLOGY, Sept. 1995, p. 5864–5868 Vol. 69, No. 9
0022-538X/95/$04.00+0

Sequences within the Parvovirus Minute Virus of Mice NS2-Specific Exon Are Required for Inclusion of This Exon into Spliced Steady-State RNA

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Received 16 March 1995/Accepted 15 May 1995

When the minute virus of mice NS2-specific exon was modified by either substitution or deletion, most P4-generated pre-mRNA was spliced from the large-intron donor at nucleotide 514 to the small-intron acceptor at nucleotide 2377. Improvement to consensus of large-intron splice sites in such mutants did not suppress exon skipping or restore large-intron excision. Therefore, sequences within the NS2-specific exon are required for inclusion of this exon into spliced, steady-state minute virus of mice RNA.

Alternative splicing of pre-mRNA molecules represents a fundamental process of posttranscriptional gene regulation among higher eukaryotes (reviewed in references 13 and 19). This process generates distinct mRNAs from a single mRNA precursor and often results in functional diversity via production of multiple protein isoforms from a single gene. Another important consequence of alternative splicing is the regulation, in some cases, of mRNA levels in response to developmental or tissue-specific signals. Although it is known that pre-mRNAs from a large number of genes undergo alternative splicing, relatively little is known about the molecular mechanisms controlling this process. Many DNA viruses have compact genomes and extensively utilize alternative splicing to increase their coding capacity from overlapping open reading frames (ORFs). Minute virus of mice (MVM), a member of the autonomously replicating subgroup of DNA parvoviruses, has a relatively simple transcriptional profile in which alternative splicing contributes to the determination of the relative steady-state levels of viral proteins (reviewed in references 3 and 7).

MVM is organized into two overlapping transcription units which have been shown to produce three major transcript classes, R1, R2, and R3, all of which terminate near the right-hand end of the linear 5-kb genome (Fig. 1) (1, 5, 16). Transcripts R1 (4.8 kb) and R2 (3.3 kb) are generated from a promoter (P4) at map unit (m.u.) 4 (1, 16) and encode the viral nonstructural proteins NS1 (83 kDa) and NS2 (24 kDa), respectively, utilizing the ORFs in the left half of the genome (6). Both NS1 and NS2 play essential roles in viral replication and cytotoxicity (3, 7). The 3.0-kb R3 transcripts, which encode the overlapping viral capsid proteins VP1 and VP2 utilizing the ORF in the right half of the genome, are generated from a promoter at m.u. 38 (1, 10, 12, 14, 16).

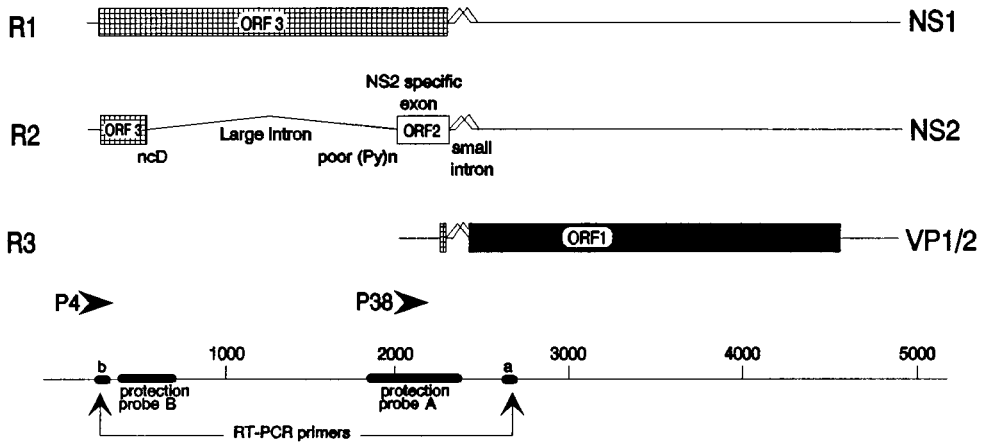

FIG. 1. Genetic map of MVM. The three major MVM transcript classes and protein-encoding ORFs are shown. The small intron, the large intron, and the NS2-specific exon are indicated. The nonconsensus donor (ncD) and the poor polypyrimidine tract [(Py)n] of the large intron are also shown. The two promoters (P4 and P38) are indicated by arrows. The bottom diagram shows nucleotide locations, the two probes (A [nt 1854 to 2378] and B [nt 385 to 650]) used for RNase protection assays, and the two primers (a [nt 2557 to 2538] and b [nt 326 to 345]) used for RT-PCR as described in the text.

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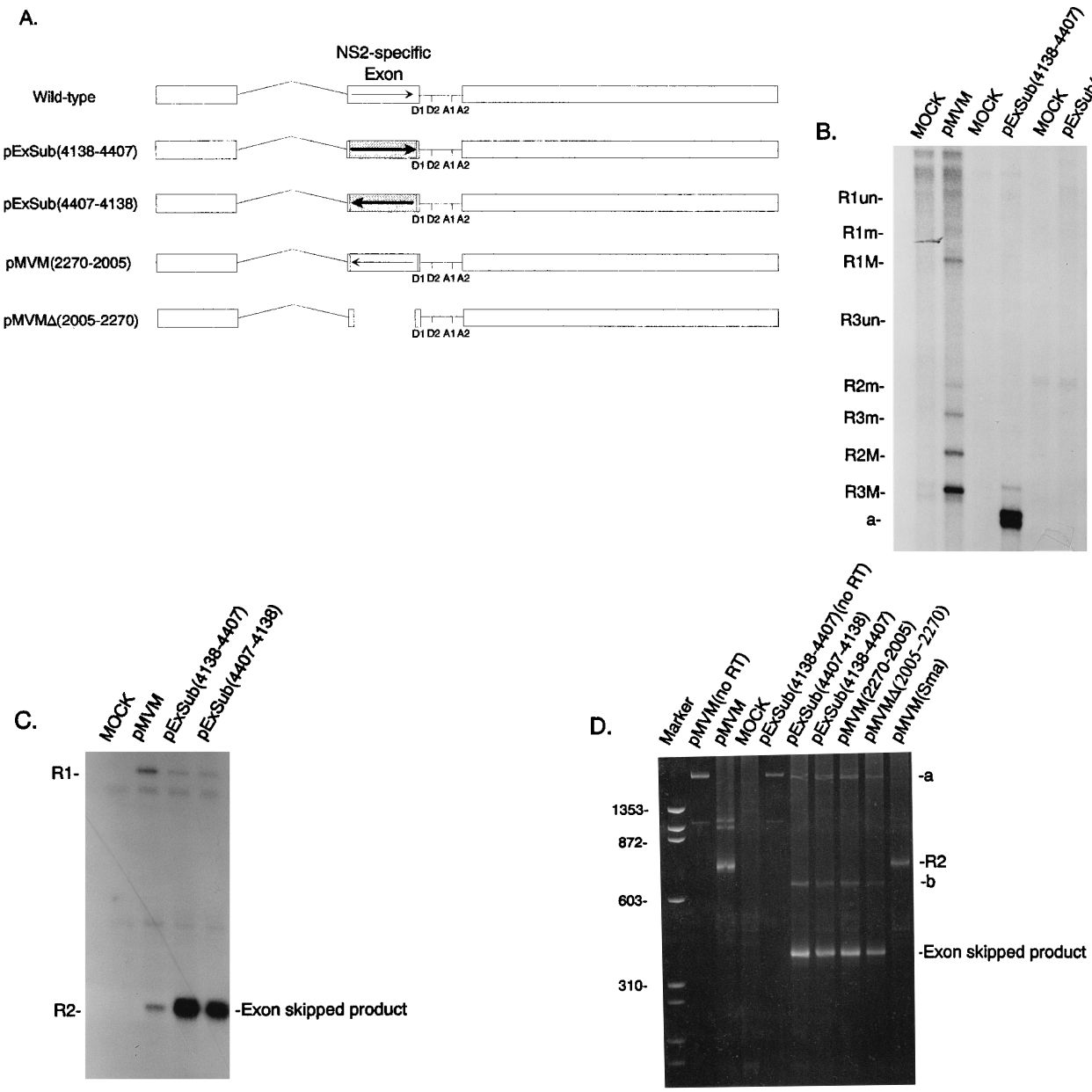

FIG. 2. Alteration of the NS2-specific exon resulted in its almost uniform exclusion. (A) Map of the wild type and mutants with an altered NS2-specific exon, as described in the text. (B) RNase protection assay, using protection probe A (Fig. 1), of 20 μg of total RNA isolated 48 h after transfection of semiconfluent A9 cells with 20 μg of wild-type MVM (pMVM) or mutant plasmid DNA per 100-cm^2 dish, or mock transfection, as indicated. For RNA generated by pExSub(4138-4407) and pExSub(4407-4138), we used derivatives of protection probe A which were homologous to the mutants examined. The identities of protected bands for unspliced (R1un and R3un) and spliced (R1m, R1M, R2m, R2M, R3m, and R3M) transcripts are shown on the left. M represents protection by those molecules that use the predominant donor at nt 2280; m represents protection by those molecules that use the less frequently used donor at nt 2317, as previously described (5). RT-PCR analysis (data not shown) suggested that band a is generated from P38. (C) RNase protection assay, using left-end protection probe B (Fig. 1), of RNA isolated after transfection with 20μg of wild-type MVM (pMVM) or mutant plasmid DNA per dish, or mock transfection, as indicated. The bands representing protection of probe B by R1 (265 nt) and by an RNA utilizing the splice donor at nt 514 (129 nt, designated R2 for the wild type and Exon skipped product for the exon substitution mutants) are indicated. The amount of RNA used for this analysis was not equalized. (D) RT-PCR analysis, using MVM primers a and b shown in Fig. 1, of 5 μg of total RNA isolated after transfection with 20 μg of wild-type MVM (pMVM) or mutant plasmid DNA per dish, or mock transfection, as indicated. pMVM(no RT) and pExSub(4138-4407)(no RT) were used as controls without reverse transcriptase. Only R2 (R2M, 658 nt) was significantly amplified from RNA generated by pMVM and by pMVM(*Sma*I), the parent plasmid for mutants shown in panel A, which contains two newly created *Sma*I sites at nt 2005 and nt 2270 in an otherwise wild-type clone of MVM (see text for a more complete description). For RNAs generated by all mutants with an altered NS2-specific exon, the exon-skipped product (mainly using A1 at nt 2377 of the small intron) was detected as a 368-nt RT-PCR product and further authenticated as described in the text. Band a probably represents PCR amplification of input MVM plasmid DNA because it also appeared in lanes for pMVM(no RT) and pExSub(4138-4407)(no RT) controls. The identity of band b is unknown. Products were run on 8% native polyacrylamide gels.

Two types of introns exist in MVM P4-generated transcripts. A small overlapping set of introns is located at m.u. 44 to 46 and is common to both P4-generated transcripts (R1 and R2) and P38-generated transcripts (R3) (1, 4, 8, 10, 14, 16). An additional upstream large intron, which is located between m.u. 10 and 39, is unique to R2 transcripts (1, 6, 10, 16). This intron utilizes a nonconsensus splice donor at nucleotide (nt) 514 (AA/GCAAGT) and has a poor polypyrimidine tract

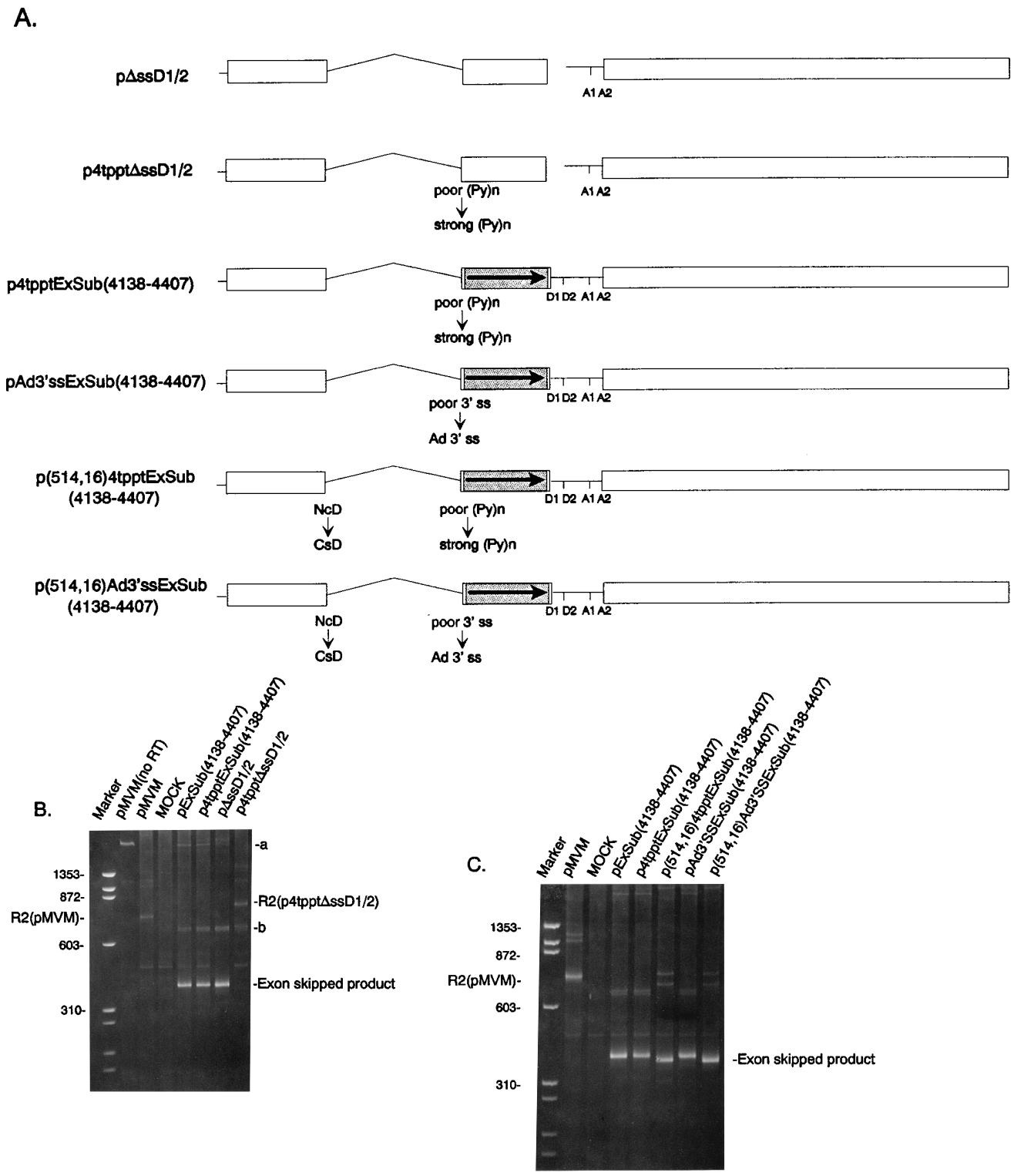

FIG. 3. Improving large-intron splice sites cannot overcome exon skipping due to alteration of the NS2-specific exon sequences. (A) Map of the mutants used for this set of experiments. The small-intron donors are deleted in pΔssD1/2 (this mutant is more fully described in reference 26, an improved large-intron polypyrimidine tract [(Py)n] is combined with the deletion of small-intron donors in p4tpptΔssD1/2, an improved large-intron polypyrimidine tract is combined with the altered NS2-specific exon in p4tpptExSub(4138-4407), an improved entire large-intron 3′ splice site (branch point, polypyrimidine tract, and AG\cleavage site, taken from the adenovirus major late intron 1; use of this 3′ splice site is also described in reference 25) is combined with the altered exon in pAd3′ssExSub(4138-4407), an improved 5′ splice site and polypyrimidine tract of the large intron are combined with the altered exon in p(514,16)4tpptExSub(4138-4407), and improved 5′ and complete 3′ splice sites of the large intron are combined with the altered exon in p(514,16)Ad3′ssExSub(4138-4407). (B) RT-PCR analysis, using primers a and b shown in Fig. 1, of 5 μg of total RNA isolated after transfection with wild-type MVM (pMVM) or mutant plasmid DNA, or mock transfection, as indicated. pMVM(no RT) was used as a control. For RNA generated by pMVM, R2M (658 nt) was detected. For RNA generated by pExSub(4138-4407), p4tpptExSub(4138-4407), and pΔssD1/2, the exon-skipped product (mainly using A1; 368 nt) was detected. For RNA generated by p4tpptΔssD1/2, only the R2 unspliced form (larger, at 712 nt, than wild-type R2M)

at its 3′ splice site (TATAAATTTACTAG\, which overlaps the TATA sequence of the capsid gene promoter P38 (deviations from the consensus are underlined). The branch point of the large intron has not yet been identified. Efficient excision of the large intron is dependent upon at least the initial presence, in P4-generated pre-mRNAs, of sequences within the downstream intron, suggesting that the small intron plays a primary role in efficient excision of the large intron (25). Improvement of the polypyrimidine tract of the upstream large intron renders its efficient excision independent of the downstream intron, suggesting that perhaps the small intron acts as the initial entry site(s) for an element(s) of the spliceosome, which stabilizes the binding of required factors to the polypyrimidine tract of the large intron (25).

Sequences within the MVM intervening NS2-specific exon are required for inclusion of this exon into spliced steady-state RNA. Since sequences within the downstream small intron are required for the efficient excision of the upstream large intron from MVM P4-generated pre-mRNAs, we chose to examine the role of the intervening (NS2-specific) exon in control of alternative splicing of MVM P4-generated transcripts. Mutations in NS2-specific exon sequences were constructed by (i) replacement of the majority of the intervening NS2-specific exon (MVM nt 2005 to 2270) with a heterologous piece of DNA of the same length containing an ORF from the MVM capsid gene (nt 4138 to 4407) in either orientation [in pExSub(4138-4407) the capsid gene ORF was inserted in frame with the NS2-encoding ORF, and in pExSub(4407-4138) it was inserted in the opposite orientation], (ii) inversion of the intervening exon sequences between nt 2005 and 2270 (pMVM 2270-2005), or (iii) deletion of the intervening exon sequences between nt 2005 and 2270 [pMVMΔ(2005-2270)] (Fig. 2A). The parent construct for these exon mutants was made by introducing two unique *Sma*I sites at nt 2005 and 2270 in the wild-type clone of MVM, by site-directed mutagenesis (15), so that the highly conserved exon sequence for the 3′ splice site (AG\G) of the upstream large intron and those for the donor site (AG/GT) of the downstream small intron were left intact. All of the exon substitution and deletion mutants failed to generate MVM P4-generated transcripts that contained the altered exon as assayed by RNase protection analysis (18) utilizing homologous antisense probes which span the center of the mutant genomes (Fig. 2B; data shown only for the pExSub mutants), whereas RNA generated by the parent construct containing the two *Sma*I sites was spliced like that of the wild type (data not shown). The exon substitution mutants did, however, produce abundant amounts of MVM P4-generated transcripts that utilized the large-intron splice donor at nt 514, as well as a relatively small amount of R1, as assayed by RNase protection analysis using an antisense probe spanning nt 385 to 650. RNA using the large-intron donor at nt 514 generated a band of 129 nt (Fig. 2C; designated R2 for the wild type and "Exon skipped product" for the mutants). These results suggested that these NS2-specific exon mutants generated an abundant, spliced, P4-generated RNA product that excluded the modified intervening NS2-specific exon. Reverse transcriptase PCR (RT-PCR) analysis, using established protocols (11) and MVM-specific primers a (nt 2557 to 2538) and b (nt 326 to 345) (diagrammed in Fig. 1), demonstrated that these MVM mutants (but not the wild type or the parent construct) generated an RNA whose size was consistent with a spliced RNA in which the large-intron donor at nt 514 was joined to the downstream small-intron acceptors (Fig. 2D; the joining to A1 at nt 2377 was at least 10 to 15 times more frequent than was that to A2 at nt 2399). The identity of this 368-nt product was further authenticated by Northern (RNA) analysis (16) (an abundant 2.8-kb RNA species generated by these mutants was detected with a DNA probe spanning MVM nt 147 to 650 [data not shown]), by an RNase protection assay using an antisense cDNA probe homologous to the large-intron donor/small-intron acceptor junction (data not shown), and by direct sequencing of the PCR product across the splice junction (data not shown). These results demonstrated that alteration of the nature of the intervening exon gives rise to almost uniform exclusion of the intervening exon and that sequences within the intervening exon are required for its inclusion into spliced steady-state RNA.

Improvement to consensus of the large-intron splice sites cannot overcome the requirement of sequences within the NS2-specific exon for inclusion of this exon in RNA. We have previously shown that improvement of the polypyrimidine tract of the upstream large intron renders its excision independent of downstream intron sequences (25). We have also recently found (26), and show here again, that when both small-intron donors are deleted (Fig. 3A), the NS2-specific exon is skipped, and that exon skipping in this situation can be overcome by improvement of the upstream large-intron polypyrimidine tract (the large intron is again efficiently excised and R2 is generated [Fig. 3B] [26]). However, when the large-intron polypyrimidine tract or complete 3′ splice site of an exon substitution mutant was improved [p4tpptExSub(4138-4407) and pAd3′ssExSub(4138-4407), respectively, diagrammed in Fig. 3A], exon skipping was not suppressed (Fig. 3B). Improvements of both the large-intron 5′ donor site and 3′ splice site polypyrimide tract together [p(514,16)4tpptExSub(4138-4407), Fig. 3A] or both the large-intron 5′ donor site and the complete 3′ splice site together [p(514,16)Ad3′ssExSub(4138-4407), Fig. 3A] were also unable to suppress exon skipping that was the consequence of NS2-specific exon substitution (Fig. 3C). The RT-PCR analysis shown was further substantiated by RNase protection assays that failed to detect significant R1 or R2 production from these mutants with homologous probes spanning nt 1854 to 2377 (probe A in Fig. 1; data not shown). These results demonstrate that the requirement for sequences within the NS2-specific exon is dominant over large-intron splice site signals, since improvement of these signals could neither suppress exon skipping nor restore large-intron excision in these mutants. This also suggested that for wild-type MVM it is unlikely that NS2-specific exon sequences affect inclusion of this exon by effectively strengthening the upstream large-intron splice sites. In addition, destruction of the 3′ splice site of the upstream intron in an otherwise wild-type clone did not result in detectable exon skipping (data not shown). Change of the large-intron nonconsensus donor to consensus in p(514,16)4tpptExSub(4138-4407) and p(514,16)Ad3′ssExSub(4138-4407) led to an exon-skipped product that ran slightly faster on 8% neutral acrylamide gels (Fig. 3C). These RT-PCR products have been sequenced and are spliced be-

was detected. (C) RT-PCR analysis, using primers a and b shown in Fig. 1, of 5 μg of total RNA isolated after transfection with 20 μg of pMVM or mutant plasmids per dish, or mock transfection, as indicated. For RNA generated by p4tpptExSub(4138-4407) and pAd3′ssExSub(4138-4407), the exon-skipped product (mainly using A1; 368 nt) was detected. For RNA generated by p(514,16)4tpptExSub(4138-4407) and p(514,16)Ad3′ssExSub(4138-4407), an exon-skipped product of slightly smaller size was generated; however, sequencing of this product indicated that it contained the same splice junction (nt 514 joined to nt 2377) as do pExSub(4138-4407), p4tpptExSub(4138-4407), and pAd3′ssExSub(4138-4407) (see text for further explanation). PCR products were run on 8% native polyacrylamide gels.

tween the same large-intron donor (at nt 514) and small-intron acceptor (at nt 2377). The reason for the difference in mobility is not known. The constructs in which the large-intron donor has been converted to the consensus, however, have a C rather than an A residue at nt 513 in the spliced product (25); perhaps this results in a change in RNA secondary structure that may account for the difference in electrophoretic mobility.

The MVM-specific exon joins a growing list of examples in which exon sequences have been shown to program their inclusion into mRNAs (9, 17, 21–23). In one prominent example, the *Drosophila doublesex* pre-mRNA, both in vivo and in vitro studies have shown that female-specific exon sequences bind *trans*-acting factors which stimulate splicing of the upstream intron by strengthening the binding of essential splicing factors to its suboptimal polypyrimidine tract (9, 20). How might sequences within the NS2-specific exon program its inclusion into final spliced mRNA (R2)? As suggested above, since improvement of large-intron splice sites cannot overcome exon skipping due to loss of wild-type exon sequences, it is unlikely that the effect of exon sequences is to directly improve the efficiency of excision of the upstream intron. At least two possibilities remain to account for the role of sequences within the NS2-specific exon. Wild-type exon sequences could function to greatly enhance the competitiveness of downstream small-intron excision, relative to an exon-skipping event. Initial small-intron excision would then prevent subsequent exon skipping, which requires at least one small-intron acceptor. Alternatively, wild-type exon sequences might form a secondary structure that imposes a physical barrier to the exon-skipping event. N2-specific exon sequences are predicted on the basis of computer calculations to form a very tight secondary structure. We are currently testing these possibilities.

We have not as yet determined whether the exon-skipped product generated by exon substitution mutants is either polyadenylated or transported from the nucleus, although we have determined that it is a very stable species (24), as are all MVM mRNAs. We have not detected the exon-skipped product at significant levels during wild-type virus infection; therefore, it is unlikely that it encodes a protein that is important in virus replication in murine fibroblasts, although it remains possible that such a product is generated by wild-type MVM in other cell types. A recent report demonstrates the presence of a similar exon-skipped product during infection by porcine parvovirus, which has a transcription profile similar to that of MVM (2).

A growing body of evidence has suggested that sequences other than those defined for the 5′ splice site (donor) and 3′ splice site (the branch point, polypyrimidine tract, and the AG site) play essential roles in determining the selection of splice sites in vertebrates. Here we have shown that the inclusion of the intervening NS2-specific exon is dependent upon the intervening exon sequences in addition to the downstream small-intron donors. Further investigation of the nature and the roles of these sequence elements and their interaction with cellular *trans*-acting factors in the control of alternative splicing of MVM P4-generated pre-mRNAs will be useful not only for understanding the essential roles alternative splicing plays in the efficient expression of the MVM genome but also for studying the mechanisms of exon definition in mammalian cells.

This work was supported by PHS grant AI-21302 from NIAID and funds from the University of Missouri Research Board. Q.Z. was supported by the University of Missouri Molecular Biology Program for a portion of this work.

We thank Mark Hannink and members of our laboratory for helpful discussions.

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