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Interactions among SR Proteins, an Exonic Splicing Enhancer, and a Lentivirus Rev Protein Regulate Alternative Splicing

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We examine here the roles of cellular splicing factors and virus regulatory proteins in coordinately regulating alternative splicing of the *tat*/*rev* mRNA of equine infectious anemia virus (EIAV). This bicistronic mRNA contains four exons; exons 1 and 2 encode Tat, and exons 3 and 4 encode Rev. In the absence of Rev expression, the four-exon mRNA is synthesized exclusively, but when Rev is expressed, exon 3 is skipped to produce an mRNA that contains only exons 1, 2, and 4. We identify a purine-rich exonic splicing enhancer (ESE) in exon 3 that promotes exon inclusion. Similar to other cellular ESEs that have been identified by other laboratories, the EIAV ESE interacted specifically with SR proteins, a group of serine/arginine-rich splicing factors that function in constitutive and alternative mRNA splicing. Substitution of purines with pyrimidines in the ESE resulted in a switch from exon inclusion to exon skipping in vivo and abolished binding of SR proteins in vitro. Exon skipping was also induced by expression of EIAV Rev. We show that Rev binds to exon 3 RNA in vitro, and while the precise determinants have not been mapped, Rev function in vivo and RNA binding in vitro indicate that the RNA element necessary for Rev responsiveness overlaps or is adjacent to the ESE. We suggest that EIAV Rev promotes exon skipping by interfering with SR protein interactions with RNA or with other splicing factors.

Although the biochemical events involved in constitutive pre-mRNA splicing are being elucidated, the complex regulation underlying the selection of alternative exons is less well understood (for reviews of alternative splicing, see references 17 and 37). The cleavage and ligation reactions required for the splicing of pre-mRNAs are catalyzed by a 60S complex called the spliceosome, which consists of the five spliceosomal small nuclear ribonucleoproteins (U1, U2, U4, U5, and U6 snRNPs) and numerous protein cofactors (for a review, see reference 32). The precision and efficiency of the splicing reaction are determined by numerous RNA-RNA, protein-protein, and protein-RNA contacts. Splice site strength, or similarity to splice site consensus sequences, is one important level involved in defining splice sites (33, 34). Because sequence similarity is not sufficient to guarantee correct selection of proper 5′ splice sites (42), splice site recognition must be regulated by additional sequence elements as well. A number of laboratories have shown the existence of purine-rich exon sequences, called exonic splicing enhancers (ESEs), that positively affect exon recognition (5, 12, 18, 25, 44, 46, 48–51). These elements are generally 6 to 13 nucleotides (nt) in length and contain multiple repeats of the motif GAR, where R is a purine (50). Several studies have shown that these enhancer elements are capable of binding members of a family of serine/arginine-rich splicing factors known as SR proteins (23, 35, 45, 47).

SR proteins are essential for constitutive pre-mRNA splicing and are involved in splice site selection (13–15, 28, 52, 53; for a review of SR proteins, see reference 11). The SR protein family consists of at least six structurally related proteins (SRp20, SRp30a [SF2/ASF], SRp30b [SC35], SRp40, SRp55, and SRp75) that have similar activities in vitro: they are all capable of complementing a splicing-deficient S100 extract and function in a concentration-dependent manner to affect usage of alternative 5′ and 3′ splice sites (13, 15, 22, 28, 52, 53). Recent evidence indicates that SR proteins potentiate splicing by promoting the associations between the 5′ splice site and U1 snRNP and between the 3′ splice site and U2 snRNP (10, 21, 40).

Alternative splicing plays an important role in the regulation of retroviral gene expression. For all retroviruses, a primary unspliced mRNA serves as mRNA for the Gag and Pol gene products, as well as pre-mRNA for the alternatively spliced mRNAs encoding Env and regulatory proteins. Because most cellular pre-mRNAs are spliced to completion before exiting the nucleus, mechanisms that ensure a proper accumulation of unspliced retroviral mRNA for translation and packaging must exist. For simple retroviruses like Rous sarcoma virus, poor splice sites and *cis*-acting elements that negatively regulate splicing provide signals that modulate the efficiency with which splice sites are utilized (12, 16, 29, 30, 43). In addition, retroviruses may contain *cis*-acting transport elements that allow cytoplasmic accumulation of intron-containing mRNAs (3). The more complex retroviruses like human immunodeficiency virus type 1 (HIV-1) and human T-cell leukemia virus type 1 have evolved more elaborate mechanisms to regulate the expression of their mRNAs, including complex patterns of alternative mRNA splicing and differential mRNA transport. These viruses encode transactivating proteins, known as Rev or Rex, that bind RNA and function in the nuclear-cytoplasmic transport of the unspliced and singly spliced viral mRNAs (for a review, see reference 7). Equine infectious anemia virus (EIAV) is a retrovirus distantly related to HIV. EIAV contains all of the genes common to retroviruses that encode virion structural proteins and enzymes required for viral replication, and like other lentiviruses, it encodes transcriptional (Tat) and posttranscriptional (Rev) regulatory proteins. In addition to its

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involvement in viral mRNA stability and transport, EIAV Rev is unique in that it appears to be involved in alternative splicing as well (26). It was shown previously that *rev*-minus EIAV proviruses expressed a four-exon *tat-rev* mRNA at high levels; when Rev was expressed in *trans*, levels of the four-exon mRNA decreased, and a new mRNA coding only for *tat*, in which exon 3 was skipped, appeared (26). EIAV Rev thus down-regulates expression of its own mRNA.

We have extended these observations to examine alternative splicing and the interactions between splicing factors and proteins of the Rev/Rex family. We have identified a purine-rich ESE in EIAV that promotes exon inclusion in vivo and show that this ESE binds SR proteins in vitro. A mutation that results in exon skipping in vivo fails to bind SR proteins in vitro, strongly supporting a functional correlation between exon skipping and SR protein binding to the ESE. Expression of EIAV Rev also promotes exon skipping in vivo and mimics the effects of the ESE mutation. Previous functional analyses indicated that a Rev-responsive element was located near the 3′ end of exon 3. We report here that Rev binds to exon 3 RNA in vitro at a site that overlaps or is adjacent to the ESE. Thus, EIAV Rev may promote exon skipping by interfering with SR protein-RNA or SR protein-snRNP interactions.

MATERIALS AND METHODS

Cells and transfections. The canine osteosarcoma cell line D17 was maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum. Cells were plated at a density of 3×10^5 per well in six-well cluster dishes 24 h prior to transfection. Cells were transfected by the calcium phosphate procedure. Two days later, cells were collected for analysis of viral mRNAs.

Plasmids. The *rev*-minus EIAV provirus clone pFL8511 has been described elsewhere (26). pFL8511 EL1Py was constructed by recombinatorial PCR in the following manner. By using a plasmid containing the *Pst*I-*Eco*RI fragment of pFL85 (41) cloned into pUC18 as a template, primer RSP was used along with primer E3L1Py(−) to synthesize a 1,151-bp fragment, fragment 1; primer E3L1Py(+) was used along with primer E5761 to synthesize a 330-bp fragment, fragment 2. Fragment 1 and fragment 2 were denatured and allowed to reanneal; the annealed fragments were then filled in and used as templates with primers RSP and E5761 to generate a 1,441-bp proviral DNA fragment containing a substitution of pyrimidines for purines in exon 3 loop 1. The recombinatorial PCR fragment was cut with *Pst*I and *Sph*I and substituted for the *Pst*I-*Sph*I fragment of pFL-8511. Plasmid pFL8511 EL2Py was constructed in the same manner, using primers RSP and E3L2Py(−) to generate fragment 3 and primers E3L2Py(+) and E5761 to generate fragment 4. Plasmid pSK-ERE1, containing EIAV nt 5275 to 5832 driven by a T7 RNA polymerase promoter, was provided by Tom Hope (The Salk Institute). EIAV nt 5275 to 5832 were PCR amplified from pFL8511 EL1Py and pFL8511 EL2Py and cloned into the *Eco*RV site of pKSII+ (Stratagene) to yield pKS-EREL1Py and pKS-EREL2Py.

Construction and bacterial expression of GST-ERev. By using a plasmid containing the EIAV Rev cDNA as a template (41), primers Rev(+) and Rev(−) were used to amplify the Rev coding region. After digestion of the PCR product with *Bam*HI and *Eco*RI, the fragment was cloned into the *Bam*HI-*Eco*RI sites of pGEX2T (Pharmacia) to yield plasmid pGST-ERev. A bacterial culture containing pGST-ERev was induced with isopropylthiogalactopyranoside (IPTG) for 30 min at 37°C. The bacterial pellet was resuspended in 10 ml of lysis buffer (20 mM $NaHPO_4$ [pH 7.0], 2 mM dithiothreitol, Boehringer protease inhibitor cocktail) and kept on ice for 10 min. The cell lysate was sonicated twice with 30-s bursts, and Triton X-100 was added to a final concentration of 1%. The lysate was then centrifuged at 15,000 rpm for 20 min in an SS-34 rotor. One-tenth volume of 10× phosphate-buffered saline (PBS) was added to the cleared lysate, as was 1 ml of glutathione-Sepharose (Pharmacia). After a 1-h incubation at 4°C with gentle rocking, the mix was then washed twice with 25 ml of cold PBS. The glutathione-Sepharose was transferred to a small column, and the glutathione *S*-transferase–EIAV Rev (GST-ERev) fusion protein was then eluted with 50 mM Tris-HCl (pH 8.0)–25 mM reduced glutathione–100 mM KCl. Peak fractions were pooled and dialyzed against 20 mM Tris-HCl (pH 8.0)–100 mM KCl–5 mM dithiothreitol. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and immunoblotting with anti-EIAV Rev sera (a gift from Nancy Rice) revealed expression of full-length GST-ERev protein (~46 kDa) as well as several minor bands (data not shown).

Reverse transcription (RT)-PCR. Total cellular RNA was prepared from transfected cells by the RNA-STAT reagent (Tel-Test, Inc.) and dissolved in 100 μl of water. cDNA synthesis reactions were done as described previously (26), and the reaction mixtures were diluted to 100 μl with water and heated to 95°C for 10 min. PCRs were set up in a total volume of 50 μl containing 5 μl of the diluted cDNA, 200 mM deoxynucleoside triphosphates, 200 ng of each oligonucleotide primer, 50 mM KCl, 2 mM $MgCl_2$, and 2 U of *Taq* polymerase (Boehringer). Reactions were carried out at the following conditions: 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min for 30 cycles. The reaction products were then resolved on nondenaturing 8% polyacrylamide gels and visualized by staining with ethidium bromide. The PCR primers used were previously described (26); primers ex-2 (positions 5138 to 5157) and ex-4 (positions 7264 to 7245) yield a 267-bp PCR product representing the constitutively spliced message and a 167-bp PCR product which represents the message in which exon 3 is skipped.

UV cross-linking assays. HeLa nuclear extracts were prepared essentially as described previously (9). In vitro binding reactions were carried out with 7.5 μg of HeLa nuclear proteins or 2.5 μg of GST-ERev in a reaction volume of 24 μl containing 8.8 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.9), 8.8% glycerol, 60 mM KCl, 3.2 mM $MgCl_2$, 1.0 mM creatine phosphate, 0.37 mM dithiothreitol, 0.9% polyethylene glycol, and 0.2 mM EDTA. Total SR proteins were prepared from 10^{10} HeLa cells as described previously (52), and relative purity was assessed by Coomassie blue staining of proteins subjected to SDS-PAGE. Binding reactions with SR proteins were done as described above except that 50 ng of total SR proteins was incubated with test RNAs at 200 mM KCl. In vitro binding reactions containing 1 fmol of labeled RNA were done at 30°C for 15 min. The reaction mixtures were then transferred to Parafilm on ice and exposed to UV light (254 nm) at a distance of 4 cm for 10 min. Following treatment with 1 mg of RNase A per ml at 37°C for 15 min, the samples were resolved by SDS-PAGE on 10% gels. For UV cross-linking and immunoprecipitation assays, 300 μl of anti-SF2/ASF monoclonal antibody culture supernatant (a generous gift from A. Krainer, Cold Spring Harbor Laboratory) was prebound to 20 μl of protein G-Sepharose (Gammabind beads; Pharmacia) for 1 h at room temperature in IP (immunoprecipitation) buffer (10 mM $NaPO_4$ [pH 7.0], 150 mM NaCl, 10 mM EDTA). In vitro binding reactions were carried out as described above, using HeLa nuclear extracts, and incubated with prebound antibody in 40-μl reaction mixtures containing IP buffer at room temperature for 2 h. After four washes with IP buffer, bound proteins were removed from the beads by addition of 40 μl of SDS loading buffer and heating to 75°C for 4 min and were then resolved by SDS-PAGE on a 10% gel.

PCR primers. The PCR primers used were as follows: E3L1Py(+) (nt 5461 to 5490), 5′-CCTTCGTTCTCTCCAAGAAATGAACCTGAA-3′; E3L1Py(−) (nt 5443 to 5472), 5′-AGAGAACGA GGTTCGATTCTGCCATGCTG-3′; E3L2Py (+) (nt 5504 to 5542), 5′-CTTCTTTTTTCTT CTTTACTGTGGTGGAAAATA GGTATG-3′; E3L2Py(−) (nt 5486 to 5524), 5′-CAGTAAAGAAGAAAAAAG AAGTTTAGATTCTTCTTTCAG-3′; RSP, 5′-AACAGCTATGACCATG-3′; E5761 (nt 5761 to 5775), 5′-GCTTCTAATAATGTAGCA-3′; Rev(+), 5′-C GCGGATCCATGGCAGAATCGAAGGAAGC-3′; and Rev(−), 5′-CGG AATTCTCATAAATGTTTCCTCCTTCG-3′.

Synthesis of labeled RNA. Radiolabeled RNAs were synthesized by in vitro transcription, using T7 or T3 RNA polymerase (31), of plasmids linearized with *Hin*dIII. Nonspecific control RNA was synthesized by using pKSII+ (pKS) that was linearized with *Afl*III, yielding a 510-nt RNA.

RESULTS

RNA sequence and predicted secondary structure of EIAV exon 3. Previous studies revealed that the region containing EIAV exon 3 and its flanking intron sequences was necessary for both Rev-dependent exon skipping and viral mRNA transport (26). The nucleotide sequence of this region (EIAV nt 5400 to 5600) shows that exon 3 contains two distinct tracts composed almost entirely of purines (nt 5462 to 5473 and 5504 to 5542; Fig. 1A). A computer-assisted RNA folding analysis (8, 54) was done to gain insights into the secondary structure of the RNA (Fig. 1B). A striking feature of this predicted structure is that the two purine-rich regions form large single-stranded loops (loop 1 and loop 2). Loop 1 (EIAV nt 5462 to 5473) consists of 12 nt and contains 10 purines, and loop 2 (EIAV nt 5504 to 5524) contains a 21-nt purine-rich sequence interrupted only twice by pyrimidines. Analysis of larger EIAV fragments containing exon 3 (e.g., nt 5277 to 5834) retained the same secondary structure features shown in Fig. 1B (data not shown). While confirmation of this predicted secondary structure will require further experimentation, the purine tracts will be referred to here as loop 1 and loop 2.

Roles of exon elements on splicing in vivo. Both loop 1 and loop 2 (Fig. 1B) have repeated sequence elements (GAR) that resemble the general consensus sequence of purine-rich splicing enhancers, GARGARGAR (where R is any purine) (50). To determine whether these sequence elements function as

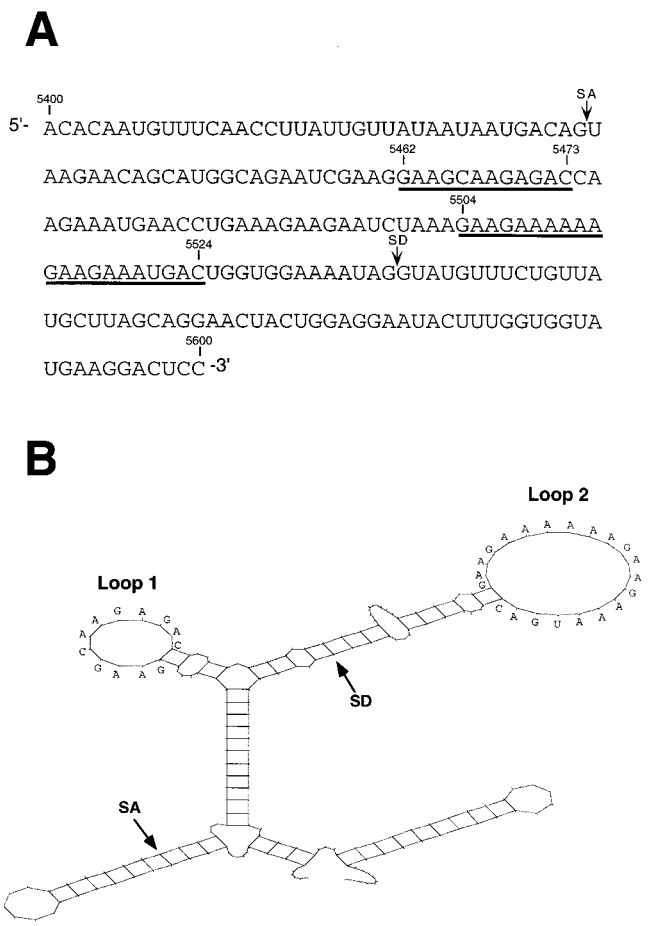

FIG. 1. Sequence and predicted secondary structure of EIAV exon 3 and flanking intron sequences. (A) Sequence of EIAV nt 5400 to 5600, spanning exon 3 and flanking intron sequences. Underlined sequences indicate purine-rich regions. Splice acceptor (SA) and splice donor (SD) sites are shown. (B) EIAV exon 3 and flanking intron sequences (nt 5400 to 5600) were subjected to a computer-predicted secondary structure determination according to the method of Zuker and Steigler (54) as modified by the Genetics Computer Group software package (8). The structure is adapted from the output of the Squiggles program. The calculated free energy of the structure is −29.1 kcal (ca. −121.8 kJ). The splice sites flanking exon 3 are indicated with arrows. Two large RNA loops composed almost entirely of purines (loop 1 and loop 2) are predicted to form.

ESEs, the purines in loop 1 and loop 2 were changed to pyrimidines and then substituted into the *rev*-minus EIAV provirus pFL8511 (26). The resulting constructs are shown in Fig. 2A. These plasmids were transfected into D17 cells in the absence or presence of a Rev expression plasmid, and 48 h posttransfection, total cellular RNA was harvested and subjected to RT-PCR analysis using primers designed to detect spliced messages (Fig. 2B).

The *rev*-minus provirus pFL8511 expressed only the four-exon spliced *tat*/*rev* mRNA containing exon 3 in transfected cells (Fig. 2B, lane 1). When Rev was supplied in *trans* (lane 2), levels of the four-exon mRNA decreased and the *tat* mRNA lacking exon 3 appeared, consistent with previous results (26). Substitution of pyrimidines for purines in loop 1 (pFL8511 EL1Py) had no effect on exon skipping (lanes 3 and 4) and exhibited the same splicing pattern as pFL8511. In contrast, substitution of pyrimidines for purines in the second purine tract (pFL8511 EL2Py) resulted in expression of the mRNA lacking exon 3 in the presence or absence of Rev (lanes 5 and 6). Mutation of exon 3 loop 2, then, results in exon skipping. Since increasing exon inclusion is a common hallmark of ESEs, this region by definition functions as an ESE because the purines in loop 2 are necessary for the recognition of exon 3 as a unit and its subsequent inclusion in the spliced message. Although these results are not quantitative, the amount of the skipped product observed from this provirus mutant in the absence of Rev was comparable to the amount of skipped product generated by the wild-type (wt) provirus in the presence of Rev (compare lanes 5 and 2), suggesting that the observed exon skipping in both instances occurs via a common underlying mechanism.

Several HeLa nuclear proteins specifically cross-link to EIAV ESE. To identify nuclear factors interacting with EIAV ESE sequences, radiolabeled ESE RNA (nt 5277 to 5834) and a nonspecific RNA derived from pKS were incubated with HeLa cell nuclear extracts under splicing conditions. The reaction mixtures were incubated in the presence of UV light to form covalent cross-links between the labeled RNA and proteins, treated with RNase A to degrade any unbound RNA, and analyzed by SDS-PAGE. Autoradiography revealed several HeLa nuclear proteins bound to ESE RNA with apparent molecular sizes of 30, 55, 75, and 90 kDa (Fig. 3, lane 1). The binding of the 30- and 55-kDa proteins was specific for the ESE RNA, since these proteins did not bind appreciably to the pKS RNA (compare lane 1 with lane 8). When radiolabeled ESE was incubated in reactions with 30-, 300-, and 3,000-fold molar excesses of unlabeled ESE RNA (lanes 2, 3, and 4, respectively), the binding of the 75- and 90-kDa proteins was diminished. In contrast, when reactions contained 30-, 300-, and 3,000-fold molar excesses of unlabeled pKS RNA (lanes 5, 6, and 7, respectively), binding of the 75- and 90-kDa proteins was not competed for. From these results, it can be concluded that the 30- and 55-kDa HeLa nuclear proteins specifically cross-link to the ESE RNA, while the 75- and 90-kDa proteins cross-link to the ESE RNA with lower specificity.

SR proteins specifically cross-link to EIAV ESE. Because the sizes of three of the proteins observed to specifically cross-link to EIAV ESE (p30, p55, and p75) are consistent with the sizes of three members of the family of SR proteins (SRp30a/b, SRp55, and SRp75 [36, 52]), we examined whether purified SR proteins could bind to the EIAV ESE. Total SR proteins were purified from HeLa cells according to published procedures (52) and used in cross-linking experiments with wt and mutated ESE RNAs (Fig. 4A). When wt ESE RNA was incubated with SR proteins (Fig. 4A, lane 1), four distinct cross-linked bands with various levels of intensity, corresponding to SRp20, SRp30a/b, SRp40, and SRp55, were detected; cross-linking of SRp75 was found to be very weak. This finding result suggested that the 30- and 55-kDa HeLa nuclear proteins detected cross-linking to ESE RNA in Fig. 3 were most likely SRp30a/b and SRp55. ESE RNA containing a substitution of pyrimidines for purines in loop 1 (ESEL1Py; lane 2) cross-linked the same set of SR proteins as did the wild-type ESE. Note that in both cases, the cross-linked band corresponding to SRp30a/b was the most intense. When ESEL2Py RNA (containing a substitution of pyrimidines for purines in loop 2) was used as a substrate, no detectable cross-linking of any SR proteins was evident (lane 3). Further, cross-linking to the nonspecific RNA derived from pKS (lane 4) was at low background levels, indicating sequence-specific binding of SR proteins to the EIAV ESE. Because the EL2Py mutation resulted in exon skipping in vivo (Fig. 2), this result strongly suggests a functional correlation between exon skipping in vivo and binding of SR proteins to the ESE in vitro.

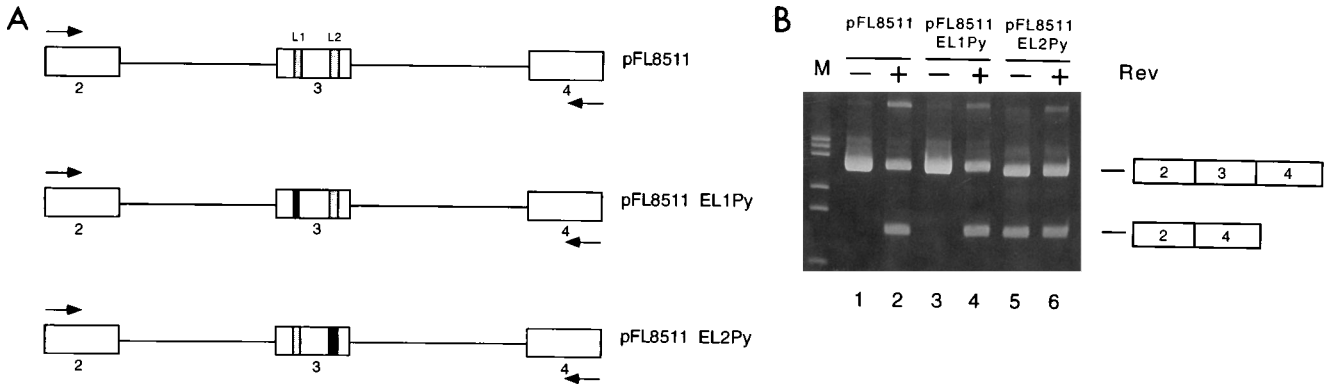

FIG. 2. A mutation in EIAV exon 3 results in Rev-independent exon skipping. (A) Schematic drawing of EIAV provirus constructs. EIAV exons 2, 3, and 4 (boxes) and introns 2 and 3 (lines) are shown, along with locations of primers used for RT-PCR (arrows). pFL8511 is a *rev*-minus provirus (26); stippled boxes indicate relative locations of exon 3 loops 1 and 2 (L1 and L2). Dark boxes indicate substitution of pyrimidines for purines in loop 1 (p8511 EL1Py) and in loop 2 (pFL8511 EL2Py). (B) RT-PCR analysis of exon skipping in EIAV provirus constructs. Cells were transfected with 3 μg of pFL8511 (lanes 1 and 2) or provirus mutants pFL8511 EL1Py (lanes 3 and 4) and pFL8511 EL2Py (lanes 5 and 6) in the absence (−) or presence (+) of 1 μg of pRS-ERev. Two days after transfection, total cellular RNA was prepared and converted to cDNA. PCR amplification of cDNA was done with primers to EIAV exon 2 and exon 4, which were designed to detect the skipped and constitutively spliced mRNAs (shown at the right). PCR products were resolved on an 8% polyacrylamide gel and visualized by ethidium bromide staining. Lane M, ϕX-*Hae*III DNA molecular weight markers.

Because SRp30a and SRp30b cannot be distinguished on the gel shown in Fig. 4A, we next performed a UV cross-linking and immunoprecipitation assay using a monoclonal antibody specific for SRp30a (anti-SF2/ASF; generously provided by Adrian Krainer, Cold Spring Harbor Laboratory). Figure 4B shows that this antibody immunoprecipitated a doublet of the expected size for UV cross-linked SRp30a when wt ESE was used as a binding substrate (lane 1). In contrast, no cross-linked adduct was immunoprecipitated when ESEL2Py RNA was used (lane 2). Competition experiments revealed that unlabeled ESE was able to efficiently compete for the binding of SRp30a, while ESEL2Py was not (lanes 3 and 4). These results indicate that SRp20, SRp30a, SRp40, and SRp55 are able to interact with the wt but not the mutated ESE.

GST-ERev specifically cross-links to exon 3 RNA. Previous studies revealed that the region containing EIAV exon 3 and its flanking intron sequences is necessary for viral mRNA transport (26), suggesting that EIAV Rev recognizes an RNA element within this region. We wished to test directly whether EIAV Rev could interact with exon 3 RNA in vitro. GST-ERev was purified from bacteria and used in cross-linking assays with wt and mutated ESE RNAs (Fig. 5). When GST-ERev was incubated with wt ESE or ESEL1Py RNA (lanes 1 and 2), a cross-linked band of approximately 46 kDa (corresponding to the size of the GST-ERev fusion protein) was observed. When ESEL2Py RNA or RNA derived from pKS was used (lanes 3 and 4), no detectable cross-linking of GST-ERev was evident. When wt ESE RNA was incubated with bacterially expressed GST protein (lane 5), the 46-kDa cross-linked band was not observed, indicating that cross-linking of GST-ERev to the ESE was not due to the GST portion of the fusion protein. Thus, GST-ERev binds to RNA within a region previously shown to contain the Rev-responsive element. Rev did not bind to a mutated RNA containing pyrimidines instead of purines in loop 2. Since this substitution also affects the predicted secondary structure adjacent to this loop (16a), the Rev binding site may overlap or flank the ESE. The proximity of SR protein and Rev binding sites suggests possible mechanisms for their effects on exon 3 splicing.

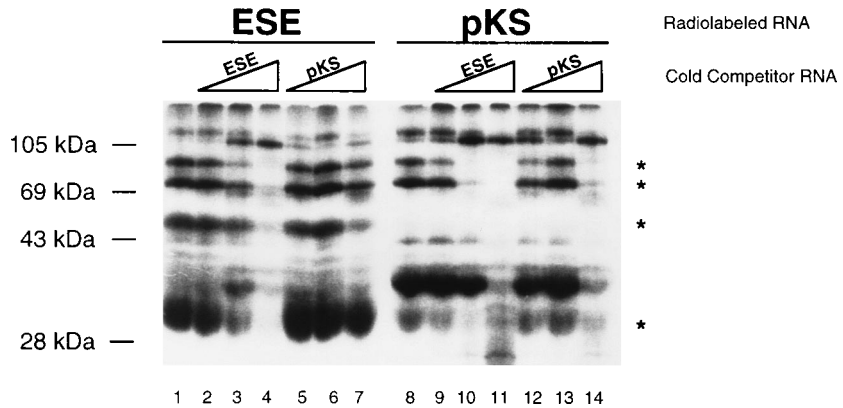

FIG. 3. Several HeLa nuclear proteins specifically cross-link to EIAV ESE. Radiolabeled ESE RNA (lanes 1 to 7) or nonspecific RNA derived from pKS (lanes 8 to 14) were incubated in HeLa cell nuclear extracts under splicing conditions. Lanes 2 to 4 and 9 to 11 contain a 30-, 300-, or 3,000-fold molar excess of unlabeled ESE RNA, and lanes 5 to 7 and 12 to 14 contain a 30-, 300-, or 3,000-fold molar excess of unlabeled pKS RNA. The reactions were incubated in the presence of UV light to form covalent cross-links between the labeled RNA and proteins, treated with RNase A, and fractionated by SDS-PAGE on a 10% gel. The positions of the molecular weight markers are shown at the left. Asterisks indicate nuclear proteins specifically cross-linking to EIAV ESE element.

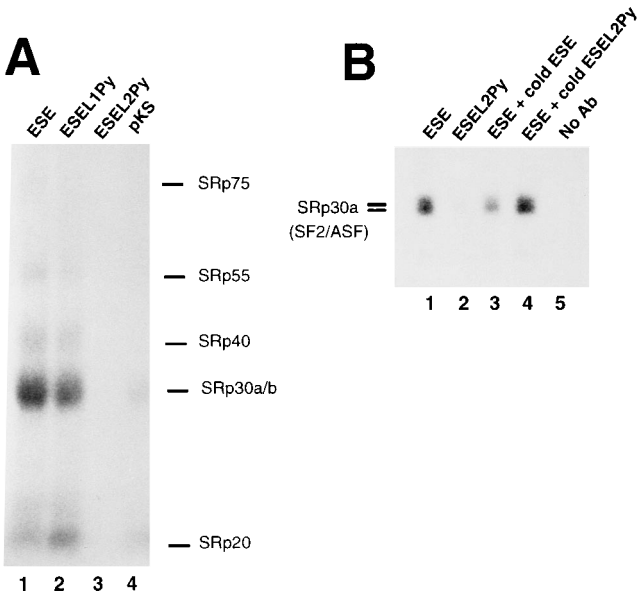

FIG. 4. SR proteins specifically bind to EIAV ESE sequences. (A) Radiolabeled RNAs corresponding to wt ESE (lane 1), ESEL1Py (lane 2), ESEL2Py (lane 3), and pKS (lane 4) were incubated with total HeLa SR proteins and subjected to UV cross-linking as described in the legend to Fig. 3. The positions of the SR proteins are indicated at the right. (B) Sequence-specific binding of SRp30a (SF2/ASF) to the EIAV ESE. UV cross-linking and immunoprecipitation reactions using anti-SF2/ASF were performed with wt ESE alone (lane 1), ESEL2Py alone (lane 2), or wt ESE and the indicated competitor RNA (lanes 3 and 4). Lane 5 represents a mock immunoprecipitation without antibody (Ab).

DISCUSSION

We have identified a purine-rich ESE element within exon 3 of EIAV that binds members of the SR family of splicing factors. A mutation in this splicing enhancer results in exon skipping in vivo and no longer binds SR proteins in vitro, strongly suggesting an important role for SR protein-ESE interactions in the control of EIAV splicing. Furthermore, EIAV Rev binds to exon 3 sequences at or near the ESE in vitro. The EL2Py substitution mutation abolished Rev binding in vitro (Fig. 5) and Rev-mediated RNA transport in vivo (16a). These data suggest mechanisms for Rev-mediated exon skipping depicted in Fig. 6. In the absence of Rev (Fig. 6A), SR proteins can bind to the ESE and promote or stabilize spliceosomal complex formation on exon 3, resulting in exon inclusion. If SR proteins are displaced by Rev (Fig. 6B), the exon is not recognized and is skipped. The amount of the skipped mRNA product observed from the proviral mutant containing a mutation in the ESE was comparable to the amount of skipped product generated by the wt provirus in the presence of Rev (Fig. 2; compare lanes 5 and 2), suggesting that mutation of the ESE has the same overall effect on alternative splicing as supplying Rev in *trans*. These results and the proximity of binding sites for Rev and SR proteins suggest that Rev may displace SR proteins from the ESE. This could result from either steric competition or perturbations of RNA secondary structure. It is possible that binding of Rev and binding of SR proteins are not mutually exclusive, in which case Rev might interfere with the subsequent protein-protein interactions required for spliceosome assembly (Fig. 6C). Interestingly, mutations in the effector domain of EIAV Rev that abolish RNA transport did not impair Rev-dependent exon skipping (16b). Thus, if Rev interacts directly with SR proteins, it does not do so via its effector domain.

Other laboratories have shown binding of SR proteins to purine-rich ESEs (23, 35, 45, 47). For example, Lavigueur et al. (23) used RNA gel mobility shift experiments to show that SR proteins interact specifically with an ESE in the human fibronectin ED1 exon. In addition to showing that the ED1 ESE could stimulate the splicing of a heterologous pre-mRNA in vitro, these studies revealed that the mechanism of action of the ED1 ESE appeared to be at the level of early splicing complex assembly, allowing a more efficient interaction of the U2 snRNP with the branch point. As for the EIAV ESE, binding of SR proteins to the ED1 ESE appeared to be a functional requirement of enhancer activity. Sun et al. (45) report the binding of SF2/ASF (SRp30a) to an ESE located in the last exon of bovine growth hormone pre-mRNA; while binding of SF2/ASF to the ESE was required for the stimulation of splicing in vitro of the bovine growth hormone intron, the addition of hetereogeneous ribonucleoprotein A1 counteracted these effects. A splicing enhancer in cardiac troponin T pre-mRNA that is necessary for exon inclusion was recently shown to bind a subset of SR proteins (35). Further, a series of mutations in the cardiac troponin ESE was used to correlate enhancer strength in vivo with SR protein binding in vitro. Because cross-linking efficiency does not necessarily reflect protein binding affinity, it is difficult to assess the relative binding affinities of the different SR proteins for the EIAV ESE on the basis of signal intensities in Fig. 4A. We are currently attempting to test binding of purified, individual SR proteins to the ESE. Thus, it is possible that while some ESEs are promiscuous with respect to SR protein binding, others may bind distinct subsets or individual members of the SR family of splicing factors. This may represent another level of tuning of the constitutive splicing machinery in the alternative splicing of complex pre-mRNAs.

SR proteins have been shown to enhance interactions between the U2 snRNP and branch point sequences (23), interactions between U1 and U2 snRNPs at the 3′ splice site (13), and interactions between the U1 snRNP and the 5′ splice site (10, 21, 40). These activities may account for the ability of SR proteins, in particular SF2/ASF (SRp30a), to promote exon inclusion (4, 27). Staknis and Reed (40) propose that splicing enhancers function by promoting the assembly of an enhancer complex on pre-mRNAs containing weak splicing signals. It seems likely that such an enhancer complex is promoted by the EIAV ESE and that Rev may somehow perturb this complex. The proximity of the exon 3 splice donor, the Rev-responsive element, and the ESE suggests effects at or near the 5′ splice site. In support of this view, it was previously observed that a deletion of the exon 3 splice acceptor displayed wt Rev responsiveness (26). Further characterization of these splicing signals in EIAV may help to define the exact mechanism of action of

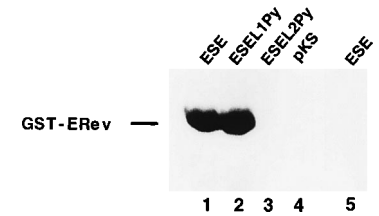

FIG. 5. GST-ERev specifically binds to exon 3 RNA. Radiolabeled RNAs corresponding to wt ESE (lane 1), ESEL1Py (lane 2), ESEL2Py (lane 3), and pKS (lane 4) were incubated with bacterially expressed GST-ERev and subjected to UV cross-linking as described in the legend to Fig. 3. Lane 5 shows ESE RNA incubated with GST protein. The band corresponding to GST-ERev (~46 kDa) is indicated.

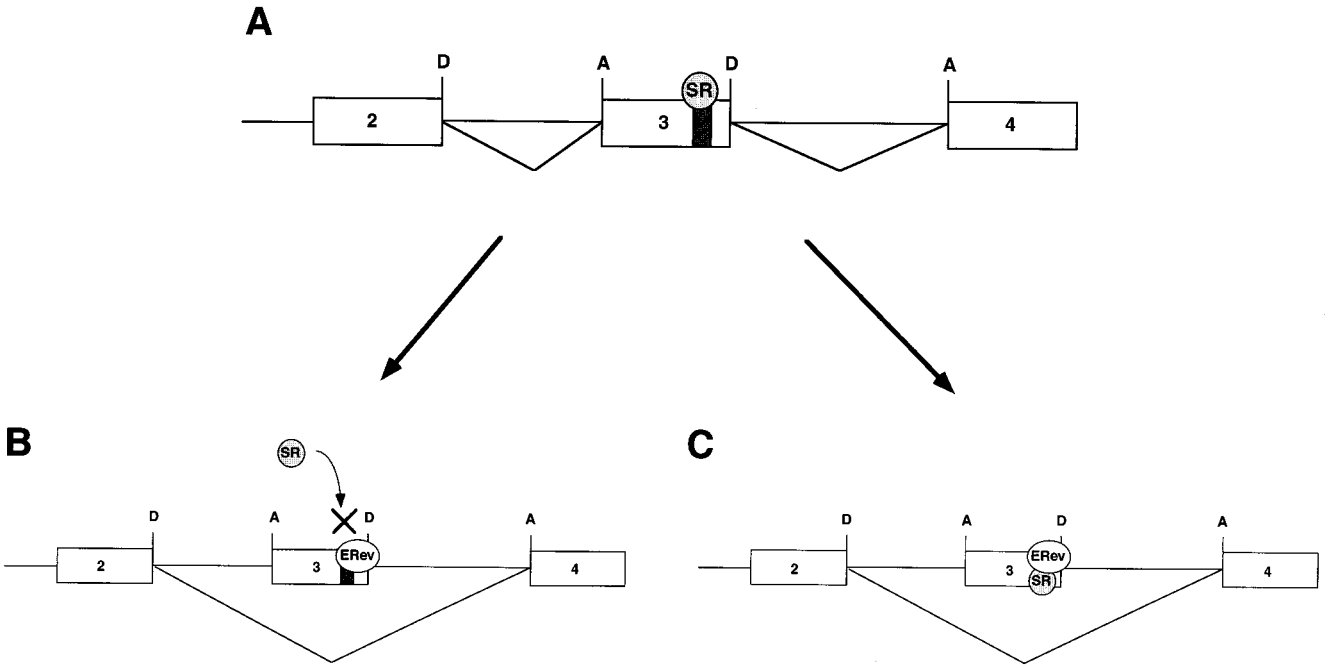

FIG. 6. Model of EIAV Rev-dependent exon skipping mediated by ESE. (A) In the absence of Rev, SR proteins can recognize and bind to the ESE, promoting recognition and inclusion of exon 3. (B) In this scenario, Rev binding and SR protein binding are mutually exclusive. When Rev is present, it binds at a site overlapping or adjacent to the site of SR protein binding; the SR proteins are then displaced so that they cannot bind, resulting in exon skipping. (C) Here, Rev binding and SR protein binding are not mutually exclusive; binding of Rev interferes with SR protein function by preventing the subsequent protein-protein and protein-snRNP interactions required for spliceosome assembly.

the ESE and the role of Rev. In particular, it will be of interest to test whether the EIAV ESE can confer Rev-dependent exon skipping upon heterologous alternatively spliced pre-mRNAs that contain suboptimal splicing signals and, more importantly, to test whether EIAV Rev can affect the splicing pattern of alternatively spliced cellular pre-mRNAs that may contain ESEs.

The identification of an ESE in EIAV extends the repertoire of retroviral *cis*-acting elements that modulate splicing efficiency. Other laboratories have described exonic elements in HIV-1 that negatively regulate splicing; analogous to ESEs, these sequences are called exon splicing silencers (1, 2, 39). Interestingly, one of these exon splicing silencer sequences in HIV-1 exon 3 is juxtaposed to a purine-rich ESE, forming a bipartite splicing regulatory element that may control the overall efficiency of the *tat/rev* 3′ splice site (2, 39). In the case of HIV-1, these elements appear to be necessary to augment function of suboptimal splice site sequences (38). Thus, while negative regulatory elements and suboptimal splicing signals in retroviruses help to maintain an appropriate balance of unspliced RNA that is necessary for viral replication, other elements (ESEs) are required to assure that constitutive splicing does take place.

While it is clear that HIV-1 Rev is essential for transport of intron-containing mRNAs, additional effects on splicing have been reported (6, 19, 20, 24). The identification of ESE elements in HIV-1 (2, 39) suggests that SR proteins may be involved in regulating alternative splicing in this system. Because the results presented in this report suggest interactions among components of the splicing machinery, ESE signals, and EIAV Rev, it is likely that more subtle interactions between SR proteins and other members of the Rev/Rex family exist. The EIAV system may help us to better understand the functions of these viral transactivators, as well as the host factors with which they interact. This information will be useful in understanding the general principles that underlie gene regulation of individual viruses.

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