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Efficient Excision of the Upstream Large Intron from P4-Generated Pre-mRNA of the Parvovirus Minute Virus of Mice Requires at Least One Donor and the 3′ Splice Site of the Small Downstream Intron

QIHONG ZHAO,† ANAND GERSAPPE, AND DAVID J. PINTEL*

Department of Molecular Microbiology and Immunology, University of Missouri School of Medicine, Columbia, Missouri 65212

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We have previously shown that efficient excision of the upstream large intron from P4-generated pre-mRNA of the autonomous parvovirus minute virus of mice depends upon at least the initial presence of sequences within the downstream small intron (Q. Zhao, R. V. Schoborg, and D. J. Pintel, J. Virol. 68:2849–2859, 1994). In this report, we show that the requirement of downstream small intron sequences is complex and that efficient excision of the upstream intron requires at least one small intron donor and the 3′ splice site. In the absence of both small intron donors, a new spliced product is produced in which the intervening exon is skipped and the large intron donor at nucleotide 514 is joined to a small intron acceptor. Exon skipping caused by the loss of the two small intron donors can be overcome, and the excision of the large intron can be regained by mutations that improve the large intron polypyrimidine tract. These results are consistent with a model in which the binding of multiple splicing factors that assemble at both a downstream donor and acceptor facilitates the binding of splicing factors to the weak polypyrimidine tract of the upstream large intron, thereby defining the intervening exon and promoting excision of the upstream intron.

Alternative splicing of mRNA precursors (pre-mRNAs) plays a fundamental role in the regulation of gene expression in eukaryotic organisms. Frequently, it provides a mechanism to produce structurally related but distinct proteins from a single gene (25, 36). In some cases, patterns of alternative splicing are regulated developmentally or in a cell-type-specific fashion, whereas in others, they are constitutive.

Alternative splicing is accomplished by alternative selection of splice sites that can be located at either the 5′ or 3′ end of an intron. Comparison of intron sequences and subsequent mutational analysis have demonstrated that the 5′ and 3′ ends of introns are conserved (35); however, while the strength of a splice site, as determined by its similarity to the consensus, plays an important role in its selection, it is not sufficient to determine its choice. It has been observed that splice sites with great homology to the consensus sequence are not always used as authentic sites, whereas seemingly weak splice sites are chosen instead, suggesting that sequences other than the conserved 5′ (AG/GTAAGT) or 3′ (CAG\G cleavage site, polypyrimidine tract, and branchpoint) splice site sequences also play essential roles in splice site selection (6). It has also been demonstrated, in a growing number of cases, that sequences within the adjacent exon (23, 32, 38), those within the adjacent intron (other than those identified as the 5′ or 3′ splice site) (5, 15, 17, 19, 20, 41), and RNA secondary structure (13, 14, 37) are important determinants for splice site selection.

We have been interested in examining the features that govern the alternative splicing of the parvovirus minute virus of mice (MVM) P4 promoter-generated pre-mRNAs. MVM is organized into two overlapping transcription units which 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, 8, 31). Transcripts R1 (4.8 kb) and R2 (3.3 kb) are generated from a promoter (P4) at map unit 4 (31) and encode the viral nonstructural proteins NS1 (83 kDa) and NS2 (24 kDa), respectively, utilizing the open reading frame in the left half of the genome (11). Both NS1 and NS2 play essential roles in viral replication and cytotoxicity (12).

Two types of introns exist in MVM P4-generated transcripts. An overlapping set of downstream small introns, which is located at map units 44 to 46, is common to both P4-generated transcripts (R1 and R2) and P38-generated transcripts (R3). Two small intron donors, denoted as D1 (AG/GTACGA) and D2 (AG/GTAAGG), are located at nucleotides (nt) 2280 and 2317, respectively, and are very similar to the donor sequence consensus (only the underlined residues are not conserved). Two acceptors, denoted as A1 (TTACCTGTTTTACAG\G) and A2 (AATCACTTGGTTTAG\G), are located at nt 2377 and 2399, respectively. Both have well-conserved AG cleavage sites but numerous purine interruptions (underlined) of their polypyrimidine tracts (there are more interruptions in A2 than in A1). Three splicing patterns (major [M], minor [m] and rare [r]) are alternatively used to excise this small intron from each transcript class, resulting in nine steady-state spliced MVM mRNA species produced during infection (7, 21, 26). The major splicing pattern (found in approximately 70% of the spliced molecules of each transcript class) joins D1 to A1, a minor splicing pattern (found in approximately 25% of the molecules) joins D2 to A2, and a rare splicing pattern joins D1 to A2 (5%). The fourth splicing pattern, D2-A1, is not detected in vivo (26), presumably because the distance between these sites (60 nt) is shorter than the minimum suggested to be required for successful excision of introns in mammalian cells (30). Unspliced, polyadenylated R1 and R3 comprise a signif-

* Corresponding author. Phone: (314) 882-3920. Fax: (314) 882-4287. Electronic mail address: pintel@medsci.mbp.missouri.edu.

† Present address: Division of Rheumatology and Immunology, School of Medicine, University of California, San Francisco, CA 94143-0724.

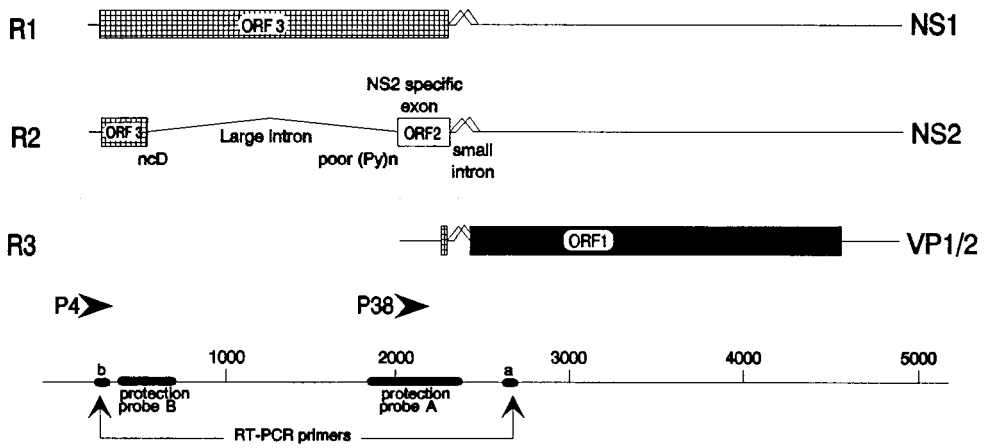

FIG. 1. Genetic map of MVM. The three major transcript classes and protein-encoding open reading frames (ORFs) are shown. The small intron, large intron, and NS2-specific exon are indicated. The nonconsensus donor (ncD) and the poor polypyrimidine tract [(Py)n] of the large intron are also shown. The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

icant portion of viral RNA detected in both nuclear and total RNA preparations throughout MVM infection and after transfection of MVM genomic plasmid clones (28, 34, 41).

An upstream large intron, which is located between map units 10 and 39, is unique to P4-generated transcripts. This intron utilizes a nonconsensus splice donor at nt 514 (AA/GCAAGT) and has a poor polypyrimidine tract at its 3′ splice site (TATAAATTTACTAG\), which overlaps the TATA sequence of the capsid gene promoter P38 (deviations from polypyrimidine tract consensus are underlined). We have previously shown that efficient excision of this intron depends upon at least the initial presence of sequences within the downstream small intron in P4-generated pre-mRNAs (41). Improvement of the polypyrimidine tract of the upstream large intron renders its efficient excision independent of the downstream intron, and prior excision of the small intron is unnecessary. These results suggest that the small intron plays a primary role in efficient excision of the large intron, perhaps 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.

In this study, we have performed an extensive mutational analysis of both the upstream large intron polypyrimidine tract and the downstream small intron in order to further characterize their interaction. Our results show that both the small intron 3′ and 5′ splice sites affect the efficient excision of the upstream large intron from MVM P4-generated pre-mRNA, suggesting that there is a complex interaction between the two introns. These results are consistent with a model in which the binding of multiple splicing factors to the downstream small intron facilitates the binding of splicing factors to the weak polypyrimidine tract of the upstream large intron (perhaps via a network of exon-bridging protein-protein interactions), thereby defining the intervening exon and promoting excision of the upstream intron.

MATERIALS AND METHODS

Mutant construction. Construction of pNS2-1989, p4Tppt, p4TpptΔN/P, pΔS/P, pΔA/P, pΔN/P, and pssD1(−) has been previously described (27, 39, 41). Other mutations were constructed with m13-based oligonucleotide mutagenesis as previously described (27) with the mutant oligonucleotides described below. The template used to make the following mutations was an MVM fragment from nt 1086 to 3521 cloned into m13mp19. After mutagenesis, a small fragment containing the mutation was substituted back into the plasmid clone of MVM, or in some cases pΔN/P, by standard techniques and sequenced to ensure that only the desired mutations were introduced. To make the following mutants, the following oligonucleotides were used: 3Tppt and 3TpptΔN/P, 5′GCCGAACCTAGTAAAAAAATAGGAGTTGGCACC3′ (MVM nt 2000 to 1964); 2Tppt and 2TpptΔN/P, 5′GCCGAACCTAGTAAAAATATAGGAGTTGGCACC3′ (MVM nt 2000 to 1964); 1Tppt and 1TpptΔN/P, 5′GAACCTAGTAAAATTATAGGAGTTGGC3′ (MVM nt 1993 to 1967); 5Appt, 5′GCCGAACCTAGTTTTTTTTTTGGAGTTGGCACC3′ (MVM nt 2000 to 1964); pΔssA1/2, an oligonucleotide spanning MVM nt 2411 to 2340 with a deletion from nt 2372 to 2359; pssD2(−), 5′CCCTTAAACCATTTGGTCTTTTAGC3′ (MVM nt 2331 to 2307); and pΔssD1/2, an oligonucleotide spanning MVM nt 2335 to 2262 with a deletion from nt 2322 to 2278.

The m13 template used to construct pMVM(2322–2348) and pA1ppt(−) was prepared as follows. The *Xho*I-*Hpa*I fragment (nt 2072 to 3757) from pΔS/P (which lacks nt 2377 to 3636) was cloned into the wild-type MVM m13 template described above (containing MVM nt 1086 to 3521), between the *Xho*I (nt 2072) and *Stu*I (nt 2500) sites. After mutagenesis, the *Xho*I-*Bgl*II fragment (nt 2072 to 3453) from m13 replicative-form DNA was substituted back into the plasmid clone of MVM. (Note that this final construct contains an additional copy of nt 3636 to 3757, rather than wild-type sequence, between the two *Stu*I sites at nt 2377 to 2500. Since it has been previously demonstrated that deletion of MVM sequences between nt 2652 and 3996 has no effect on MVM pre-mRNA splicing [28], it is unlikely that the additional copy of MVM nt 3636 to 3757 present in these constructs has any effect upon excision of the large or small intron.) pA1ppt(−) was made with the following oligonucleotide that spanned MVM nt 3649 to 3636/2377 to 2342: 5′GGTTGGTTTGCCACCCTCTTTTTCTCCTCCTTAAACATTAATACCCCACC3′. Mutant 2322-2348 required two oligonucleotides; the first was 5′CCCCACCAACCAAGTAGTCAGTTGATCCTTACCTCTTTTAGC3′ (MVM nt 2348 to 2316) and the second was 5′AATTAAACATTAATATGACGTTAGTAATGTAGTCAGTTGATCCTTACC3′ (MVM nt 2363 to 2325). pssD1(−) and pssD2(−) were combined to generate pssD1/2(−), and p4Tppt and pΔssD1/2 were combined to generate p4TpptΔssD1/2, by standard recombinant DNA techniques.

Transfections, RNA isolation, and RNA analysis. Murine A92L cells, the normal tissue culture host for MVM(p), were grown as previously described (31) and transfected with wild-type and mutant MVM plasmids with DEAE-dextran exactly as previously described (28). RNA was typically isolated 48 h posttransfection, after lysis in guanidinium hydrochloride, by centrifugation through CsCl exactly as previously described (34). RNase protection assays were performed as previously described (34). The probe used for RNase protection assays was an [α-^{32}P]UTP-labelled Sp6-generated antisense MVM RNA from nt 1854 to 2378 (corresponding to an MVM *Hae*III restriction fragment [Fig. 1]). This probe extends from before the acceptor site of the large intron to within the small intron common to all MVM RNAs at map units 44 to 46 and distinguishes RNA species with either of the alternative small intron donors, designated M for the major splice donor (D1) at nt 2280 and m for the minor splice donor (D2) at nt 2317 (9). For analysis of RNA produced after transfection with pNS2-1989, p5Appt, p4Tppt, p3Tppt, p2Tppt, and p1Tppt (and their pΔN/P derivatives) and pssD1(−), pssD2(−), pssD1/2(−), pΔD1/2(−), and p4TpptΔD1/2, antisense RNase protection probes (nt 1854 to 2377) that were homologous to the mutants being analyzed were constructed and used. In some cases, probe B (MVM nt 385 to 650 [Fig. 1]) was used. RNase protection assays were analyzed on a Betagen

B-scanning phosphorimage analyzer, and molar ratios of MVM RNA were determined by standardization to the number of uridines in each protected fragment. Unless otherwise stated in the figure legends, total R1 and R2 (unspliced and spliced R1 and R2) were included in the quantitation of each analysis. Reverse transcriptase (RT)-PCR assays were done as previously described (39).

RESULTS

Excision of the small intron is independent of sequences within the upstream large intron 3′ splice site. Sequences within the downstream small intron are required to strengthen the weak polypyrimidine tract of the upstream large intron in order to facilitate its efficient excision (41). In contrast, however, analysis of RNA generated after transfection of a mutant in which all of the pyrimidines in the large intron polypyrimidine tract were changed to purines (p5Appt) and of a previously constructed mutant in which the cleavage site of the large intron 3′ splice site was changed from AG to CG (pNS2-1989 [27]) demonstrated that efficient excision of the downstream small intron does not depend on the large intron 3′ splice site polypyrimidine tract and cleavage site. Although both large intron 3′ splice site mutations prevented detectable large intron excision (as monitored by the absence of R2), both the efficiency and pattern of excision of the downstream small intron from pre-mRNA generated by these mutants were similar to those of RNA generated by wild-type MVM(p) (Fig. 2; compare the ratio of R1un relative to R1m plus R1M in pMVM with that in p5Appt or in pNS2-1989 [The ratio of unspliced R3 {R3un} relative to R3m plus R3M generated by pNS2-1989 was also like that of the wild type.]). Deletion of the entire 3′ splice site (nt 1886 to 2005, including the putative branchpoint, polypyrimidine tract, and cleavage site) also had no detectable effect upon either the pattern or efficiency of excision of the downstream small intron (data not shown). These results demonstrate that although efficient excision of the upstream large intron is dependent upon downstream small intron sequences (41), splicing of the downstream small intron in P4-generated pre-mRNAs is independent of sequences within the 3′ splice site of the upstream large intron.

Improvement of the large intron polypyrimidine tract by two pyrimidines renders its efficient excision independent of sequences within the downstream small intron. We have previously demonstrated that increasing the pyrimidine content by four in the purine-rich polypyrimidine tract of the large intron 3′ splice site both increased its excision to levels greater than those seen for the wild type and rendered its efficient excision independent of the downstream small intron (41). Improvement of the large intron donor 5′ splice site at nt 514 to consensus (AA/GCA to A**G**/G**T**A), however, did not render efficient excision of the large intron independent of downstream small intron sequences, although this change also increased large intron excision to levels greater than those seen for the wild type (41). A more detailed mutational analysis of the large intron polypyrimidine tract has now shown that an increase in the number of pyrimidines in the large intron polypyrimidine tract by as few as two rendered efficient excision of the large intron independent of downstream small intron sequences (Fig. 3, left panel), while this change only modestly increased its excision in an otherwise wild-type clone (as measured by the steady-state ratio of R1 to R2 [Fig. 3, right panel; compare p2Tppt and pMVM]). Moreover, even a single change from A to T at nt 1981 was able to make large intron excision significantly less dependent upon downstream intron sequences (Fig. 3, left panel).

Sequences within the small intron 3′ splice site facilitate efficient excision of the upstream large intron. Previous analysis of small intron cDNA constructs and deletion mutants suggested that the nature of the small intron sequences necessary for efficient upstream intron excision is complex (41). Therefore, we have performed the following mutational analysis to more precisely define those *cis*-acting sequences within the small intron that are required for efficient upstream intron excision.

Deletion of sequences downstream of the small intron acceptor A1 has previously been shown to have no effect upon the excision of the upstream large intron (shown for the previously constructed mutant pΔS/P [41] and shown again in Fig. 4C). Examination of RNA generated by mutant pΔssA1/2, in which both the 3′ acceptor cleavage sites (CAG\) of the small intron and the sequences in between have been deleted (nt 2375 to 2440 [see Fig. 4A]), however, demonstrated the requirement of a small intron 3′ acceptor cleavage site for the efficient excision of the upstream large intron. pΔssA1/2 generated two classes of RNA: one in which there was no excision of the small intron (because of the lack of both small intron acceptor cleavage sites) and a second in which the small intron donor D1 was used, presumably joined to a cryptic downstream acceptor (Fig. 4B [Interestingly, under these conditions only D1 is used.]). The large intron was excised to reduced levels from pre-mRNA from which the small intron region was not excised [compare R1un(pssΔA1/2) with R2un(pssΔA1/2); Fig. 4B]; however, it was excised at wild-type levels of efficiency from pre-mRNA in which the small intron donor D1 was joined to a cryptic acceptor site [compare R1M(pΔssA1/2) with R2M(pΔssA1/2); Fig. 4B]. Alteration of the polypyrimidine tract of the remaining small intron 3′ splice site A1 of pΔS/P [mutant pA1ppt(−); Fig. 4A] also resulted in decreased levels of large intron excision [compare R1pA1ppt(−) with R2pA1ppt(−); Fig. 4C]. Both pA1ppt(−) and pΔA/P also produced a small amount of P4- and P38-generated RNA, which was spliced by utilizing small intron donor D2 (Fig. 4C, bands a and b, respectively), presumably joined to a cryptic acceptor downstream. The large intron was efficiently excised from P4-generated molecules that utilized a small intron donor [only R2m (band a) and not R1m was detected in RNA generated by pΔA/P and pA1ppt(−)], which is similar to what was described above for RNA generated by pΔssA1/2 (Fig. 4B). These results suggest that efficient excision of the upstream intron requires factors that assemble at both a downstream donor site and an acceptor site. It is interesting to note, however, that those RNA molecules generated by pΔssA1/2 that were spliced with a small intron donor utilized only D1 and were relatively abundant, while those RNA molecules generated by pΔA/P and pA1ppt(−) that were spliced with a small intron donor utilized only D2 and were relatively less abundant. Perhaps the presence of the small intron polypyrimidine tract in pre-mRNA generated by pΔssA1/2 permits a level of cryptic splicing greater than can be achieved in its absence. The small intron polypyrimidine tract may also specify the use of D1 under these circumstances; splicing then shifts to D2 (which is somewhat more consensus than D1) in its absence.

The effects of these alterations are specific, since an alteration of 22 nt within the 26-nt region 2322 to 2348 of the downstream small intron had no effect upon excision of the upstream large intron [compare R1un plus R1M and R2M generated by pMVM(2322–2348); Fig. 4A and C].

Alteration of the two small intron donors resulted in skipping of the NS2-specific exon and joining of the large intron donor at nt 514 to the small intron acceptors. When either small intron donor was individually altered (Fig. 5A), small intron splicing was restricted to the remaining donor, and yet the large intron was excised at wild-type levels (Fig. 5B). When

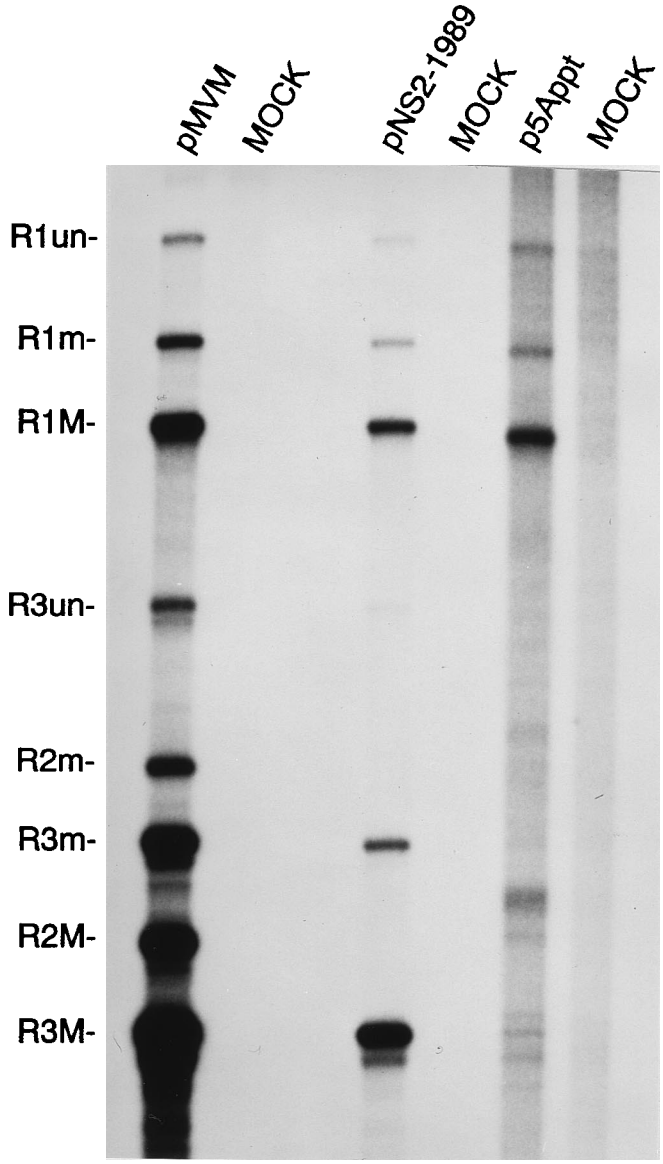

FIG. 2. Excision of the small intron is independent of upstream large intron 3′ splice site sequences. The upper part of the figure shows maps of the two mutants used in this experiment. The lower panel shows an RNase protection assay with either wild-type probe A (nt 1854 to 2378; Fig. 1) for pMVM or versions of probe A homologous to the mutants being analyzed. The identities of the protected bands are designated on the left. Neither mutant generates R2 RNA, and p5Appt does not generate R3 RNA because of changes to the amino acid composition of NS1 (underlined in boldface italic) and alteration of the P38 TATA box.

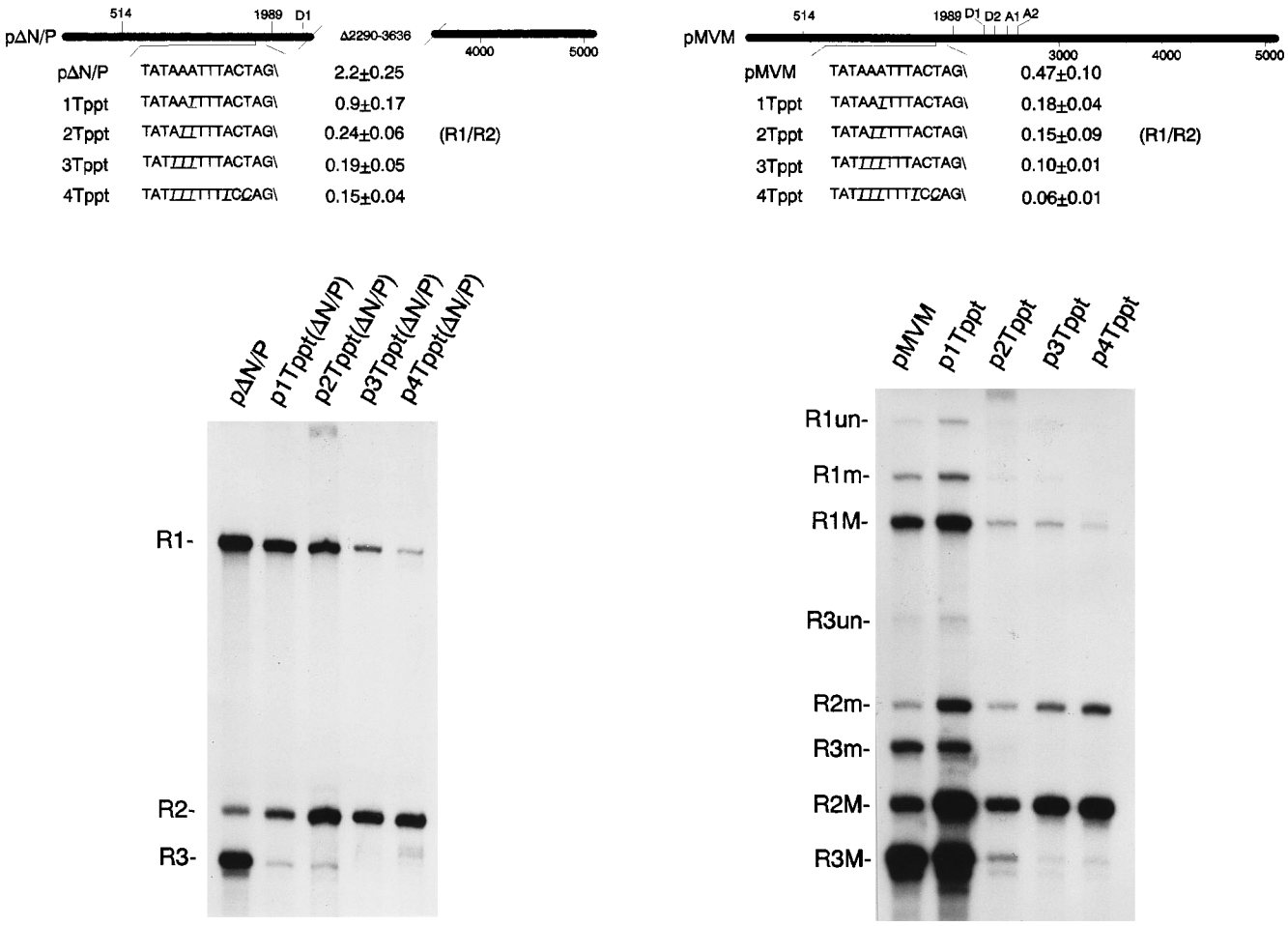

FIG. 3. Minor changes in the large intron polypyrimidine tract render its efficient excision independent of sequences within the downstream small intron. The upper part of the figure shows a map of the mutations in the large intron polypyrimidine tract, either in the wild type (right) or the ΔN/P background (left). The lower panel shows RNase protection assays with either wild-type probe A for pMVM and pΔN/P or versions of probe A homologous to the mutants being analyzed. The identities of the protected bands are also shown. The 2T, 3T, and 4T mutants generate very little R3 because of changes to both the P38 TATA box and the NS1 coding sequence. The deficiency in steady-state levels of R3 generated by pΔN/P has been previously reported (41) and is likely due to decreased NS1 production because of inefficient transport of unspliced R1 to the cytoplasm. The 1t mutation, which alters the P38 TATA box and a single amino acid of NS1, shows a much greater reduction in steady-state levels of R3 in the ΔN/P deletion background than in a wild-type background for reasons that are not yet clear but that may be related to the lower steady-state levels of NS1 seen in ΔN/P versus wild-type background. The average steady-state levels of R1 versus R2 (R1/R2) are shown for each construct. The averages of four individual transfections for each construct are shown.

both small intron donors were altered, however, either by point mutation or by deletion (Fig. 5A), excision of the large intron was not detected, as monitored by the absence of R2 as assayed by RNase protection with a homologous probe, A, that spans the center of the genome (Fig. 5B [probe diagrammed in Fig. 1]). Surprisingly, however, the relative amounts of unspliced R1 (containing the large intron) generated by these mutants were not concomitantly increased (Fig. 5B). RNase protection assays of RNA generated by pΔssD1/2 with a left-end probe B (see Fig. 1 for probe diagram), however, identified abundant amounts of RNA which utilized the large intron donor at nt 514 (Fig. 5C; the 129-nt band designated exon-skipped product for pΔssD1/2), suggesting that an abundant P4 transcript that utilized the large intron donor was present. Northern (RNA) hybridization analysis of RNA generated by this mutant demonstrated a transcript approximately 2.8 kb in size that hybridized with the left-end probe, B (data not shown). RT-PCR analysis of RNA produced by this mutant, with the primers diagrammed in Fig. 1, detected a product consistent in size with a novel spliced product that joined the large intron donor at nt 514 to the small intron acceptor, A1, at nt 2377 (Fig. 5D, see lane marked as pΔssD1/2). The identity of this product was further verified by sequence analysis across the splice junction (39). Therefore, in the absence of the two small intron donors, the large intron was not retained in the P4-generated product, yielding increased levels of unspliced R1 as may have been expected, but instead a novel splicing event occurred in which the NS2-specific exon was skipped. Exon-skipped products utilizing the small intron acceptor A2 at nt 2399 were at least 10-fold less abundant than those using A1 in RNA generated by pΔssD1/2.

Exon skipping caused by loss of the two small intron donors can be overcome by mutations that improve the polypyrimidine tract of the upstream large intron. Since improvement of the large intron polypyrimidine tract renders its efficient excision independent of the downstream small intron, could such a mutation also overcome exclusion of the NS2-specific exon in pΔssD1/2 and restore large intron excision? RNase protection analysis of RNA generated by such a double mutant (p4TpptΔssD1/2) with homologous probe A detected un-

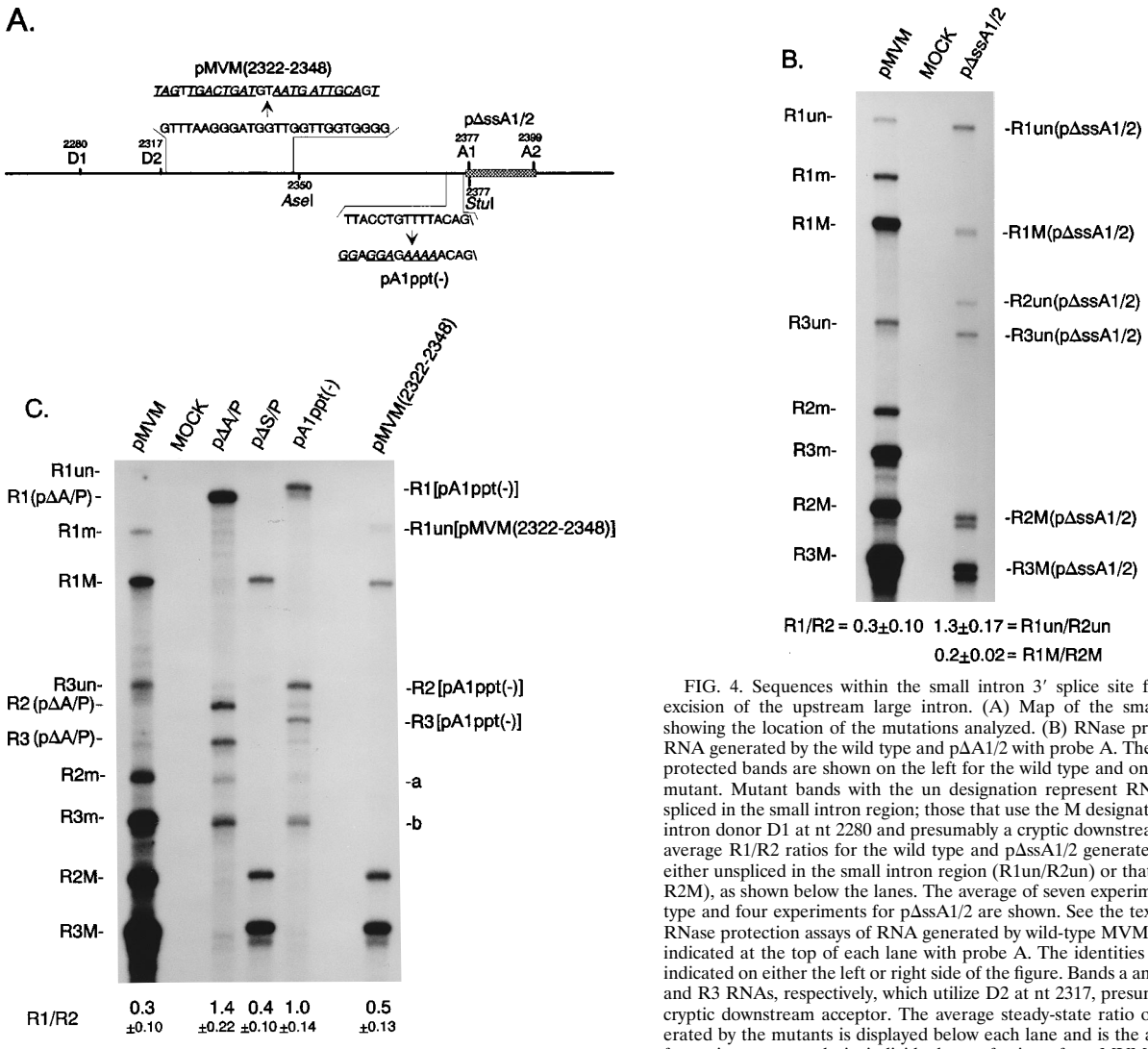

FIG. 4. Sequences within the small intron 3′ splice site facilitate efficient excision of the upstream large intron. (A) Map of the small intron region showing the location of the mutations analyzed. (B) RNase protection assay of RNA generated by the wild type and pΔA1/2 with probe A. The identities of the protected bands are shown on the left for the wild type and on the right for the mutant. Mutant bands with the un designation represent RNAs that are unspliced in the small intron region; those that use the M designation use the small intron donor D1 at nt 2280 and presumably a cryptic downstream acceptor. The average R1/R2 ratios for the wild type and pΔssA1/2 generated RNA that was either unspliced in the small intron region (R1un/R2un) or that used D1 (R1M/R2M), as shown below the lanes. The average of seven experiments for the wild type and four experiments for pΔssA1/2 are shown. See the text for details. (C) RNase protection assays of RNA generated by wild-type MVM and the mutants indicated at the top of each lane with probe A. The identities of the bands are indicated on either the left or right side of the figure. Bands a and b represent R2 and R3 RNAs, respectively, which utilize D2 at nt 2317, presumably joined to a cryptic downstream acceptor. The average steady-state ratio of R1 to R2 generated by the mutants is displayed below each lane and is the average of seven, four, six, seven, and six individual transfections for pMVM, pΔA/P, pΔS/P, pA1ppt(−), and pMVM(2322–2348), respectively. R1/R2 ratios for pΔA/P and pA1ppt(−) include only unspliced R2 [R2 (pΔA/P)] and spliced R2 [pA1ppt(−)], respectively. The decreased levels of R3 generated by pΔA/P are likely due to a decrease in NS1 production because of inefficient transport of unspliced R1 (41).

spliced R1 and unspliced R2 from which the large intron (but not the small intron) was excised, and the ratio of R1 relative to R2 was like that in p4Tppt (Fig. 6 [41]). The generation of R2 was confirmed by RT-PCR, which also failed to detect an exon-skipped product (Fig. 5D [see lane marked as p4TpptΔssD1/2]). These results demonstrate that improvement of the large intron polypyrimidine tract of a mutant in which both small intron donors have been removed restored efficient excision of the large intron and inclusion of the intervening NS2-specific exon. They are consistent with our previous findings that interactions between sequences within the small intron and the weak polypyrimidine tract of the upstream large intron may help define the intervening NS2-specific exon (41).

DISCUSSION

The P4-generated pre-mRNAs of the autonomous parvovirus MVM undergo alternative splicing which determines the ratio of the accumulated levels of mRNAs R1 relative to R2 (9, 26, 28, 31, 34). This event is critical in determining the relative steady-state levels of the viral nonstructural proteins NS1 and NS2 and therefore in optimizing the balance between the essential roles that these two proteins play in viral replication and cytotoxicity. No viral protein participates in this process (28), which must therefore be accomplished solely by the interactions between cellular factors and viral *cis*-acting signals.

Previous results have led us to propose a model regarding alternative splicing of P4-generated pre-mRNA in which the small intron plays a primary role in efficient excision of the large intron, perhaps 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 (41). In the present study, we have further characterized the interaction between the upstream large intron and the downstream small intron. We show that while the small intron is required for efficient upstream intron excision, small intron excision in P4-generated pre-mRNAs is independent of sequences within the upstream intron 3′ splice site. We have also demonstrated that the requirement of downstream small intron sequences is complex; efficient excision of the upstream intron requires at least one small intron donor and the 3′ splice

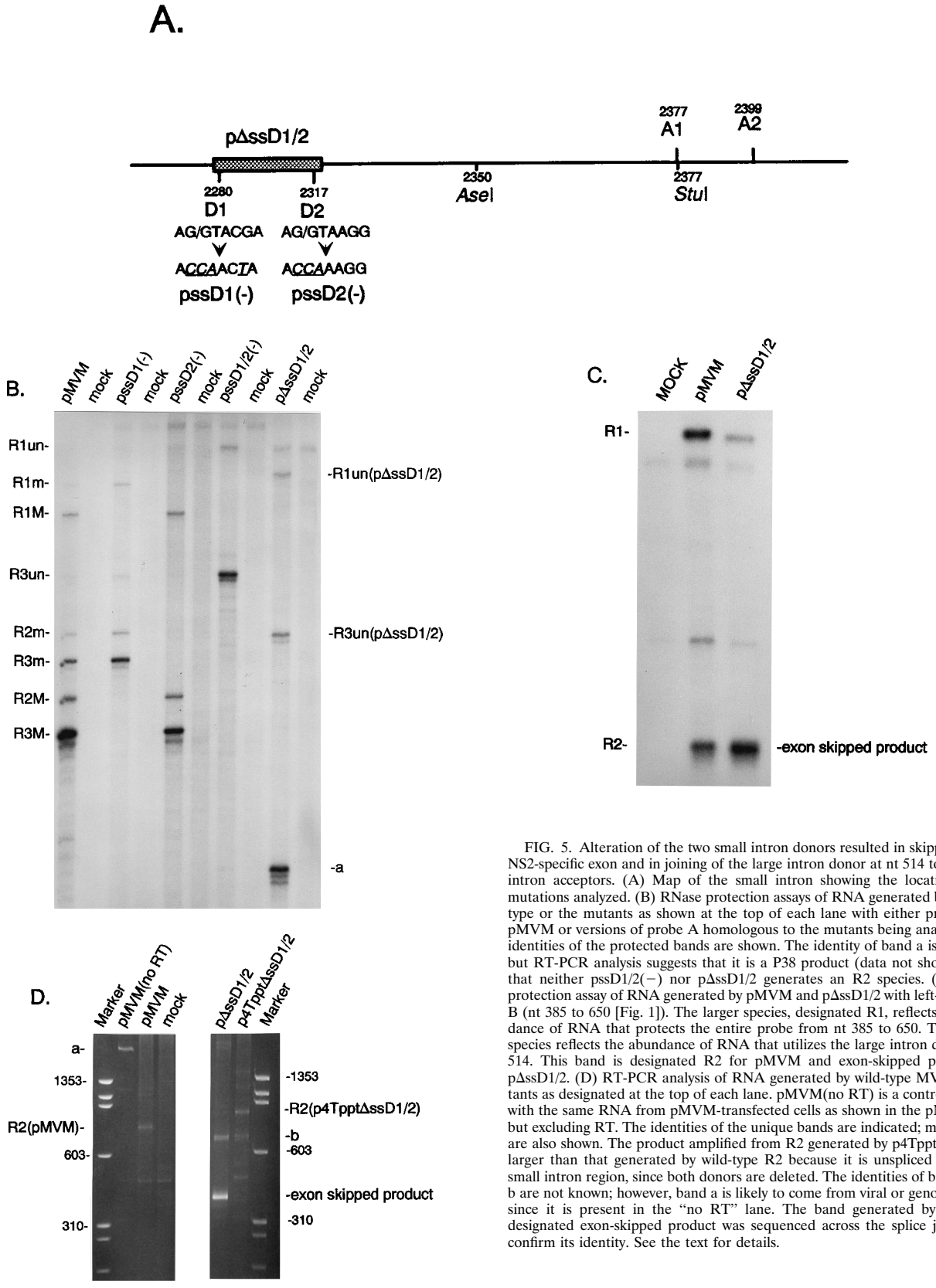

FIG. 5. Alteration of the two small intron donors resulted in skipping of the NS2-specific exon and in joining of the large intron donor at nt 514 to the small intron acceptors. (A) Map of the small intron showing the location of the mutations analyzed. (B) RNase protection assays of RNA generated by the wild type or the mutants as shown at the top of each lane with either probe A for pMVM or versions of probe A homologous to the mutants being analyzed. The identities of the protected bands are shown. The identity of band a is unknown, but RT-PCR analysis suggests that it is a P38 product (data not shown). Note that neither pssD1/2(−) nor pΔssD1/2 generates an R2 species. (C) RNase protection assay of RNA generated by pMVM and pΔssD1/2 with left-end probe B (nt 385 to 650 [Fig. 1]). The larger species, designated R1, reflects the abundance of RNA that protects the entire probe from nt 385 to 650. The smaller species reflects the abundance of RNA that utilizes the large intron donor at nt 514. This band is designated R2 for pMVM and exon-skipped product for pΔssD1/2. (D) RT-PCR analysis of RNA generated by wild-type MVM or mutants as designated at the top of each lane. pMVM(no RT) is a control reaction with the same RNA from pMVM-transfected cells as shown in the pMVM lane but excluding RT. The identities of the unique bands are indicated; marker sizes are also shown. The product amplified from R2 generated by p4TpptΔssD1/2 is larger than that generated by wild-type R2 because it is unspliced across the small intron region, since both donors are deleted. The identities of bands a and b are not known; however, band a is likely to come from viral or genomic DNA, since it is present in the "no RT" lane. The band generated by pΔssD1/2 designated exon-skipped product was sequenced across the splice junction to confirm its identity. See the text for details.

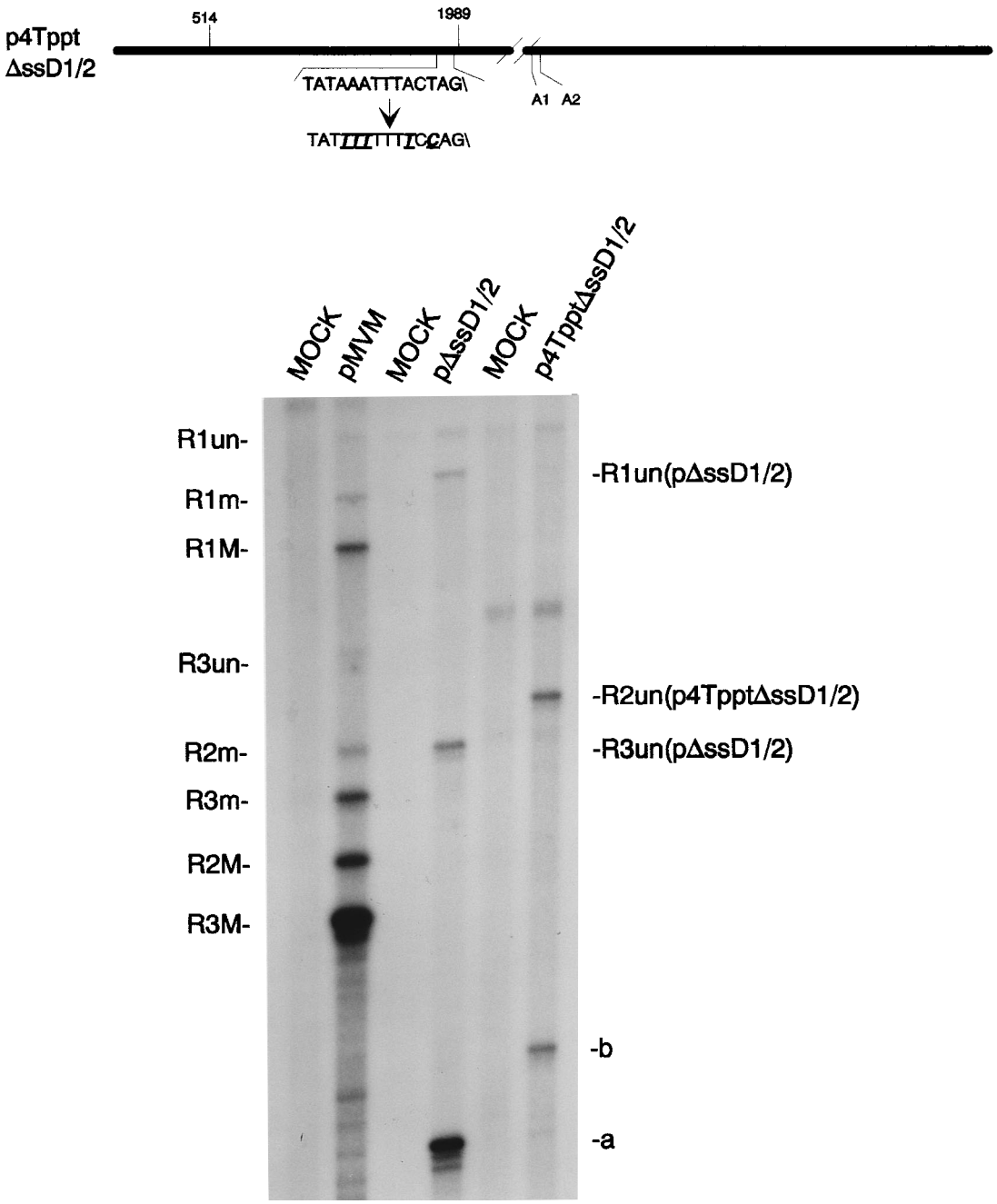

FIG. 6. Exon skipping caused by loss of the two small intron donors can be overcome by mutations that improve the polypyrimidine tract of the upstream large intron. The top of the figure shows a map of p4TpptΔssD1/2. The lower part shows an RNase protection assay of RNA generated by the wild type or the mutants shown at the top of the lanes with probe A or a version of probe A homologous to the mutants being analyzed. The identities of the bands are designated to the right and left of the panel. The identities of bands a and b are unknown, although as described above, RT-PCR analysis suggests that band a is a P38 product. Note that production of R2 is restored when the polypyrimidine tract of the small intron donor deletion mutant is improved (p4TpptΔssD1/2). The generation of R2 was confirmed by RT-PCR, which also failed to detect an exon-skipped product (Fig. 5).

site (including the polypyrimidine tract and cleavage site). In addition, we have shown that even slight improvement in the large intron polypyrimidine tract can render efficient excision of the large intron independent of the small intron.

While at least one donor site of the downstream small intron is necessary for efficient excision of the upstream large intron, loss of both small intron donors did not result in the accumulation of unspliced R1 from which the large intron had failed to be removed, as had been expected. Rather, P4-generated pre-mRNAs produced by pΔssD1/2 were spliced between the large intron donor and small intron acceptors, excluding the NS2-specific exon from spliced steady-state RNA. The small intron donors thus act as part of a complex element (that includes the 3′ splice site) that strengthens the weak polypyrimidine tract of the upstream large intron, which facilitates the excision of this intron and results in the inclusion of the intervening exon.

Our results, which underscore the primary role of the small intron in the alternative splicing of P4-generated pre-mRNAs,

are consistent with the "exon definition" model proposed by Berget and coworkers, in which exons are recognized as units by the interaction of factors bound at the 3′ splice site (5′ end of an exon) and also at the 5′ splice site (3′ end of an exon) (4, 33). This model predicts that loss of both small intron donors would result in NS2-specific exon skipping and that such exon skipping could be suppressed by improvement of the large intron polypyrimidine tract. The role of a downstream donor site in stimulating upstream intron splicing has also been demonstrated both in vivo and in vitro for the rat preprotachykinin gene pre-mRNA (18, 22, 29). Using this system, Grabowski and coworkers have suggested a model in which exon selection is positively regulated by the communication of U1 small nuclear ribonucleoprotein and the U2 small nuclear ribonucleoprotein auxiliary factor U2AF. That is, a natural deficiency in binding of U2AF to a weak 3′ splice site, which leads to the exclusion of the exon immediately downstream, may be overcome by a mechanism in which U1 small nuclear ribonucleoprotein facilitates the binding of U2AF through a network of exon-bridging protein-protein interactions. In this example, only the downstream 5′ donor site is required for efficient excision of the upstream intron. For MVM P4-generated pre-mRNA, however, efficient excision of the upstream large intron requires both a downstream 5′ splice site and a downstream 3′ splice site, suggesting that the factors that assemble at both sites are needed. This does not necessarily imply that a prior splicing event is required to facilitate upstream intron excision and remains consistent with our previous finding that the presence of *cis*-acting elements within the small intron is sufficient for efficient upstream intron excision (41).

Perhaps for wild-type MVM, exon skipping is not favored because the small intron donors are more competitive than the large intron donor for joining to the small intron acceptors, and only in the absence of the small intron donors can the exon-skipped spliced product be generated. This is almost certainly an oversimplification, however, because changing the large intron donor to the consensus in an otherwise wild-type clone does not promote exon skipping (40). In addition, we have recently shown that sequences within the NS2-specific exon play a role in the inclusion of this exon in steady-state RNA (39).

Although there is no indication that the alternative splicing of MVM pre-mRNAs is regulated during the viral life cycle, it is clearly different in cells with a different host origin (16), and the relative steady-state ratios of R1 to R2 differ for MVM(i) versus MVM(p) (2, 10, 24). In addition, other related parvoviruses utilize different patterns of alternative splicing; for example, wild-type porcine parvovirus generates an exon-skipped product during infection that is similar to the exon-skipped product generated by the MVM mutant lacking the two small intron donors (3). Thus, the constitutive efficiencies of these alternative splicing patterns are likely to be an adaptation by parvoviruses for growth in certain environments.

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