The equine infectious anemia virus (EIAV) Rev protein (ERev) negatively regulates its own synthesis by inducing alternative splicing of its mRNA. This bicistronic mRNA contains four exons; exons 1 and 2 encode Tat, and exons 3 and 4 encode Rev. When Rev is expressed, exon 3 is skipped to produce an mRNA that contains only exons 1, 2, and 4. The interaction of ERev with its cis-acting RNA response element, the RRE, is also essential for nuclear export of intron-containing viral mRNAs that encode structural and enzymatic gene products. The primary ERev binding site and the manner in which ERev interacts with RNA or cellular proteins to exert its regulatory function have not been defined. We have performed in vitro RNA binding experiments to show that recombinant ERev binds to a 55-nucleotide, purine-rich tract proximal to the 5′ splice site of exon 3. Because of its proximity to the 5′ splice site and since it contains elements related to consensus exonic splicing enhancer sequences, we asked whether cellular proteins recognize the EIAV RRE. The cellular protein, ASF/SF2, a member of the serine- and arginine-rich family of splicing factors (SR proteins) bound to repeated sequences within the 55-nucleotide RRE region. Electrophoretic mobility shift and UV cross-linking experiments indicated that ERev and SR proteins bind simultaneously to the RRE. Furthermore, in vitro protein-protein interaction studies revealed an association between ERev and SR proteins. These data suggest that EIAV Rev-induced exon skipping observed in vivo may be initiated by simultaneous binding of Rev and SR proteins to the RRE that alter the subsequent assembly or catalytic activity of the spliceosomal complex.

The Rev and Rex proteins encoded by lentiviruses and other complex retroviruses mediate the nucleocytoplasmic transport of intron-containing, viral pre-mRNAs that would otherwise be retained in the nucleus (1–4). In addition to their roles in RNA transport, some members of this protein family have been shown to affect pre-mRNA splicing (5–8). Our current understanding of Rev function and mechanism derives largely from the HIV type 1 (HIV-1) system; Rex proteins encoded by the human T-cell leukemia virus group of retroviruses (HTLV-I, HTLV-II, and bovine leukemia virus) have been extensively studied as well (9–16). These and other retroviruses share conserved mechanisms to regulate RNA transport, but the sequences and structures of viral cis- and trans-acting components display considerable differences. Rev and Rex proteins contain a nuclear localization signal and a leucine-rich nuclear export signal, as well as an arginine-rich RNA binding domain, that together allow the proteins to shuttle between the nucleus and cytoplasm and tether viral pre-mRNAs to a cellular protein-transport pathway (11, 17–22). The specificity of RNA recognition by the proteins is determined, in part, by cis-acting RNA sequences called the Rev- or Rex-responsive elements (RRE/RxRE) (23). Even though HIV-1 RRE and HTLV-1 RxRE do not show significant sequence homology, both form large, stable secondary structures (24). The HIV-1 RRE is a complex 234-nucleotide stem-loop structure located in the envelope gene, which contains a 30-nucleotide high affinity binding site for Rev (25, 26). A 255-nucleotide stem-loop structure in the 3′ long terminal repeat of HTLV-1 functions as the RxRE and contains a 33-nucleotide element, which represents the primary binding site for Rex (24). In these viruses and others, there are secondary, lower affinity Rev/Rex binding sites that cooperate with the primary binding elements to effect Rev/Rex response (25, 27, 28).

Equine infectious anemia virus (EIAV) is a lentivirus distantly related to HIV-1. As in other Rev/Rex systems, EIAV Rev (ERev) was shown to mediate the nuclear export of unspliced viral RNAs and to be essential for virus structural protein synthesis (20). Our previous studies showed that, in addition to regulating viral mRNA transport, ERev induced alternative splicing of EIAV mRNA in vivo (6, 8). The ERev protein has little sequence similarity with other Rev or Rex proteins and does not recognize heterologous response elements (20). The EIAV RRE is also distinct in that computer-assisted RNA folding does not predict the formation of a large, stable secondary structure (6). Previous results indicated that the EIAV RRE was located in an exon near the 5′ end of the env gene (8, 29), but the primary ERev binding site and the manner in which ERev interacts with RNA have not yet been defined.

In addition to the viral trans-regulatory proteins, a variety of cellular proteins bind to retrovirus RNA sequences and thereby control RNA processing and transport. These protein-RNA associations can also influence the outcome of Rev-RRE interactions. For example, cellular protein interactions with cis-acting repressor elements and 5′ splice sites (11, 30, 31) act to retain pre-mRNAs in the nucleus, which can be circumvented by Rev (32–34). Furthermore, cellular protein interactions with splice sites, exonic splicing enhancer (ESE) and exonic splicing silencer elements affect the efficiency and rate of viral pre-mRNA splicing (33, 35–37). Cellular proteins have been identified that bind directly to RRE of HIV-1 and RxRE of...
HTLV-II (32). A purine-rich ESE was previously identified in EIAV, which binds members of the SR-family of splicing factors (6). In this report, we show that a 55-nucleotide region at the 3’ end of exon 3 contains both an ESE, which binds ASF/SF2, and an RRE, which binds ERev. ERev and ASF/SF2 interactions with this composite RNA element were not mutually exclusive, but rather cell and virus proteins appeared to bind simultaneously.

EXPERIMENTAL PROCEDURES

**Plasmids**—The plasmids, pEX312 and pEX322, were constructed by PCR amplification of EIAV exon 3 sequences and contain all of exon 3 (positions 5435–5541) or the 3’ half of exon 3 (positions 5487–5541), respectively. PCR primers used to amplify the exon 3 and 3’ half of exon 3 (‘Ex3’) fragments were: exon 3, 5’ primer (5’-CCGAGCTCCTGAA-AAGACAGCTGAGGAGCAG-3’), 3’ Ex3, 5’ primer (5’-CCCGGAGCTCCTGAAA-GAAGAATCCAAAGA-3’), and exon 3, 3’ primer (5’-GGGGGATC- CACATACCTTCTCACC-3’). The PCR fragments were cloned into ScaI and BamHI sites of pBluescript KS II (+). The plasmids, pEXB1, pEXL21, pEXL22, pEXS11, and pEXS12, which contain mutated versions of the 3’ half of exon 3, were constructed by PCR and cloned into ScaI and BamHI site of pBluescript KS II (+). In vitro transcription of plasmids with T7 RNA polymerase yields RNAs of 139 and 88 base-pairs for exon 3 and 3’Ex3, respectively; both RNAs contain 33 nucleotides derived from plasmid sequences. All plasmids were confirmed by DNA sequence analysis.

**Bacterial Expression and Purification of ERev**—ERev coding sequences were PCR amplified from pEEx-Rev (8) with oligonucleotide primers. The PCR product was cloned as an NcoI to Smal fragment into pT7B4 (New England Biolabs) to yield pERev-Intein. Escherichia coli strain ER2566, containing pERev-Intein, was induced with isopropyl-pTYB4 (New England Biolabs) to yield pERev-Intein.

**Translation of EIAV provirus and the**

**RESULTS**

**Rev Binds to the 3’ Half of Exon 3 RNA in Vitro**—The genetic organization and splicing pattern of the EIAV provirus and the exons utilized to produce the bicistronic tat/rev mRNA are shown in Fig. 1 (A and B). Previous results suggested that sequences in exon 3 (Fig. 1C) were necessary for both Rev-dependent viral mRNA transport and alternative splicing in vivo (6, 8). With respect to the latter, Rev was shown to induce a shift from the multiply spliced, tat/rev mRNA to the synthesis and accumulation of the singly spliced env mRNA and to RNAs in which exon 3 was skipped. In order to identify the minimal sequences within exon 3 that are required for ERev binding, 32P-labeled RNA containing part of exon 3 was incubated with recombinant ERev protein and the resulting complexes were examined by EMSA. The 58-nucleotide 3’ half of exon 3 RNA (‘Ex3’), which was purified from a single band by denaturing gel electrophoresis, migrated as two bands under native conditions, designated as RNA I and RNA II (Fig. 2A, lane 1). That these represent different structural isoforms was shown in denaturation/renaturation experiments described below. Addition of recombinant ERev protein to the RNA resulted in the appearance of a protein-RNA complex, which migrated above the RNA II band (Fig. 3A, lanes 2–4). As the amount of ERev was increased, there was an increase in the intensity of the ERev-RNA complex accompanied by a reciprocal decrease in the RNA II band. At the highest concentration of ERev, RNA II was quantitatively shifted into complexes with ERev. Quantitation of the relative amounts of the RNAs and protein-RNA complex by phosphorimaging analysis (Fig. 2B) revealed that complex formation occurred at the expense of RNA II with no detectable change in the amounts of RNA I. These data suggested that ERev binds to a unique structural element in form II RNA. Two different antisera, raised against synthetic peptides representing ERV (a-Rev1) and anti-Rev2), abolished ERev/RNA complex formation (Fig. 2A, lane 5 and 6) but pre-immunization Sephadex G-200 supershifted Rev/RNA complex was probably due to interference with the ability of ERev to bind RNA.

**Exon 3 RNA Forms a Dimer-like Structure in Vitro**—The fact that 3’Ex3 RNA migrated as two forms was unexpected since the 3’Ex3 RNA was not predicted to form a stable, base-paired DNA sequence analysis.

**In Vitro Renaturation of Exon 3 RNA**—32P-labeled Exon 3 and 3’Ex3 RNA (1 x 10^6 cpm) were heated for 2 min at 95 °C and gradually cooled to 15 °C in the cold room for 1 h in binding buffer condition (8.8 mM Hepes, pH 7.9, 3.2 mM MgCl2, 0.2 mM dithiothreitol) containing varied concentrations of KCl. The renaturation products were analyzed by electrophoresis on native 8% polyacrylamide gel at 4 °C overnight at 5 Vcm. **In Vitro Protein-Protein Interaction Assays**—Chelating Sepharose high performance beads (Amersham Pharmacia Biotech) were charged with Ni2+ and equilibrated in binding buffer containing: 157 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4, 1.4 mM KH2PO4, 10 mM EDTA, 0.05% Triton X-100, and 10 mM imidazole. For each binding reaction, 40 µl of a 50% slurry (v/v) of beads was used. ERev-coated beads were prepared by incubating beads with 1 µg of purified His-ERev in 250 µl of binding buffer for 1 h at 4 °C and then washed four times in 500 µl of binding buffer. ERev-coated or uncoated beads were incubated with 8 µg of purified HeLa SR proteins in 500 µl of binding buffer at 4 °C for 3 h and then washed four times with 500 µl of binding buffer. Bound proteins were released from beads in SDS-sample loading buffer, and protein complexes were separated by SDS-PAGE, transferred to the Immobilon-P membranes (Millipore), and reacted with anti-ASF/SF2 monoclonal antibody. Band intensities, visualized by horseradish peroxidase-linked secondary antibody and enhanced chemiluminescence detection reagents (New England Biolabs).

**RESULTS**

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structure. Heat denaturation and rapid cooling of the 3′Ex3 RNA prior to binding reactions and native gel electrophoresis converted the slower migrating RNA II to the RNA I form (Fig. 3A, lanes 1 and 2). ERev did not form a complex with heat-denatured RNA nor did it induce a conformational change in RNA I (lane 3), but, as before, it did bind to RNA II (lane 4). In contrast to ERev binding, HeLa SR proteins bound to both forms of RNA and bound to heat-denatured 3′Ex3 RNA (Fig. 3B, see below). Thus, the ERev binding site is located in a 55-nucleotide region of exon 3 proximal to the 5′ splice site and ERev recognized an unusual structure in that region.

We next asked whether the form II RNA resulted from intramolecular or intermolecular RNA interactions. A 139-nucleotide exon 3 RNA and the 88-nucleotide 3′Ex3 RNA were heat-denatured, mixed together, and slowly renatured in buffer containing varied KCl concentrations. The size difference between exon 3 and 3′Ex3 RNAs allowed each species to be distinguished on non-denaturing polyacrylamide gels (Fig. 3C, lane 4).
Fig. 3. Exon 3 RNAs form a dimer-like structure which binds ERev. A, heat denaturation of 3′Ex3 RNA converts RNA II to RNA I. 32P-Labeled 3′Ex3 RNA probe (3.2 fmol) was denatured by heating at 95 °C then quick-chilled on ice. Denatured (D) or non-denatured (N) RNAs were incubated in the absence of ERev (lanes 1 and 2) or in the presence of 1.4 nM ERev (lanes 3 and 4), and complexes were resolved by non-denaturing polyacrylamide gel electrophoresis. B, native (N) and heat-denatured (D) RNAs were incubated in the absence of SR proteins (lanes 1 and 3) or in the presence of 3.8 μg of SR proteins (lanes 2 and 4). RNA probe preparation and electrophoresis were carried out as in panel A. C, 32P-labeled exon 3 RNA (139 nucleotides; 2 × 10⁴ cpm) and 3′Ex3 RNA (88 nucleotides; 2 × 10⁴ cpm) were denatured by heating at 95 °C, mixed together, and slowly cooled to 4 °C in buffers containing KCl at 60 mM (lane 1), 160 mM (lane 2), 260 mM (lane 3), and 360 mM (lane 4). Non-denatured 3′Ex3 and exon 3 RNAs are shown in lanes 5 and 6, respectively. The reaction mixtures were subjected to electrophoresis on 8% non-denaturing polyacrylamide gel. RNA bands corresponding to 3′Ex3 and exon 3 RNA I and RNA II are designated as RNA Iₘₚ, RNA Iₘₚ, RNA Iₜₚ, and RNA IIₜₚ, respectively. A dimer-like structure of 3′Ex3 and exon 3 RNAs is designated as RNA IIₜₘₚ. The molar ratio of 3′Ex3 RNA to exon 3 RNA was 2:1.

lanes 5 and 6). Conversion of denatured form I RNA to form II RNA was enhanced with increasing concentrations of KCl in the renaturation buffer (lanes 1–4). In addition to the expected bands corresponding to each form II RNA, a new band appeared that migrated at a position intermediate between exon 3 form II RNA and 3′Ex3 form II RNA. These data were consistent with an intermolecular association between the 139-nucleotide exon 3 RNA and the 88-nucleotide 3′Ex3 RNA. In this reaction mixture, 3′Ex3 RNA was present at a 2-fold excess over exon 3 RNA. Therefore, the exon 3 RNA II band was fainter than the 3′ Ex3 RNA II band, since most of the exon 3 form II RNA was in the “heterodimer-like” form.

The ability of the exon 3 RNAs to form a slower migrating dimer-like RNA species that was bound by ERev was perplexing, since computer analysis failed to predict stable inter- or intramolecular structures based on Watson-Crick base pairing. In order to define nucleotides essential for the RNA-RNA interaction, we generated mutated transcripts with base substitutions within 3′Ex3 RNA (Fig. 4B). 32P-Labeled RNAs were incubated in the presence or in the absence of recombinant ERev protein, and the reaction complexes were examined by EMSA (Fig. 4A). In L21 and L22 RNAs, two adenine to uridine substitutions within a 20-nucleotide-long purine tract prevented interactions necessary to generate form II RNAs (Fig. 4A, lanes 5–8). Substitution of the AAAUU sequence with UUUUA near the 3′ end of S12 RNA also abolished formation of RNA II (Fig. 4A, lanes 11 and 12). In contrast, the substitutions in B1 and S11 RNAs had no effect on RNA structure. Mutations that abolished RNA II formation also prevented Rev-RNA complex formation. Rev binding to B1 and S11 RNAs appeared to be weaker compared with wild type RNA (Fig. 4A, lanes 3 and 4 and lanes 9 and 10). pKS RNA also migrated as two bands, but ERev protein did not affect migration of either (lanes 13 and 14). Taken together, the data indicated that ERev specifically recognized a unique structure in exon 3 RNA, which appeared to be mediated by purine-purine interactions.

Binding of Cellular Proteins to Sequences in the 3′ Half of Exon 3 RNA—The presence of ESE elements within exon 3 and their proximity to the 5′ splice site (6) suggested that cellular proteins may bind to RNA sequences juxtaposed with the Rev binding site. Three tandem purine-rich repeats, denoted as A repeats and composed of the sequence AAAGAAGAA, were identified in the 3′ half of exon 3 (Fig. 5A). Binding of purified HeLa SR proteins to RNAs containing individual or composite repeat elements was examined in electrophoretic mobility shift assays. The 3′Ex3 RNA, which contains three copies of the A repeat (Fig. 5A), was shifted into a very slow migrating complex by purified HeLa SR proteins (Fig. 5B, lane 2). Factors in the SR protein preparation bound to both the fast (RNA I) and slow (RNA II) migrating forms of the 3′Ex3 RNA, since both forms were shifted into a protein-RNA complex (Fig. 5B). Furthermore, SR proteins shifted heat-denatured form I RNA (Fig. 5B). This contrasts with the binding of ERev, which bound exclusively to the slower migrating form of 3′Ex3 RNA. The oligoribonucleotide probe L2 is shorter by 9 bases from the 5′ end and 10 bases from the 3′ end compared with 3′Ex3 and contains two A repeats (Fig. 5A). Unlike the 3′Ex3 RNA, which migrated as two bands on non-denaturing gels, the L2 RNA migrated as a single species, implying that the terminal bases in 3′Ex3 RNA contribute to the formation of higher order structures (Fig. 5B, lanes 1–3). Proteins in the purified HeLa SR protein sample shifted the L2 RNA probe, yielding a protein-RNA complex, which migrated faster than the 3′Ex3 RNA-protein complex (Fig. 5B, lanes 3 and 4). The oligoribonucleotide probe A (Fig. 5A), which contains a single copy of the A repeat, formed complexes that migrated faster than those formed on the longer RNA probes (Fig. 5B, lanes 5 and 6). To delineate the specific sequence elements in the 3′Ex3 region required for SR protein
interactions, we examined the relative binding activities of SR proteins to the L2 RNA probe by UV cross-linking in the presence of various cold competitor RNAs (Fig. 5A). Protein and RNA mixtures were irradiated with UV light, digested with RNase A, resolved by SDS-gel electrophoresis, and analyzed by phosphorimage analysis. L2 RNA was cross-linked to two proteins in the SR protein preparation of ∼35 and 50 kDa (Fig. 5C, lane 2). The 50-kDa product is a nonspecific RNA-binding protein that cross-links to a wide variety of RNAs (39). Addition of cold homologous L2 RNA competitor reduced formation of the 35-kDa cross-linked product by 50% when added at 10-fold excess relative to the L2 RNA probe (Fig. 5C, lane 4). The unlabeled A RNA, which contains a consensus site for ASF/SF2, reduced complex formation on the L2 probe by 60% (Fig. 5C, lane 7). The other competitors, which contain consensus binding sites for SC35 and SRp40 proteins or the KS negative control sequence failed to compete with L2 for binding to the 35-kDa polypeptide. Thus, the A repeat containing the ASF/SF2 consensus binding element appeared to be the minimal sequence in exon 3 recognized by the 35-kDa protein.

In order to determine whether the 35-kDa polypeptide was ASF/SF2, as previous data suggested (6), purified HeLa SR proteins were mixed with 5′-end-labeled A- or KS RNAs and subjected to UV cross-linking, followed by immunoprecipitation with an anti-ASF/SF2 monoclonal antibody and SDS-gel electrophoresis (Fig. 5D). A UV cross-linked protein product of 35 kDa was detected with the A RNA probe (lane 1) but not with the KS RNA probe (lane 2). In summary, EMSA, UV cross-linking competition, and immunoprecipitation experiments indicated that the SR protein, ASF/SF2, binds to repeated RNA elements proximal to the 5′ splice site of exon 3. Although ERev and SR protein binding sites appeared to overlap, we believe that they are not identical. Therefore, an important question is whether both viral and cellular proteins can bind simultaneously to this 55-nucleotide purine-rich region.

Rev and SR Protein Interactions on the 3′ Exon 3 RNA—Having shown that ERev and SR proteins bind independently to cis-acting RNA elements located in the same region of exon 3, we next asked whether the viral and cellular proteins bind RNA competitively or simultaneously. Purified SR proteins and 32P-labeled 3′Ex3 RNA were cross-linked by UV irradiation in the presence of increasing amounts of ERev protein. After digestion with RNase A, the complexes were fractionated by SDS-PAGE and visualized by autoradiography. ERev did not interfere with ASF/SF2 cross-linking to 3′Ex3 RNA in this assay, but rather, caused a modest but reproducible increase in ASF/SF2 binding (Fig. 6A, lanes 3–5). ASF/SF2 was not cross-linked to the control KS RNA (Fig. 6A, lane 1).

We next performed EMSA experiments with labeled 3′Ex3 RNA and SR proteins in combination with either wild type or mutated ERev proteins. The ERev mutant, M27, has a 4-amino acid alanine substitution (ERLE93–96AAAA) and was shown to be defective for viral RNA transport and alternative splicing in vivo (20). The mutation in ERev-M27 disrupts a predicted α-helical region and was previously suggested to be defective for RNA binding. This prediction was confirmed by the observation that recombinant ERev-M27 protein, in contrast to wild type ERev, failed to bind 3′Ex3 RNA in vitro (Fig. 6B, compare lanes 2 and 3). Addition of 100 ng of purified HeLa SR proteins to the binding reaction yielded diffuse complexes that migrated near the position of RNA II and higher (Fig. 6B, lane 4). Addition of 100 ng of purified HeLa SR proteins in combination with 1 μg of ERev resulted in the formation of a new complex, which migrated more slowly than the ERev-RNA complex (Fig. 6B, lane 5; designated as Complex) and which was absent in reactions containing SR proteins and ERev-M27 (lane 6). These data indicated that ERev bound to RNA in association with factors present in the SR protein fraction and that complex formation was dependent on the ability of ERev to bind RNA. Although EMSA and UV cross-linking studies indicated that ASF/SF2 and ERev bind to 3′Ex3 RNA, we cannot rule out the possible involvement of additional proteins in formation of the novel complex. We are currently in the process of identifying the cellular proteins that bind exon 3 RNA in association with ERev.

We next asked whether there was an interaction between...
recombinant ERev and SR proteins in the absence of exon 3 RNA with an affinity pull-down assay. Purified HeLa SR proteins were incubated with Ni²⁺-chelate Sepharose beads in the absence or presence of His-tagged ERev protein. After incubation, the beads were washed extensively and proteins that were bound to the affinity matrix were fractionated by SDS-PAGE electrophoresis and visualized by Western blotting using anti-ASF/SF2 monoclonal antibody and SDS-polyacrylamide gel electrophoresis. KS is the nonspecific RNA control. The positions of the molecular size markers are shown at the left.

FIG. 5. Cellular SR proteins bind to sequence elements in the 3' half of exon 3 RNA. A, sequences of RNA probes and competitors. The purine-rich repeat elements designated as A are indicated. B, EMSA analysis was performed with [³²P]-labeled 3' Ex3 RNA and [³²P]-end-labeled synthetic oligoribonucleotides designated L2 and A in the absence (lanes 1, 3, and 5) or presence (lanes 2, 4, 6) of 3.8 μg of purified HeLa SR proteins. The [³²P]-end-labeled oligoribonucleotide probes migrated as one band identified as RNA oligo at the bottom of the gel. The RNA-SR protein and oligoRNA-SR protein complexes are indicated as RNA/SR or RNA oligo/SR. C, UV cross-linking competition binding assay of SR proteins to the [³²P]GTP-labeled L2 oligoribonucleotide with unlabeled oligoribonucleotides. Binding reactions were performed as in panel B except that the indicated unlabeled oligoribonucleotides were present at 5-fold (lanes 3, 6, 9, 12, and 15) or 50-fold (lanes 4, 7, 10, 15, and 16) molar excess compared with labeled L2 oligoribonucleotide. Reactions were UV-irradiated and protein resolved by SDS-PAGE and analyzed on phosphorimager. D, UV cross-linking of HeLa SR proteins to 5' end-labeled A or KS RNA probes followed by immunoprecipitation with anti-ASF/SF2 monoclonal antibody and SDS-polyacrylamide gel electrophoresis. KS is the nonspecific RNA control. The positions of the molecular size markers are shown at the left.

DISCUSSION

In this report we have identified the binding site for EIAV Rev and show that it is located in a 55-nucleotide region proximal to the 5' splice site of exon 3 RNA. We also show that elements within this relatively short purine-rich region were bound by cellular SR proteins. Indeed, ASF/SF2 was shown to bind a 34-nucleotide sequence (L2) contained within the longer purine-rich tract. Exon 3 RNAs that contained the 55-nucleotide purine-rich tract formed a higher order intermolecular structure and it was this form of RNA, which was specifically bound by ERev in vitro. Heat denaturation of the RNA prior to EMSA reactions, or base substitutions that prevented formation of the dimer-like structure, abolished ERev binding. In contrast, SR protein binding was independent of structural features in exon 3 RNA. These differences in recognition of the RNA by the individual proteins suggested that Rev and ASF/SF2 binding sites may overlap but are nevertheless distinct entities. In combination, ERev and SR proteins formed a novel complex on exon 3 RNA and, concomitantly, complexes that were seen with the individual proteins were diminished. The new complex was not formed when SR proteins were combined with an ERev mutant, which was defective for RNA binding activity. UV cross-linking experiments confirmed that binding of ERev and SR proteins to the RNA was not mutually exclusive. Furthermore, ERev was shown to interact with SR proteins in the absence of RNA in vitro. Thus, EIAV presents a subtle variation on the HIV-1 Rev system, where ASF/SF2 does not bind to the RRE alone but is recruited to the RRE subsequent to Rev binding (46).

Other RREs are large, stem-loop structures, which contain a small, discrete element that binds Rev with high affinity; adjacent lower affinity Rev binding sites cooperate with the primary binding site to support optimal Rev/RRE function. HIV-1 Rev binds to a purine-rich structure, which is stabilized by non-Watson-Crick G-A and G-G base pairs (40, 41). Although computer-assisted folding of the high affinity binding site for EIAV Rev did not predict a stable secondary structure, native gel electrophoresis of this region indicated the presence of two
discrete forms, only one of which was recognized by ERev in vivo. It is likely that unusual base pairing in this purine-rich region forms a unique structural feature recognized by ERev. It seems unlikely that this particular dimer-like structure is required for EIAV Rev/RRE function in vivo. On the other hand, purine-rich RNA elements elsewhere in EIAV RNA (for example, in exon 4) could interact with the RRE in exon 3 to provide a similar structured element in vivo. Since ERev does not interact with RNA in the absence of cellular proteins in vivo, it is possible that association of ERev with cellular factors modifies its binding affinity and specificity. The analogy of ERev-ASF/SF2-RRE associations to Tat-cyclin T-TAR RNA interactions is noteworthy. In the latter case, various lentivirus Tat proteins bind to RNA in association with cellular cyclin T proteins; the latter cooperate with Tat to stably bind RNA and subsequently act on the transcription complex (42).

Although the distantly related, genetically complex retroviruses employ conserved mechanisms and similar cellular pathways to control virus gene expression, they display variation in the ways that the components are recognized and assembled. For example, the Rev proteins encoded by EIAV and HIV-1 have no sequence similarity, yet both bind to their cognate RNA elements, associate with CRM-1 (exportin 1), and couple viral pre-mRNA to a protein export pathway (3, 43–45). Furthermore, the RNA elements with which Rev proteins interact are quite diverse with respect to their sequences, structures, and genomic locations. The HIV-1 RRE forms a large secondary structure with multiple stem-loop elements and is located in the middle of the env gene. The HTLV-I RxRE also forms a large stem-loop structure but is located at the 3′ end of the viral RNA. In contrast, the EIAV Rev binding site lies in a relatively short purine-rich region located in exon 3 adjacent to a 5′ splice site and is not predicted to form a typical base-paired structure. The various locations of the Rev binding sites among the viruses may be related to differences in their genetic organization and gene expression programs. For example, the location of the ERev binding site next to a splice site in EIAV exon 3 could facilitate alternative splicing and negative autoregulation of ERev synthesis. The varied structures of the RREs would also determine the manner in which both Rev and cellular proteins interact with these RNAs. The RNA binding domains of HIV-1 Rev and HTLV-I Rex proteins are arginine-rich, whereas that of EIAV Rev has few and interspersed arginine residues. The short, purine-rich EIAV RRE and the distinct RNA binding domain of ERev suggest that this ERev-RRE interaction would be different compared with other retroviruses and might require cellular cofactors for optimal stability.

In a previous study, we suggested that the juxtaposition of ERev and SR protein binding sites in EIAV RNA could influence two alternative mechanisms for Rev-induced alternative splicing (6) EIAV Rev-induced exon 3 skipping was proposed to result either from mutually exclusive binding of ERev and SR proteins to RNA or from simultaneous binding and subsequent disruption of spliceosomal complex assembly or activity. The data presented here are consistent with the latter mechanism. It is of particular interest that Rev proteins from distantly related viruses such as EIAV and HIV-1 bind to RNA in association with particular SR proteins and have been reported to inhibit splicing in vivo and in vitro (46). Although SR proteins, especially ASF/SF2, have been studied primarily in the context

![Image](http://www.jbc.org/)

**FIG. 6.** ERev and HeLa SR proteins bind simultaneously to a composite element in exon 3 in vitro. **A,** UV cross-linking of 100 ng of HeLa SR proteins to the 32P-labeled 3′Ex3 RNA in the absence (lane 2) and in the presence of 100, 200, and 500 ng of ERev protein (lanes 3, 4, and 5, respectively). The complexes were separated by SDS-PAGE and visualized by autoradiography. pKS RNA is the nonspecific control (lane 1). **B,** wild type ERev or mutant ERev-M27 were combined with HeLa SR proteins and complex formation with 32P-labeled 3′Ex3 RNA (88 bases; 1 × 104 cpm) was examined by EMSA. The ERev-M27 mutant was previously predicted to be defective for RNA binding (20). RNA was incubated in the absence of protein (lane 1), or with individual proteins: 1 μg of ERev (lane 2), 1 μg of ERev-M27 (lane 3), or 100 ng of HeLa SR proteins (lane 4). Complex formation was examined with combinations of ERev (1 μg) and HeLa SR proteins (100 ng) (lane 5) or mutant ERev-M27 plus HeLa SR proteins (lane 6). The novel RNA-ERev-SR protein complexes in lane 5 is identified as Complex C. A, pull-down assay was performed with 1 μg of Ni2+-bead-immobilized His-tagged ERev (lane 1) and 8 μg of purified SR proteins, and then SDS-polyacrylamide gel electrophoresis was carried out for fractionation of the bound proteins. The protein retained by interaction with ERev were visualized by Western blotting (WB) using anti-ASF/SF2 antibody (lanes 2 and 3) or anti-monoclonal antibody 104 (lanes 4 and 5).
of constitutive and alternative splicing in the presence of an exonic splicing enhancer element (47), it has been reported that ASF/SF2 can act as a negative regulator of splicing in some contexts (48, 49). It is likely that the inhibitory effects of SR proteins in these situations may be analogous to Rev-SR protein effects at the RRE. We are currently testing the possibility that ERev-ASF/SF2 interactions interfere with the assembly or catalytic activity of the spliceosomal complex.

Acknowledgments—We thank Gisela Heidecker, Barry Morse, and Huey-Jane Liao for helpful discussions.

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