The role of Rev-SR protein interactions in the regulation of equine infectious anemia virus replication

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The role of Rev-SR protein interactions in the regulation of equine infectious anemia virus replication

by

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A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

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Program of Study Committee:
Susan L. Carpenter, Major Professor
Norman F. Cheville
W. Allen Miller
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Daniel F. Voytas

Iowa State University
Ames, Iowa
2003

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This is to certify that the doctoral dissertation of

Gregory Saang Park

has met the dissertation requirements of Iowa State University

Signature was redacted for privacy.

Major Professor

Signature was redacted for privacy.

For the Major Program
Dedication

For my father, Dr. Yong Ho Park

For my mother, Sharon Park

You gave me life and shaped my mind.
You did the best with what you had to work with.

Thank you.
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ABSTRACT

Equine infectious anemia virus (EIAV) is a member of the lentivirus subfamily of retroviruses that produces a variable clinical disease course characterized as acute, chronic, and inapparent. The clinical signs can vary according to the stage of disease, and generally correlate with levels of virus replication. As with other retroviruses, EIAV utilizes both RNA and proteins to produce alternatively spliced transcripts required for virus replication. EIAV encodes a protein called Rev, which functions by binding unspliced and singly spliced viral mRNAs in the nucleus at a sequence called the Rev responsive element (RRE) and exporting them into the cytoplasm. Rev is absolutely required for virus replication, and factors that inhibit Rev function would be expected to inhibit virus replication. EIAV Rev is encoded in exons 3 and 4 of a bicistronic, four-exon mRNA, which also encodes the protein Tat in exons 1 and 2. The presence of Rev results in the expression of an alternatively spliced viral mRNA that differs from the four-exon mRNA by lacking exon 3. Exon 3 contains cis-acting sequences that function as both an exon splicing enhancer (ESE) and a RRE. ESEs bind cellular SR proteins to assist in the recognition and inclusion of exons. Therefore, the EIAV ESE/RRE sequences bind both SR proteins and Rev. The goal of this research is to characterize the interactions between EIAV and cellular SR proteins that modulate virus replication. I first show that Rev-mediated alternative splicing of exon 3 is not a mechanism to up-regulate Tat activity. I demonstrate that SF2/ASF inhibits Rev-mediated nuclear export activity and EIAV replication in vitro. I show that the RNA binding domain of SF2/ASF is necessary and sufficient for the inhibition of Rev nuclear export activity and EIAV replication. Further, the inhibition of both Rev activity and virus
replication correlated with the SR protein RNA binding specificity. These results suggest that SR proteins and Rev compete for binding viral RNAs at the ESE/RRE. Therefore, factors that modulate intracellular concentrations of SR proteins may play a role in regulating Rev nuclear export activity and EIAV replication.
CHAPTER 1. GENERAL INTRODUCTION

Dissertation Organization

This dissertation biologically characterizes the interactions of virus and host cell factors in equine infectious anemia virus (EIAV) alternative splicing. This dissertation is in the alternative format and has four chapters and two appendices. The first chapter provides a general background of retroviruses, the importance of splicing in retroviral gene expression, and introduces the retrovirus EIAV. There is a description of splicing and the SR protein family of splicing regulators, with an emphasis on the SR protein SF2/ASF. Finally, there is a detailed description of SF2/ASF and EIAV Rev interactions with EIA viral RNAs. Chapter 2 is a paper to be submitted for publication in the journal *Virus Genes* that is a functional characterization of the monocistronic Tat transcripts of EIAV. This work was done in collaboration with Michael Belshan and Susan Schommer. Susan Schommer and Michael performed the cloning of the EIAV cDNAs. The monocistronic Tat transcript is an alternatively spliced variant of a bicistronic transcript that encodes Tat and Rev. The presented data indicates that Tat activity from either monocistronic or bicistronic viral mRNAs was not significantly different, suggesting that RNA of the downstream rev gene does not affect translation initiation at the CUG start codon of the upstream tat gene. Chapter 3 is a paper to be submitted for publication in the journal *Molecular and Cellular Biology* that characterizes SF2/ASF inhibition of both EIAV Rev activity and EIAV replication. All of the work in this manuscript was performed by myself. The RNA binding domain of SF2/ASF was found to be necessary and sufficient for the inhibition of Rev activity and virus replication. The results suggest that SF2/ASF competes with Rev for
binding viral RNAs, inhibiting Rev activity, and subsequently, EIAV replication. Chapter 4 includes the general conclusions of my dissertation research as well as my recommendations for future studies. Appendix A is a detailed description of the methods used and the progress made towards purifying the protein EIAV Rev, which will be used in RNA binding competition studies with SF2/ASF, and in mapping the Rev RNA binding domain. Appendix B is a paper published in the journal *Molecular and Cellular Biology* by Michael Belshan that is a molecular characterization of Rev-mediated alternative splicing. Whereas the majority of the paper was written and performed by Michael Belshan, I performed the protein purification of Rev and the *in vitro* assays in which SF2/ASF inhibited Rev-mediated nuclear export. The purification of Rev was essential to much of the work in the paper. The protein was used in the *in vitro* binding assays for the identification of the RRE, and in the *in vitro* splicing assays to show that Rev inhibits exon 3 splicing.

**Introduction**

Retroviruses are a family of enveloped, ssRNA viruses, whose hallmark is the use of a viral encoded RNA-dependent DNA polymerase, or reverse transcriptase, to produce a linear, dsDNA copy from a ssRNA genome (reviewed in 19). In retroviral replication, a retrovirus attaches to a host-cell, fuses with the lipid bi-layer, and the viral core enters the cytoplasm (Figure 1). The viral core goes through a process of uncoating and the packaged reverse transcriptase creates the intermediate dsDNA genome, which then translocates into the nucleus and integrates into the host-cell genome. From the integrated viral DNA copy called the provirus, transcription at the 5'-long terminal repeat (LTR) produces full-length transcripts that are the source of all viral mRNAs necessary for replication. All retroviruses
Figure 1. General retroviral replication cycle. After attachment, the retroviral and cellular membranes fuse, releasing the core into the cytoplasm. The core goes through a process of uncoating, and the ssRNA genome is reverse transcribed into dsDNA with the packaged reverse transcriptase. The gray/black/white boxes indicate the U3 (gray), R (black), and U5 (white) sequences that make up the long terminal repeats (LTRs) of the retrovirus. The dsDNA copy of the viral genome translocates into the nucleus, integrates into the host cell genome, and transcription from the single viral promoter in the 5' LTR produces unspliced transcripts. The unspliced transcripts encode gag and pol and serve as new virion genomes. For retroviral replication, some unspliced transcripts must be spliced to produce the singly spliced transcripts encoding env. Virion proteins and genomes are assembled at the cell membrane and new infectious viruses are produced by budding and proteolytic maturation. Figure adapted from Coffin et al. 1997 (19).

produce mRNAs that encode three major coding regions for the polyproteins gag, pol, and env. The group-specific antigen gene, gag, encodes the major internal core structural proteins: matrix, capsid, and nucleocapsid. The pol gene encodes minimally the reverse
transcriptase and integrase enzymes, and env encodes the envelope glycoproteins made up of surface and transmembrane components, which have roles in cell attachment and virus entry. In addition, all retroviruses encode a protease in the gag and/or pol ORFs, which plays a role in virion maturation. Because retroviruses have a limited genome size and a single promoter, they utilize the host-cell splicing machinery that acts on unspliced viral transcripts to produce multiply spliced transcripts that are necessary for replication.

Figure 2. Molony murine leukemia virus genome and transcripts. The boxes indicate the open reading frames, the horizontal dark lines indicate exon sequences, and the diagonal lines indicate the spliced out RNA sequence.

For some retroviruses, such as Moloney murine leukemia virus, inefficient splicing of the unspliced transcript produces a second transcript that encodes the env (Figure 2). For other retroviruses, virally encoded proteins regulate RNA synthesis and expression. For example, the Lentiviridae subfamily of retroviruses encodes two proteins Tat and Rev (reviewed in 26,85). Tat functions by entering the nucleus and binding to a bulged, stem-loop structure found at the 5' terminus of all viral mRNAs called the Tat activation region (TAR) (22,28,42,74,75). Tat binds TAR in association with cyclin T1 (91) and cyclin-dependent kinase 9 (Cdk9) (7). In this complex called P-TEFb, Cdk9 phosphorylates the C-
terminal domain of RNA polymerase II (RNAPII) (59,94,98), which shifts transcription from initiation to elongation. Therefore, Tat up-regulates transcription.

Figure 3. Mechanism of Rev function. Rev enters the nucleus, binds singly spliced and unspliced viral mRNAs, multimerizes, and exports the mRNAs into the cytoplasm. The singly spliced and unspliced viral mRNAs encode the structural proteins of the virus and the unspliced mRNA serves as the genomes for new virions. The cytoplasmic expression of the structural proteins is dependent on Rev, and therefore, Rev is absolutely required for virus replication.

A second lentiviral regulatory protein is Rev. The prototypical and most-characterized Rev is human immunodeficiency virus (HIV) Rev (reviewed in 26,48,72). Rev functions by entering the nucleus, binding singly and unspliced viral mRNAs at a sequence in the env called the Rev responsive element (RRE) (20,97), multimerizing (71,96), and exporting the mRNAs into the cytoplasm through the exportin 1 (Crm1) nuclear export pathway (Figure 3) (29,30). Because Rev is necessary for the cytoplasmic expression of the unspliced and incompletely spliced viral mRNAs, Rev is absolutely required for virus replication.
An in vitro assay has been developed to measure Rev nuclear export activity (4,5,44), which uses a chloramphenicol acetyltransferase (CAT) reporter plasmid (Figure 4). When transfected into cells, the CAT-based Rev reporter expresses mRNAs that, from 5’ to 3’, consist of a splice donor, a CAT gene, a RRE, and a splice acceptor. Therefore, the CAT gene and RRE are within RNA sequence that is recognized as an intron. In the absence of Rev, the CAT gene and RRE are spliced out of the transcript and no CAT is expressed. However, if the reporter is in the presence of Rev, Rev binds the RRE and exports the unspliced reporter transcript into the cytoplasm, and CAT is expressed. CAT can then be assayed as a quantification of Rev nuclear export activity.

Figure 4. In vitro Rev nuclear export reporter. The Rev reporter plasmid produces transcripts that contain a CAT gene and a RRE flanked by a splice donor (SD) and a splice acceptor (SA). In the absence of Rev, the CAT gene and RRE are spliced out of mRNAs, whereas in the presence of Rev, Rev binds the RRE, exports reporter transcripts into the cytoplasm, and CAT is expressed.

**Equine infectious anemia virus**

Equine infectious anemia virus (EIAV) is a lentivirus that encodes both Rev and Tat proteins. EIAV is closely related to caprine arthritis-encephalitis virus as well as the human, simian, and feline immunodeficiency viruses (HIV-1 and -2, SIV, FIV). Infection with
EIAV produces a persistent, lifelong infection in horses and other members of the family *Equidae* (reviewed in 77,78). Most EIAV-infected horses show little to no sign of infection (45), but the disease course may vary to include periods of acute, chronic, and/or inapparent disease. Clinical signs include cycles of high fever, thrombocytopenia and/or anemia, and each febrile episode is associated with viremia. Interestingly, there is no single immune parameter that correlates to the control of virus replication or the clinical disease in EIAV infection (36-38). EIAV is transmitted through blood or blood products, most commonly by the large biting insects of the *Tabanidae* family (horseflies); another common method of transmission is through the use of contaminated blood transfusions, needles, or surgical equipment. EIAV is tropic to cells of the monocyte/macrophage lineage, with the majority of virus replication occurring in the tissue macrophages (70,79), and the highest titers of virus found in the serum, liver, spleen, bone marrow, lung and kidney (79).

There are five major transcripts that have been identified in EIAV replication (68). A fully spliced, four-exon, bicistronic mRNA encodes both Tat and Rev (mRNA1, Figure 5) (67,81). Tat is translated from exons 1 and 2, and Rev is translated from exons 3 and 4 (23,24,67,69,81), though the known functional domains of Rev are wholly encoded in exon 4 (31,40,60). In the absence of Rev, only the bicistronic mRNA is expressed in the cytoplasm (62,76), and thus, Rev mediates the cytoplasmic expression of the other EIAV mRNAs. One of the four mRNAs is an alternatively spliced mRNA identical to the fully spliced, four-exon mRNA, but lacking *rev* exon 1 and encoding only Tat (mRNA 2, Figure 5) (62). As with other lentiviral Rev's, EIAV Rev binds and exports the unspliced and singly spliced viral mRNAs, which encode the structural genes (mRNA 4 and mRNA 5, Figure 5).
EIAV Rev is a 165 amino acid protein that is functionally homologous to HIV Rev, yet shares little amino acid homology. EIAV Rev is not as well characterized as HIV Rev, but some functional domains have been identified, including the nuclear localization signal (NLS) (amino acids 160-165) (65) and the nuclear export signal (NES) (amino acids 32-55) (31). The RNA binding domain (RBD) has not been mapped, but recent studies suggested that a region of the protein that mediates alternative splicing (amino acids 75-127) may be the RNA binding domain (65). Finally, some studies suggest that EIAV Rev also multimerizes, but the domain has not been mapped (88). Interestingly, the EIAV functional domains differ in their structural organization compared to HIV Rev (Figure 6). The residues of the HIV Rev RBD/NLS are located toward the amino end of the protein and are flanked
by the residues of the multimerization domain, and the residues of the HIV Rev NES are located toward the carboxy end.

![Diagram of EIAV Rev and HIV Rev domain organization.](image)

Figure 6. The domain organization of both EIAV Rev and HIV Rev. Though they are functionally homologous, the domain organization is different between EIAV Rev and HIV Rev. Both proteins contain nuclear export signals (NES) and nuclear localization signals (NLS), though the HIV Rev NLS is also the RNA binding domain (RBD). In contrast, the RBD of EIAV Rev has not been well characterized, but may be the identified alternative splicing domain. Finally, the multimerization domain of HIV Rev flanks the NLS/RBD, whereas EIAV Rev multimerization has not been well characterized.

**Pre-mRNA Splicing**

There is growing evidence that many of the major processes within the nucleus including transcription, splicing, and nuclear export, are not separate, but intricately connected (reviewed in 6,25,33). Splicing is a post-transcriptional modification performed on pre-messenger RNA (pre-mRNA) to remove introns and join segments of RNA as exons (reviewed in 33,41,52). The splicing reaction is carried out by the splicing machinery, and involves many interactions between a number of cis-acting pre-mRNA sequences and a larger number of trans-acting factors.

Three *cis*-RNA sequences are necessary in splicing. These are the 5' splice site, the branch point, and the 3' splice site. The 5' splice site is also called the splice donor, and it has
the consensus RNA sequence $^{\text{AG}}|\text{GUPuAGU}^{|}$, where the line between the AG and GUPu marks the border between the upstream exon and downstream intron, and the Pu represents either purine nucleotide (G or A). The branch point is downstream of the 5' splice site and has a consensus RNA sequence CUPuAPy, where Py denotes either C or U, and also is the beginning of a polypyrimidine tract. The 3' splice site is also called the splice acceptor, and it has the consensus RNA sequence $^{\text{NPyAG|PuN}}^{|}$. The line between the AG and PuN marks the border between the upstream intron and the downstream exon, and the N represents any RNA nucleotide. Thus, the 5' splice donor defines the downstream boundary of one exon, and the 3' splice acceptor defines the upstream border of another exon.

The trans-acting splicing machinery includes, amongst other proteins, a multi-protein, catalytic RNA-protein complex called the spliceosome. The spliceosome is made up of a large number of proteins that are divided into two groups: the small nuclear ribonucleoprotein complexes (snRNPs), which are RNA-protein complexes, and the non-snRNPs, which are also called general splicing factors (GSFs). Spliceosome assembly is initiated through snRNP components at the cis-RNA sequences. During assembly, major complex formations are made by an ordered interaction of other snRNPs (Figure 7). In the commitment or early (E) complex, the pre-mRNA is bound at the 5' splice site by the U1 snRNP, at the branch point by splicing factor 1 and 65 kDa subunit of the heterodimeric factor U2AF, and at the 3' splice site by the 35 kDa subunit of U2AF. It is thought that the E complex defines exons such that the formation of the E complex commits the pre-mRNA to a particular splicing choice. Complex A is formed when the U2 snRNP displaces splicing factor 1 and U2AF. Complex B1 and then Complex B2, the mature spliceosome, follow Complex A when the U5/U4-U6 tri-snRNP and a number of GSFs interact with the pre-
mRNA. During the interaction, the RNA component of U6 base pairs with the 5' splice donor, and the protein component of U6 associates with the U2 snRNP. The U5 snRNP and GSFs remain associated with the spliceosome, and both the U4 and U1 snRNPs are released. Many of the GSFs involved in spliceosome assembly belong to a family of proteins called the SR proteins.

Figure 7. Spliceosomal assembly. The commitment complex, or E complex, forms when the pre-mRNA is bound by the U1 snRNP at the 5' splice donor, by both splicing factor 1 (SF1) and the 65 kDa subunit of U2AF at the branch point (BP), and by the 35 kDa subunit of U2AF at the 3' splice acceptor. The U2 snRNP binds the branch point and displaces SF1 and U2AF to form Complex A. The mature spliceosome, Complex B2, is formed when the U5/U4-U6 tri-snRNP interacts with the pre-mRNA, releasing the U4 and U1 snRNPs. The figure was adapted from Murray and Jarrell, 1999 (66).
SR Proteins

SR proteins are a family of proteins that are involved in the process of splicing during pre-mRNA processing, and are also involved in other stages of gene expression including transcription, 5' capping, polyadenylation, and nucleocytoplasmic transport (reviewed in 8,9,14,33,58,84). SR proteins function in both exon-independent and exon-dependent splicing (43). In exon-independent splicing, SR proteins assist in protein-protein interactions during the splicing reaction, but they do not directly interact with the pre-mRNA. In exon-dependent splicing, SR proteins bind pre-mRNA to aid splice site recognition by the components of the splicing machinery. The necessity for exon-dependent splicing is typically conditional on the strength of the splice site, which is judged by the splice site's conformity to a consensus sequence.

Members of the SR protein family are generally characterized by the presence of one or two amino-terminal RNA recognition motifs (RRMs) and a carboxy-terminal RS domain. The RRM conforms to a consensus RNP-type RNA binding domain, whereas the RS domain consists of a number of arginine-serine dipeptide repeats. A second defining characteristic of SR proteins is the ability to activate splicing in a SR protein-depleted in vitro splicing assay. To date, 10 human SR proteins have been identified (SRp20, SF2/ASF, SC35, SRp30c, 9G8, SRp40, SRp46, SRp55, SRp75, p54), and numerous highly conserved homologs, orthologs, and other SR family members have been identified in a wide variety of species of both plants and animals including: Arabidopsis thaliana, Chironomas tentans, Caenorhabditis elegans, Mus musculus, and Drosophila melanogaster (1,2,13,56,57). The two prototypical SR proteins that separate the one and two RRM-containing protein members are SC35 and SF2/ASF (99).
The SR Protein Functional Domains

The main function of the SR protein RRM is in determining substrate-specificity by binding to RNA (17,46,55,63,64,84,87). The consensus RNA binding sequence of specific SR protein RRMs has been determined using a procedure called SELEX (sequential evolution of ligands by exponential enrichment) (86). The results of SELEX analyses shows that each RRM can bind a number of different RNA sequences, but there are specific RNA sequences that distinct SR proteins will bind. In addition, SR proteins that have two RRMs require both to bind a specific RNA sequence (99). Therefore, sequence-specific RNA binding depends not only on the identity of the RRM, but also on the number of RRMs (83).

The other functional domain of the SR proteins is the RS domain. The major functional role of the RS domain is in mediating the protein-protein interactions among SR proteins and the components of the splicing machinery (49,92). These interactions include, but are not limited to, phosphorylation, nuclear localization, and direct interactions with other SR proteins as well as other splicing proteins. RS domains may also have some minor role in determining substrate specificity, but they contribute little to RNA binding (17,63,83). RS domains are somewhat conserved at the amino acid level, and for some SR proteins, are functionally interchangeable (17,90). Phosphorylation and dephosphorylation of the RS domain modulates the activity of SR proteins, and phosphorylation is mediated by the interaction of the RS domain with two SR protein kinases (SRPK1 and SRPK2) (50). The phosphorylation state of the RS domain also affects SR protein intracellular movement and subcellular localization. RS domains function as sufficient nuclear localization signals, and interact with two importin-β family nuclear import proteins called transportin-SR (TRN-SR) and transportin-SR2 (TRN-SR2) (47,53). For some SR proteins, the RS domain also confers
an ability of the SR protein to shuttle from the nucleus back into the cytoplasm (11,16). In addition, RS domains are required for SR protein movement between the nuclear speckles (storage sites) and sites of active transcription (50,64).

**SR Protein Regulated Alternative Splicing**

SR proteins play a role in both constitutive and alternative splicing (reviewed in 14,35,61). Constitutive splicing is simply the removal of an intron between two exons, whereas alternative splicing is the differential use of multiple splice sites in a pre-mRNA to construct different mRNAs consisting of various exons. The four most common modes of alternative splicing are: exon exclusion/inclusion, use of alternative 3' splice acceptors, use of alternative 5' splice donors, and mutually exclusive exons (Figure 8). SR proteins function in a parallel and concentration-dependent manner in splicing. For example, a particular SR protein may be sufficient but not necessary to assist in the splicing of a substrate, as it can be replaced by a different SR protein. In addition, SR proteins may assist in exon inclusion during alternative splicing at a certain concentration, but an increase or decrease in that concentration may change the choice of splice site utilization and result in a different mRNA. SR protein expression varies in a number of tissues and cellular activation states (39,54,95), and it has been shown that alternative splicing occurs in a tissue-specific manner (80).

It is generally agreed that SR proteins function in alternative splicing by binding to RNA sequences within pre-mRNAs and assisting in the recognition of non-consensus splice sites, which leads to the inclusion of exons (reviewed in 8,14,84). In particular, SR proteins are involved during E complex formation, which is the complex that commits the pre-mRNA
to a splicing pattern. For example, the SR proteins SF2/ASF and SC35 interact with the U1-70kDa protein subunit of the U1 snRNP, and assist in stabilizing the U1 snRNP interaction with the 5' splice donor (12,27,49,93). In addition, SR proteins can bind U2AF and assist in the recognition of 3' splice acceptor (92).

![Exon exclusion/inclusion](image)

![Alternative 3' splice acceptors](image)

![Alternative 5' splice donors](image)

![Mutually exclusive exons](image)

Figure 8. Four common modes of alternative splicing. Each mode shows two possible splicing choices, and are represented by either the top diagonal lines or bottom diagonal lines. For example, in exon exclusion/inclusion, the mRNA may be spliced to include the exon (top diagonal lines), or may be spliced to exclude the exon (bottom diagonal lines). The boxes indicate the exon sequences, the horizontal lines indicate the intron sequences, and the diagonal lines indicate the spliced intron. Adapted from Cartegni et al., 2002 (14).

In alternative splicing, the prevalent theory of exon exclusion/inclusion is called exon definition, which proposes that the exon, not the intron, is the unit recognized by the splicing machinery (73). Thus, there is communication between the 3’ splice acceptor and the 5’ splice donor that flank an exon in order for the spliceosome to recognize the exon and include it during pre-mRNA processing (73). Because SR proteins can interact with both the U1-70k and U2AF proteins simultaneously (92), it is thought that SR proteins assist in the recognition of the splice sites as well as bridge the interactions between the proteins at the
upstream 3' and downstream 5' splice sites. Indeed, many intraexonic RNA binding sites have been discovered, and have an active role in regulating alternative splicing.

In addition to the 5' splice donor, branch point, and 3' splice acceptor, there are two groups of cis-RNA elements that also modulate splicing. Those that mediate the inclusion of an exon during constitutive or alternative splicing are referred to as exon splicing enhancers (ESEs). Those that mediate the exclusion of an exon are referred to as exon splicing silencers (ESSs) (3,82). SR proteins function by binding ESEs and recruiting the splicing machinery to non-consensus (weak) splicing signals through RS domain-mediated protein-protein interactions. SR proteins may also function by binding RNA and antagonizing the action of nearby ESSs. Investigation has identified a large number ESEs that are purine-rich and intraexonic, but ESEs are not strictly purine-rich (15,21).

The SR Protein SF2/ASF

One particular SR protein that is extensively studied in the process of splicing is the protein Splicing Factor 2/Alternative Splicing Factor (SF2/ASF). SF2/ASF is also referred to as ASF/SF2 (32,51). SF2/ASF is highly conserved among mammals, and numerous homologs and orthologs have been discovered in other species including birds, plants, worms, and insects (1,2,13,56,57,95). For example, between humans and mice, the amino acid sequence of SF2/ASF is 100% identical. SF2/ASF is also an essential gene for cell viability (89). Some of the major effects of SF2/ASF in splicing are to (i) assist the U1 snRNP bind the 5' splice donor, (ii) to assist the U2AF bind the 3' splice acceptor, and (iii) to play a role in the first part of the splicing reaction (12,49,92).
SF2/ASF is a 248 amino acid protein with an apparent molecular weight of 33 kDa and a predicted molecular weight of 27.7 kDa. The functional domains of SF2/ASF include two amino-terminal RRMs and a carboxy-terminal RS domain (Figure 9) (10,99). Amino acids 1-97 constitute RRM1, amino acids 107-197 constitute RRM2, and amino acids 198-248 constitute the RS domain. Between the two RRMs is a glycine rich region (amino acids 98-106) that is suggested to serve as a hinge between the two RRMs. SF2/ASF is considered the prototype of a class of SR proteins that have two RRMs (99).

Using various SELEX protocols, three different consensus RNA binding motifs have been identified for SF2/ASF: RGAAGAAC, AGGACAGAGC, and SRSASGA (R=G/A and S=C/G) (55,83). SELEX analyses with only RRM1 of SF2/ASF resulted in the consensus RNA sequence ACGCGCA. ESE sequences that conform to the SF2/ASF RNA binding motifs and specifically bind wild-type SF2/ASF also bind the amino-terminal RRMs (amino acids 1-197). Thus, substrate specificity depends on the presence of both RRMs (17,99), and the RS domain is not a major determinant of binding specificity (83).
SF2/ASF and EIAV Rev

As with other retroviruses, EIAV utilizes the splicing machinery to produce the viral RNAs necessary for replication. EIAV is also a lentivirus and produces the regulatory protein Rev, which is necessary for the cytoplasmic expression of the unspliced and singly spliced viral mRNAs. Interestingly, exon 3 of the four-exon, bicistronic viral mRNA (mRNA1, Figure 4) contains a purine-rich sequence that functions as both an ESE and as an EIAV RRE (ESE/RRE) (5,34). This ESE/RRE was sufficient for SR protein binding (18), and was necessary for exon inclusion, Rev binding, and Rev-dependent nuclear export (5). The SR protein that specifically binds the ESE/RRE is SF2/ASF (18,34), and the ESE/RRE contains multiple sequences that conform to the SF2/ASF consensus RNA binding motif RGAAGAAC.

A mutational analysis of the ESE/RRE has shown that the sequences that bind Rev also strongly assist in ESE-mediated splicing, and mutations that inhibit ESE function also inhibit RRE function (3). However, mutation of the ESE/RRE does not exclusively knock out the function of both the ESE and RRE. Thus, it is thought that the RNA binding sites for SF2/ASF and Rev on the ESE/RRE overlap, but are not the same. It is clear that Rev can inhibit exon 3 inclusion in the bicistronic viral mRNA (5,62), and SF2/ASF can inhibit Rev-mediated nuclear export activity (5). These results suggest that Rev and SF2/ASF competitively bind the ESE/RRE, which regulates splicing and viral mRNA expression. Because Rev is absolutely required for virus replication, SF2/ASF may also inhibit EIAV replication. Therefore, the interaction of Rev and SF2/ASF with the ESE/RRE may modulate EIAV replication, and ultimately contribute to viral persistence and pathogenesis.
Overall Goal

The lentiviral protein Rev is absolutely required for EIAV replication, and factors that inhibit Rev function would be expected to inhibit virus replication. The cellular SR protein SF2/ASF inhibits EIAV Rev function (5). Rev and SF2/ASF are regulators of viral mRNA splicing and expression, and they both play a role in EIAV alternative splicing. The goal of this research is to characterize the interactions between EIAV and cellular SR proteins that modulate virus replication. It is our hypothesis that SF2/ASF inhibits Rev nuclear export activity and virus replication. To test this hypothesis, we have undertaken the following specific aims:

Specific Aims:

1. Determine the effect of Rev-mediated alternative splicing on EIAV Tat activity.
   (Chapter 2)
2. Determine the effect of SF2/ASF expression on Rev-dependent nuclear export.
   (Chapter 3)
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CHAPTER 2. FUNCTIONAL CHARACTERIZATION OF TAT ACTIVITY FROM EIAV ALTERNATIVELY SPLICED MESSENGER RNAs

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Abstract

Similar to other lentiviruses, equine infectious anemia virus (EIAV) encodes the regulatory proteins Tat and Rev, which regulate virus gene expression. Tat up-regulates transcription and Rev binds at a region of viral RNA called the Rev responsive element (RRE), and exports incompletely spliced viral transcripts into the cytoplasm. Tat and Rev are translated from a four-exon bicistronic mRNA, which is the predominate mRNA expressed early after infection. Tat translation is initiated at a CUG in exon 1, and only through a leaky scanning mechanism is Rev translation initiated at the first AUG located in exon 3. Exon 3 also contains the EIAV RRE. Rev mediates the expression of a three-exon, alternatively spliced mRNA that lacks exon 3 and encodes only Tat. To determine the effect of Rev-mediated alternative splicing on Tat expression, we used transient assays to functionally analyze alternative spliced mRNAs. There was no significant difference in Tat activity between monocistronic and bicistronic cDNAs, suggesting that Tat expression is not different between the three-exon and four-exon EIAV mRNAs. Thus, the presence or absence of Exon 3 did not affect Tat activity, suggesting that neither the RRE in rev exon 1 nor translation initiation of Rev affect Tat expression.
Introduction

Retroviruses are single stranded, positive sense RNA viruses that use reverse transcriptase to create dsDNA from their ssRNA genome. The single viral promoter of the integrated proviral dsDNA produces unspliced viral pre-mRNA. Retroviruses utilize both viral and host cell factors to express unspliced and incompletely spliced viral mRNAs. Members of the lentivirus subfamily of retroviruses encode two proteins, Tat and Rev, which regulate viral gene expression (reviewed in 11,27,32). Rev acts post-transcriptionally to facilitate the nuclear export of unspliced and incompletely spliced mRNAs, and is absolutely required for virus replication (reviewed in 11,17,27). Rev functions by entering the nucleus, binding viral mRNAs at a region of RNA called the Rev responsive element (RRE), and exporting the viral mRNAs into the cytoplasm (7,13,14,26,36,37). Tat functions by entering the nucleus and, in association with cyclin T1 and cyclin-dependent kinase 9 (Cdk9) (3,34), binds to a bulged, stem-loop structure found at the 5' terminus of all viral mRNAs called the Tat activation region (TAR) (8,12,16,28,29). Cdk9 phosphorylates the C-terminal domain of RNA polymerase II (RNAPII) to shift transcription from initiation to elongation (22,35,38). Therefore, Tat promotes RNAPII elongation, and can enhance transcription of viral mRNA 50-100 fold.

Equine infectious anemia virus is a member of the lentivirus subfamily of retroviruses that produces Tat and Rev from a four-exon, bicistronic mRNA (Figure 1) (24,31). Tat is translated from exon 1 and exon 2, and Rev is translated from exon 3 and exon 4 (9,10,24,25,31). Interestingly, Tat translation is initiated at a CUG start codon in exon 1 (4,9,25,30), whereas Rev translation initiates at an AUG start codon in exon 3 through a leaky ribosomal scanning mechanism (4). In the absence of Rev, the four-exon mRNA is the
only cytoplasmically expressed mRNA (mRNA1, Figure 1) (23,30). The presence of Rev results in the expression of the other EIAV mRNAs (mRNA3, mRNA4, mRNA5) including a monocistronic, three-exon mRNA, encoding only Tat (mRNA 2) (23). The monocistronic mRNA is identical to the bicistronic mRNA, except it lacks exon 3.

Leaky ribosomal scanning proposes that only some ribosomes stop to initiate translation at non-AUG start codons or AUG start codons in a weak context (A or G not at position –3 and G not at +4), while most continue scanning downstream (20). There are factors that affect leaky scanning translation other than the context of the start codon. RNA secondary structures can affect translation initiation in bicistronic messages, depending on their location. RNA secondary structures located downstream of the first initiation site can enhance translation of the first cistron by slowing ribosomes enough for codon/anti-codon base pairing to occur (19).

Exon 3 of the four-exon bicistronic mRNA contains not only the translation initiation site for Rev, but also contains the EIAV RRE, which is suggested to have secondary structure (6,15). Secondary structure would be consistent with other RREs, such as HIV’s RRE, which is a large secondary structure (5,18,21). Thus, the three-exon alternatively spliced RNA may differ from the four-exon mRNA in Tat expression due to the absence of exon 3, which may contain RNA factors that affect translation of the upstream cistron. To determine the effects of EIAV Rev-mediated alternative splicing on Tat expression, transient expression assays were used to compare the functional activity of Tat among alternatively spliced EIAV cDNAs. There was no significant difference in Tat activity in cells transfected with the monocistronic or the bicistronic cDNAs, suggesting that Tat expression is not different between the monocistronic and bicistronic EIAV mRNAs that encode Tat. Therefore, the
results suggest that neither the RRE nor translation initiation at the Rev AUG affect Tat translation.

**Materials and Methods**

**Plasmids.** EIAV MA-1 cDNAs (4x+, 3x+, 4x-) were amplified by RT-PCR of total RNA isolated from Cf2Th cells transfected with Rev+ or Rev- proviral DNA using the 5' primer, CGCAGACCCCTACCTGTTG and the 3' primer, TAGCCTGCTATGCGTCCTAC (Figure 2). The cDNA products were TA-cloned into pCR3.1 (Invitrogen, Carlsbad, Calif.) and confirmed by sequence analysis (DNA Sequencing and Synthesis Facility, Iowa State University). Transcription of the cDNAs in pCR3.1 is under control of the CMV promoter. ETat-M was constructed by David Derse in pRSPA-S (pRSETAT-M) (9). Exon 1 of the Tat cDNA in pRSETAT-M is missing the first 38 nucleotides and starts at an engineered AUG initiation site. Transcription of pRSETAT-M is under control of the RSV promoter. The plasmid pCH110 (Amersham Pharmacia, Buckinghamshire, UK) produces β-galactosidase. The LTR-CAT reporter plasmid pCATLTERIAV-1 contains the EIAV LTR upstream of a chloramphenicol acetyltransferase (CAT) gene. The plasmid pcDNA3 (Invitrogen) was used as a negative control and produces no Tat.

**Transient transfection assays.** Transient transfections were performed in canine fetal thymus (Cf2Th) cells, which support both EIAV Tat activity and EIAV replication (33). Cf2Th cells were maintained in Dulbecco’s modified Eagle media supplemented with 10% fetal calf serum and penicillin/streptomycin. All transfections were performed with the transfection reagent LipofectAMINE (Life Technologies/Invitrogen, Carlsbad, CA) according to reagent protocols. Briefly, 0.2 μg of individual cDNA plasmids were co-
transfected into cells with 0.2 \( \mu g \) pCH110, 1.0 \( \mu g \) of pCATLTREIAV-1, and an amount of pUC19 to equalize the amount of DNA used in all transfections. Two days post-transfection, cells were harvested, lysed by freeze/thaw, and clarified lysates were assayed for transfection efficiency by their \( \beta \)-galactosidase activity. Normalized amounts of cell lysate were then assayed for CAT expression with a commercially available CAT enzyme-linked immunosorbent assay (ELISA) kit (Roche Molecular Biochemicals, Indianapolis, IN). Pilot assays quantified CAT from pRSETAT-M transfected cells, and results were used to determine the parameters to assay all the transfected cell lysates. Initial assays determined a range of Tat activity from co-transfection of increasing amounts of pRSETAT-M or p3x+ with pCATLTREIAV-1 (data not shown). Results were used to determine the working amount of Tat cDNA plasmid.

Results and Discussion

EIAV Tat translation is initiated at a non-standard CUG in exon 1 of both the three-exon and four-exon EIAV mRNAs. Rev translation initiates in exon 3 at the first AUG of the EIAV four-exon, bicistronic viral mRNA. Thus, leaky scanning of the CUG initiation codon permits translation of Rev (4). Interestingly, Rev mediates the expression of the three-exon mRNAs that lack exon 3 and encode only Tat. Exon 3 contains a purine-rich sequence that functions as both an ESE and an RRE. Typical lentiviral RRE’s are highly structured RNA elements. It is possible that initiation at the Tat CUG is altered depending on the presence or absence of the AUG initiation site and/or RNA secondary structure in exon 3. If so, Rev-mediated alternative splicing may be a novel mechanism to increase Tat activity. To determine if EIAV alternative splicing results in mRNAs that differ in Tat activity, cDNAs
were constructed and tested for Tat activity in an *in vitro* transient expression assay. EIAV Tat constructs, 3x+ and 4x+ (Figure 2), represent the three-exon and four-exon mRNAs, respectively, and differ in the absence and presence of exon 3. Thus, 3x+ encodes only Tat, and 4x+ encodes both Tat and Rev. We also constructed a cDNA that contain exon 3, but produce no Rev due to a stop codon in exon 4. This would allow us to differentiate any effect due to Rev or exon 3. Thus, 4x- encodes a truncated Rev protein (amino acids 1-79) that contains only the identified nuclear export signal. The truncated Rev protein lacks amino acids 80-165, which includes the domains required for RNA binding and nuclear localization, and the protein is expected to be non-functional. The ETat-M construct has an engineered AUG start codon for Tat instead of the wild-type CUG, and was used as a positive control for Tat activity.

Less than 2-fold differences in levels of CAT were observed among the cDNA constructs. There was no significant difference in Tat activity between the 3x+ and 4x+ constructs ($P>0.05$) (Figure 3), which indicates that Tat activity is the same between the monocistronic and bicistronic EIAV Tat mRNAs. Co-transfection of the CAT reporter with the 4x- construct resulted in levels of CAT not significantly different from 3x+ ($P>0.05$). Together, these data suggest that the presence of exon 3 has little to no effect on Tat activity (Figure 3). Interestingly, the 4x- construct had significantly higher Tat activity than the 4x+ construct ($P<0.05$). While this suggests that Rev inhibited Tat activity, there is no significant difference in Tat activity between the 3x+ and 4x+ constructs. In addition, trans-complementation experiments showed that Rev does not significantly affect Tat activity (data not shown). It is possible that the presence of a truncated Rev protein may affect Tat activity. Together, the data indicate that there is no difference in Tat activity between the
monocistronic and bicistronic EIAV mRNAs, and suggest that exon 3 does not affect Tat expression. Thus, EIAV Rev-mediated alternative splicing may be a mechanism to express mRNAs that produce Tat without producing Rev, which may play a role in regulating both viral protein and mRNA expression.

Rev mediates exclusion of exon 3, and the subsequent expression of the alternatively spliced, three-exon mRNAs that encode only Tat. Recently, an exon splicing enhancer has been mapped to exon 3 (2,6,15). The ESE sequence also functions as a RRE (ESE/RRE) and the sequences of the ESE/RRE bind Rev. The cellular splicing protein SF2/ASF also binds the ESE/RRE and assists in exon 3 inclusion (2,15). The mechanism that results in expression of the alternatively spliced, three-exon mRNA is not clear, but current models suggest that exon 3 exclusion is the result of the inhibition of splicing, due to the binding of Rev at the ESE/RRE. The biological significance of the three-exon mRNA in virus replication is not known, but our data indicate that exon 3 had no effect on Tat activity. This does not necessarily mean that production of a second mRNA species encoding Tat plays no role in EIAV replication. Indeed, Tat is produced at very low levels, yet has profound affects on viral transcription (3). Our data suggests that Rev-mediated alternative splicing is not a mechanism to increase Tat expression. Thus, further investigations are necessary to better understand if the generation of the alternatively spliced mRNA is important in EIAV replication.
References


Figure Legends

Figure 1. The five major mRNAs of EIAV and their encoded proteins. Messenger RNA1 and mRNA2 encode the regulatory proteins Tat and Rev. Messenger RNA3 encodes a truncated transmembrane protein (Ttm) (1). Rev facilitates the nuclear export of incompletely spliced mRNA4 and unspliced mRNA5, which encode the structural proteins of the virus. The unspliced mRNA5 also serves as the genome for new virions. The boxes of the genome indicate the open reading frames. The thick, horizontal lines of the mRNAs indicate the exon sequences of the mRNAs, whereas the diagonal lines indicate the intron sequences that are spliced out. The proteins the mRNAs encode are listed.

Figures 2. EIAV genome and cDNA constructs. (A) EIAV genome and four-exon mRNA that encodes both Tat and Rev. Exons 1 and 2 encode Tat and exons 3 and 4 encode Rev. The boxes of the genome indicate the open reading frames, whereas the boxes of the RNA indicate the exons. (B) Expression plasmids used to evaluate the effect of alternative splicing on Tat activity. Promoters and protein products produced from the constructs created are shown. The 4x+ construct produces both Tat and Rev. The 3x+ construct is the same as the 4x+ construct except that it lacks exon 3, and produces only Tat. The diagonal lines of 3x+ indicate exon 3 exclusion in the cDNA. The 4x- construct contains a stop codon in exon 4 and encodes a truncated Rev protein that contains only the nuclear export signal. The ETat-M construct produces Tat from an engineered AUG start codon instead of the wild-type CUG, and transcription is under control of the RSV promoter. The boxes of the genome indicate the open reading frames, whereas the boxes of the constructs indicate the exons included.
Figure 3. Tat activity of alternatively spliced mRNAs. (A) Linear range of Tat activity from transfected pRSETAT-M and p3x+. (B) EIAV cDNA constructs produce Tat that transactivates the LTR-CAT reporter to produce CAT. The plasmid pcDNA3 produces no protein, and the results indicate the background ground level of LTR-CAT reporter expression. The results represent the mean of at least nine independent transfections per construct, and the error bars represent the standard error of the mean. The asterisks indicate a significant difference, as determined by ANOVA, between the results from cells transfected with either p4x+ or p4x- (P<0.05).
Figure 1.
Figure 2.
Figure 3.
CHAPTER 3. SF2/ASF INHIBITS EQUINE INFECTIOUS ANEMIA VIRUS REV ACTIVITY AND VIRAL REPLICATION

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Abstract

Complex retroviruses encode the essential regulatory protein Rev/Rex, which functions by entering the nucleus, binding viral mRNAs at the Rev/Rex responsive element (RRE), and exporting the singly spliced and unspliced viral mRNAs necessary for virus replication into the cytoplasm. Equine infectious anemia virus (EIAV) encodes Rev in exons 3 and 4 of a four exon, bicistronic viral mRNA. A purine-rich sequence in exon 3 functions as an exon splicing enhancer (ESE), and binds the cellular SR protein SF2/ASF. The ESE also binds Rev and functions as the EIAV RRE (ESE/RRE). Here, we show that SF2/ASF inhibits Rev-dependent nuclear export activity and EIAV replication. Inhibition mapped to the RNA binding domain of SF2/ASF and correlated with the RNA binding specificity. These results indicate that SF2/ASF binding viral mRNAs inhibits Rev-dependent nuclear export activity and EIAV replication, and suggest that the SF2/ASF and Rev compete for binding the ESE/RRE.
Introduction

Retroviruses have a limited genome size and employ a variety of mechanisms to express unspliced or differentially spliced viral mRNAs transcribed from a single promoter. One such mechanism involves the use of non-consensus splice sites and cis-regulatory sequences to produce unspliced and singly spliced viral mRNAs by inefficient splicing. Other mechanisms utilize viral and cellular proteins that act in trans to regulate the cytoplasmic expression of incompletely spliced viral mRNAs. Members of the lentivirus subfamily of retroviruses encode the protein Rev to assist in the cytoplasmic expression of the singly and unspliced viral mRNAs. The prototypical Rev is human immunodeficiency virus type 1 (HIV-1) Rev, which functions by entering the nucleus, binding singly and unspliced viral mRNAs at a sequence in the env called the Rev responsive element (RRE) (14,55), multimerizing (37,54), and exporting the mRNAs into the cytoplasm through the exportin 1 (Crm1) nuclear export pathway (16,18). The RRE-containing viral mRNAs encode the structural proteins necessary for replication, and the unspliced mRNAs also serve as the genome for new virions. The cytoplasmic expression of these viral RNAs is dependent on Rev and, therefore, Rev is absolutely required for virus replication.

Equine infectious anemia virus (EIAV) is a lentivirus similar to HIV, and also encodes a Rev that it is necessary in replication. Early in infection, EIAV expresses a multiply spliced, four-exon, bicistronic mRNA (mRNA1, Figure 1). The first and second exons encode the transcriptional activator protein Tat, and the third and fourth exons encode Rev (46). EIAV Rev (ERev) is a 165 amino acid protein with little amino acid homology to other lentiviral Rev proteins. It is functionally homologous to HIV-1 Rev, yet not as well characterized (19,32). As with other Rev's, ERev enters the nucleus, binds RRE-containing
mRNAs, and is necessary for the nuclear export of the singly and unspliced transcripts (mRNAs 3&4, Figure 1) through an exportin 1-mediated pathway (16-18,45). In addition, the presence of ERev results in the expression of an alternatively spliced viral mRNA that lacks exon 3. The rev AUG start codon is located in exon three and the alternatively spliced mRNA2 (Figure 1) encodes only Tat.

Exon 3 contains a purine-rich sequence that functions as both an exon splicing enhancer (ESE) and as a RRE (ESE/RRE). Exon splicing enhancers are cis-acting, intraexonic sequences that assist in exon recognition and inclusion during splicing by interacting with a family of cellular proteins, called the SR proteins (reviewed in 5,20,33). Members of the SR protein family share a modular, functional domain structure of one or two amino-terminal RNA recognition motifs (RRMs) and a carboxy-terminal RS domain that consists of a number of arginine-serine dipeptide repeats (12). SR proteins function by binding ESEs and assisting components of the splicing machinery to recognize non-consensus splice sites through protein-protein interactions (reviewed in 5,10,49). SR protein activity is both substrate and concentration-dependent, and their intracellular concentration has been shown to vary in a number of tissues and cellular activation states (22,25,53). Many SR proteins have been identified in a variety of both plants and animals. One SR protein, SF2/ASF, is highly conserved in mammals and has numerous homologs in other diverse species including birds, plants, worms, and insects (1,2,8,29,30,53). SF2/ASF binds the ESE/RRE in EIAV rev exon 1, which suggests it is the SR protein partner to the ESE (13,21).

In vitro splicing assays demonstrated that the purine-rich ESE motif was required for exon three splicing, and that splicing was inhibited by GST-ERev fusion protein (4). The
current model for alternative splicing of mRNA2 is that binding of ERev to the ESE/RRE inhibits SF2/ASF-dependent exon 3 inclusion (4,13). Interestingly, our previous results suggested that SF2/ASF can inhibit Rev-dependent nuclear export (4). Here, we further investigate the effects of SF2/ASF on EIAV Rev-dependent nuclear export. SF2/ASF did not affect the expression of Rev, but inhibited Rev-dependent nuclear export activity and EIAV replication in a dose-dependent manner. Further, the inhibition mapped to the RNA binding domain and correlated with RNA substrate specificity. These data indicate that SF2/ASF binding the viral RNA inhibits both Rev activity and EIAV replication, and suggest that Rev and SF2/ASF are functionally competitive. If so, the intracellular concentrations of SR proteins may be an important factor in the regulation of EIAV replication.

Materials and Methods

PCR and Plasmid Construction. All plasmids and constructs were confirmed by sequence analysis (Iowa State University DNA Synthesis and Sequencing Facility). PCRs for the construction of plasmids were carried out using 1 μM primers and standard protocols as per the manufacturer (Perkin Elmer, Foster City, CA). Unless otherwise indicated, PCRs consisted of 30 cycles of 2 min of denaturation at 94°C, 1 min of annealing at 55°C, and 1 min of extension at 72°C, followed by an additional cycle with a 7 min extension.

The expression plasmid pERev contains a Rev cDNA derived from a MA-1 isolate of EIAV previously described (9). pΔERev was constructed by the digestion and ligation of two Rev fragments to produce a cDNA that encodes ERev without amino acids 19-23. The upstream fragment, which encodes amino acids 1-18, was PCR amplified using the 5’ primer AATACGACTCACTATAG and the 3’ primer GAGTCTAGATTCTTCTTTTCAG. The
downstream fragment, which encodes amino acids 24-165, was PCR amplified using the 5' primer GAGTCTAGAGACTGGTGAAA and the 3' primer GAGGAATTCTCATAATGTTTCCTCCT. The underlined nucleotides indicate an introduced XbaI site at the end and beginning of the two respective fragments. The resulting PCR products were digested with XbaI, ligated together using standard protocols, and amplified with the 5' primer TAAGAACAGCATGGCAGAATCGAAG and the 3' primer GAGGAATTCTCATAATGTTTCCTCCT. The resulting cDNA was TA cloned into the eukaryotic expression vector pCR3.1 (Invitrogen, Carlsbad, CA).

A recombinant baculovirus that expresses SF2/ASF was generously provided by Tom Maniatis (Harvard University). The SF2/ASF cDNA was amplified from purified baculovirus DNA using the 5' primer ACCGCCATGTGAGAGGT and the 3' primer ATCTTATGTACGAGAGCG, and TA cloned into pCR3.1. The SF2/ASF deletion mutants pSF2ARS and pSF2ARRMS were also constructed by PCR amplification from recombinant baculovirus DNA and TA cloning into pCR3.1. The SF2ARS cDNA was constructed by amplifying a SF2/ASF fragment that encodes amino acids 1-195 using the 5' primer ACCGCCATGTGAGAGGT and the 3' primer GGGCTAATCAACTTTAACCCGG. The underlined codon of the 3' primer indicates the anti-sense introduced stop codon after amino acid 195. The SF2ARRMS cDNA was constructed by amplifying a SF2/ASF fragment that encodes amino acids 196-248 using the 5' primer ACCGCCATGTGGCGGAGGT and the 3' primer ATCTTATGTACGAGAGCG. The underlined codon in the 5' primer indicates a start codon introduced before amino acid 196.
Both pSF2ΔRRM1 and pSF2ΔRRM2 were constructed by PCR amplification from pSF2/ASF and TA cloning into pCR3.1. The SF2ΔRRM1 cDNA was constructed by amplifying a SF2/ASF fragment that encodes amino acids 107-248 using the 5' primer ACCGCCATGGCTCCCCGAGGTCGC and the 3' primer GGATCCATCTTATGTACGAGAGCG. The SF2/ASF deletion mutant SF2ΔRRM2 was constructed by amplifying the RRM1 and RS domain cDNAs with primers that introduce a *Pvu*I restriction site into both. The RRM1 cDNA encoding amino acids 1-97 was amplified with the 5' primer ACCGCCATGTCGGGAGGT and 3' primer ATCGATCGTCGGCCTGTCC. The RS domain cDNA encoding amino acids 198-248 was amplified with the 5' primer TACGATCGCCAAGTTATGGA and 3' primer GGATCCATCTTATGTACGAGAGCG. The underlined nucleotides in the primers indicate the *Pvu*I restriction site. The PCR products were digested with *Pvu*I and ligated together using standard protocols. The full length SF2ΔRRM2 cDNA was then amplified using the 5' primer ACCGCCATGTCGGGAGGT and the 3' primer GGATCCATCTTATGTACGAGAGCG, and the resulting cDNA was cloned into pCR3.1.

Subcellular localization of SF2/ASF and the SF2/ASF deletion mutants were assessed using GFP fusion proteins. Restriction digestion of the flanking *EcoR*I and *BamH*I sites in the multiple cloning site of pCR3.1 produced cDNA fragments that were cloned into the multiple cloning site of an *EcoR*I and *BamH*I digested GFP vector, pEGFP-C2 (BD Biosciences Clontech, Palo Alto, CA). One μg of each GFP fusion construct was transfected into Cf2Th cells, and 48 hours post-transfection, nuclear localization was observed by light microscopy using an inverted Nikon Diaphot fluorescence microscope with a 40X objective, a 100W high-pressure mercury lamp, and epifluorescence filters.
A plasmid containing the cDNA of SC35 was obtained from ATCC (ATCC#95691), and the cDNA was amplified and TA cloned into pCR3.1 using the 5' primer GATTTAGGTGACACTATAG and the 3' primer TAATACGACTCACTATAGGG.

**Transfections.** Transfections were performed according to the reagent protocol with TransIT-LT1 transfection reagent (Mirus, Madison, WI) and canine fetal thymus (Cf2Th) cells. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and penicillin-streptomycin. In all transfections, pcDNA3 (Invitrogen, Carlsbad, CA) was used to equalize the amount of DNA per transfection, and TransIT-LT1 was used at a ratio of 2 µl:1 µg DNA.

**Western blot analyses.** Cf2Th cells were transfected with 100 ng of pΔERev, 200 ng of the pCH110, increasing concentrations (50, 100, or 500 ng) of pSF2/ASF, and a varying concentration of pcDNA3. Two days post-transfection, cells were harvested, resuspended in a 100 µl of 0.25 M Tris-HCl (pH 7.5), and 50 µl was lysed by freeze/thawing and assayed for β-galactosidase activity. The remaining volume was lysed by adding 2X lysis buffer (63 mM Tris-HCl, pH 6.8, 1% (w/v) SDS, 10% (v/v) β-mercaptoethanol, 0.004% (w/v) bromophenol blue). Samples were normalized for β-galactosidase activity, and electrophoresed in a 12% SDS-PAGE gel. Duplicate gels were blotted, blocked with a 1% gelatin solution, and reacted with either EIAV convalescent horse serum or a monoclonal anti-SF2/ASF antibody (Zymed Laboratories, San Francisco, CA). Blots were then reacted with 125I-protein G (Amersham Biosciences, Allington Heights, IL) or 125I-anti-mouse Ig (Amersham Biosciences, Allington Heights, IL) and analyzed with a Personal Molecular Imager FX PhosphorImager (Biorad, Hercules, CA) using Quantity One (Biorad, Hercules, CA) imaging and analysis software. For Western blot analyses of SC35, a commercially
available anti-SC35 monoclonal antibody (Sigma, St. Louis, MO) was used. One μg of pSC35 was transfected into Cf2Th cells, and two days post-transfection Western blot analyses of transfected cell lysates showed protein expression of SC35 higher than background levels of non-transfected cell lysates.

**CAT assays.** The Rev nuclear export reporter pERRE-1A has been previously described (4). Briefly, pERRE-1A contains EIAV nucleotides 5281 to 5795 downstream of a CAT gene. Thus, pERRE-1A includes the identified EIAV RRE (nucleotides 5485 to 5540), (Figure 1B). The CAT gene and RRE sequences are flanked by an upstream splice donor and a downstream splice acceptor. In the presence of Rev, reporter transcripts are bound by Rev, exported into the cytoplasm, and CAT is expressed. In the absence of Rev, reporter transcripts are spliced, removing the CAT gene and RRE. Thus, no CAT is expressed. Cf2Th cells were transfected with 1 μg of pERRE-1A, 0.2 μg of pCH110 and the required expression plasmids. Two days post-transfection cells were harvested and resuspended in 0.5 ml of 0.25 M Tris-HCl (pH 7.5). Cells were lysed by freeze/thawing, and lysates were assayed for β-galactosidase activity to normalize for transfection efficiency. Normalized lysates were assayed for CAT expression with a commercially available CAT enzyme-linked immunosorbent assay (ELISA) kit (Roche Molecular Biochemicals, Indianapolis, IN). Pilot assays were performed with pΔERev and pERRE-1A co-transfected cell lysates to determine assay conditions within the linear range of the CAT ELISA. The results of pilot assays were used to adjust the normalized amounts of cell lysates to assay conditions, and all sample lysates were subsequently assayed for CAT.

**Virus replication assays.** The EIAV infectious molecular clone pSPEIAV-19R was kindly provided by Susan Payne (University of Texas, Arlington) and has been previously
described (38). Cf2Th cells were co-transfected with 2 µg of pSPEIAV-19R, 0.2 µg of pCH110, and the various expression plasmids. Supernatant was collected and cells were harvested, lysed, and assayed for β-galactosidase activity. Cell-free, infectious virus was titered on equine dermal (ED) cells with a focal immunoassay using a chromogenic method of detection as previously described (44). Briefly, harvested culture supernatant samples were clarified by centrifugation at 2000 x g for 5 min, serially diluted in DMEM containing polybrene (8 µg/ml), and added to cultures of ED cells. Five days post-inoculation, ED cells were washed with TNE buffer (10 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, pH 7.4), and fixed with 100% methanol. Cells were incubated with an EIAV convalescent horse serum, followed by incubation with a horseradish peroxidase-conjugated goat anti-horse IgG polyclonal antibody, and incubation with the AEC substrate (0.2 mg/ml 3-amino-9-ethylcarbazole, 0.01% H₂O₂, 0.05 M NaOAc, pH 5.0). Focus forming units (FFUs) were counted and results were normalized to β-galactosidase activity.

Reverse transcriptase (RT) assays were performed as previously described (9). Briefly, 10 µl of clarified culture supernatant was mixed with 30 µl RT cocktail (50 mM Tris-HCl, pH 7.9, 75 mM KCl, 2 mM dithiothreitol, 5 mM MgCl₂, 0.05% Nonidet P-40, 4 µg/ml oligo(dT), 5 µg/ml polyriboadenylic acid, 20 µCi/ml [α-³²P]dTTP), incubated at 37°C for 2 hours, and 8 µl was spotted onto DE81 paper. After washing, the DE81 paper was analyzed using a phosphorimager and software as described above. Results were normalized to β-galactosidase activity.
Results

SF2/ASF inhibits EIAV Rev-dependent nuclear export. In previous studies, we determined that increasing concentrations of the SR protein SF2/ASF resulted in a dose-dependent decrease in Rev nuclear export activity (4). The first exon of the Rev cDNA contains the EIAV RRE which binds SF2/ASF (13) and functions as an ESE (ESE/RRE) (4). Therefore, it is possible that SF2/ASF binding to rev mRNA could affect Rev expression in transient assays. The transient assays are done in the linear range of Rev activity, in which the levels of Rev protein are below the limits of our detection. To rule out the possibility that the observed decrease in activity was due to SF2/ASF inhibition of Rev protein expression rather than an inhibition of Rev function, we constructed a Rev cDNA expression plasmid in which we deleted the poly-purine stretch in the ESE/RRE, thus eliminating the SF2/ASF binding site (ΔERev). Cf2Th cells were co-transfected with pΔERev in the presence of increasing amounts of pSF2/ASF, and cell lysates were analyzed by Western blot analysis. Increasing concentrations of pSF2/ASF did not decrease levels of ΔERev protein (Figure 2A). The nuclear export activity of ΔERev was comparable to wild-type Rev (Figure 2B), although it was less efficient in the lower range of concentrations tested. Therefore, ΔERev was used to further examine the effect of SF2/ASF on Rev function.

Increasing concentrations of pSF2/ASF resulted in a significant, dose-dependent decrease in Rev nuclear export activity ($P<0.05$) (Figure 3A). In the absence of ΔERev, there was little change in the expression of CAT over the range of pSF2/ASF concentrations tested. Therefore, the inhibition of nuclear export by SF2/ASF was not due to an independent effect on the CAT reporter plasmid, but to a specific inhibition of Rev function. CAT levels were restored when increasing amounts of pΔERev were co-transfected with an
inhibitory amount of pSF2/ASF (Figure 3B). This suggested that ΔERev and SF2/ASF compete for a factor in the Rev export pathway.

**The amino-terminal RNA recognition motifs of SF2/ASF are necessary and sufficient to inhibit Rev-dependent nuclear export.** SR proteins are comprised of modular, functional domains, including one or two amino-terminal RRM domains and a carboxy-terminal RS domain (12). The RRM domains function typically in RNA binding, whereas the RS domain mediates protein-protein interactions. SELEX analyses of individual members of the SR protein family have shown differences in RNA binding specificity, depending on the identity and number of RRM domains (Figure 4). For example, the SR protein SF2/ASF contains two RRM domains, and the RNA binding specificity differs depending on whether one or both RRM domains are present (48). The ESE/RRE in EIAV rev exon 1 contains multiple copies of a near perfect binding site for SF2/ASF. To determine if binding to the ESE/RRE plays a role in the inhibition of Rev dependent nuclear export activity, we constructed a series of SF2/ASF deletion mutants that lack either the RS domain or one or both of the RRM domains (Figure 5A). Nuclear localization of each construct was confirmed using GFP-fusion proteins (data not shown).

Each SF2/ASF mutant was tested for effect on Rev nuclear export activity in transient expression assays (Figure 5B). In the absence of ΔERev, none of the constructs had an inhibitory effect on CAT expression from the reporter plasmid (data not shown). Deletion of the RS domain (SF2ΔRS) resulted in a significant inhibition of Rev activity to levels equivalent to that of wild-type SF2/ASF (Figure 5B). However, deletion of one or both of the amino-terminal RRM domains (SF2ΔRRMS, SF2ΔRRM1, SF2ΔRRM2) had no significant effect on Rev activity (P>0.05). To rule out that these results reflected differences in levels of
protein expression, assays were done using increasing concentrations of deletion plasmids. Co-transfection of up to 10 fold more of either pSF2ΔRRM1 or pSF2ΔRRM2 resulted in no significant inhibition of Rev activity (data not shown). Therefore, the presence of RRM1/RRM2 was both necessary and sufficient for SF2/ASF inhibition of Rev activity. The RNA binding specificity of both SF2/ASF and SF2ΔRS is conferred by RRM1/RRM2, and distinct from the RNA consensus binding specificity conferred by RRM1 alone (Figure 4). Moreover, the consensus RRM1/RRM2 binding site is nearly identical to multiple parts of the EIAV ESE/RRE. The correlation between RNA binding specificity and Rev inhibition suggests that binding to the viral RNAs at the ESE/RRE is required for SF2/ASF inhibition of Rev activity.

The amino-terminal RNA recognition motifs of SF2/ASF are necessary and sufficient to inhibit EIAV replication. Rev is absolutely required for virus replication, and factors that inhibit Rev would be predicted to inhibit EIAV replication. To determine if SF2/ASF could inhibit production of infectious virus, Cf2Th cells were transfected with an infectious molecular clone in the presence or absence of pSF2/ASF. In cells transfected with the proviral clone and pcDNA3, the supernatant reverse transcriptase (RT) activity increased over 48 hours. In the presence of pSF2/ASF, RT activity was only slightly higher than background levels over the same time period (Figure 6A), such that by 48 hours, there was 15-fold less RT activity in pSF2/ASF-transfected cells as compared to cells transfected with pcDNA3. When increasing amounts of pSF2/ASF were co-transfected, we observed a dose-dependent decrease in virus titer, with nearly a 2-log reduction in virus titer in the presence of 1 µg of transfected pSF2/ASF (Figure 6B). To map the domains of SF2/ASF that inhibit EIAV replication, each SF2/ASF deletion construct was co-transfected with the proviral
clone. SF2ΔRS inhibited virus replication similar to wild-type SF2/ASF, whereas no significant inhibition was observed with SF2ΔRRMS, SF2ΔRRM1, or SF2ΔRRM2 (P>0.05) (Figure 6C). Therefore, the presence of RRM1/RRM2 was necessary and sufficient to inhibit virus replication, indicating that the inhibition of EIAV replication correlated with SR protein RNA binding specificity.

Results using SF2/ASF deletion constructs indicated that inhibition of EIAV Rev activity correlated with RNA binding specificity of the SR protein. SC35 is another member of the SR protein family, but SC35 has only one RRM and differs from SF2/ASF in RNA binding specificity (Figure 4) (7,12,23,34,47,51,52). To rule out that the SR protein inhibition of EIAV replication was due to an excess of SR proteins, we co-transfected the EIAV molecular clone with either pSF2/ASF or pSC35. As before, co-transfection with pSF2/ASF resulted in a significant reduction in virus titer (Figure 7A). SC35 did inhibit EIAV replication, but the reduction was less than 10% of the inhibition observed with SF2/ASF (6-fold verses 68-fold) (Figure 7A). Western blot analyses of cells transfected with pSF2/ASF or pSC35 showed increased expression of both SR proteins above constitutive levels (data not shown). Thus, while some inhibition of virus replication might be attributed to excess SR protein, it appeared that the primary effect of SF2/ASF on EIAV replication was specific to SF2/ASF and correlated with the RNA binding specificity of the SR protein.

To determine if the inhibition of EIAV replication was due to the inhibition of Rev activity, we used an in vitro system that requires the presence of Rev in trans to produce virus. Cf2Th/51 is a stably transfected cell line containing a Rev-defective provirus, and RT activity is produced only in the presence of Rev (3). When Cf2Th/51 cells were trans-complemented with ΔERev and SF2/ASF, there was nearly a 3-fold decrease in supernatant
RT activity (Figure 7B). In contrast, when ΔERev was expressed in Cf2Th/51 cells with SC35, there was only a modest 20% reduction in supernatant RT activity. Thus, the marked inhibition of EIAV replication appeared to be due to a SF2/ASF-specific inhibition of Rev activity. Together, the results further support a model wherein SF2/ASF inhibits EIAV replication by competing with Rev for binding viral mRNAs and inhibiting Rev function.

Discussion

One function of SR proteins is to bind exon splicing enhancers (ESEs) and enhance splice site recognition and exon inclusion in pre-mRNA splicing. The lentiviral protein Rev binds the Rev responsive element (RRE) and mediates the nuclear export of unspliced and incompletely spliced viral mRNAs, and is necessary for viral replication. In EIAV, the first exon of rev contains a purine-rich sequence that functions as both a ESE and as a RRE (ESE/RRE) (4,21). The ESE/RRE binds both SF2/ASF and ERev (4,13,21), and ERev inhibits exon inclusion in in vitro splicing assays (4). In the present study, we examined SF2/ASF inhibition of ERev-dependent nuclear export activity. SF2/ASF did not inhibit the expression of Rev protein, but caused a dose-dependent decrease in Rev-dependent nuclear export activity and EIAV replication. Rev activity could be restored by increasing concentrations of pΔERev, suggesting that SF2/ASF and Rev compete for some limiting factor in the Rev-dependent nuclear export pathway. Deletion analyses indicated that the RRM1/RRM2 of SF2/ASF was both necessary and sufficient to inhibit Rev activity and EIAV replication. There was correlation between RNA binding specificity of SF2/ASF and Rev inhibition, which suggested that SF2/ASF binding viral mRNAs inhibits both Rev activity and virus replication. Together, these results support a model wherein SF2/ASF and
Rev compete for binding to viral mRNAs at the ESE/RRE. Therefore, factors that modulate intracellular concentrations of SF2/ASF may be important in the regulation of EIAV Rev-dependent nuclear export activity and virus replication.

The primary function of an SR protein RRM is to determine substrate specificity by binding RNA in a sequence specific manner (12,34,49,51). Our data indicate that SF2/ASF binding the ESE/RRE is required for the inhibition of Rev-dependent nuclear export activity and EIAV replication, and suggest that SF2/ASF competes with Rev in Rev-dependent nuclear export. Together, this supports a model wherein SF2/ASF competes with Rev for binding the ESE/RRE. However, it remains unclear if the binding of SF2/ASF inhibits the binding of Rev, or just Rev-dependent nuclear export activity. Rev binding to the ESE/RRE inhibits ESE-dependent EIAV splicing (4,21). Therefore, it appears that binding the ESE/RRE by either Rev or SF2/ASF inhibits the function of the other protein. Thus, it remains to be determined if the SF2/ASF and Rev functional competition is the result of the binding competition. Further studies must be done to elucidate the mechanism by which SF2/ASF and Rev bind the ESE/RRE and to better understand the role of SF2/ASF in rev exon 1 inclusion during splicing.

There are three, nearly consecutive AAAGAAGAA sequence motifs in the ESE/RRE that mediate Rev binding, Rev-dependent nuclear export, SF2/ASF binding, and *in vitro* splicing (Table 2) (4,13). SF2/ASF can bind RNA that contains either one, two, or three motifs (13), whereas the two 3' motifs of the ESE/RRE appear to be necessary to promote exon inclusion in an *in vitro* splicing assay (Table 2) (4). Interestingly, Rev binds RNA containing either or both of the two 3' motifs, yet wild-type Rev-dependent nuclear export activity depends on the presence of all three motifs (4). While it is not clear if both SF2/ASF
and Rev bind the exact same sequences, it is apparent that binding of one inhibits function of the other. Chung and Derse have suggested that the ESE and RRE sequences are distinct, yet overlap, and they have shown that Rev and SF2/ASF can bind the ESE/RRE simultaneously (13). This suggests that the requirements for binding may be less stringent than the requirements for functional activity, as data presented here suggested that Rev and SF2/ASF are functionally competitive. If Rev nuclear export activity is dependent on presence of all three motifs, it is possible that SF2/ASF binding at any one of the ESE/RRE motifs would inhibit Rev nuclear export activity, but not binding. Further investigations are necessary to characterize the specific interactions among SF2/ASF, Rev and the ESE/RRE that are required for exon inclusion and nuclear export.

The SR proteins play a significant role in the regulation of gene expression in a number of viruses. In adenovirus replication, the early and late phases of transcript expression are regulated by the phosphorylation state of the SR proteins in infected cells (24,26,36). Early transcripts express the viral protein E4-ORF4, which preferentially interacts with hyperphosphorylated SR proteins as well as the cellular protein phosphatase PP2A (24). The interactions among E4-ORF4, SR proteins, and PP2A induce the dephosphorylation and inactivation of the SR proteins (6,36), which shifts the phase of transcript expression from early to late. Thus, exogenous, hyperphosphorylated SR proteins added during the early phase of mRNA expression inhibit the shift to the late phase and subsequently, inhibit virus replication (35). In the lentivirus HIV, SF2/ASF has been reported to bind the HIV RRE in a Rev-dependent manner and inhibit both Rev nuclear export activity and replication (41). The HIV RRE is neither an ESE nor is it located with the Rev gene, however, the RRM1/RRM2 of SF2/ASF was sufficient to inhibit HIV Rev
activity (40,41). Other HIV studies suggest that there may be a number of other proteins that interact with SF2/ASF, HIV Rev, and the HIV RNA to regulate Rev activity and virus replication (31,39,40,50). The finding that SF2/ASF can modulate EIAV Rev activity provides an additional example of how viruses utilize complex cellular mechanisms of gene regulation to regulate viral gene expression.

SR protein expression varies in different tissues and cellular activation states, which can result in the expression of alternatively spliced mRNAs in a tissue or cell-specific manner (22,43,53). For example, SF2/ASF expression increases in T lymphocytes in response to activation, resulting in the alternative splicing of CD45 mRNAs (25). Our results show that SF2/ASF inhibits EIAV replication in a dose-dependent manner. EIAV replicates in monocytes/macrophages, and we have previously shown that activated and infected macrophages impair viral replication through an, as yet, unidentified post-transcriptional mechanism (44). It is possible that the activation of macrophages increases the cellular expression of SF2/ASF, thereby decreasing viral replication by binding viral RNAs at the ESE/RRE and inhibiting Rev activity. Indeed, SR protein expression varies during development, differentiation, and proliferation (15). Further investigations to examine intracellular concentrations of SF2/ASF during EIAV infection may identify a role for SF2/ASF in regulating EIAV replication in vivo.
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### Table 1. Summary of Rev, SF2/ASF, and ESE/RRE Interactions.

<table>
<thead>
<tr>
<th>RNA Motifs of the ESE/RRE</th>
<th>RNA Functional Activities</th>
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<td>Rev&lt;sup&gt;a&lt;/sup&gt; Binding</td>
</tr>
<tr>
<td>AAAGAAGAAATTCAAAGAAGAAAAAGAAGAA</td>
<td>+++&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
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<td>AAAGAAGAA</td>
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<sup>a</sup> RNA binding studies were performed using Rev and the ESE/RRE as a substrate in previous studies (4). The symbols indicate the binding activity of the motif(s).

<sup>b</sup> Transient Rev-dependent nuclear export assays were performed with Rev reporter plasmids containing the ESE/RRE in previous studies (4). The symbols indicate the Rev-dependent nuclear export activity of the motif(s).

<sup>c</sup> RNA binding studies were performed in previous studies using SF2/ASF and the RNA sequences listed as substrates (13). The symbols indicate the ability of the motif(s) to bind SF2/ASF. ND indicates not determined.

<sup>d</sup> *In vitro* splicing assays were performed in previous studies with RNA substrates containing the motif listed (4). The symbols indicate the effect of the motif(s) on splicing of the substrate.

<sup>e</sup> The activity of the RNA motifs on protein binding, nuclear export, or *in vitro* splicing activity of the RNA motifs are indicated by symbols representing a gradation of effects from no activity (-) to highest activity (+++).
**Figure Legends**

Figure 1. The EIAV genome, mRNAs, proteins, and rev exon 1 sequence. (A) The EIAV genome, the mRNAs produced during replication, and the proteins they encode. The boxes indicate the open reading frames. The horizontal, thick lines indicate the exon sequences of the mRNA, and the diagonal thin lines indicate the spliced out RNA sequence. Messenger RNA1 is a bicistronic, four exon mRNA that encodes both Tat (t) and Rev (r). Messenger RNA2 is similar to mRNA1, but lacks rev exon 1 and encodes only Tat. The structural genes of EIAV, including gag, pol, and env, are encoded by the singly spliced mRNA3 and the unspliced mRNA4. (B) The sequence of rev exon 1. The Rev start codon is underlined, and the light grey box indicates the identified EIAV RRE (light grey box)(4). The dark gray boxes indicate the purine-rich sequences that function in both Rev binding, SF2/ASF binding, Rev-mediated nuclear export, and ESE function. SA indicates the upstream splice acceptor and SD indicates the downstream splice donor of the exon.

Figure 2. Characterization of ΔERev expression and activity. (A) Western blot analysis of ΔERev expression in the presence of increasing concentrations of SF2/ASF. The bar graph represents the counts of ΔERev obtained from phosphorimager analysis of the blot shown. (B) Comparison of Rev nuclear export activity between wild-type Rev (white bars) and ΔERev (gray bars). Results represent the mean of two independent transfections for wild-type pERev and five independent transfections for pΔERev. The error bars of the figure and subsequent figures represent the standard error of the mean. Asterisks indicate the concentration at which there is a significant difference in nuclear export activity between wild-type Rev and ΔERev ($P<0.01$).
Figure 3. SF2/ASF inhibits Rev-dependent nuclear export activity. (A) Cf2Th cells were co-transfected with 1 µg pERRE-1A and increasing concentrations of pSF2/ASF in the presence (−) or absence (---) of 50 ng pΔERev. The results represent the mean value of at least six independent transfections in the presence of pΔERev and three independent transfections in the absence of pΔERev. The asterisk indicates the concentration at which there was no significant reduction of Rev nuclear export activity as compared to cells transfected in the absence of pSF2/ASF (P>0.05). All other concentrations of pSF2/ASF above 1 ng resulted in a significant reduction of Rev nuclear export activity as compared to cells transfected in the absence of pSF2/ASF (P<0.05). (B) Cf2Th cells were co-transfected with 1 µg pERRE-1A, 50 ng pSF2/ASF, and increasing concentrations of pΔERev. The results represent the mean of at least three independent transfections. All concentrations above 0 ng pΔERev resulted in a significant difference from cells transfected in the absence of pΔERev (P<0.05).

Figure 4. Consensus RNA sequences bound by SR protein RRMs. SELEX analyses of the individual RRMs of SF2/ASF and SC35 result in a number of RNA sequences that both SR proteins will bind. The consensus sequence motifs of those RNA sequences are listed. The symbols for alternative bases are: R=A/G, S=C/G, M=A/C, W=A/U, Y=C/U, and K=G/U. The SF2/ASF consensus RNA binding motif RGAAGAAC nearly matches the three AGAAGAAA motifs in the ESE/RRE.

Figure 5. RRM1/RRM2 of SF2/ASF is necessary and sufficient to inhibit Rev-dependent nuclear export activity. (A) SF2/ASF consists of two amino-terminal RRMs and a carboxy-terminal RS domain. SF2/ASF deletion mutants were constructed and lack the RS domain or
one or both of the RRM1s. (B) Cf2Th cells were co-transfected with 1 µg of pERRE-1A, 50 ng of pΔERRev (if indicated) and 50 ng of SF2/ASF construct. Results represent the mean value of at least six independent transfections and are expressed as a percentage of the ΔERRev activity. The asterisks indicate significant differences from cells co-transfected with only pERRE-1A and pΔERRev ($P<0.001$). There is no significant difference between values from transfections with either pSF2/ASF or pSF2/ARS ($P>0.05$).

Figure 6. SF2/ASF and RRM1/RRM2 inhibit EIAV replication. (A) 2 µg of either pSF2/ASF (---) or pcDNA3 (—) were co-transfected into cells with the EIAV infectious molecular clone pSPEIAV-19R. (A) Supernatant RT activity over 48 hours. The results represent the mean of six independent transfections as determined by phosphorimager analyses of RT assays. (B) Infectious virus collected at 48 hours after co-transfection with 2 µg of pSPEIAV-19R and increasing concentrations of pSF2/ASF was titrated in equine dermal (ED) cells. The results represent the mean of three independent transfections from one of two experiments and are reported as focus forming units per mL of clarified supernatant (FFU/mL). Values from all three concentrations of pSF2/ASF were significantly different from control transfections with only pSPEIAV-19R ($P<0.0001$). (C) 1 µg of each SF2/ASF expression construct was co-transfected into cells with 2 µg of pSPEIAV-19R, and 48 hours post-transfection clarified supernatants were titrated for infectious virus on ED cells. The results represent the mean of three independent transfections from one of two experiments. The asterisks represent values significantly different from cells transfected with only pSPEIAV-19R ($P<10^{-8}$). There is no significant difference between values from transfections with either pSF2/ASF or pSF2ARS ($P>0.05$).
Figure 7. SF2/ASF inhibition of EIAV replication is due to a specific inhibition of Rev activity. (A) Cf2Th cells were co-transfected with 2 µg of pSPEIAV-19R and 1 µg of either pcDNA3, pSF2/ASF, or pSC35. At 48 hours post-transfection, clarified supernatants were assayed for infectious virus. The results represent the mean of three independent transfections from one of two experiments, and the error bars represent the standard error of the mean. The asterisks represent values significantly different from cells transfected with only pSPEIAV-19R ($P<0.001$). (B) Cf2Th/51 cells were co-transfected with 100 ng of pΔERrev and 1 µg of either pcDNA3, pSF2/ASF, or pSC35. 48 hours post-transfection, clarified supernatants were assayed for RT activity, and quantified by phosphorimager analysis. The results represent the mean of at least six independent transfections. The asterisks indicate a significant difference in values from cell transfected with pcDNA3 ($P<0.01$).
A. mRNA

| 1 | 2 | 3 | 4 |

Protein

Tat, Rev

Tat

Env

Gag, Pol

Figure 1.
Figure 2.
Figure 3.
<table>
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<tr>
<th>SR Protein</th>
<th>Domain(s)</th>
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<td>RRM1/RRM2</td>
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Figure 4.
A.  

SF2/ASF
SF2ΔRS
SF2ΔRRMS
SF2ΔRRM1
SF2ΔRRM2

B.  

Figure 5.
CHAPTER 4. GENERAL CONCLUSIONS

Retroviruses utilize the post-transcriptional mechanism of splicing to produce the necessary viral mRNAs for replication. Splicing during replication of simple retroviruses produces only a singly spliced viral mRNA. Splicing during replication of complex retroviruses, such as HIV, may produce more than 30 multiply spliced viral mRNAs (6). Splicing in EIAV replication results in four major mRNA transcripts. As with other lentiviral Rev proteins, EIAV Rev binds and exports the unspliced and singly spliced viral mRNAs into the cytoplasm. Because Rev is absolutely required for virus replication, factors that can inhibit Rev function would be expected to inhibit virus replication. EIAV produces a persistent, life-long infection, and most infected animals show no sign of infection. Unlike other lentiviral infections, which result in a chronic disease course, EIAV infection results in a variable disease course, including periods of acute, chronic and/or inapparent disease. The acute and chronic disease courses are characterized by periods of high virus replication associated with cycles of fever, thrombocytopenia, and/or anemia (7,8). Therefore, virus and host cell factors that affect virus replication, may also play a role in EIAV pathogenesis.

Alternatively Spliced Tat Transcripts

Splicing of the EIAV primary transcript produces, among other transcripts, a four-exon, bicistronic mRNA that encodes Tat and Rev. The presence of Rev results in exon exclusion and the subsequent expression of an alternatively spliced mRNA similar to four-exon mRNA, but lacking exon 3. Exon 3 is also rev exon 1, and thus, Rev mediates production of mRNAs that only encode Tat. Interestingly, the Tat start codon is a CUG, and
only by leaky scanning through the CUG is translation initiated at the Rev start codon, which is the first AUG of the bicistronic message (2). Though the presence of a non-AUG start codon upstream of an AUG start codon of a bicistronic message is not uncommon, the fact that the start codon is a CUG and the fact that is the transcript is viral makes the bicistronic EIAV mRNA uncommon.

We have found that the bicistronic and monocistronic Tat transcripts have the same Tat activity, suggesting that the RNA sequences of exon 3 do not affect translation initiation at the Tat CUG (Chapter 2). Our results also suggest that initiation at the Rev AUG and the RRE of exon 3 do not have an effect on Tat translation. This is consistent with the mechanism of leaky scanning, as Kozak states that translation initiation of the first start codon is not influenced by a better initiation site downstream (5). Thus, the findings from this work indicate that exclusion of exon 3 is not a mechanism to directly regulate Tat expression, but may be a mechanism to produce Tat without producing more Rev.

**SF2/ASF Inhibits EIAV Rev-mediated Nuclear Export and EIAV Replication**

Interestingly, rev exon 1 contains a sequence that is both an ESE and an RRE (ESE/RRE). This is unique among the lentiviruses as other lentiviral RREs are not ESEs and are not located within RNA that encodes Rev. Though the ESE/RRE binds the SR protein called SF2/ASF as well as Rev, the role of each in exon 3 inclusion/exclusion is not fully described (1,3). Earlier work established that Rev inhibits ESE activity (1,4). We have found that SF2/ASF inhibits Rev nuclear export activity in a dose-dependent manner, but not by inhibiting Rev expression (Chapter 3) (1). Further, Rev and SF2/ASF appear to functionally compete with one another.
As might be expected of a protein that inhibits Rev function, SF2/ASF also inhibited EIAV replication. In fact, SF2/ASF potently inhibited EIAV replication. The inhibition of both Rev nuclear export activity and EIAV replication mapped to the RNA binding domain, made up of both RNA recognition motifs of SF2/ASF. These results suggest that SF2/ASF and Rev may compete for binding the ESE/RRE. Because functional activity is dependent on binding for Rev and typically so for SF2/ASF, a model of competitive binding would support the *in vitro* functional competition observed.

Viruses do interact with SR proteins in many ways, and can exploit many of their functions, which may influence both virus replication and host cells. Interestingly, the impact of viruses on host cell gene expression through the exploitation of the SR proteins has not been studied and may be an important area to investigate. Our results suggest that Rev and SF2/ASF compete for binding the ESE/RRE, and it is possible that Rev and SF2/ASF bind the same sequences. If so, Rev may bind cellular RNA sequences that are normally bound by SF2/ASF, and could potentially affect cellular gene expression.

**Future studies**

The studies of this dissertation biologically characterize the effects of the interactions of EIAV with Rev and SF2/ASF. The identification of SF2/ASF as a potent inhibitor of virus replication *in vitro* suggests that the protein may be a cellular factor involved in EIAV pathogenesis. There are a number of questions that need to be answered as to the role of SF2/ASF in EIAV replication. Though we show that SF2/ASF inhibited both Rev nuclear export activity and EIAV replication, we have not directly tested if the inhibition of EIAV replication is due to an inhibition of Rev nuclear export activity. Therefore, future studies
should address this through Northern analysis of cytoplasmic and nuclear viral RNAs in the presence of SF2/ASF.

From the research of this dissertation, the most logical, next step is to characterize the specific interactions of Rev and SF2/ASF with the ESE/RRE. Only a few mutations have been made in the ESE/RRE to investigate the RNA requirements for protein binding. Therefore, purification of both Rev and SF2/ASF and their use in RNA gel shift assays will determine if Rev and SF2/ASF compete for binding the ESE/RRE. A variety of RNA substrates should also be used to determine if the ESE/RRE has important secondary structure for protein binding. The results of these assays will assist in determining the mechanism behind the functional competition apparent between Rev and SF2/ASF. I and others have put a major effort into the purification of Rev protein for use in the assays, and the protocol to purify Rev is nearly complete. Therefore, the purification of Rev should also be pursued in the future, and the successful purification of functional Rev will be necessary to directly test the hypothesis that Rev competes with SF2/ASF for binding the ESE/RRE.

In addition, the exact role of SF2/ASF in the activity of the ESE of exon 3 is unknown, and future work should determine the role of SF2/ASF in exon 3 inclusion. SF2/ASF binds the ESE in in vitro binding assays, but purified SF2/ASF has never been used. The protein used in binding assays was from purified, total SR proteins (3). In addition, in vitro splicing assays that have shown the Rev inhibition of ESE function have always used purified, total SR proteins (1). Therefore, it will be important to use purified SF2/ASF in in vitro splicing assays to determine if SF2/ASF is sufficient to promote exon 3 inclusion, and if purified Rev can inhibit exon inclusion.
It will also be important to determine if the SF2/ASF inhibition of EIAV replication is biologically significant in *in vivo* virus replication. Though SF2/ASF expression has been shown to vary among different tissues and cellular states, the levels of cellular expression during replication and/or during different stages of disease are not known. Therefore, the examination of SF2/ASF expression in cells before and after virus infection will assist in determining if endogenous levels of SF2/ASF affect EIAV replication.

**References**


APPENDIX A. THE PURIFICATION OF EIAV REV

Introduction

Members of the lentivirus subfamily of retroviruses produce viral proteins during replication that function in regulating the expression of viral mRNAs. The two most common regulatory proteins are called Tat and Rev. Where Tat assists RNA polymerase II in elongation to up-regulate viral transcription, Rev is an essential protein that functions by localizing in the nucleus, binding unspliced or incompletely spliced viral mRNAs at a nucleotide sequence called the Rev responsive element (RRE), and exporting the RNAs to the cytoplasm through the exportin 1 pathway of nuclear export (reviewed in 4). The structural proteins necessary for replication are encoded by the unspliced and incompletely spliced viral mRNAs, and in the absence of Rev, these mRNAs are not expressed in the cytoplasm. Thus, Rev is absolutely required for virus replication.

The lentivirus equine infectious anemia virus (EIAV) produces a Rev protein from the third and fourth exons of a bicistronic, four-exon mRNA. Interestingly, rev exon 1 contains an EIAV RRE and binds Rev. The sequences of the RRE also function as an exon splicing enhancer (ESE), which is necessary in the recognition and inclusion of rev exon 1 during splicing. In the presence of Rev, an alternatively spliced mRNA is produced identical to the four-exon mRNA except lacking rev exon 1. The mechanism that results in Rev-mediated alternative splicing has not been fully elucidated. Previous studies suggest that the exclusion of rev exon 1 is due to competition between Rev and the cellular splicing protein SF2/ASF for binding the sequences of the ESE and RRE. However, the exact sequences bound by either protein are not known, and it appears they are not the same, but overlap.
(ESE/RRE). Therefore, it was of interest to directly test the hypothesis that SF2/ASF and Rev compete for binding the sequences of the ESE/RRE.

The 165 amino acid EIAV Rev protein (Figure 1) is functionally homologous to other lentiviral Revs, but has little amino acid homology to them. Many of the functional domains of EIAV Rev have yet to be fully defined, and those that have been mapped, are in different locations of the protein than in other lentiviral Revs. For example, the amino acids responsible for the nuclear export activity of the Rev (NES) are located toward the amino-terminus of EIAV Rev (amino acids 32-55), whereas in other lentiviral Revs, the NES is located near the carboxy-terminus. In addition, in vitro studies in our laboratory describe a role for EIAV Rev amino acids 75-127 in alternative splicing (3), as scanning deletion mutagenesis within amino acids 75-127 reduce Rev-mediated alternative splicing. Because Rev and the SF2/ASF bind the ESE/RRE region, we hypothesize that the Rev alternative splicing domain may be the RNA binding domain. To further define the functional domains of EIAV Rev, it was of interest to map the RNA binding domain. By better understanding how Rev and SF2/ASF bind the ESE/RRE, and by mapping the of the Rev RNA binding domain, the role of Rev in EIAV alternative splicing will be better understood. Therefore, to further characterize the functional role of Rev in EIAV alternative splicing, the first specific aim was to purify functional EIAV Rev. Purification of Rev would result in a protocol for use in the additional purification of a number of Rev deletion mutants, which could be used in RNA binding assays to map the RNA binding domain.
Methods and Results

Glutathione S-transferase (GST) Gene Fusion System. Initial studies to purify Rev utilized a prokaryotic GST-fusion protein expression system from Pharmacia Biotech. cDNA fragments of an EIAV Rev clone (H21) (1) and a H21 Rev-derived mutant lacking amino acids 32-55 (RevΔNES) were cloned into two vectors, pGEX-2T and pGEX-3X (Amersham Biosciences), using the flanking 5' BamHI and 3' EcoRI sites. Each resulting vector was transformed into both BL21 (Novagen, Madison, WI) and JM109 (New England Biolabs, Beverly, MA) E. coli strains. The pGEX-2T vector encodes a GST protein upstream of Rev with a six amino acid thrombin protease cleavage site immediately upstream of the multiple cloning site of the vector. The pGEX-3X vector encodes a GST protein upstream of Rev with four amino acids between the two proteins that are a Factor Xa protease cleavage site. GST-Rev RNA transcription is induced from the tac promoter by addition of IPTG to a bacterial culture. In protein purification, after inducing expression of GST-Rev, the bacteria are harvested and lysed, and the lysate is added to a column containing a fixed glutathione substrate, which binds to the GST portion of GST-Rev. Multiple washes remove the unwanted proteins, and the purified fusion protein is eluted from the column with a reduced glutathione buffer.

Initial studies optimized protein expression from the GST-H21 Rev plasmid; examining a number of factors including the IPTG concentration, induction time, and the method of lysis. Concentrations from 0.1 mM to 1 mM IPTG, induction times from one to five hours, and lysis including sonication and the commercial buffer Bacterial Protein Extraction Reagent (B-PER) (Pierce, Rockford, IL) were tested. Two different methods using glutathione-fixed substrates were tested for binding, including a pre-packed column.
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(Pierce) and a batch purification (Amersham Biosciences). These experiments determined that the optimal induction of GST-Rev was with 1 mM IPTG in log growth cells for 5 hours. The best method for lysis was resuspension of the harvested pellet in B-PER. The best purification was obtained by using the Glutathione Sepharose 4B bulk matrix. After washing, GST-Rev protein was eluted with a 15 mM reduced glutathione buffer (50 mM Tris-HCl, pH 8.0), dialyzed with TN buffer (50 mM Tris-HCl, 100 mM NaCl, pH 8.0), and quantified with a BCA assay (Pierce). Protein purification from bacteria consistently resulted in low yields (~15 ng/ml bacterial culture) and degradation products. The GST-Rev degradation products were used successfully in RNA binding assays and in *in vitro* splicing assays to identify the EIAV RRE and ESE (Appendix B). Figure 2 shows a Western blot of the protein products from the purification that were used in the experiments of the Appendix B (2).

Because the goal of these studies was to purify Rev for use in RNA competitive binding assays, a higher yield of pure protein was required. A variety of methods were investigated to increase yield and decrease Rev degradation products. The first included the use of the RevΔNES protein, which would eliminate a number of charged amino acids from the protein that may have effect on induced GST-Rev expression (as Rev has always empirically seemed poisonous to cells). A second variable tested was the bacterial cell type, therefore, the protease deficient BL21 strain was used. The results indicated that the use of RevΔNES protein did not improve yield, and that changing the host strain to BL21 did not decrease the degradation.

In addition to other products, Rev appeared to cleave from GST-Rev in earlier studies (Figure 2), and therefore, a number of studies were initiated to purify Rev from the degraded
sample. Initial studies with a size exclusion column did not result in purified Rev. However, the results could have been due to the low yield, as no protein was detected by either protein quantification assays or Western blot analysis. Therefore, to improve the yield of Rev from the degraded preparation, protease cleavage of GST-Rev was investigated using the specific protease sites between GST and Rev. Protease cleavage was performed on GST-Rev while on the column, as well as on the eluted GST-Rev preparation. Results of protease cleavage assays showed that Thrombin did not cleave GST-Rev, and Factor Xa cleaved GST-Rev at a number of sites within Rev.

**Rev purification using a baculovirus expression system.** The goal of Rev protein purification was to prepare purified Rev and Rev deletion constructs for use in RNA binding assays. Studies of protein expression in a prokaryotic system resulted in low-yield and degraded protein products. Protein purification was switched to a eukaryotic, baculovirus expression system in hopes of increasing yield and purity, with an added benefit of expressed protein that is post-translationally modified.

The MaxBac 2.0 Transfection and Expression kit was utilized (Invitrogen, Carlsbad, CA). All kit protocols were used with the provided reagents, unless otherwise stated. Briefly, a desired gene is cloned into the multiple cloning site of a baculovirus transfer vector downstream of a sequence encoding six tandem histidine residues (6-His) and an enterokinase cleavage (Xpress) tag. The transfer vector containing the desired gene is transfected into insect cells with linearized *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) DNA. Through recombination, the transfer vector provides the essential sequence needed for replication, including the desired gene. Recombinant virus is selected, amplified, and when the virus infects cells, the desired protein is expressed.
The H21 Rev cDNA was cloned into the baculovirus transfer vector pBlueBacHis2-C (Invitrogen) using flanking 5' BamHI and 3' EcoRI restriction enzyme sites (pBBH-Rev21). Standard kit protocols were followed to create a recombinant baculovirus that expresses Rev. Linearized viral DNA (Bac-N-Blue DNA) was transfected with pBBH-Rev21 into the Spodoptera frugiperda derived insect cell line Sf9. At 72 hours post-transfection, supernatant was harvested and assayed for budded, recombinant virus by a viral plaque assay. During the assay, 5-bromo-4-chloro-3-indolyl-β-D-galactoside was added to the growth media, and recombinant viral plaques appeared blue in color. Individual plaques from the plaque assay were removed and placed in separate wells of a 12 well microtiter plate with 5 X 10⁵ Sf9 cells/well resulting in a P-1 stock. After 72 hours, viral DNA was isolated, and PCR was performed with primers flanking the Rev gene to confirm recombinant virus contained Rev. From individual PCR positive wells, 20 µl of the P-1 viral stock was used to infect a 25 cm² flask with 2X10⁶ Sf9 cells to generate a high-titer P-2 viral stock. From the P-2 stock a high-titer, large-volume P-3 stock was generated and titered for use in Rev expression and purification studies.

Initial studies determined the optimal multiplicity of infection (MOI), expression time post-inoculation, and preparation of cell lysate. In addition, Rev expression was confirmed by Western blot analysis of infected cell lysates using polyclonal rabbit anti-Rev sera and a mouse monoclonal antibody to the 6-His tag portion of the protein. The optimal MOI was 1 and the optimal expression time post-infection was 4 days. The 6-His Tag allowed protein purification to be done in either native or denaturing conditions, and the Xpress System Protein Purification kit (Invitrogen) was used. In both native and denaturing conditions, the lysate was added to a column containing the nickel affinity resin ProBond (Invitrogen).
After multiple washes, the protein is eluted using either an imidazole buffer (3M imidazole, 500 mM NaCl, 20 mM NaPO₄, pH 6.0) for native elution, or a low pH buffer (8M Urea, 20 mM Na₂HP0₄, 500 mM NaCl, pH 4.0) for denaturing elution.

Initial analyses of Rev purification using freeze/thaw for lysis in native conditions showed that freeze/thaw did not release Rev into the lysate, and the majority of Rev was located in an insoluble fraction. Therefore, we investigated other lysis methods to improve the soluble fraction of Rev. Results of the studies showed that the addition of mild-detergents could increase the amount of soluble Rev in the lysate, but the 6-His Rev did not bind the column. Therefore, we next investigated purification of Rev in denaturing conditions.

As per the denaturing conditions purification protocol, the cell pellets were resuspended in 10 mls of Guanidinium Lysis Buffer. Cell lysates were passed through an 18 gauge needle four times and then centrifuged at 20,000 X g for 10 minutes. Supernatants were added to a pre-equilibrated ProBond resin column in two, 5 mls aliquots. After addition of each aliquot, the resin was resuspended and the column was rocked for 10 minutes to allow the protein to bind. The resin was then settled by centrifugation at 800 X g for 2 minutes, and the supernatant was removed. The column was washed twice with 4 mls of Denaturing Binding Buffer, five times with 4 mls Denaturing Wash Buffer 6, and four times with 4 mls of Denaturing Wash Buffer 5.3. Each wash cycle consists of resuspending the resin in the wash solution, rocking the column for 2 minutes and settling the resin by centrifugation at 800 Xg for 2 minutes. To confirm the efficacy of each wash, absorbance values at 260 nm were monitored by a spectrophotometer. After washing, the protein was eluted from the column, by adding 7 mls of Denaturing Elution Buffer and collecting 1 ml
aliquots. Protein aliquots were snap frozen in N\textsubscript{2} (l) and stored at -80°C. Protein elution was again monitored by spectrophotometer with absorbance values at 260 nm. Fractions that contained protein were analyzed by Western blot analysis, and results showed that the fractions contained pure \textit{Rev}.

Large amounts (1 mg/ 1.0X10\textsuperscript{6} cells) of pure \textit{Rev} were successfully purified under denaturing conditions (Figure 3). Because the protein was denatured, it is expected to have no functional activity. Denatured \textit{Rev} functional activity could not be tested, because the denaturing buffer inhibited \textit{in vitro} functional assays. Therefore, a number of studies were performed to renature the protein, using both gradient and step-dialysis. Briefly, 8 M Urea was added to a base buffer containing 20 mM Hepes, pH 7.9, 2.5 mM MgCl\textsubscript{2}, 1 M KCl, 1 mM EDTA. During the renaturation, protein dialysis occurred in either buffers with half molar decreasing steps in Urea or in the base buffer containing 8M urea, and increasing amounts of base buffer over time. In either gradient or step dialysis, the results showed that \textit{Rev} precipitated when the Urea concentration decreased below 3 M. To improve the solubilization of \textit{Rev}, further studies used buffer containing the base reagents and varying concentrations of other reagents to reduce protein-protein interactions. These included: NaCl (< 1 M), MgCl\textsubscript{2} (< 10 mM), \(\beta\)-mercaptoethanol (BME)(<6 mM), DTT (0.5 mM), and tRNA (~100 µg/µl). The results showed some success, and the best result in which \textit{Rev} stayed soluble to 1 M Urea resulted from renaturation by step dialysis in a buffer containing a variable concentration of Urea (6M to 1M) in 20 mM Hepes, pH 7.9, 1 M KCl, 10 mM MgCl\textsubscript{2}, 1 mM EDTA, and 6 mM BME. The same result was also observed in other studies using a low pH buffer (50 mM NaOAc, 1 M KCl, pH 5.0).
Recent studies have investigated the possibility of renaturation while on the nickel column, and those studies showed that the protein was released from the column as the Urea concentration decreased. Finally, studies have shown that renaturation of a Rev mutant expressed by the baculovirus system in which a large number of charged amino acids were deleted (Δ144-165), also precipitates at 1 M Urea. Future studies will be in purification of Rev amino acids 75-127, as it our hypothesis that these amino acids contain the RNA binding domain.

Acknowledgements

I would like to thank Michael Belshan and Sean Murphy, who initiated these studies and cloned the Rev cDNA into pGEX-2T. In addition, I thank Yuanbin Ru for the most recent studies of protein renaturation on the column and the renaturation attempt of the RevΔ144-165.

References


Figure Legends

Figure 1. EIAV Rev amino acid sequence. The amino acid sequences of a cDNA isolated from Th-1 infected horse macrophage cultures (1). NES indicates the identified nuclear export signal (amino acids 32-55). RBD/ASD indicates the putative RNA binding domain/alternative splicing domain (amino acids 75-125). NLS indicates the nuclear localization signal (amino acids 160-165).

Figure 2. Bacterial expressed GST-Rev. GST-Rev has an estimated molecular weight of ~46 kDa. GST has a estimated molecular weight of ~29 kDa, and Rev has an estimated molecular weight of ~18 kDa. Convalescent horse serum was used for the detection of Rev, and a monoclonal antibody against GST was used for the detection of GST. Uninduced cell lysate and GST-induced cell lysate were run a separate blot and are not shown.

Figure 3. Baculovirus expressed 6-His Rev. Rev was nickel affinity purified. Anti-Xpress is a mouse monoclonal antibody that recognizes an epitope within the enterokinase cleavage site. The tagged Rev protein has an estimated molecular weight of 24 kDa. The primary Rev detection reagent is an anti-Rev polyclonal sera made from Rev-peptide injected rabbits.
Figure 1
Figure 2

GST-Rev

Degraded GST-Rev

Rev

anti-EIAV   anti-GST

KDa

- 43.9-

- 32.1-

- 18.1-
Figure 3
APPENDIX B. BINDING OF EQUINE INFECTIOUS ANEMIA VIRUS REV TO AN EXON SPlicing ENHANCER MEDIATES ALTERNATIVE SPLICING AND NUCLEAR EXPORT OF VIRAL MRNAS


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Abstract

In addition to facilitating the nuclear export of incompletely spliced viral mRNAs, equine infectious anemia virus (EIAV) Rev regulates alternative splicing of the third exon of the tat/rev mRNA. In the presence of Rev, this exon of the bicistronic RNA is skipped in a fraction of the spliced mRNAs. In this report, the cis-acting requirements for exon 3 usage were correlated with sequences necessary for Rev binding and transport of incompletely spliced RNA. The presence of a purine-rich exon splicing enhancer (ESE) was required for exon 3 recognition and the addition of Rev inhibited exon 3 splicing. GST-Rev bound to probes containing the ESE, and mutation of GAA repeats to GCA within the ESE inhibited both exon 3 recognition in RNA splicing experiments and GST-Rev binding in vitro. These results suggest that Rev regulates alternative splicing by binding at or near the ESE to block

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SR protein-ESE interactions. A 57 nt sequence containing the ESE was sufficient to mediate Rev dependent nuclear export of incompletely spliced RNAs. Rev export activity was significantly inhibited by mutation of the ESE, or by trans-complementation with SF2/ASF. These results indicate that the ESE functions as a Rev responsive element (RRE), and demonstrate that EIAV Rev mediates exon 3 exclusion through protein-RNA interactions required for efficient export of incompletely spliced viral RNAs.

**Introduction**

Retroviruses utilize a variety of mechanisms to differentially express numerous proteins from relatively small genomes which possess a single transcriptional start site. These mechanisms include, but are not limited to, the use of polyprotein precursors, ribosomal frameshifting (19), alternate start codons (6), bicistronic mRNAs (6), and alternative splicing (29). Alternative splicing allows for the production of multiple viral mRNAs from a single RNA precursor. The simplest retroviruses, such as murine leukemia virus, express only two mRNAs, an unspliced RNA which serves as both mRNA for the gag and pol proteins and as progeny viral RNA, and a singly spliced mRNA which encodes the env gene. In contrast, complex retroviruses, such as human immunodeficiency virus type 1 (HIV-1) produce at least 20 different mRNAs, including several multiply spliced RNAs that encode small regulatory proteins (29).

In all retroviruses, alternative splicing requires the presence of suboptimal splice sites, allowing for expression of several mRNAs from a single pre-RNA. Regulation of suboptimal splice sites is a complex process mediated in part by cis-acting RNA sequences that can either enhance or repress recognition of a splice site by the splicing machinery. Exon
splicing enhancers (ESEs) and silencers (ESSs) have been described for many virus and cellular RNAs (2, 23). ESEs typically are purine rich sequences embedded within alternatively spliced exons. The purine rich sequences mediate exon recognition through interactions with members of the SR protein family of splicing factors. SR proteins are both essential splicing factors and regulators of alternative splicing (reviewed in (16)). Binding of SR proteins to an ESE recruits essential splicing factors to suboptimal splice sites near ESE sequences, resulting in exon inclusion of alternatively spliced exons.

In addition to the above mechanisms, complex retroviruses utilize Rev-like pathways to differentially regulate expression of incompletely spliced RNAs encoding for virion structural and enzymatic proteins and progeny RNA molecules (reviewed in (9)). The prototype member of this family, HIV-1 Rev, binds to the viral pre-mRNA at a specific sequence called the Rev-responsive element (RRE) (8, 35), multimerizes (24, 34), and facilitates export of incompletely spliced RNAs from the nucleus via a Crm1-mediated pathway (13, 14). Equine infectious anemia virus (EIAV) Rev is functionally homologous to HIV-1 Rev (15), but is less well characterized. EIAV Rev is a 165 amino acid protein translated from exons 3 and 4 of a multiply spliced, 4-exon, bicistronic mRNA which also encodes the trans-activating protein, Tat (Fig. 1) (6). In addition to promoting nuclear export of incompletely spliced RNA, EIAV Rev also regulates inclusion of exon 3 of the multiply spliced RNA. In the presence of Rev, a multiply spliced mRNA lacking exon 3 is produced (22). Rev variants which are NES-defective have been shown to mediate alternative splicing (4, 18); however, it is not known if the alternative splicing function is required for nuclear export activity. Exon 3 is flanked by a suboptimal splice acceptor and contains a purine rich, exon splicing enhancer-like motif which has been shown to interact with the SR protein.
Derse and co-workers have proposed that EIAV Rev-mediated skipping of exon 3 is a consequence of Rev-RNA interactions which directly or indirectly inhibit SF2/ASF (17). We previously mapped a RRE of Rev to a 534 nt region containing exon 3 (4), suggesting the possibility that Rev mediates exon 3 skipping by binding at or near the purine rich sequence to disrupt SR protein interactions necessary for exon 3 recognition.

Here, we further delineate the role of Rev in exon 3 alternative splicing. Our results indicate that the purine rich sequence in exon 3 is required for the utilization of the exon 3 splice acceptor, confirming the presence of an ESE within exon 3. RNA gel mobility shift assays and nuclear export assays demonstrate that Rev binds to the ESE, and that this binding facilitates RNA export. Together, these results indicate the exon 3 ESE is a RRE of EIAV. Therefore, Rev mediates exon 3 alternative splicing by binding the viral pre-mRNA at the ESE/RRE and interfering with SR protein – ESE interactions.

**Materials and Methods**

**PCR and Plasmid construction.** All plasmid constructs were confirmed by sequence analysis (Iowa State University DNA Synthesis and Sequencing Facility). DNA templates for splicing substrates were amplified from p33k, a subclone of the p26 EIAV proviral clone previously described (5). Unless otherwise indicated, PCR reactions were performed as directed by the manufacturer (Perkin Elmer, Foster City, CA) using 1 μM primers. Standard PCR reactions consisted of 25 cycles of one minute denaturation at 94° C, one minute annealing at 50° C, and one minute extension at 72° C, followed by an addition cycle with a prolonged, five minute extension. All DNA templates for splicing substrates used a common 5’ primer, CTGAAGGCAATCCAACAAGG; and individual 3’ primers to generate the
substrates shown in figure 2A. The 3’ primers used and the region of EIAV amplified were: CTCTCTATGATAAGCTTC, EIAV nt 5233-5793; CCAGTAGTTCTGCTAAGCA, nt 5233-5573; TTTCCACCAGTCATTCTTCTTC, nt 5233-5535; CAGGTTCATTTCTTGGTCT, nt 5233-5490. All nucleotide numbering is based on that of Kawakami et al. (20). After PCR, fragments were TA cloned into the pGEM-T easy vector as directed by the manufacturer (Promega, Madison, WI).

The expression plasmid pRevWT was described previously as pcH21 (4). pDM138, pERRE-All (EIAV nt 5280-7534), and pERRE-1 (nt 5280-5834) have also been previously described (4). To construct pERRE-1A, primers containing a ClaI restriction site were used to amplify EIAV nt 5281-5795. ERRE-1A 5’ primer:
GGATCGATTTGATATATGGGATTATT; 3’ primer:
GGATCGATCTCTCTATGATAAGCTTC (ClaI sites are underlined). The minRRE sequence (EIAV nt 5485-5540) was synthesized as complementary oligonucleotides with ClaI extensions on the 5’ and 3’ ends. The oligonucleotides were heated at 95° C for 5 minutes and annealed by slow cooling. The fragment was phosphorylated, then ligated with pDM138. The pGST-Rev expression vector contains a cDNA cloned in frame into the BamH1 site of the GST fusion vector pGEX-3X (Amersham Pharmacia Biotech, Piscataway, NJ).

ESE mutants were constructed by PCR-Ligation-PCR mutagenesis according to the methods described by Ali et al. (1) using internal primers designed with the specified mutations shown in figure 4A. The two regions were amplified with Vent DNA polymerase (New England Biolabs, Beverly, MA). The 3’ fragment was phosphorylated, ligated with the 5’ fragment, and 2 µl of the ligation reaction was PCR amplified with outer primers described
above to amplify EIAV nt 5233-5793. Amplicons were cloned into pGEM-T. To construct the mutant pDM138 constructs, the pERRE-1A primers described above were used to PCR amplify the respective mutants from the pGEM-T background and clone into the ClaI restriction site of pDM138.

pSF2/ASF was generated from the PET9c-SF2 plasmid obtained from Dr. Adrian Krainer, Cold Spring Harbor Laboratory (21). The cDNA region corresponding to SF2/ASF was cloned as two fragments: a BgIII to KpnI (-100 to +242; numbering based on +1 at initiation AUG), and KpnI to BamH1 (+242 to a Bam HI site downstream of the UAA terminator). These were inserted into the eukaryotic expression vector pCMV5 (provided by Mark Stinski, University of Iowa) which was cleaved by BgIII and BamH1.

**Synthesis of RNA substrates.** The plasmids containing the splicing substrates were digested with Spe I (GIBCO BRL, Rockville, MD) to create linearized templates for transcription of RNA splicing substrates. In vitro run-off RNA transcripts labeled with [³²P]UTP (Amersham Pharmacia Biotech) were generated as previously described (2). DNA templates for RNA binding analysis were amplified by PCR from p33k using 5' primers containing a T7 promoter site (a diagram of the substrates is in Fig. 3A). The primers used for the substrates were: 5' primer:

TAATACGACTCACTATAGGGAGGAACAGCATGGCAGAATCG, 3' primer:
TTTCCACCAGTCATTTCTTC (RREp1, nt 5443-5535); 5' primer:
TAATACGACTCACTATAGGGAGGTGAAGAAGAATCTAAAG, 3' primer:
CCACCAAAGTATTCCTCC (RREp2, nt 5489-5589); 5' primer:
TAATACGACTCACTATAGGGAGGTGACTGGTGGAAAATAGG,3' primer:
CCCTATATAATGTGCTG (RREp3, nt 5523-5622); 5’ primer:

TAATACGACTCACTATAGGGAGGCGGAGGAAGCAAGAGACC, 3’ primer:

CCTGCTAAGCATAACAGA (RREp4, nt 5458-5565). The T7 promoter is underlined in the 5’ primers. Amplified DNA was phenol:chloroform extracted, ethanol precipitated, and resuspended in RNase-free dH₂O. The RREp5 DNA fragment was synthesized as two complementary oligonucleotides containing the T7 promoter attached to EIAV nt 5485–5540. Complementary DNA fragments were combined at equal molar amounts, heated at 95° C for 5 minutes, then slow cooled to anneal.

**Expression/Purification of GST-Rev.** BL21 *Escherichia coli* transformed with the pGST-Rev expression vector were grown overnight at 1/10 final culture volume in NZY broth containing 0.1 mg/ml ampicillin. The next day, cells were brought up to final volume, grown an additional 3 hours, then induced with 1 mM IPTG for 5 hours. After induction, cells were washed 3 times and resuspended in 50 mM Tris (pH 8.0), 50 mM NaCl (TN buffer). Cells were lysed by sonication and the supernatant clarified by centrifugation at 10,000 xg. GST-Rev was purified by binding to glutathione sepharose 4B beads (Amersham Pharmacia Biotech) overnight and washed 3X with TN buffer. The fusion protein was eluted with 15 mM reduced glutathione in 50 mM Tris, pH 8.0, concentrated with a 30 kDa MWCO filter concentrator (Millipore, Bedford, MA), and dialyzed against TN buffer. Protein expression was confirmed by SDS-PAGE and immunoblotting with convalescent anti-EIAV antisera or anti-GST antisera (Amersham Pharmacia Biotech) which detected expression of the GST-Rev fusion protein and several minor bands, including GST alone (data not shown). In some cases, the fusion protein was digested with 4 units/bead bed volume of Factor Xa protease (Amersham Pharmacia Biotech) while bound to glutathione sepharose beads. Excess
GST and GST-Rev fusion protein were removed with the glutathione sepharose beads, and the supernatant, containing cut Rev protein, concentrated and dialyzed as described above.

**In vitro splicing, and gel electrophoresis.** Splicing reactions were carried out as previously described (2). In brief, approximately 8 fmol of EIAV RNA substrates were incubated for 2 hours at 30°C with 60% (v/v) nuclear extract in Dignam’s buffer D (12), 20 mM creatine phosphate, 3mM MgCl₂, 0.8 mM ATP and 2.6% (w/v) polyvinyl alcohol. In some experiments, EIAV Rev protein was diluted in buffer D (12) and added to the splicing reactions at the indicated concentrations. RNAs were analyzed on 4% polyacrylamide gels containing 7 M urea.

**RNA binding Assays and gel electrophoresis.** RNA – protein interactions were performed in 1X RNA binding buffer containing 10 mM HEPES-KOH (pH 7.5), 100 mM KCl, 1 mM MgCl₂, 0.5 mM EDTA, 1 mM DTT, 50 μg/μl E. coli tRNA, and 10% glycerol. RNA was in vitro transcribed in the presence of [³²P]UTP as described above. 100 ng to 2 mg of GST or GST-Rev fusion protein was incubated with approximately 1x10⁶ cpm RNA probe on ice for 15 minutes. The reactions were directly loaded onto a 8% native 100 mM tris-glycine polyacrylamide gel (37.5:1 acrylamide:bis cross-linking ratio) which had been pre-run 1 hour. The samples were electrophoresed an additional 3 hours. The gel was fixed in 20% ethanol, 10% acetic acid for 15 minutes, dried, and exposed to X-ray film with an intensifying screen.

**CAT Assays.** Transient transfections and CAT assays were performed using human embryonic kidney (293) cells and canine fetal thymus (Cf2th) cells. Cells were maintained in Dulbecco’s modified eagle medium supplemented with 10% fetal calf serum and penicillin/streptomycin. CAT assays using 293 cells were performed as previously described.
(4). Briefly, 1 µg of either pcDNA3 (Invitrogen, Carlsbad, CA) or pRevWT was transfected by calcium phosphate co-precipitation with 0.2 µg of pDM138 reporter plasmid, 0.2 µg of pCH110, and 0.6 µg of pUC19. Two days post transfection, cells were harvested, resuspended in 0.3 ml 0.25 M Tris (pH 7.5), lysed by freeze/thawing, and assayed for β-galactosidase activity to normalize CAT assays for transfection efficiency. Normalized lysates were assayed for CAT activity using 3 µl [14C]chloramphenicol and 1 mM acetyl CoA. Acetylated products were separated by thin layer chromatography and the percentage acetylation was quantified by phosphorimager (Molecular Dynamics, Sunnyvale, CA). Experiments were performed in triplicate and results summarize a minimum of six independent transfections.

Cf2th cells were used for in vivo competition assays. Cells were transfected with 0.5 ng pRevWT, 0.3 µg of pERRE-1 reporter plasmid, and ten-fold increasing concentrations (0-100 ng) of pSF2/ASF using 4 µl TransIT-LT reagent (Mirus Corporation, Madison, WI). Each reaction also included 0.2 µg of pCH110 and pUC19 to equalize the total DNA per reaction. Two days post-transfections, cells were harvested and lysates were assayed for β-galactosidase activity as above. Normalized lysates were assayed for CAT using a commercially available CAT-ELISA kit (Roche Molecular Biochemicals).

Results

The purine rich sequence is required for exon 3 recognition. Exon 3 of the bicistronic, four exon equine infectious anemia virus (EIAV) mRNA contains a purine rich sequence which resembles an exon splicing enhancer (ESE). Previous reports showed that the SR protein SF2/ASF cross-links to the ESE-like sequence in vitro and suggested the ESE-
like sequence may enhance exon 3 inclusion during pre-mRNA splicing (17). To further investigate the cis-acting requirements for exon 3 inclusion, we constructed a series of DNA templates to generate radiolabelled RNA substrates for in vitro splicing. All substrates contained the exon 2 splice donor, the intervening intron, exon 3, and downstream sequences. Nested 3’ deletions were made to identify splicing enhancer sequences present within or downstream of exon 3 (Fig. 2A). Splicing of radiolabelled substrates was assayed in vitro using HeLa cell nuclear extracts, which include all SR proteins. All constructs containing the purine rich sequence were spliced (Fig. 2B, lanes 3-5), whereas no splicing was observed using the substrate lacking the purine rich sequence (Fig. 2B, lane 2). This is consistent with the hypothesis that the purine sequence functions as an ESE and is required for exon 3 inclusion in the multiply spliced four exon transcript. Taken together with previous work (17), this suggests exon 3 recognition requires SF2/ASF interactions at the ESE. The addition of as little as 100 ng of Rev to a splicing reaction containing the largest splicing substrate inhibited exon 3 recognition (Fig. 3). This confirms earlier, in vivo observations of Rev-mediated changes of alternative splicing (17), and indicates that Rev is the only viral protein necessary for exon 3 skipping.

**Rev binds the ESE.** Derse has suggested that binding of EIAV Rev to a region of the viral pre-mRNA near the ESE results in either a direct or indirect inhibition of SR protein function (17). In previous work, we identified an RRE region spanning exon 3 (nt 5280 to 5834) (4). This favors a mechanism wherein Rev-RRE interactions disrupt SF2/ASF binding at the ESE. To examine whether Rev binds at or near the ESE, we generated a series of RNA probes and tested for Rev-RNA interactions by RNA gel mobility shift assays using a bacterially expressed and purified GST-Rev fusion protein. The location of the RNA probes
relative to exon 3 and the ESE is shown in Fig. 4A. GST-Rev bound to exon 3 probes RREp1 and RREp2, which both contain the ESE; however, no binding was observed with the GST negative control (Fig. 4B, lanes 1 to 4). Minor binding was observed with RREp3 (Fig. 4B, lane 6), which has sequences immediately downstream of the ESE (nt 5523 to 5622), but lacks the purine rich region. The binding site was further delineated to a 57 nt region of viral RNA using two smaller ESE-containing probes, RREp4 and RREp5. GST-Rev interacted with both probes (Fig. 4B, lanes 8 and 11), further suggesting that Rev binds at or near the ESE. To confirm the specificity of binding, gel shift assays were performed with RREp2 in the presence of excess cold competitor RREp2 or RREp3 (Fig. 4C). Excess cold RREp2 inhibited GST-Rev binding (Fig. 4C, lanes 2 and 3), whereas no inhibition was observed with RREp3 (Fig. 4C, lanes 4 and 5), demonstrating that the binding of GST-Rev to the ESE-containing RREp2 is specific. No slower migrating bands, indicative of Rev multimerization, were observed in any of the RNA binding analysis, although multimerization was readily observed with HIV-1 Rev when used as a positive control (data not shown). The minor bands in Fig. 4C represent GST-Rev and degraded by-products. Overall, these results demonstrate that Rev specifically interacts with the viral RNA at or near the ESE.

Mutagenesis of the ESE reduces exon 3 splicing and Rev binding. The finding that GST-Rev bound to a 57 nt region containing the ESE suggested that a Rev - RNA interaction was directly competing with SF2/ASF for binding at the ESE. If so, there should be similar sequence requirements for exon 3 recognition and Rev binding. The ESE contains two purine rich sequences (designated A and B), which include seven GAA repeats (Fig. 5A). GAA repeats have been shown to be important for SF2/ASF recognition of ESE sequences in other systems (reviewed in 16). Therefore, we constructed five ESE mutants in the largest splicing
construct and the RREp4 RNA probe fragment which contained various GAA to GCA mutations (Fig. 5A). The mutant templates were tested for in vitro splicing and GST-Rev binding. Mutation of all GAA motifs (mutAll) or the B purine stretch (mutB) resulted in a decrease in both exon 3 in vitro splicing (figure 5B, lanes 3 and 5) and in GST-Rev binding (figure 5C, lanes 2 and 4). Mutation of the GAA repeats in only the B purine stretch resulted in a more modest reduction in both in vitro splicing and GST-Rev binding (Fig. 5B, lanes 6 and 7 and 5C, lanes 5 and 6). Mutation of GAA repeats in the A region (mutA) did not appear to significantly affect either exon 3 splicing or GST-Rev binding in vitro (Fig. 5B, lane 4 and 4C, lane 3), suggesting the B purine stretch alone contains cis-acting sequences necessary for exon 3 recognition and GST-Rev binding. The finding that each mutant had comparable effects in both assays suggests similar requirements in the ESE for both exon 3 recognition and Rev binding, further supporting a model of Rev inhibition of splicing through direct competition with SR proteins for binding at the ESE.

The ESE can function as a RRE to mediate RNA nuclear export. In other complex retroviruses, Rev functions to regulate the export of incompletely spliced RNAs via interaction with the viral pre-mRNA at a specific sequence called the Rev responsive element (RRE). We had previously used the HIV-1 derived pDM138 reporter system to preliminarily map the RRE of EIAV to a region which overlapped exon 3 (ERRE-1) (4). However, this fragment possessed only 52% of the activity of a reporter containing a much larger fragment of EIAV (ERRE-All), suggesting further downstream sequences enhanced Rev-mediated export. The RNA binding data given above suggested that the functional sequence within ERRE-1 was the ESE sequence. To test this, we constructed a pDM138 reporter plasmid, miniRRE, which contains only 57 nt of EIAV (nt 5485–5540, RRe5), spanning the ESE and
the remainder of exon 3. Transient transfection assays in 293 cells demonstrated the minRRE reporter produced levels of CAT activity comparable to ERRE-1 (Fig. 6A), but only 35% of the activity ERRE-All. This indicates minRRE contains the functional RRE in ERRE-1, however additional elements outside ERRE-1 may be required for full export activity.

To confirm the ESE is the RRE within minRRE, we introduced the GAA to GCA mutations used for in vitro splicing and RNA binding assays (Fig. 5A) into a reporter vector containing the same sequences present in the largest splicing substrate (Fig. 2A). This vector, ERRE-1A, is 41 nt shorter than ERRE-1, but exhibited similar levels of activity as ERRE-1 (Fig. 6A). In all cases, mutation of the GAA repeats in the ESE significantly reduced Rev dependent nuclear export activity (p < 0.01) (Fig. 6B). The greatest reduction in activity was seen in constructs containing mutations of all seven GAA repeats (mutAll) or the three repeats in the A purine stretch (mutA). The reduction in activity in mutA indicates this region, while not necessary for GST-Rev binding, is required for RNA nuclear export. Mutations in the B region (mutB, B12, B34) also significantly reduced activity. Therefore, we conclude that the B purine stretch functions in GST-Rev binding, exon 3 inclusion, and Rev-dependent nuclear export. Together, these results indicate the ESE sequence acts as an RRE and that Rev mediates alternative splicing by binding at or near the ESE to disrupt SF2/ASF interactions.

SR proteins inhibit Rev-dependent nuclear export. The finding that similar cis-acting sequences mediate nuclear export, RNA binding, and exon inclusion suggested that EIAV Rev directly competes with SF2/ASF for binding at a similar site on the viral premRNA. If so, increasing concentrations of SF2/ASF would inhibit Rev-dependent nuclear export activity. To test this, initial studies were done to determine the linear range of Rev-
dependent nuclear export activity. Based on these results, Cf2th cells were transfected with
0.5 ng pRevWT and ten-fold increasing concentrations of pSF2/ASF. Results indicated a
significant, dose-dependent decrease in CAT levels in the presence of pSF2/ASF (Fig. 7).
This suggests that SF2/ASF and Rev are mutually competitive and is consistent with the
conclusion that both proteins bind to nearly identical sequences on the viral pre-mRNA.
Western blot analyses confirmed that SF2/ASF protein levels increased concomitant with
increased amounts of transfected plasmid DNA (data not shown). The level of Rev produced
by transfected Rev cDNA was below the limits of detection by Western blot. Thus, we could
not eliminate the possibility that the decrease in Rev-mediated nuclear export activity resulted
from quantitative changes in Rev levels rather than changes in Rev binding.

Discussion

In addition to its role in nuclear export of incompletely spliced viral mRNAs, EIAV
Rev mediates alternative splicing of the four-exon multiply spliced EIAV mRNA (22).
Derse and co-workers (17) demonstrated that both GST-Rev and the SR protein SF2/ASF
cross-link in vitro with exon 3, and proposed a model wherein Rev disrupts SR protein
interactions required for exon inclusion. Consistent with this previous data, we show the
purine rich sequence is required for GST-Rev binding and for exon 3 recognition in splicing
reactions in vitro. In addition, a 57 nt sequence containing the ESE was shown to act as a
functional RRE in a heterologous nuclear export assay system. Mutation of GAA nucleotide
repeats in the ESE reduced GST-Rev binding, exon 3 splicing in vitro, and nuclear export of
ESE containing pre-mRNA. Trans-complementation assays demonstrated that SF2/ASF
inhibited Rev-dependent nuclear export in a dose-dependent manner. Therefore, both
SF2/ASF-mediated exon 3 splicing and Rev-mediated RNA export have similar cis-acting RNA requirements, and EIAV Rev and SF2/ASF appear to be mutually competitive. From these data, we conclude that the purine rich sequence within exon 3 of EIAV is both an ESE and a functional RRE. Extending Derse's model, we propose that Rev-mediated nuclear export requires binding at or near the ESE, and that this results in skipping of exon 3 through direct inhibition of SF2/ASF- ESE interactions required for recognition of exon 3 by the host cell splicing machinery. The use of an ESE as an RRE is unprecedented among complex retroviruses.

It is interesting that mutation of the 5' purine stretch (mutA) decreased nuclear export but appeared to have little effect on GST-Rev binding in vitro. Studies with HIV-1 Rev also indicate that sequences in the HIV-1 RRE are required for nuclear export but not RNA binding (25, 26). While these observations have not been fully explained, it is likely that RNA secondary structure may play a role. Secondary structure is a key determinant for HIV-1 binding, multimerization, and function (7, 8, 10, 11, 24, 25, 27). No biochemical data is available to date to confirm the proposed structure (17) of the EIAV ESE/RRE, and it is not possible to predict the structural effects of the mutations used in our study. Our data would suggest the mutation of the 5' purine stretch does not affect the primary binding site of Rev, but may alter distant structures required for Rev-mediated nuclear export. It has been demonstrated that HIV-1 Rev multimerization occurs only after binding to a primary site on the RNA and furthermore, other regions of the RNA are important for secondary binding (8, 10, 32). However, we were unable to observe Rev multimerization in our RNA binding assays, including those assays containing the ESE. Therefore, it remains unclear why mutA exhibited reduced activity with no apparent defect in RNA binding. It is also possible that
binding of host cell proteins to the RRE may be required to facilitate Rev export activity. Further studies will be necessary to delineate the role of this purine region in Rev-mediated nuclear export.

Interactions of Rev-like proteins with SR proteins have been demonstrated in other complex retroviruses. SR proteins have been shown to bind the HIV-1 RRE in a Rev-dependent manner (28). The same study also demonstrated that excess exogenous SF2/ASF could produce a dose-dependent inhibition of HIV-1 Rev function in vivo. We have previously reported an inhibition of EIAV replication in activated macrophages associated with a delay in the appearance of incompletely spliced RNAs (31). The data presented here would suggest this inhibition may be a result of competition of SF2/ASF with Rev for binding at the ESE. This hypothesis is supported by our data showing that excess SF2/ASF provided in trans can inhibit Rev function in transient transfection assays. Together, these results suggest the inhibition in activated macrophages may be due to an increase in the level of SR proteins. It is known that expression of SR proteins varies in cells at different states of activation and differentiation (16, 30, 33), including an increased expression of the SR protein SRp30c in activated T-cells (30). However, little is yet known about the phenotype of SR proteins in monocyte cells. Also, our data cannot rule out the possibility that in addition to competing for binding at the RRE, Rev may also inhibit function via protein-protein interactions.

Previous reports have demonstrated that mutations in the NES do not affect the alternative splicing activity of EIAV Rev (4, 18). To date, no laboratory has identified a Rev protein which is competent in nuclear export, but deficient in alternative splicing. Therefore, it is not clear whether alternative splicing of exon 3 is merely a consequence of Rev-
mediated nuclear export, or if it plays a separate role in virus replication. The RREs of most complex retroviruses are located near the SU-TM cleavage site or in the 3' end of env. The location of EIAV RRE in the 5' env is unique, and may be explained by the requirement of the ESE for exon 3 inclusion. The env mRNA is spliced using the exon 2 splice acceptor (Fig. 1). A singly spliced mRNA using the exon 3 splice acceptor has not been observed in infected cells, and would encode a truncated Env protein lacking the signal peptide. A singly spliced mRNA using the exon 4 splice acceptor is observed, which produces a truncated transmembrane protein from the alternate start codon present in exon 1. Therefore, the use of an ESE as an RRE may function to silence recognition of exon 3 to eliminate another singly spliced transcript. Although a number of retroviruses utilize cis-acting sequences such as ESEs to take advantage of cellular mechanisms of alternative splicing, EIAV appears to be the only retrovirus to encode a trans-acting protein that directly modulates SR-mediated alternative splicing. EIAV was the first described lentivirus and is smaller and genetically less complex than the other lentiviruses. It is possible that the EIAV Rev-ESE interaction may represent an transitional step in the evolution of the Rev-Rex pathway utilized by most complex retroviruses. Interestingly, previous work in our laboratory and others suggested that EIAV may possess two separate RREs (4, 22). In the current study, reporter constructs containing the 57 nt ESE region showed significantly reduced activity as compared to the ERRE-All reporter construct containing a larger portion of the env gene (Fig. 6A). However, a second RRE element has not been identified, nor is it clear that such an element can function independently of the ESE to mediate export of viral pre-mRNAs. Additional studies will help to fully understand the biological and evolutionary significance of the EIAV Rev-mediated export pathway.
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References


Figure Legends

Figure 1. Organization and splicing patterns of EIAV. Schematic of EIAV genome with ORFs. The tat ORFs are indicated with a 't', the first exon of rev with a 'r' (location in
genome is indicated by the shaded region). Splicing patterns and genes expressed are indicated. The ttm ORF encodes a truncated transmembrane protein of unknown function (3).

**Figure 2.** Exon 3 splicing requires the purine rich sequence. (A) Diagram of RNA substrates used for in vitro splicing showing the location of exons 2 and 3. All substrates contain the exon 2 splice donor and exon 3 splice acceptor. Approximate location of purine rich sequence is highlighted. (B) After incubation for 2 hr in HeLa cell splicing extracts, RNA products were electrophoresed through 4% polyacrylamide gels and visualized by autoradiography. Location of spliced and unspliced products is shown. The fastest migrating products in lanes 3-5 are intron products resulting from splicing.

**Figure 3.** Rev inhibits exon 3 splicing. GST-Rev or GST was added at the indicated concentrations to the splicing reactions. Location of the splicing products is indicated.

**Figure 4.** GST-Rev binds the ESE in exon 3. (A) Location of RNA probes, relative to exon 3, used in RNA gel mobility shift assays. The purine rich ESE sequence is highlighted. (B) After incubation with GST or GST-Rev, radiolabelled RNAs were electrophoresed through 8% native polyacrylamide gels. Location of GST-Rev-RNA complexes is indicated with an arrow. (C) Competition assays were performed with either 0.5 or 1 μg of the indicated excess cold competitor RNAs. Competitors were mixed with GST-Rev 10 min. prior to the addition of radiolabelled probe.
**Figure 5.** In vitro splicing and RNA binding of ESE mutants. (A) Sequence of two purine stretches (designated A and B) in exon 3. GAA repeats were mutated to GCA in largest splicing construct (Fig. 2A) and RNA probe RREp4 (Fig. 4A). (B) In vitro splicing analysis of mutant ESE constructs. Location of splicing products is indicated. (C) RNA gel mobility shift assays detecting GST-Rev binding to the mutant probes.

**Figure 6.** EIAV ESE can function as an RRE. (A) pDM138-derived reporter vectors containing various regions of the EIAV genome. Transient transfections and CAT assays were performed in 293 cells as described in Materials and Methods. The results are presented as the percentage acetylation. Experiments were performed in triplicate and the results represents at least nine independent transfections. Error bars denote the standard error of the mean. (B) ESE mutations indicated in figure 5A were also introduced in the ERRE-1A reporter vector and assayed for CAT activity in the presence or absence of Rev as described above.

**Figure 7.** SF2/ASF inhibits Rev-dependent nuclear export. pERRE-1 reporter plasmid was co-transfected with 0.5 ng pRevWT and increasing amounts of pSF2/ASF. CAT levels were quantified by ELISA and are reported as pg CAT per normalized lysate. Results represent the mean of six independent transfections, and the error bars denote the standard error of the mean. Asterisks indicate values significantly different (p < 0.05) from control transfections which contained no pSF2/ASF.
Figure 1
Figure 2
Figure 3

- Lanes 2, 3, 4, 5, 6, and 1
- Labeled bands at 154, 201, 220, 296, 344, 396, and 506/517
- Markers: +200 ng Rev, +300 ng GST, +300 ng GST
- Lanes 2, 3, 4, 5, 6, and 1
Figure 5
Figure 6
Figure 7