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Determinants that govern alternative splicing of parvovirus pre-mRNAs

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Parvoviruses extensively utilize alternative splicing to increase the coding capacity from overlapping reading frames in their compact genomes. Recently, a considerable amount of attention has been paid to characterizing the determinants that govern alternative splicing of pre-mRNAs generated by the parvovirus minute virus of mice (MVM). These studies have revealed that MVM alternative splicing is governed both by factors interacting between introns and by determinants within exons. Comparison with other autonomous parvoviruses with known transcript maps suggest means by which such determinants may function. Parvoviruses have thus become a promising, and in some respects novel, model for understanding the process of alternative splicing and exon definition in mammalian cells.

Key words: parvovirus / alternative splicing

ALTERNATIVE SPLICING of messenger RNA 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.[1,2] 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 either at 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.[3] However, while the strength of a splice site, as determined by its similarity to 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 the branchpoint) splice site sequences also play essential roles in splice site selection.[4] It has also been demonstrated, in a growing number of cases, that sequences within the adjacent exon,[5-7] those within the adjacent intron (other than those identified as the 5' or 3' splice site)[8-13] and RNA secondary structure[14-16] are important determinants for splice site selection.

In some cases, alternative splice site selection is determined by the interaction of the general splicing machinery with non-consensus *cis*-acting signals,[17,18] with *cis*-acting signals lying at suboptimal positions,[19,20] or with secondary structures within the pre-mRNA molecule.[16,21] In other cases, alternative splice site selection has been shown to be mediated by proteins that are not constituents of the general splicing machinery; these proteins act either in a positive or negative manner to determine alternative splice site selection.[22-24] Among the proteins implicated in the biochemistry of splicing, many have been shown to affect splice site selection *in vitro*,[25-29] which suggests that differential concentrations of some of the general splicing factors may also regulate some alternative splicing events *in vivo*.[22] Therefore there are many parameters that can affect alternative splicing in ways that are complex and not readily predictable.

DNA viruses extensively utilize alternative splicing to increase the coding capacity from overlapping reading frames in their compact genomes, and provide excellent models to study this aspect of gene regulation. Although parvoviruses utilize such a complex pattern of alternative splicing, very little is known about the determinants that govern this process which determines the relative steady-state abundances of their mRNAs. However, characterization of the process of alternative splicing of parvovirus pre-mRNAs offers a number of advantages. First, parvovirus alternative splicing is relatively simple so that its analysis can be approached in the context of the

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complete viral genome and therefore as a whole system, rather than an isolated exon-intron-exon segment from a multi-intron cellular gene. Second, parvovirus alternative splicing is governed by both interactions between introns and determinants within exons. This permits an analysis of these determinants individually and an analysis of how they may be interrelated. Finally, parvoviruses are very amenable to mutational analysis, and RNA is readily analysed *in vivo* following transfection. For these reasons, parvoviruses have become a promising, and in some respects novel model for understanding the process of alternative splicing and exon definition in mammalian cells. Recently, a considerable amount of attention has been paid to characterizing the determinants that govern alternative splicing of pre-mRNAs generated by the parvovirus minute virus of mice (MVM).[11,30,31]

Transcript organization of minute virus of mice (MVM)

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 (Figure 1).[32-34] Transcripts R1 (4.8 Kb in size) and R2 (3.3 Kb) are generated from a promoter (P4) at map unit (mu) 4[32] and encode the viral nonstructural proteins NS1 (83 KD) and NS2 (24KD), respectively, utilizing the open reading frame in the left half of the genome.[35] Both NS1 and NS2 play essential roles in viral replication and cytotoxicity.[36]

Two types of introns exist in MVM P4-generated transcripts. An overlapping set of downstream small introns, which is located at mu 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 nt2280 and 2317, respectively, and are very similar to the donor sequence consensus (only the underlined nucleotides are not conserved). Two acceptors, denoted as A1 (TTACCTGTTTTACAG\G) and A2 (AATCACTTGGTTTAG\G), are located at 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.[37-39] 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*,[38] presumably because the distance between these sites (60 nts) is shorter than the minimum suggested to be required for successful excision of introns in mammalian cells.[40] Unspliced, polyadenylated R1 and R3 comprise a significant portion of viral RNA detected in both nuclear and total RNA preparations throughout MVM infection and following transfection of MVM genomic plasmid clones, however unspliced molecules are not found in the cytoplasm.[11,41,42]

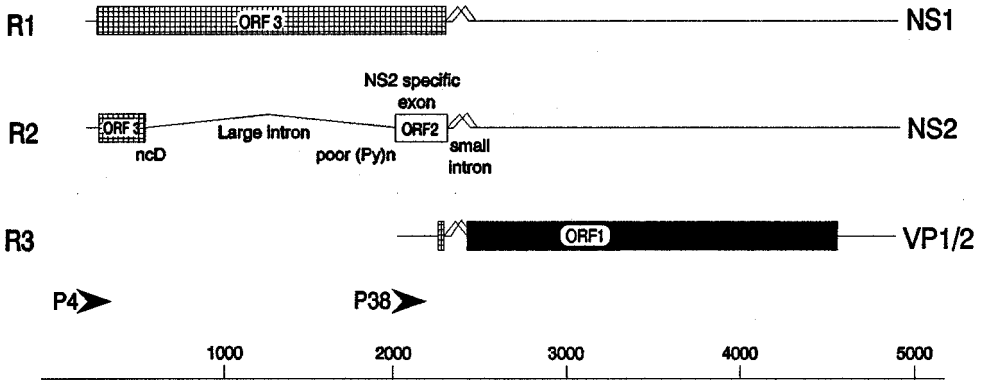

Figure 1. Genetic map of MVM showing the three major transcript classes, R1, R2 and R3, the locations of the two promoters, P4 and P38, the open reading frame used by each transcript, and the proteins that each transcript encodes. The small intron, the large intron and the 'NS2-specific' exon are indicated. The large intron nonconsensus donor and acceptor are also shown.

An upstream large intron, which is located between mu 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). This promoter generates the R3 (2.8 kb) transcripts, which encode the overlapping viral capsid proteins VP1 and VP2, utilizing the open reading frame in the right half of the genome. The P4-generated pre-mRNAs precede the P38-generated pre-mRNAs during highly synchronous infection, and polyadenylation of MVM RNAs precedes splicing.[43] No MVM proteins are required to achieve wild-type patterns of alternative splicing of MVM pre-mRNAs.[41]

The relative steady-state levels of the nonstructural proteins NS1 and NS2, which are of critical importance for efficient viral replication, are controlled by both protein stability and the relative steady-state levels of R1 and R2.[42,44] There is approximately twice the accumulated steady-state level of R2 relative to that of R1 during MVM infection.[32,42] Since transcripts R1 and R2 are both generated from P4,[32] have similar stabilities,[42] and are transported similarly to the cytoplasm,[41] the ratio of accumulated steady-state levels of R1 relative to R2 is dependent upon the percentage of P4-generated RNA molecules from which the large intron is excised. Therefore excision of this intron, which utilizes non-consensus 5' and 3' splice sites, is critical in determining the relative steady-state levels of NS1 and NS2,[42,44] and thus the optimal balance between the essential roles these proteins play in viral replication and cytotoxicity. In addition, while NS1 terminates prior to the small intron, alternate splicing of this region results in three isoforms of NS2 that vary at their carboxy termini.[38,44,45] The relative abundances of the viral capsid proteins VP1 and VP2 is also determined by alternative splicing of the small intron. The joining of donor D1 to acceptor A1 generates an mRNA that encodes the major capsid protein VP2, while the joining of D2 and A2 generates an mRNA that encodes the minor capsid protein VP1.[33,37,38,46]

Determinants that govern the alternative splicing of MVM pre-mRNAs

The determinants that govern the alternative splicing of the P4-generated pre-mRNAs of MVM are complex: there are interactions between the large and small intron,[11,30] and sequences within the NS2-specific exon itself are required for its inclusion.[31]

Efficient excision of the upstream large intron from MVM P4-generated pre-mRNAs depend upon at least the initial presence of sequences within the downstream small intron. Prior or concurrent small intron splicing is not required for large intron excision, but rather small intron sequences must act in *cis*. This suggests that the small intron acts as a binding site, and is probably the primary entry site for the splicing apparatus. The MVM large intron polypyrimidine tract overlaps the purine-rich P38 TATA box, and improvement of the large intron polypyrimidine tract (the binding site for the required splicing factor U2AF) can overcome the requirement for downstream intron sequences. This is consistent with a model in which the binding of splicing factors to downstream intron sequences stabilize or permit the binding of factor(s) required for efficient excision of the large intron, to the large intron polypyrimidine tract. Although the large intron can be efficiently excised in the absence of a small intron splicing event under certain experimental conditions, it is not yet known whether or not the large intron is excised from spliced or unspliced R1 during infection. The sequences within the small intron that facilitate upstream intron excision are complex. For optimum facilitation, at least one small intron donor and complete 3' splice site (including the polypyrimidine tract, AG\ cleavage site and probably the branch point) are required.

The available evidence, therefore, suggests that excision of the large intron depends upon initial definition of the NS2 specific exon, per the model proposed by Berget and colleagues to explain the mechanism of excision of introns adjacent to internal exons.[47,48] This model proposes that exons are the units of definition (and thus recognition) during the splicing process, and that they are defined by the binding of splicing factors to the donors and acceptors at their termini. After such definition (which permits the identification of small exons in very large pre-mRNAs, and prevents the promiscuous use of cryptic splice sites), defined exons are juxtaposed. A direct prediction of the exon definition model is that if the downstream donor of an internal exon were deleted, that exon would not be defined and thus be skipped.[47,48] This has been shown for the NS2-specific exon of MVM P4-generated pre-mRNA.[30] When the small intron donors which form the 3' boundary of the NS2-specific exon are deleted, the NS2 intron is

almost uniformly skipped, and the large intron donor is joined to the small intron acceptor at nucleotide 2377. Interestingly, NS2-specific exon skipping resulting from the loss of the small intron donors can be efficiently suppressed by improving the pyrimidine content of the large intron polypyrimidine tract. This suggests that one of the functions of the small intron donors in the definition of the NS2-specific exon is to strengthen interactions at the upstream, large intron 3' splice site. Deletion of both small intron acceptors also results in a reduction in the excision of the upstream large intron, but does not lead to exon skipping to cryptic acceptors. This observation suggests that, in contrast to the role of the small intron donors, factors binding to small intron acceptor sequences are likely to be involved in a step subsequent to exon definition.

Alternative splicing of the small intron from P4-generated pre-mRNA leads to the production of three isoforms of NS2, and alternative small intron splicing of P38-generated pre-mRNA leads to the generation of VP1 and VP2. There are few known examples of small overlapping introns with two donors and two acceptors,[2,22,49] and the determinants that govern the excision of the small intron from MVM pre-mRNAs are as yet poorly understood.

There are a growing number of examples in which exon sequences themselves have been shown to be important for excision of introns which are immediately upstream.[5-7,12,13,50] This is the case for the NS2-specific exon of MVM as well.[30] When NS2-specific sequences are replaced or deleted, the modified NS2-specific exon is skipped at high frequency. Interestingly, such exon skipping cannot be overcome by improvements to the large intron polypyrimidine tract. This suggests that a dominant element within the NS2 specific exon is required for its inclusion, and that this exon does not act directly to strengthen the large intron 3' splice site. Whether the NS2-specific exon forms a secondary structural determinant preventing exon skipping in wild-type, or is a binding site for a positively acting splicing factor(s) has yet to be determined.

Comparisons with other autonomous parvoviruses

Amongst the autonomous parvoviruses other than MVM, a detailed transcript profile that includes both the primary structure and the steady-state abundances of mRNAs produced, has been determined only for B19[51,52] and Aleutian disease virus (ADV).[53] The collection of alternatively spliced mRNAs generated by B19 and ADV are quite complex and significantly different compared to the other autonomous parvoviruses. In addition, internal polyadenylation also plays a significant role in the processing of these viruses' pre-mRNAs.

The rodent(RV)-like parvoviruses MVM, H1, canine parvovirus (CPV), and porcine parvovirus (PPV), however, seem to share generally similar transcription profiles, although there are significant and potentially informative differences. A detailed picture of the primary structure of the mRNAs generated by PPV has recently been obtained,[54] however, the relative steady-state abundances of these mRNAs has not yet been determined. In contrast to MVM, the PPV small intron has two donor sites and only a single acceptor site, and both the NS1 and NS2 coding regions of PPV terminate prior to the small intron splice sites. Perhaps the most striking difference between the splicing pattern of MVM and PPV, however, is that PPV encodes an abundant mRNA (R3) which joins the large intron donor to the single small intron acceptor, skipping the NS2 specific exon. The small nonstructural protein NS3, which is encoded by the PPV R3 mRNA, may be required to compensate for the absence of multiple isoforms of PPV NS2.[54] For MVM, an mRNA in which the NS2-specific exon is skipped is not seen during infection, but only after mutagenesis of the small intron donors or the NS2-specific exon itself. Both the PPV large intron donor (AC/GGCAAG) and 3' polypyrimidine tract (TAAA-TACACCAACAG/AC) are further from consensus than the comparable MVM sequences, and, as is the case for MVM, the PPV small intron acceptor appears significantly stronger than the PPV large intron acceptor (Figure 2). Finally, the PPV NS2 specific exon is only 75% the size of the MVM NS2 specific exon, and computer aided structural predictions of the sequences within this exon [using the energy dot plot analysis of Jacobsen and Zucker[55]] are quite different from the other RV-like parvoviruses. It is likely that some or all of these features may favor the skipping of the NS2 specific exon of PPV.

The splicing signals for H1 are very similar to MVM and while the final spliced products are likely to be identical, the relative abundance of these mRNAs has not yet been firmly established. CPV is similar to PPV in that its small intron seems to utilize two donors and a single small intron acceptor (C. Parrish, personal communication). The CPV small intron 3' polypyrimidine tract is very strong relative to its large

intron acceptor. Therefore, while MVM, H1, CPV and PPV have similarly consensus small intron donor sites, their small intron acceptor sites — and the relative strength of these sites compared to their large intron 3' splice sites — vary significantly.

Another interesting feature regarding splicing of the P4-generated pre-mRNAs is that although all the RV-like parvoviruses utilize a weak large intron 5' donor site, each has a strong 5' donor site 4–5 nts downstream that is not used. Use of this downstream donor would join the NS2-specific exon out of frame, and an understanding of the molecular constraints that prevent the use of this seemingly more favorable site will be of general interest.

Is parvovirus alternative splicing regulated?

The determinants that govern alternative splicing of parvoviruses are clearly complex, but are these alternative splicing patterns constitutive or are they regulated, either temporally during infection, or in a cell type specific manner, in order to modulate the relative levels of the viral proteins at different times?

At the moment there is little evidence to suggest that parvovirus alternative splicing is regulated in a temporal fashion during infection, however, some evidence is available that suggests that it may be regulated in a cell-type specific manner. Perhaps the clearest example is for parvovirus B19. A variability in the splice boundaries of exon 2 of the 500- and 600-nt class of RNA was observed in transfected COS cells compared to that seen in B19-infected human leukemic cells.[52] Although COS cells do not support full B19 replication, these differently spliced RNAs do encode protein products in these cells, suggesting that they may be functional under certain circumstances or cell types. It has also been observed, that the large intron of MVM(p) is excised less efficiently in human HeLa and HT1080 cells compared to

Large Intron

	Donor	Acceptor
Consensus	AG/GTAAGT $^{64}_{73}$ $^{99}_{99}$ $^{62}_{68}$ $^{84}_{63}$	⟵ (Py)n ⟶⊣ NCAG/NN $^{65}_{99}$ ^{99}
MVM	AA/GCAAGT	CCTATAAATTTACTAG/GT
H1	AA/GCAAGT	CCTATAAATTCGCTAG/GT
CPV	AA/GCAAGT	GGTATAAATTCACCAG/GT
PPV	AC/GGCAAG	ATAAATACACCAACAG/AC

Small Intron

	D1	D2	A1	A2
Consensus	AG/GTAAGT	AG/GTAAGT	⟵ (Py)n ⟶⊣ NCAG/NN	⟵ (Py)n ⟶⊣ NCAG/NN
MVM	AG/GTACGA	AG/GTAAGG	ATTACCTGTTTTACAG/GC	AATCACTTGGTTTTAG/GT
H1	AG/GTACAA	AG/GTAAGG	ACTACCTGTTTTACAG/GC	AATCACTTGGTTCTAG/GT
CPV	AG/GTACGA	AG/GTAAGG	ATGTGTTTTTTTATAG/GA	
PPV	AG/GTAGGA	AG/GTAAGG	TTTTTATATATTACAG/GA	

Figure 2. Top. A comparison of the sequences at the large intron donor and acceptor used to generate the R2 mRNA for various RV-like parvoviruses. The consensus sequence for all vertebrate introns is showed for comparison, with the percent-utilization shown under critical nucleotides. **Bottom.** A similar comparison of the sequences at the small intron donors and acceptors. The consensus sequence shown is for all vertebrate introns. The donors and acceptors for CPV are those likely to be used (C. Parrish, personal communication), but have not yet been unequivocally established.

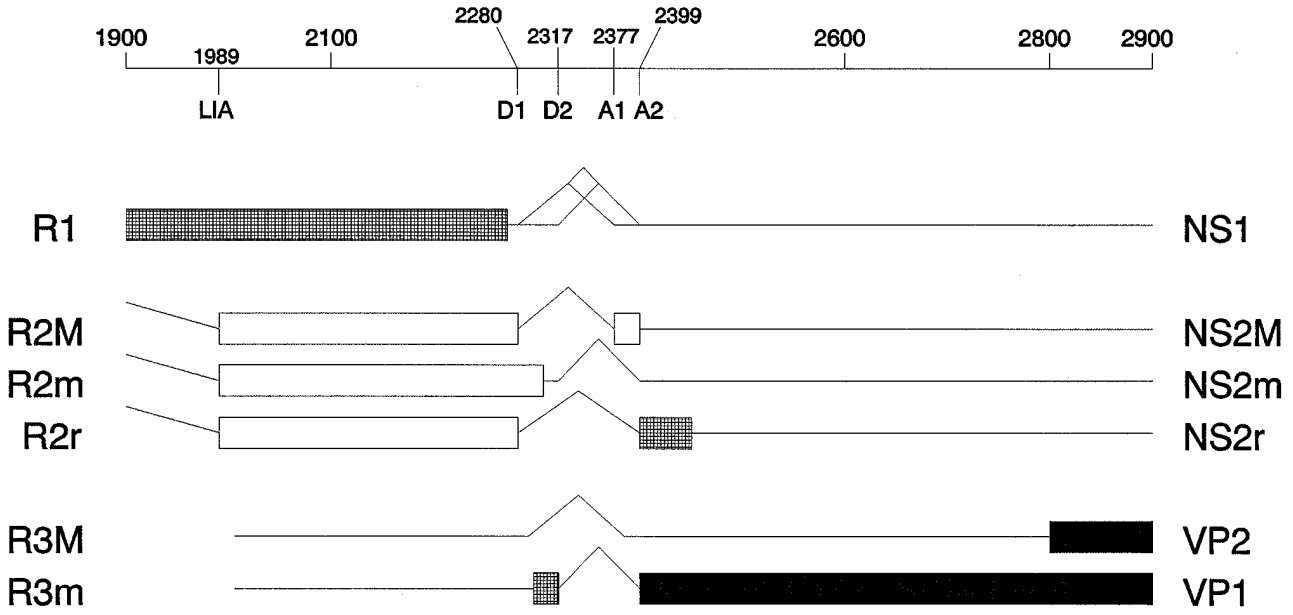

Figure 3. A detailed map of the MVM genome in the region of the NS2-specific exon and small intron. The upstream large intron donor (LIA) and downstream small intron donors and acceptors (D1, D2, A1, A2) are shown. The three major classes of transcripts are shown, with M, m and r designating those RNAs that use the major (D1-A1), minor (D2-A2), and rare (A1-D2), small intron splice, respectively. NS2 isoforms generated from such alternatively spliced NS2 mRNAs are designated NS2M, NS2m, and NS2r. Sequences used to encode NS1 (which terminates before the overlapping small intron as shown), and the capsid proteins VP1 and VP2 are also shown. Open reading frames 1, 2 and 3 [as described in ref 33] are designated by solid, open and hatched boxes, respectively.

murine cells or the SV40 transformed human cell line NB324K (D. Haut and D.J. Pintel, unpublished). While HeLa is not a supportive host for MVM, these results suggest that this intron can be excised differently under various in-vivo conditions. Excision of the large intron can also be strain specific. The large intron is excised less efficiently from P4-generated pre-mRNA produced by the lymphotropic strain of MVM, MVM(i), than seen for its counterpart MVM(p), in NB324K cells [refs 56,57, S. Mathur and D.J. Pintel, unpublished]. The only discernible sequence difference in a defined splicing signal between the two strains is an A to G transition in the putative large intron branch point. It is tempting to speculate that MVM(p) has acquired an A in this position to enhance the excision of the large intron, so that more NS2 can be produced to facilitate growth in fibroblasts.

It has been established that no viral proteins are required to accomplish the complicated pattern of alternative splicing of parvovirus pre-mRNAs.[41] An understanding of the cellular determinants that govern this process will thus be important in illuminating the mechanism of alternative splicing and exon definition in mammalian cells.

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