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cis-Acting and trans-acting modulation of equine infectious anemia virus alternative RNA splicing

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Abstract

Equine infectious anemia virus (EIAV), a lentivirus distantly related to HIV-1, encodes regulatory proteins, EIAV Tat (ETat) and Rev (ERev), from a four-exon mRNA. Exon 3 of the tat/rev mRNA contains a 30-nucleotide purine-rich element (PRE) which binds both ERev and SF2/ASF, a member of the SR family of RNA splicing factors. To better understand the role of this element in the regulation of EIAV pre-mRNA splicing, we quantified the effects of mutation or deletion of the PRE on exon 3 splicing in vitro and on alternative splicing in vivo. We also determined the branch point elements upstream of exons 3 and 4. In vitro splicing of exon 3 to exon 4 was not affected by mutation of the PRE, and addition of purified SR proteins enhanced splicing independently of the PRE. In vitro splicing of exon 2 to exon 3 was dependent on the PRE; under conditions of excess SR proteins, either the PRE or the 5′ splice site of exon 3 was sufficient to activate splicing. We applied isoform-specific primers in real-time RT-PCR reactions to quantitatively analyze alternative splicing in cells transfected with rev-minus EIAV provirus constructs. In the context of provirus with wild-type exon 3, greater than 80% of the viral mRNAs were multiply spliced, and of these, less than 1% excluded exon 3. Deletion of the PRE resulted in a decrease in the relative amount of multiply spliced mRNA to about 40% of the total and approximately 39% of the viral mRNA excluded exon 3. Ectopic expression of ERev caused a decrease in the relative amount of multiply spliced mRNA to approximately 50% of the total and increased mRNAs that excluded exon 3 to about 4%. Over-expression of SF2/ASF in cells transfected with wild-type provirus constructs inhibited splicing but did not significantly alter exon 3 skipping.
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Keywords: Splicing; Purine-rich element; EIAV

Introduction

Retrovirus gene expression depends on both alternative splicing and diverse mechanisms to export intron-containing mRNAs to the cytoplasm. Alternative splicing is controlled by essential elements such as splice sites and branch point sequences (BPS) as well as exonic splicing enhancer (ESE), exonic splicing suppressor (ESS), and intronic splicing suppressor (ISS) elements (Amendt et al., 1994, 1995; Bilodeau et al., 2001; Damgaard et al., 2002; Jacquenet et al., 2001; Ladd and Cooper, 2002; Schaal and Maniatis, 1999; Sun et al., 1993; Tange and Kjems, 2001; Tange et al., 2001). RNA transport is regulated by interactions of a cis-acting RNA element (CTE, constitutive transport element) with cellular proteins or, in lentiviruses, a Rev-responsive element (RRE) with the viral Rev protein (Harris and Hope, 2000; Wodrich and Krausslich, 2001). In the lentivirus equine infectious anemia virus (EIAV), the RRE is embedded in a 30-nucleotide-long purine-rich element at the 3′ end of exon 3 and has been shown to affect RNA export through interaction with viral Rev protein (Gontarek and Derse, 1996; Harris et al., 1998; Martarano et al., 1994). EIAV Tat (ETat) and Rev (ERev) proteins are encoded in a bicistronic mRNA containing four exons. ETat is encoded in exons 1 and 2; ERev is encoded in exons 3 and 4. It was shown previously that expression of ERev induced exon 3 skipping (Martarano et al., 1994). Mutation of the exon 3 purine-rich

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element also caused skipping of exon 3 (Gontarek and Derse, 1996). In addition to binding ERev, this purine-rich element (PRE) was shown to interact with cellular SR proteins and was proposed to function as an ESE (Belshan et al., 2000; Chung and Derse, 2001; Gontarek and Derse, 1996). However, because the earlier methods did not quantify alternatively spliced mRNAs, the extent to which exon 3 inclusion was dependent on the purine-rich element is unknown.

SR proteins belong to a family of splicing factors containing RNA recognition motifs in the amino-terminal region and a serine/arginine-rich domain in the carboxyl-terminal region. SR proteins are essential for both constitutive and alternative pre-mRNA splicing (Fu, 1995; Graveley, 2000; Tacke and Manley, 1999). In the early catalytic step of the splicing reaction, the first nucleotide at the 5′ end of an intron forms a 2′–5′ phosphodiester linkage with the adenosine at the branch site and generates lariat intermediates. The base pairing between U2 snRNA and the branch point sequence (BPS) plays an important role in accomplishing this step (Wu and Manley, 1989; Zhuang and Weiner, 1989). The BPS is generally located 18–37 nucleotides upstream of the 3′ splice site with the consensus sequence YNYURAY (where Y is a pyrimidine, N is any nucleotide, and R is a purine) in which only the branch adenosine is invariant (Reed and Maniatis, 1985). Deviation from the consensus BPS affects pre-mRNA splicing efficiency and 3′ splice-site selection (Reed and Maniatis, 1988). The 3′ splice site includes the 3′ AG dinucleotide and an adjacent upstream polypyrimidine tract with which $U2AF^{35}$ and $U2AF^{65}$ interact, respectively (Merendino et al., 1999; Wu et al., 1999; Zamore et al., 1992; Zorio and Blumenthal, 1999). The sequence of the BPS and of the polypyrimidine tract determines their recognition by, and therefore, interactions with splicing factors, which in turn contribute to the regulation of alternative splicing (Garcia-Blanco et al., 1989).

In the following studies we asked how the purine-rich tract in exon 3 and SR proteins affect the alternative splicing of exon 3. We first examined the splicing of exon 3 with upstream exon 2 and downstream exon 4 in simple two-exon models in vitro. Because the functional significance of the PRE with respect to alternative splicing of exon 3 has not been assessed by quantitative means, we applied isoform-specific primers in real-time QRT-PCR to quantify RNA splicing in cells transfected with wild type and mutant proviruses. We also asked how expression of EIAV Rev protein or over-expression of cellular SF2/ASF and hnRNP A1 proteins affects basal and alternative splicing of EIAV pre-mRNA. In addition, we determined the branch point elements upstream of exons 3 and 4 that influence splice acceptor site utilization.

Results

In vitro splicing reactions of EIAV pre-mRNA

To understand the regulation of EIAV pre-mRNA alternative splicing and the role of the purine-rich element within exon 3, we constructed minigene plasmids that contained exons 2 and 3 or exons 3 and 4 (pKS-Ex23 and pKS-Ex34, respectively). Their corresponding introns and essential cis-acting sequence elements (5′ splice sites, 3′ splice sites, branch point sequences, and polypyrimidine tract regions) were present as illustrated in Fig. 1. The minigene RNAs

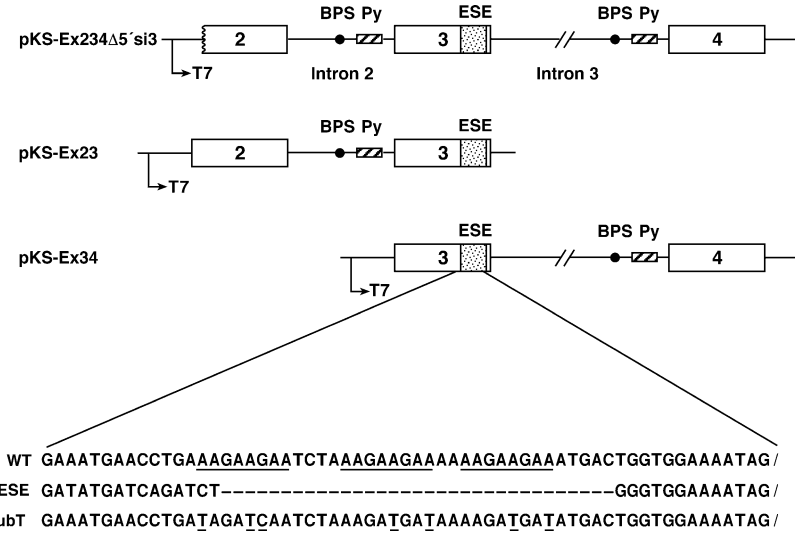

Fig. 1. A schematic representation of the EIAV pre-mRNA minigenes: pKS-Ex23, pKS-Ex34, and pKS-Ex234Δ5′si3. Open boxes represent exons; horizontal lines represent introns; stippled boxes represent exonic splicing enhancer (ESE) regions; solid circles represent branch point sequences (BPS); hatched boxes represent the polypyrimidine tract. The native intron 3 was shortened in the minigene constructs. The splice-acceptor site of exon 2 in pKS-Ex234Δ5′si3 was deleted. RNAs were transcribed in vitro from the T7 promoters at the 5′ end of the minigenes as indicated. The nucleotide sequence of the purine-rich element in exon 3 is shown at the bottom with the three repeats, matching SF2/ASF consensus binding sites, underlined. Sequences for deleted (ΔESE) or mutated (B11/SubT) versions of the purine-rich element are shown below the wild-type element.

were generated by in vitro transcription using T7 RNA polymerase. Optimal conditions for in vitro splicing reactions were defined with 3–4 RNA. Splicing activity was first detected after 1 h of reaction, approached the maximum at 2 h, and leveled off thereafter (data not shown). Splicing activity reached a maximum in the presence of 2.0 mM Mg^{2+}. Splicing products and intermediates were identified by examining various EIAV RNAs containing different insertions or deletions in exon 3, intron 3, or exon 4 and comparing migration patterns of reaction products in denaturing gels (data not shown).

SR proteins promote EIAV pre-mRNA splicing in vitro

We first examined the in vitro splicing of EIAV mRNA in the context of simple, two-exon RNA substrates. SR proteins

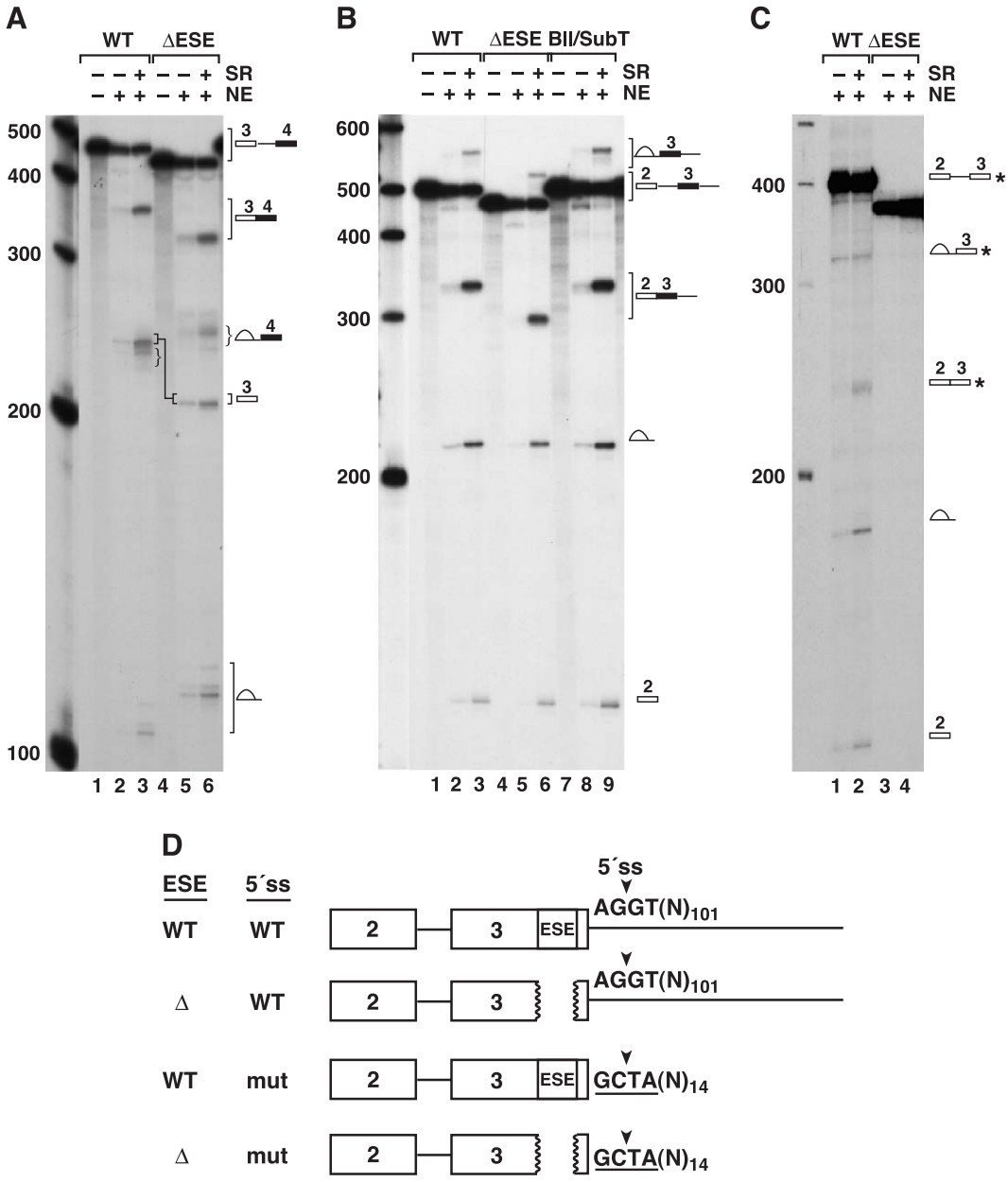

Fig. 2. Influence of the purine-rich element and SR proteins on splicing of two-exon EIAV RNA substrates in vitro. In vitro splicing reactions were examined by denaturing gel electrophoresis and autoradiography. (A) Splicing of exon 3 to exon 4 with minigene RNAs containing wild-type exon 3 (lanes 1–3) or exon 3 without the PRE (ΔESE, lanes 4–6). Splicing reactions were carried out in the presence or absence of HeLa nuclear extract (NE) or addition of purified SR proteins (SR) as indicated. Splicing products are depicted to the right and molecular size standards are shown to the left of the panel. (B) Splicing of exon 2 to exon 3 with minigene RNA substrates containing wild-type exon 3 (lanes 1–3), exon 3 without the PRE (ΔESE, lanes 4–6), or exon 3 containing nucleotide substitutions in the PRE (B11/SubT). Nuclear extract and SR proteins were present as indicated. (C) Splicing of exon 2 to exon 3 with RNA substrates in which the 5′ splice site of exon 3 was mutated and contained either the wild-type PRE (WT, lanes 1–2) or were deleted of the PRE (ΔESE, lanes 3–4). (D) Schematic representation of wild type and mutated exon 2–3 RNA substrates used in panels B and C.

purified from HeLa cells enhanced in vitro splicing of exon 3 to exon 4 (Fig. 2A, lanes 1–3). To determine whether the purine-rich element in exon 3 is required for this activation by SR proteins, we examined RNA substrates with a deletion of the PRE that are designated as ΔESE RNAs (Fig. 2A, lanes 4–6). In vitro splicing of wild type and ΔESE RNAs was comparable both in the presence or absence of added SR proteins (Fig. 2A, lanes 3 and 6). These results indicated that the exon 3 purine-rich element was not essential for SR protein activation of exon 3 to exon 4 splicing.

We next examined the effects of SR proteins on exon 2 to exon 3 splicing in vitro with RNA substrates containing a wild type, deleted (ΔESE), or mutated (B11/SubT) exon 3 PRE (Fig. 1). In the absence of added SR proteins, deletion of the PRE caused a modest decrease in the basal level of splicing of exon 2 to exon 3 (Fig. 2B, lanes 2 and 5), but the B11/SubT mutation had no effect (lanes 7–9). Addition of purified SR proteins to splicing reactions activated splicing on all three substrates to the same extent (Fig. 2B, lanes 3, 6, and 9), indicating that excess SR proteins enhanced splicing independently of the PRE. A 5′ splice site has been reported to enhance splicing and affect the binding of $U2AF^{65}$ to the upstream 3′ splice site of the alternatively spliced exon (Hoffman and Grabowski, 1992; Kuo et al., 1991). Therefore, wild type and ΔESE RNA substrates containing mutations of the 5′ splice site of exon 3 were examined (Fig. 2C). When the purine-rich element was present, splicing occurred and was activated by addition of SR proteins (Fig. 2C, lanes 1 and 2). In contrast, exon 2 to exon 3 splicing was not detected in the double mutant (ΔESE plus 5′ splice site mutation), and addition of SR proteins did not activate splicing (Fig. 2C, lanes 3 and 4). Because the RNA substrates used in panels B and C differed in the length of their 3′ termini (Fig. 2D), we also examined splicing of RNAs like those used in panel C but which contained a wild type 5′ splice site. These RNAs responded to SR proteins in a similar way as the RNAs shown in panel B (data not shown). Thus, either the purine-rich tract or the 5′ splice site of exon 3 was required for efficient splicing of exon 2 to exon 3 in vitro.

Real-time QRT-PCR analysis of pre-mRNA splicing with isoform-specific primers

PCR primers were designed that would specifically and differentially amplify unspliced pre-mRNA, spliced mRNA in which exon 3 was included (2/3/4 mRNA), or spliced mRNA in which exon 3 was excluded (2/4 mRNA) (Fig. 3A). The unspliced template was selectively amplified by positioning the 5′ primer (USP) within intron 3. The spliced-isoform-specific primers were designed such that they are complementary to one exon and have four bases at their 3′ end that are complementary to the adjoining exon. Plasmids containing provirus, 3/4-spliced cDNA, or 2/4-spliced cDNA were used to establish standard curves for real-time QRT-PCR reactions. In addition, the specificity of each primer pair was determined by testing their ability to amplify 10 pg of each of the three plasmid DNAs. In every case, only the cognate plasmid was amplified; for example, E4 plus 3/4-spliced-isoform-specific primers did not amplify plasmid DNA encoding unspliced or 2/4-spliced cDNAs (unpublished observation).

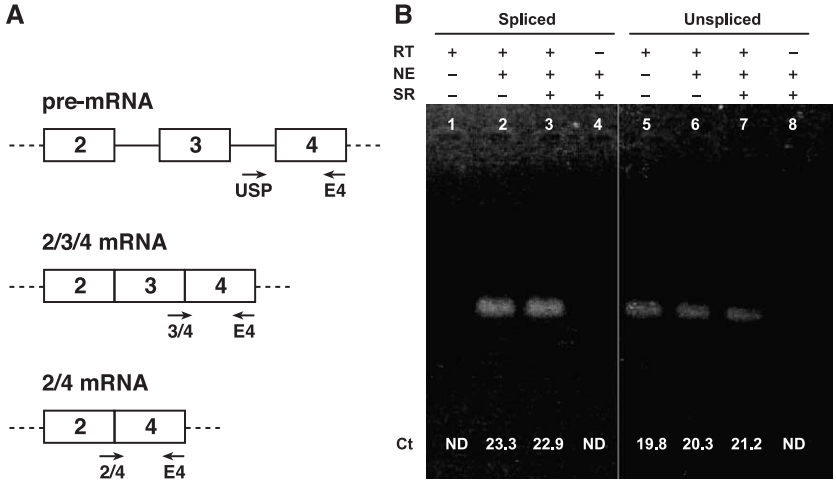

Fig. 3. Real-time QRT-PCR analysis of in vitro splicing reactions. (A) Primer pairs, USP and E4, 3/4 and E4, and 2/4 and E4, were designed to monitor specifically pre-mRNA, 3/4-spliced mRNA, and 2/4-spliced mRNA, respectively. The 5′ region of the 3/4 primer is complimentary to the 3′ end of exon 3 with a four-nucleotide overhang at the 3′ end of the primer that is complementary to the first four nucleotides in exon 4. The 2/4 primer is based on the same principle as the 3/4 primer. Primer locations relative to introns and exons are shown under the RNA isoforms that they detect. (B) Gel electrophoresis of the end products from real-time QRT-PCR reactions. Lanes 1–4 show the products from real-time QRT-PCR reactions using 3/4 spliced-specific primers; lanes 5–8 show the end products using unspliced-specific primers. In vitro splicing reactions were performed without HeLa nuclear extract or SR proteins (lanes 1 and 5), with HeLa nuclear extract alone (lanes 2 and 6), and with HeLa nuclear extract and SR proteins (lanes 3, 4, 7, and 8). Reverse transcriptase was omitted from the reverse transcription reactions as controls for carried-over DNA templates (lanes 4 and 8). The threshold cycle (Ct) at which PCR products were first detected is shown below each lane. ND signifies that no signal was detected by the fluorescence recorder after 41 cycles of PCR.

We next tested real-time QRT-PCR in the context of in vitro RNA splicing reactions with the EIAV exon 3–4 RNA substrate. In vitro splicing reactions were performed as described above followed by reverse transcription. The cDNA generated was analyzed for 3/4-spliced molecules and unspliced RNA molecules by real-time QRT-PCR in triplicate reactions. No amplification was observed in reverse transcriptase-minus reactions after 41 PCR cycles either by gel electrophoresis (Fig. 3B, lanes 4 and 8) or by fluorescence recorder. This indicated that the detected amplicon was indeed from RNA and not from carried-over template DNA in the RNA preparations. The fact that no spliced-specific amplicon was detected from splicing reactions without nuclear extract, which would contain only the input RNA substrate, (Fig. 3B, lane1) confirmed the primer specificity. Melt curves were also routinely used to assess specificity of amplification.

To evaluate assay variation, RNA from two independent in vitro transcription reactions was used in three independent splicing reactions followed by three independent reverse transcription reactions. Two different reverse transcriptases were compared, either Omniscript (Qiagen) or Superscript II RNAseH- Reverse Transcriptase (Invitrogen). cDNAs from the three independent reverse transcription reactions were then used in six real-time QRT-PCR assays in which each reaction was run in triplicate. The results are presented as percentage of 3/4-spliced product (spliced RNA / spliced RNA + unspliced RNA) in Table 1. The mean percentage of 3/4-spliced product was 0% without nuclear extract, 15.4% with nuclear extract, and 34.4% with nuclear extract and SR proteins. The levels of 3/4-spliced products determined by real-time QRT-PCR were similar to values obtained by denaturing-polyacrylamide gel electrophoresis (Fig. 2A) and phospho-image analysis. Phospho-image analysis gave 12.2% 3/4-spliced product in reactions with nuclear extract and 30.4% for reactions containing nuclear extract plus SR proteins.

Quantitative analysis of exon 3 alternative splicing in vivo

We next applied real-time QRT-PCR to quantify the effects of cis- and trans-acting factors on EIAV alternative splicing in vivo. D17 cells were transiently transfected with rev-minus proviruses or minigene constructs containing wild type or PRE-deleted versions of exon 3. Real-time QRT-PCR analysis was performed using the primer pairs (Fig. 3A) that detect unspliced mRNA, 3/4-spliced mRNA (exon 3 inclusion), or 2/4-spliced mRNA (exon 3 exclusion). The relative amount of each viral mRNA was expressed as the percentage of the sum of all three mRNAs (Table 2). In cells transfected with the wild-type exon 3 provirus construct, p8511, 3/4-spliced mRNA constituted approximately 81% of the total viral mRNA and 2/4-spliced mRNA was 0.13%; that is, exon 3 was included in the majority of multiply spliced mRNAs. In contrast, deletion of the PRE resulted in reduced levels of viral mRNA splicing overall and the majority of multiply spliced mRNAs excluded exon 3; the 3/4-spliced mRNA made up only 1.4% of the total and the 2/4-spliced mRNA comprised 39% of the total. A provirus construct containing the B11/SubT mutation in exon 3 gave results identical to the ΔESE mutant (data not shown) Thus, in these provirus constructs, the purine-rich element was critical for exon 3 inclusion in multiply spliced mRNAs.

Expression of ERev inhibited splicing of viral mRNA and increased exon 3 exclusion in the spliced mRNAs; the 2/4-spliced mRNA increased to about 4% and the 3/4-spliced mRNA decreased to about 45% of the total. With p8511ΔESE construct, ERev did not significantly alter the splicing pattern because the PRE deletion also removed the Rev response element.

The SR protein SF2/ASF was previously shown to interact with the purine-rich element in exon 3 in vitro (Chung and Derse, 2001; Gontarek and Derse, 1996). In cells transfected with the wild-type provirus, over-expression of SF2/ASF protein depressed the total yield of spliced product (Table 2). Expression of the SR protein decreased

Table 1
Quantitative analysis of EIAV exon 3–4 RNA splicing in vitro

		1	2	3	4	5	6	Mean ± SD
NE	SR							
–	–	0.0	0.0	0.0	0.0	0.0	0.0	0.0 ± 0.0
+	–	14.2	15.6	14.5	12.9	16.4	19.1	15.4 ± 2.1
+	+	34.1	31.1	28.9	33.7	39.5	38.9	34.4 ± 4.2

cDNA derived from in vitro splicing reaction of 3–4 RNA, with or without HeLa nuclear extract (NE) or purified SR proteins (SR) as indicated, was quantitatively analyzed by real-time QRT-PCR using 3/4-spliced-specific primers and-unspliced specific primers. Values shown are the percentage of 3/4-spliced product copy number [i.e. 3/4 spliced ÷ (3/4 spliced + unspliced)]. Mean values ± SD were computed from six independent real-time QRT-PCR experiments. All reactions were done in triplicate.

Table 2
Quantitative analysis of alternative splicing in cells transfected with EIAV provirus expression plasmids, p8511 or p8511ΔESE

	Rev	SF2	A1	WT			ΔESE		
				2/4	3/4	USP	2/4	3/4	USP
p8511	–	–	–	0.13	80.6	19.3	39.0	1.35	59.9
p8511	+	–	–	4.23	44.7	51.1	38.4	0.97	60.6
p8511	–	+	–	0.21	18.0	81.8	8.84	1.66	89.5
p8511	+	+	–	0.36	17.4	82.6	9.01	2.53	87.9
p8511	–	–	+	0.31	84.8	15.3	34.8	0.29	64.9
p8511	+	–	+	5.6	44.0	50.3	42.6	0.46	56.9

Total cellular mRNA was isolated from D17 cells cotransfected with provirus plasmids in combination with expression plasmids encoding ERev, SF2/ASF (SF2), or hnRNP A1 (A1), as indicated. RNAs were analyzed by real-time RT-PCR using 2/4-spliced-specific primers, 3/4-spliced-specific primers, and unspliced-specific primers for the detection of 2/4-spliced, 3/4-spliced, and unspliced (USP) mRNAs, respectively. The percentage of each mRNA isoform, relative to the sum of the three, is shown for each transfection condition. WT indicates wild-type exon 3 and ΔESE indicates deletion of the PRE in exon 3. All PCR reactions were done in triplicate and standard deviations of at least three determinations were below 20% of the mean values.

production of the 3/4-spliced product but had little effect on the low level of 2/4 splicing (exon 3 skipping) of the WT mRNA. In contrast, on the ΔESE template that lacks the purine-rich target of the SR protein, ectopic expression of SF2/ASF protein decreased the level of the 2/4-spliced product but had little effect on the level of 3/4 splicing. The cellular protein hnRNP A1, an antagonist of SF2/ASF (Bilodeau et al., 2001; Tange et al., 2001), had no effect on the splicing of the wild type or the ΔESE proviruses.

Determination of introns 2 and 3 branch point sequences

Although several important cis-acting elements in EIAV are known, the branch point sequences (BPS) have not yet been defined. Because BPS and the flanking polypyrimidine tract are likely to be important for alternative splicing, we aimed to identify them using a RT-PCR approach. To this end, in vitro splicing of RNAs transcribed from pKS-Ex23 and pKS-Ex34 were carried out as described above. The splicing products were then reverse-transcribed utilizing the ability of Superscript II Reverse Transcriptase (Invitrogen) to read through the 2′–5′ phosphodiester linkage at the lariat branch site (Vogel et al., 1997; Zheng and Baker, 2000). As illustrated in Fig. 4A, an antisense primer complementary to the 5′ end of the intron was used for reverse transcription, followed by PCR amplification with a pair of primers that flank the BPS. Amplified products were cloned and sequenced.

Of the intron 2 BPS cDNA clones sequenced, two contained the sequence 5′-GTTTCTA-3′ and three were 5′-GTTTCAA-3′; the provirus sequence of this region is 5′-G*TTTCAAC*CTT-3′ (italic letters represent the best match to the consensus BPS) (Fig. 4B). It has been reported that although MLV reverse transcriptase was able to read through the 2′–5′ phosphodiester bond at the branch site, the enzyme tends to insert a T residue for the A at the branch point (Vogel et al., 1997; Zheng and Baker, 2000). The two BPS clones with 5′-GTTTCT-3′ would be in agreement with these observations, although the three BPS clones with 5′-GTTTCA-3′ would suggest that the A residue at the branch site had not been replaced by a T. Currently, there is not enough published data on the fidelity of reverse transcriptase with 2′–5′ phosphodiester linkage to resolve this issue. Thus, the EIAV intron 2 BPS, 5′-UUUCAAC-3′, is the best match to the consensus (YNYURAY). The intron 2 branch point adenosine is 24 nucleotides from the 3′ splice site, is interspersed with 11 purines, and hence, lacks a well-defined polypyrimidine tract (Fig. 4B).

A similar approach indicated that two distinct BPS elements were present in intron 3 (Fig. 4B). Both BPS elements, 5′-UAUUU**A**U-3′ and 5′-UGCUU2′–5′U-3′ (the branch site adenosine is shown in bold type), have a six of seven nucleotide match to the consensus branch point sequence. The identification of two BPS for intron 3 is consistent with the observation of multiple lariat signals from intron 3 on denaturing polyacrylamide gels (Fig. 2A).

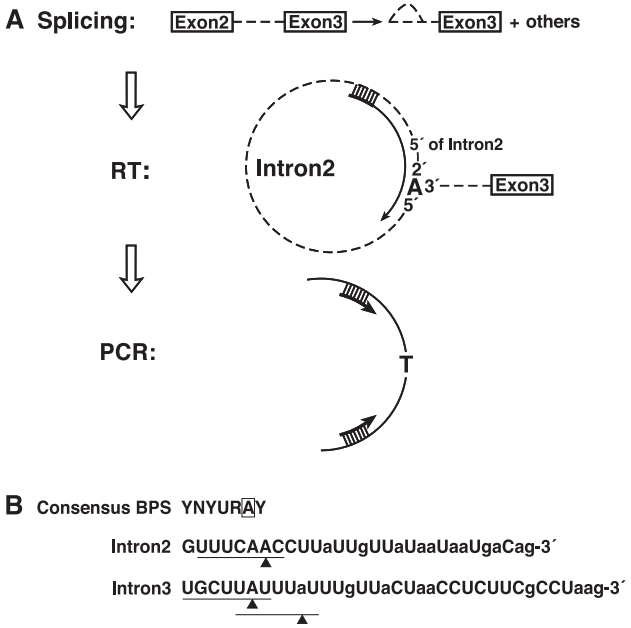

Fig. 4. Defining the branch point sequences (BPS) of intron 3 and intron 4. (A) The strategy for BPS identification is illustrated schematically. EIAV exon 2–3 pre-mRNA was subjected to in vitro splicing reaction. Lariats along with other RNA splicing products were recovered and reverse transcribed (RT) using an antisense-oligonucleotide primer near the 5′ region of intron 2 to generate cDNA (solid lines represent DNA and dashed lines represent RNA) that encompasses the branch point sequence. The cDNA was then amplified by polymerase chain reaction (PCR). PCR products were cloned and sequenced. (B) Consensus BPS, with the invariable adenosine boxed (Y = pyrimidine; N = any base; R = purine), is shown for comparison to the nucleotide sequences determined for EIAV branch point elements. The BPS of intron 2 and intron 3 are underlined. The branch point adenosine is indicated by an arrowhead. Polypyrimidine tract sequences, located between the branch point and the 3′ splice site, are displayed with purines shown in lower case letters.

Multiple BPS elements, though unconventional, have previously been reported in SV40; selection of BPS governs the SV40 pre-mRNA alternative splicing and therefore the expression of large T and small t antigens (Noble et al., 1988). Presently, the functional significance of multiple BPS in EIAV mRNA is not known. Between the BPS and the 3′ splice site in intron 3 is a stretch of 22 and 26 pyrimidines interrupted by 5 and 6 purines, respectively (Fig. 4B).

Discussion

The purine-rich element (PRE) in exon 3 RNA contains three tandemly repeated sequence elements (AAAGAA-GAA) that match the consensus SF2/ASF binding site. RNA substrates containing the PRE region were previously shown to bind SF2/ASF and recombinant ERev proteins in vitro (Chung and Derse, 2001; Gontarek and Derse, 1996). It was reported that in vitro splicing of exon 2 to exon 3 was diminished when the 3′ end of exon 3 (including the 5′ splice site and PRE) was deleted or when point mutations were introduced into the PRE (Belshan et al., 2000). Our in

vitro splicing results showed that mutation of both the 5′ splice site and PRE of exon 3 abolished exon 2 to exon 3 splicing, consistent with the earlier exon 3 deletion analyses (Belshan et al., 2000). However, in our hands, either the 5′ splice site or the PRE was sufficient to enhance splicing, which differs from the previous report that showed mutation of the PRE alone significantly decreased splicing. The ability of a 5′ splice site to enhance splicing by recruiting $U2AF^{65}$ to the upstream 3′ splice site of an alternatively spliced exon has been demonstrated previously (Kuo et al., 1991; Hoffman and Grabowski, 1992). The discordance between our results and those of Belshan et al. (2000) most likely reflects differences in the RNA substrates or composition of nuclear extracts used in the two studies. In spite of these differences, the experiments presented here and before indicate that the PRE acts as an exonic splicing enhancer element for exon 2 to exon 3 splicing in vitro.

Although we have previously shown that mutation of the PRE caused exon 3 skipping in cells transfected with EIAV proviruses (Gontarek and Derse, 1996), the extent to which exon 3 inclusion was dependent on the PRE was not determined. Moreover, the effects of viral and cellular trans-acting factors on viral mRNA splicing have not been examined by quantitative methods. To monitor the levels of individual viral mRNA species produced in transfected cells, we developed a real-time RT-PCR method to address this issue. This quantitative method was essential for determining the biological relevance of PRE function, both as an ESE and as an RRE. In cells transfected with the rev-minus provirus clone, p8511, approximately 80% of the viral mRNAs were spliced to include exon 3 and only 0.13% skipped exon 3. Deletion of the PRE resulted in a reduction in the overall levels of multiply spliced viral mRNAs and increased levels of unspliced mRNA. At the same time, levels of mRNAs that included exon 3 decreased to about 1% of the total and mRNAs that excluded exon 3 increased to approximately 40% of the total. Thus, the PRE acts as an ESE in vivo, having a strong enhancing effect on the inclusion of exon 3 in multiply spliced mRNA.

In so far as sequence comparisons of the cis-acting elements that control pre-mRNA splicing can predict splicing efficiency, the 5′ splice site and branch point sequences of exon 3 did not reveal a striking deviation from consensus sequences. The 5′ splice site of exon 3 (G/GUAUGUUU) had a two-nucleotide mismatch and the branch point had a single mismatch compared to consensus elements. Thus, although some alternatively spliced exons have suboptimal splice site or branch point sequences, this is not the case with exon 3 of EIAV. On the other hand, the sequence of the polypyrimidine tract in intron 2 may be suboptimal due to its high purine content, and may thus contribute to the regulation of exon 3 inclusion.

In addition to acting as an ESE, the exon 3 PRE also contains the Rev-responsive element (RRE) which is essential for the nuclear export of unspliced and partially spliced viral RNAs (Gontarek and Derse, 1996; Harris et al., 1998; Martarano et al., 1994). Recombinant ERev protein was shown to bind RNA containing the PRE in vitro (Belshan et al., 2000; Chung and Derse, 2001; Gontarek and Derse, 1996) and to inhibit in vitro splicing of exon 2 to exon 3 (Belshan et al., 2000). ERev expression was previously shown to induce exon 3 skipping in cells transfected with the rev-minus provirus (Gontarek and Derse, 1996; Martarano et al., 1994), but the magnitude of this effect was not determined. Quantitative analyses presented here indicated that ectopic expression of ERev caused an approximately 2-fold increase in the level of unspliced mRNA accompanied by a 2-fold decrease in spliced mRNA that included exon 3 and an increase in mRNA that excluded exon 3 – 4% of the total. It was previously suggested that ERev might negatively autoregulate its own expression because it is encoded by exons 3 and 4 of multiply spliced mRNA and induces exon 3 exclusion. The 2-fold decrease in mRNA that includes exon 3 does not support this conjecture. The more likely function of ERev with respect to viral mRNA splicing is to induce a shift from production of multiply spliced mRNAs to unspliced and singly spliced mRNAs that encode viral structural proteins.

Because both ERev and SF2/ASF bind to the PRE, two models were proposed to explain the mechanism of ERev-induced exon skipping (Gontarek and Derse, 1996). The first model suggested that the binding of ERev and SF2/ASF to the PRE is competitive, although the second model suggested that ERev and SF2/ASF bind to the PRE simultaneously and ERev inhibits a later step in the assembly or activity of the spliceosome. The second model is supported by the previous observation that SF2/ASF and ERev did not compete for binding to PRE RNA in vitro (Chung and Derse, 2001). Conversely, transient transfection experiments showing a decrease in the activity of a Rev-responsive reporter plasmid in response to SF2/ASF over-expression was interpreted to support the first model (Belshan et al., 2000). We attempted to test these models by transfecting cells with p8511 in combination with ERev plus SF2/ASF. However, we observed that over-expression of SF2/ASF alone inhibited EIAV pre-mRNA splicing but did not affect exon 3 inclusion. This effect was also seen with p8511ΔESE, indicating that the effect was independent of the ESE in exon 3 and likely due to a squelching effect; that is, high levels of SF2/ASF sequester other essential splicing factors in complexes not associated with pre-mRNA. This inhibitory effect was dominant over the effects of ERev, thus preventing a test of the proposed models and providing a cautionary note to interpretations of this type of experiment.

The RNA binding protein hnRNP A1 has been shown to act as a negative regulator of splicing in other retroviruses by binding to intronic or exonic splicing silencers (Blanchette and Chabot, 1999; Burd and Dreyfuss, 1994; Caputi et al., 1999; Del Gatto-Konczak et al., 1999). Alternative splicing of the HIV-1 tat mRNA, for example, is controlled by opposing effects of silencer elements that interact with

hnRNP A1 and enhancer elements that interact with SR proteins (Bilodeau et al., 2001; Tange et al., 2001). Overexpression of hnRNP A1 had a negligible effect on alternative splicing of either wild type or ΔESE EIAV pre-mRNA in our cotransfection experiments, indicating that the mechanism of alternative splicing for EIAV differs from HIV-1.

Materials and methods

Plasmids

The rev-minus EIAV provirus clone, p8511, and the EIAV exon 1-2-3-4 cDNA clone, pK9 have been described (Stephens et al., 1990). The pKS-24 cDNA plasmid was constructed by cloning the RT-PCR product derived from EIAV provirus-transfected cells as previously described (Martarano et al., 1994). The plasmid pKS-Ex23 was constructed by subcloning a PCR product amplified from the provirus clone (p8511) into bluescript pKS-II and contains exon 2–intron 2–exon 3 (provirus positions 5082–5780). The plasmid pKS-Ex34 contains EIAV provirus sequences from positions 5336–5557 (exon 3) joined to positions 7164–7340 (intron 3 and exon 4). Plasmids designated as ΔESE have a replacement of the purine-rich region in exon 3 between positions 5474 and 5525 with the sequence TATGATCAGATCTG. The ERev expression plasmid, pRS-ERev-P32G, was derived by site-directed mutagenesis of pRS-ERev (Martarano et al., 1994) with nucleotide sequences changed from ATAGATCCT (Ile-Asp-Pro) to ATTGACGGC (Ile-Asp-Gly). pRS-ERev-P32G displayed functional activities identical to wild-type ERev, but the mutations in ERev-P32G prevent it from being amplified by the 3/4-spliced-specific PCR primers (Liao and Derse, unpublished observation). The plasmids pCG-SF2 and pCG-hnRNP A1 were obtained from Javier Caceres (Caceres et al., 1994).

In vitro RNA synthesis and in vitro splicing

(^{32}P)-labeled RNA substrates for in vitro splicing were prepared from *Xho*I linearized DNA templates by in vitro run-off transcription using Ambion mMESSAGE mMACHINE with T7 RNA polymerase according to the manufacturer's instructions. For exon 2–3 RNAs with mutated exon 3 5′ splice sites, DNA templates for in vitro transcription were derived by PCR amplification. PCR reactions were performed using plasmids pKS-Ex23 and pKS-Ex23ΔESE as templates with forward primer (5′ GGTAATACGACTCACTATAGGGCGAATTGGCTGTGTTTCCTG-3′) in exon 2 containing a T7 promoter sequence at its 5′-end and reverse primers containing either an intact exon 3 5′ splice site sequence (5′-markusCATAACAGAAACATACCTATTTTCCACC-3′) or a mutated sequence (5′-CATAACAGAAACATTAGCATTTTCCACC-3′). HeLa cell nuclear extract preparation and in vitro splicing reactions were carried out as described by Mayeda and Krainer (1999a, 1999b) unless indicated otherwise. Typically, in vitro splicing reactions were incubated at 30 °C for 2 h in the presence of 0.5 mM ATP, 20 mM creatine phosphate, 3.2 mM $MgCl_2$, 20 mM Hepes–KOH (pH 7.3), 2.6% PVA, and 40 units of RNAsin containing 40% nuclear extract with or without the addition of purified HeLa SR proteins (40 μg/ml final concentration). SR proteins were purified from HeLa cells according to Zahler et al. (1992). Splicing reaction products were analyzed by electrophoresis on 6% polyacrylamide/7M urea gels or by real-time QRT-PCR (described below).

Transfections and RNA purification

Canine D17 cells were transfected with EIAV provirus clones in combination with ERev, SF2, or hnRNP A1 expression plasmids as previously described (Mayeda and Krainer, 1999a, 1999b). Total cellular mRNA was prepared 48 h after transfection using Qiagen RNeasy and oligotex purification methods according to the manufacturer's protocols. RNA was converted to cDNA as previously described (Mayeda and Krainer, 1999a, 1999b).

Branch point sequence mapping

Splicing products from standard in vitro splicing reactions were reverse transcribed in 20-μl reactions containing 1 μM primer (021oHJL: 5′-TGCGATGCTGACCATGTTAC-3′ or 022oHJL: 5′-CCCTTTACCAATAATCCCAT-3′ for intron 2; 012oHJL: 5′-CGCCACCAACCCTATATAAT-3′ for intron 3), 0.5 mM dNTPs, 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 5 mM MgCl2, 10 mM DTT, 2 U/μl RNAsin, and 2.5 U/μl Superscript II RNAseH- reverse transcriptase (Invitrogen). Reactions were carried out according to manufacturer′s instructions. Intron 2 branch-site primers 021oHJL or 022oHJL with primer 020oHJL (5′-CAGAAAAATCTAAGTGTGAGG-3′) and intron 3 branch-site primers 005CoHJL (5′-GAGTCCTTCATACCACCAA-3′) with 010oHJL (5′-AAGTCTTCTTGCACAGATAG-3′) and cDNA of the corresponding pre-mRNA splicing products were amplified by PCR using HotStarTaq Master Mix. Multiple clones of PCR products were then sequenced to map the branch points.

Real-time QRT-PCR

Gene-specific primer (004oHJL 5′-CCAGGTGTCTTCTCGC-3′) was used for reverse transcriptions using Qiagen Ominiscript or SuperScript II RNAseH- reverse transcriptase following manufacturer's instructions. cDNA products were purified by QIAquick PCR purification column. The 2/4-spliced-specific forward primer (2/4: 5′-AAGACAACCTCAATATTTGTTATCAGATCC-3′), 3/4-spliced-specific forward primer (3/4: 5′-GACTGGTGGAAAATAGATCC-3′), and unspliced-specific forward

primer (USP: 5′-TAGTGATGTTTTTGCTTATTTAT-3′) were used with a common reverse primer (E4: 5′-ATTTTTTCTTCAGGTAACGAC-3′) in PCR reactions to amplify exon2/4-spliced product, exon3/4-spliced product, and an amplicon encompassing intron 3 and exon 4, respectively. Real-time QRT-PCR was performed using PE Biosystems SYBR Green PCR Kit, RNAse-free dH_2O and TE were from Ambion, amplification was detected using Bio-Rad iCycler iQ Multi-Color Real Time PCR Detection System. Reactions (25 μl) contained 10 nM 6-carboxy-fluorescein (FAM), 3.0 mM $MgCl_2$ (2.0 mM for 2/4-spliced-specific primer pairs), 0.3 μM each of the forward and reverse primers, 5 U/μl of AmpliTaq Gold, 1 U/μl of AmpErase UNG, 1× SYBR Green PCR buffer (PE), and 1× dNTP blend with dUTP (PE). Reactions were carried out at 50 °C for 3 min, 95 °C for 10 min, with 41 cycles of 95 °C for 15 s and 60 °C for 60 s. Melt curves were performed for all reactions to monitor the quality of amplicons and reactions. Calibration curves were derived by running 10-fold dilutions of each specific cDNA plasmid over the range of 2.5×10^0 to 2.5×10^7 copies. The cDNA samples were run at several dilutions. Each assay included duplicate wells for each dilution of calibration plasmids, triplicate wells for each sample cDNA, and controls which included nonspecific plasmid DNA, buffer, or water, all in duplicate. The threshold cycle values (Ct) were used to plot the calibration curve. Standard curve data were graphed using Microsoft EXCEL 2000, which derived the equation for the line, and calculated the coefficient of variation (CV). All standard curves had a CV of at least 0.95. The copy number of a particular cDNA in a sample was calculated from its measured Ct value with respect to the calibration plot.

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