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Characterization of Functional Domains of Equine Infectious Anemia Virus Rev Suggests a Bipartite RNA-Binding Domain

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Equine infectious anemia virus (EIAV) Rev is an essential regulatory protein that facilitates expression of viral mRNAs encoding structural proteins and genomic RNA and regulates alternative splicing of the bicistronic *tat*/*rev* mRNA. EIAV Rev is characterized by a high rate of genetic variation in vivo, and changes in Rev genotype and phenotype have been shown to coincide with changes in clinical disease. To better understand how genetic variation alters Rev phenotype, we undertook deletion and mutational analyses to map functional domains and to identify specific motifs that are essential for EIAV Rev activity. All functional domains are contained within the second exon of EIAV Rev. The overall organization of domains within Rev exon 2 includes a nuclear export signal, a large central region required for RNA binding, a nonessential region, and a C-terminal region required for both nuclear localization and RNA binding. Subcellular localization of green fluorescent protein-Rev mutants indicated that basic residues within the KRRRK motif in the C-terminal region of Rev are necessary for targeting of Rev to the nucleus. Two separate regions of Rev were necessary for RNA binding: a central region encompassing residues 57 to 130 and a C-terminal region spanning residues 144 to 165. Within these regions were two distinct, short arginine-rich motifs essential for RNA binding, including an RRDRW motif in the central region and the KRRRK motif near the C terminus. These findings suggest that EIAV Rev utilizes a bipartite RNA-binding domain.

Equine infectious anemia virus (EIAV) infection of horses can result in a rapid, variable, and dynamic disease course. Moreover, horses that survive the early clinical episodes of disease are generally able to control virus replication and remain clinically normal, inapparent carriers of EIAV. The unique features of clinical disease, and the ability of some infected horses to eventually control virus replication, provide an excellent system for longitudinal analyses of virus and host factors important in lentivirus persistence and pathogenesis. Genetic diversity is a hallmark of lentiviruses and is considered an important mechanism of virus persistence and pathogenesis. Previous studies have identified a high rate of genetic variation in EIAV in the region overlapped by the transmembrane protein gp45 (TM) and the major exon of Rev (2, 30). Genetic variation in *rev*/*tm* can significantly alter Rev activity (7), and in vivo studies suggest that changes in Rev phenotype correlate with changes in the clinical stage of disease (4, 6). In particular, Rev is significantly less active during the inapparent compared to the chronic stage of disease, suggesting that the Rev phenotype contributes to selection of virus variants in vivo. Insight into the genetic changes and factors that contribute to Rev selection in vivo requires identification of the functional domains and motifs that mediate EIAV Rev activity.

The Rev/Rex proteins of complex retroviruses differentially regulate expression of incompletely spliced mRNAs encoding virion structural and enzymatic proteins and progeny RNA molecules (reviewed in reference 17). The prototypical member of this family, human immunodeficiency type 1 (HIV-1) Rev, binds to the viral pre-mRNA at a specific sequence called the Rev-responsive element (RRE) (15, 48), multimerizes (37, 47), and facilitates export of incompletely spliced RNAs from the nucleus via a nucleoporin pathway distinct from that used by most cellular mRNAs (18, 19). Mutational analyses indicate that the activities of HIV-1 Rev are mediated by discrete functional domains, including an N-terminal arginine-rich RNA-binding motif (ARM), which also functions as a nuclear localization signal (NLS) (24, 32), and a C-terminal leucine-rich nuclear export signal (NES) (18, 19). EIAV Rev is a 165-amino-acid protein translated from exons 3 and 4 of a multiply spliced, 4-exon, bicistronic mRNA that also encodes the *trans*-activating protein Tat (Fig. 1A) (12). EIAV Rev is functionally homologous to HIV-1 Rev (20, 33) but is less well characterized. The N-terminal leucine-rich NES, which maps to amino acids 31 to 55 (Fig. 1B) (20, 23, 33), is similar to other leucine-rich viral and cellular export proteins that interact with the nuclear exporter CRM1; however, EIAV Rev is atypical in the spacing of the leucine residues within the NES (23, 38). The C-terminal basic region has been found to be important for nuclear localization, while the central region of the protein has been implicated in RNA binding (14, 23).

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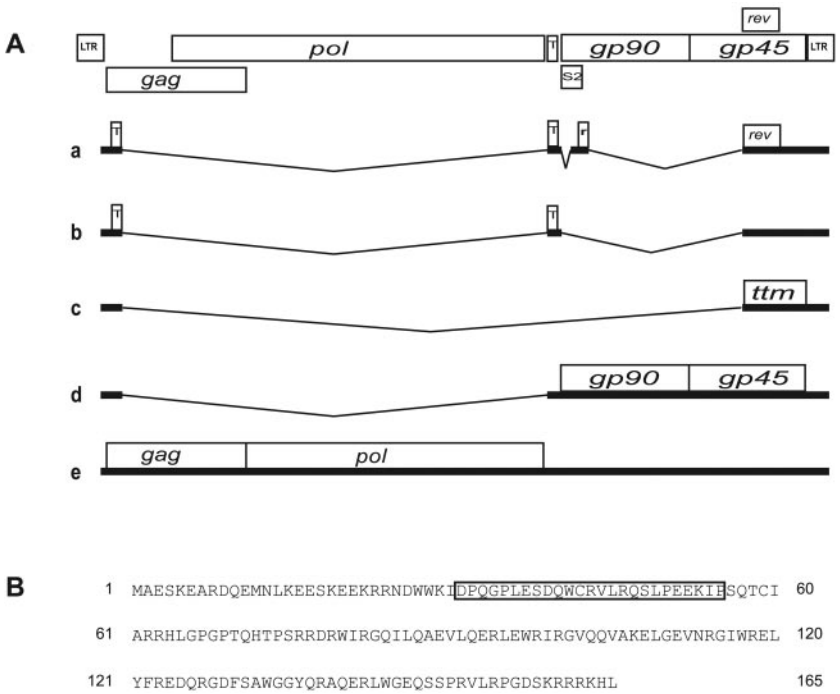

FIG. 1. Organization and splicing patterns of EIAV and the EIAV Rev amino acid sequence. (A) Schematic of the EIAV genome showing open reading frames (ORF) and predominant mRNAs (a to e) isolated from virus-infected tissue culture cells (27). Regulatory proteins Tat (T) and Rev (r, rev) are translated from the four-exon mRNA (a). In the presence of Rev, EIAV exon 3 is skipped, resulting in a three-exon multiply spliced mRNA that encodes only Tat (b). Structural proteins and progeny RNA molecules are translated from singly spliced (d) and unspliced mRNAs (e). Ttm, a protein of unknown function (5), is encoded by a two-exon mRNA (c). (B) The amino acid sequence of the wild-type EIAV Rev H21 (7) is shown and numbered 1 to 165. The nuclear export signal (aa 31 to 55) is boxed.

In addition to promoting nuclear export of incompletely spliced RNA, EIAV Rev regulates alternative splicing of the viral RNA. In the presence of Rev, the multiply spliced mRNA, which lacks exon 3 (Fig. 1A), is produced (34). Exon 3 contains the translational start site for Rev, and alternative splicing was originally proposed as a novel mechanism for autoregulation of Rev expression (21, 23). Exon 3 is flanked by a suboptimal splice acceptor and contains a purine-rich exonic splicing enhancer (ESE) that interacts with the SR protein SF2/ASF (21). The ESE also functions as an EIAV RRE (6), and we have suggested that EIAV Rev mediates alternative splicing of exon 3 through protein-RNA interactions required for efficient export of incompletely spliced viral RNAs. While the exact mechanism of alternative splicing is not known, current models (6, 14) agree that alternative splicing requires both nuclear localization and RNA binding and perhaps an as yet unidentified domain(s) of Rev that interacts directly with SF2/ASF and/or other cellular splicing factors. To date, the specific amino acids that mediate EIAV Rev nuclear import, RNA binding, and alternative splicing have not been identified. Using a series of deletions and mutations in EIAV Rev cDNA, we mapped functional domains of EIAV Rev and identified specific motifs required for nuclear localization and RNA binding. Two noncontiguous, short ARMs were required for RNA binding, suggesting that EIAV Rev contains a bipartite RNA-binding domain.

MATERIALS AND METHODS

Construction of Rev mutants. EIAV Rev cDNA deletion mutants were generated in the previously described plasmid pRevWT (7). Rev mutants containing internal deletions were constructed by PCR-ligation-PCR mutagenesis as described by Ali and Steinkasserer (3). Briefly, upstream and downstream blunt-ended cDNA fragments of each mutant were amplified from pRevWT using the primers designed from the EIAV Wyoming cell culture-adapted isolate (GenBank accession no. M16575) (27). Specific primer sequences used for cloning are available upon request. Fragments were gel purified, and downstream fragments were phosphorylated and ligated to the corresponding upstream fragment. Ligation products were amplified by PCR using wild-type 5′ (CAGCATGGCAGAATCGAAGG) and wild-type 3′ (CGAGAGTTCCTTCTTGGGA) primers. Following the second PCR, the cDNAs were TA cloned into pCR3.1 (Invitrogen, Carlsbad, CA) and transformed into *Escherichia coli* DH5-α, and transformants were screened by colony blot hybridization. The C-terminal deletion mutants were PCR amplified using the 5′ wild-type flanking primer and unique 3′ primers that generated premature stop codons. PCR was performed using standard methods, and cDNAs were TA cloned into pCR3.1 as described above. Site-specific mutations were introduced by PCR-based mutagenesis. All constructs were confirmed by sequencing, and protein expression was verified by Western blotting using EIAV convalescent horse sera (10).

Rev fusion proteins. To construct green fluorescent protein (GFP) fusion proteins, Rev sequences were PCR amplified from wild-type and deletion constructs with primers that introduced 5′ EcoRI and 3′ BamHI restriction sites. PCR products were digested with EcoRI and BamHI and cloned into the GFP expression vector pEGFP-C2 (Clontech, Palo Alto, CA). The C-terminal region of Rev (specifying amino acids 145 to 165) was synthesized as complementary oligonucleotides that created 5′ EcoRI and 3′ BamHI overhangs after annealing. Wild-type and mutant oligonucleotides were synthesized, annealed, digested with EcoRI and BamHI, and cloned into similarly restricted pGFP-RDM12. All plasmids were sequenced to verify that each mutant Rev was translated from a single open reading frame.

A series of maltose-binding protein (MBP)-Rev fusion proteins were used for RNA-binding studies. ERev fragments amplified from EIAV R1A (6) or Rev cDNA plasmids were cloned in pHMTc (43), which is based on the pMal-c2x expression vector (New England Biolabs, Beverly, MA). For most MBP-ERev constructs, Rev cDNA templates were amplified from EIAV variant R1A; MRD8 contains the Rev deletion mutant RDM11, which is based on pRevWT (see above). MBP-ERev constructs containing point mutations were cloned using amplified mutated PCR fragments based on R1A Rev cDNA template. All plasmid constructs were confirmed by DNA sequence analysis.

CAT assays. Rev nuclear export activity was quantified in transient transfection assays using a pDM138-based EIAV Rev reporter construct, pERRE-All, as previously described (7). 293T cells were seeded in triplicate at 1×10^5 to 5×10^5 cells/well in 6-well tissue culture dishes and maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS) and penicillin-streptomycin (P-S) at 37°C and 8% CO_2. One day after seeding, cells were transfected with 1.0 μg Rev cDNA or empty vector, 0.2 μg pERRE-All reporter plasmid DNA, 0.2 μg beta-galactosidase reporter plasmid DNA, and sufficient pUC19 to bring each well to 2.0 μg total transfected DNA. Medium was changed the next day, and cells were harvested at 48 h posttransfection, lysed by freezing-thawing, and normalized for transfection efficiency by measuring beta-galactosidase activity as described previously (7). Cell lysates were assayed for β-galactosidase activity and normalized reaction volumes were assayed for chloramphenicol acetyltransferase (CAT) enzyme activity or for CAT protein using thin-layer chromatography or a commercial CAT enzyme-linked immunosorbent assay kit (Roche Molecular Biochemicals, Indianapolis, IN), respectively (7, 8). All assays were performed in triplicate, and results represent at least six independent transfections. Statistical analysis was performed using analysis of variance and Student's *t* test assuming unequal variance among groups.

***Trans*-complementation assays.** Cf2Th (ATCC no. CRL-1430) clonal cell lines containing Rev (+) or Rev (−) EIAV proviral DNA were used to characterize nuclear export activity (7). Cf2Th/112 cells contain a Rev-competent proviral clone and produce supernatant reverse transcriptase (RT) activity, viral structural proteins, and all classes of viral mRNAs. Cf2Th/51 cells contain Rev-defective proviral DNA, express only fully spliced mRNA, and lack detectable levels of viral structural proteins or RT activity. *Trans*-complementation of Cf2Th/51 cells with Rev cDNA results in viral protein expression and RT activity (7). Cells were seeded at 5×10^5 cells/well in 6-well tissue culture plates and maintained in DMEM supplemented with 10% FCS and P-S at 37°C and 8% CO_2. The next day, cells were transfected with 2 μg of wild-type Rev cDNA, Rev mutant cDNA, or empty vector DNA using the liposome-mediated transfection reagent Lipofectamine (Invitrogen). At 3 days posttransfection, supernatant was collected and assayed for RT activity as previously described (11).

Nuclear localization assays and microscopy. Cf2Th cells were plated at 2×10^4 cells/cm^2 in 6- or 24-well plates or 8-well glass chamber slides (Nunc, Rochester, NY). Twenty-four hours after plating, cells were transiently transfected with 0.25 μg GFP or GFP-Rev plasmid DNA per 2×10^4 cells using the liposome-mediated transfection reagent TransIT-LT1 according to manufacturer instructions (Mirus, Madison, WI). In certain experiments, replicate cultures were treated at 20 h posttransfection with 5 nM leptomycin B (LMB) in dimethyl sulfoxide. At 24 h after transfection, cells were fixed in 3.7% formaldehyde in 10 mM phosphate-buffered saline (PBS) for 30 min at 25°C and washed twice with complete DMEM supplemented with 10% FCS and P-S. Nuclei of fixed cells were stained with 0.5 μg/ml Hoechst 33258 dye (Sigma, St. Louis, MO) in 0.5% NP-40 and 10 mM PBS for 15 min at 25°C. Cells were subsequently washed twice in complete DMEM and once in 10 mM PBS. All transfections were performed in triplicate.

Fixed cells in 6- and 24-well plates were examined with an inverted Nikon Diaphot fluorescence microscope with a 40× objective and a 100-W high-pressure mercury lamp; epifluorescence filters were used to visualize Hoechst-stained nuclei and GFP. For confocal microscopy, chambers were removed from slides and a coverslip was sealed over fixed cells; slides were examined with a Leica TCS NT laser confocal microscope using a 63× oil-immersion objective; digital filters with 400- to 480-nm and 500- to 560-nm excitation wavelengths were necessary to visualize Hoechst-stained DNA and GFP, respectively. Brightness and contrast of images obtained by confocal microscopy were adjusted with Adobe Photoshop 4.0.

RNA-binding assays. MBP-Rev fusion proteins were expressed in Rosetta-Gami DE3 (pLacI) (Novagen, Madison, WI). Harvested cells were lysed by freezing-thawing, and His-tagged fusion proteins were purified under native conditions using Ni^{2+}-charged resin (Invitrogen). The purity of fusion proteins was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie staining or Western blotting using EIAV convalescent-phase sera or polyclonal antibodies directed against the His Tag (MBL International, Woburn, MA). Purified MBP-Rev proteins were dialyzed and stored in 50 mM Tris-HCl (pH 8.0), 50 mM NaCl at 4°C. The EIAV Rev-responsive element (RRE) (nucleotides 5443 to 5565) was amplified by PCR from pERRE-All (8) using a 5′ primer containing a T7 promoter site. The PCR product was purified using QIAquick PCR purification columns (QIAGEN, Valencia, CA), and RNA was generated by in vitro transcription (T7-MEGAshortscript; Ambion, Austin, TX) in the presence of [α-^{32}P]UTP. Transcribed radiolabeled RNA was purified on a G50 column (Roche, Indianapolis, IN), denatured at 80°C for 5 min in 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, annealed by slow cooling, and stored at −80°C.

For RNA-binding reactions, 2 to 4 μg purified MBP-Rev fusion protein was incubated with 10^4 cpm of ^{32}P-labeled EIAV RRE RNA in binding buffer (10 mM HEPES-KOH, pH 7.5, 100 mM KCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 1 mM dithiothreitol, 50 μg/ml *E. coli* tRNA, and 10% glycerol) for 20 min at room temperature. After incubation, reaction samples were irradiated with 3×10^5 μJ at 254 nm for 7 min. Samples were treated with 0.1 mg/ml RNase A at 37°C for 20 min; the reaction was terminated by boiling 5 min in an equal volume of SDS and separated in SDS–12% PAGE in Tris-glycine buffer. Gels were fixed in 50% methanol–10% acetic acid, dried, and exposed to PhosphorImager screens overnight. UV cross-linked complexes were quantified using a PersonalFX scanner and Quantity One software (Bio-Rad, Hercules, CA).

RESULTS

Functional analyses of Rev deletion mutants. Lentiviral Rev proteins utilize discrete functional domains to control expression of viral mRNAs and structural proteins. To aid in identification of EIAV Rev functional domains, we constructed a nested set of Rev deletion mutants (RDM) (Fig. 2A) and tested them for nuclear export activity in transient transfection assays using the CAT reporter construct, pERRE-All (7). Nuclear export activity was highly sensitive to deletions in the Rev protein (Fig. 2B, panel 1). Mutants RDM4, RDM6-RDM10, and RDM12 showed no CAT activity, whereas activity was significantly reduced in RDM5. Only one mutant, RDM11, showed levels of CAT activity comparable to wild-type Rev. To ensure that these results were not an artifact of the CAT reporter construct, all mutants were also tested by *trans*-complementation of a Rev-defective clonal cell line, Cf2Th/51 (Fig. 2B, panel 2). Overall, there was good agreement between the two assays: transfection with mutants RDM4-RDM10 and RDM12 showed no RT activity, while cells transfected with mutant RDM11 had activity similar to that of wild-type Rev. Western blot analysis using anti-EIAV polyclonal horse sera was done to confirm that all mutants expressed Rev. Some variation in protein expression was observed; however, the levels of expression did not correlate with levels of Rev activity. Mutants RDM4, RDM5, RDM6, RDM11, and RDM12 expressed at levels equal to or higher than Rev-WT, while RDM7, RDM8, and RDM10 expression levels were somewhat lower than Rev-WT. Only RDM9 showed markedly reduced levels of protein expression compared to other mutants. With the exception of RDM9, therefore, the loss of nuclear export activity was likely due to deletion of discrete functional domains and/or the loss of tertiary structure(s) required for activity.

The KRRRK motif in the C terminus of Rev is required for nuclear localization. The highly basic C terminus of EIAV Rev contains residues essential for nuclear entry (23); however, the precise NLS has not been identified. To more specifically identify the amino acids necessary for nuclear localization, each of the Rev deletion mutants shown in Fig. 2A were used to construct GFP-Rev fusion proteins. Plasmid DNA was transiently transfected into Cf2Th cells, and subcellular localization of

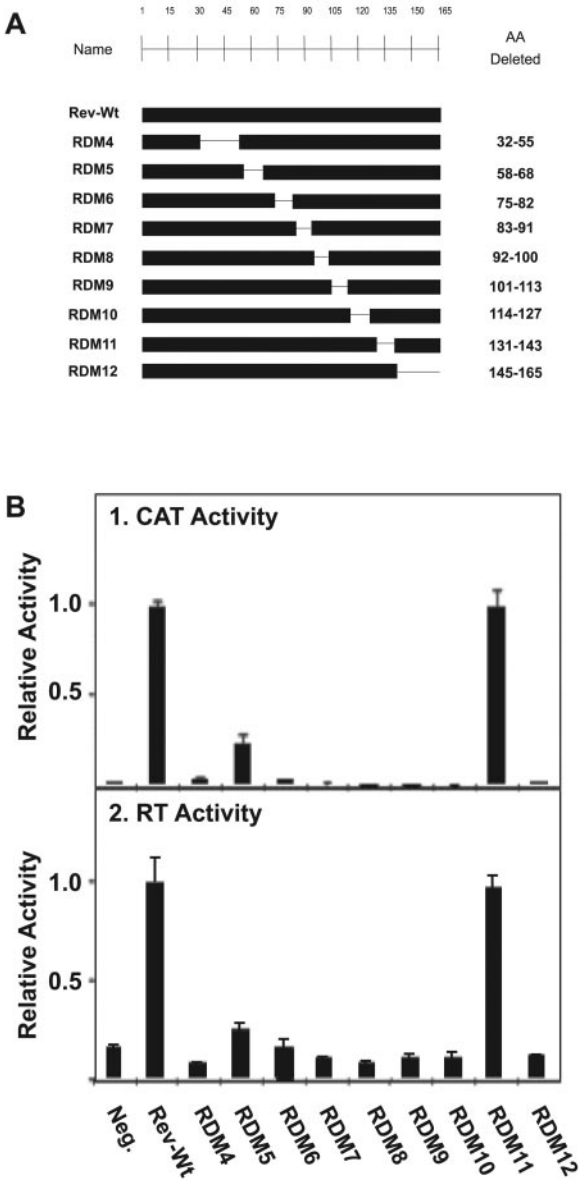

FIG. 2. Functional analyses of Rev deletion mutants. (A) Terminal and internal deletions in Rev cDNA were generated from pRevWT by PCR-ligation-PCR as described in the text. The region of amino acids deleted in each cDNA is indicated. (B) Activity of Rev deletion mutants was assayed in transient expression assays. Results are reported as activity relative to pRevWT. Cells transfected with the pCR3.1 vector DNA were used as the negative control (Neg.) in all assays. Error bars denote the standard error of the mean. Graph 1 shows nuclear export activity measured using a CAT reporter assay (7). Lysates were normalized by β-galactosidase activity, and CAT activity was measured as the percentage of acetylation. Individual experiments included triplicate wells, and the data shown represent the means of at least three separate experiments. Graph 2 shows supernatant reverse transcriptase (RT) activity following *trans*-complementation of a Rev-defective clonal cell line, Cf2Th/51, with Rev deletion mutants. Wt, wild type.

fusion proteins was assessed by fluorescence and confocal microscopy. Only two of the nine GFP-Rev deletion mutants exhibited subcellular localization patterns different from that of wild-type Rev (data not shown). GFP-RDM4, which contains a deletion in the NES, was found only in the nucleus, and GFP-RDM12, which lacks the 21 C-terminal residues of Rev, was found exclusively in the cytoplasm. The C terminus contains a strongly basic KRRRK motif that is similar to other basic NLSs (22, 25, 46) and was previously suggested to be a component of the EIAV Rev NLS (23). To identify critical residues within the C-terminal region, we introduced additional deletions or alanine substitutions across the last seven residues of GFP-Rev (Fig. 3A). Mutants were transfected into Cf2Th cells and assayed for subcellular localization in the presence and absence of leptomycin B (LMB) (Fig. 3B). Rev is a nucleocytoplasmic shuttling protein, and LMB was used to block export of fusion proteins once they had translocated to the nucleus. Deletion of six (GFP-RDM13) or two (GFP-RDM14) amino acids from the C terminus of EIAV Rev abrogated nuclear localization. Alanine substitution within the KRRRK motif indicated that mutation of the middle arginine (C3 = KRARK) or the terminal lysine (C6 = KRRRA) did not alter nuclear import; however, Rev-GFP proteins with alanine substitutions of any two adjacent basic residues within amino acids 159 to 163 (C1 = AARRK, C2 = KAARK, C4 = KRAAK, C5 = KRRAA) remained in the cytoplasm. Therefore, nuclear localization was dependent on the presence of a cluster of basic amino acids within the KRRRK motif in the C-terminal region.

Mapping the RNA-binding domain of EIAV Rev. In HIV-1 Rev, the RNA-binding domain overlaps the NLS (24). In EIAV Rev, however, the NLS is located in the C-terminal basic region, while the RNA-binding domain is thought to reside within the central region of EIAV Rev (23). To better define the RNA-binding domain of EIAV Rev, we assessed RNA binding activity of a series of truncated MBP-Rev deletion (MRD) mutants in UV cross-linking assays (Fig. 4A). The purity and integrity of the MBP-Rev fusion proteins were confirmed by SDS-PAGE (Fig. 4B). RNA-protein complexes were observed in reactions containing either MBP-Rev or MRD1 and the sense strand of the RRE but not in reactions containing the antisense RRE RNA (Fig. 4C). RNA-protein complexes included a clearly defined, faster migrating band and diffuse slower migrating products. No RNA-protein complexes were observed with either bovine serum albumin or MBP, demonstrating the specificity of Rev-RNA binding (Fig. 4C).

Our analysis of the Rev-MBP deletion mutants showed that MRD1 and MRD3 each formed an RNA-protein complex, indicating that neither exon 1 (amino acids [aa] 1 to 30) nor the N-terminal leucine-rich NES (aa 31 to 56) was required for RNA binding. Constructs lacking either the last 20 or 40 C-terminal residues (MRD2, MRD4, and MRD5) did not form a complex with the EIAV RRE (Fig. 4D). However, no RRE binding was detected using fusion proteins containing only C-terminal residues (MRD6 and MRD7). Therefore, residues within the C-terminal domain were necessary, but not sufficient, for RNA binding. To aid in identifying the boundaries of the Rev RNA binding domain, we constructed MRD8, a mutant with an internal deletion of aa 131 to 143. This is the same region deleted in RDM11 (Fig. 2), a deletion mutant with wild-type levels of nuclear export activity. MRD8 bound the RRE RNA at levels comparable to wild-type Rev and MRD1 (Fig. 4E). There appeared to be a relative increase in the slower migrating RNA-protein complexes (compare Fig. 4E

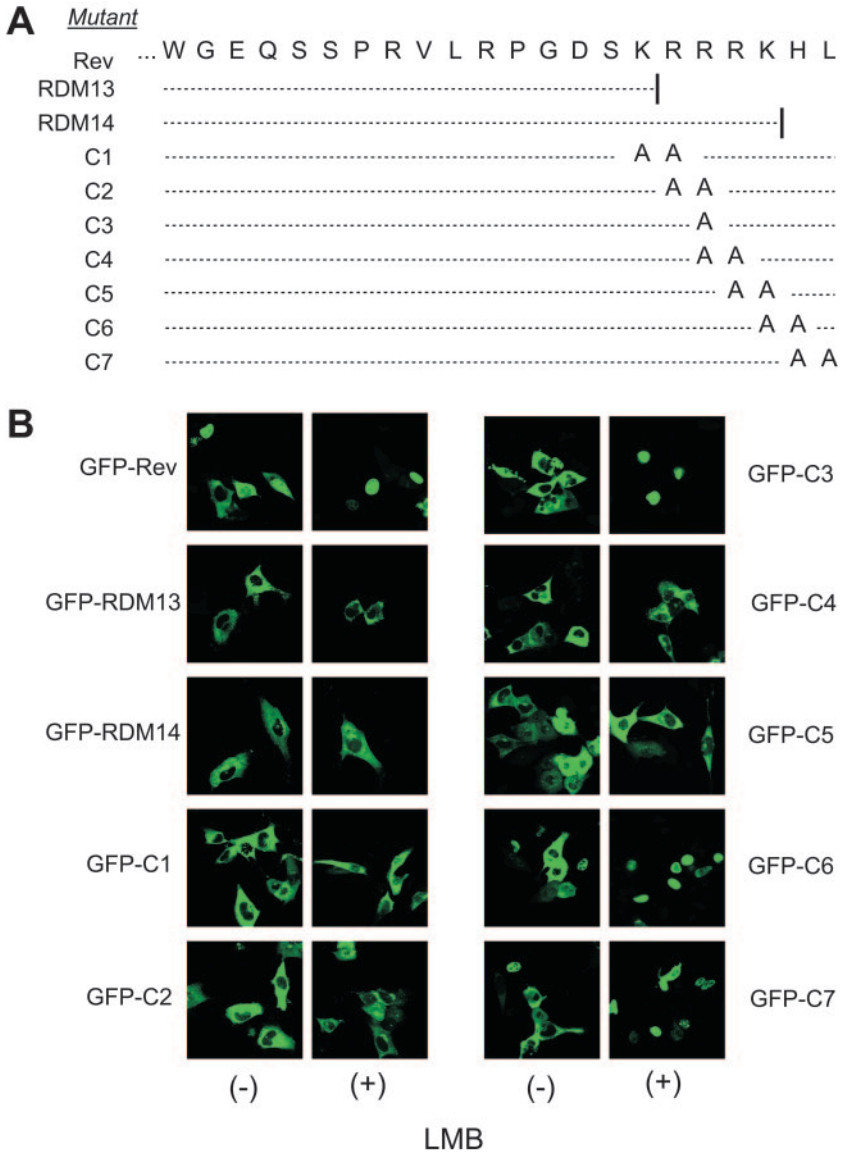

FIG. 3. The KRRRK motif in the C terminus of Rev is required for nuclear localization. (A) Amino acid sequence of the C terminus of wild-type Rev (aa 144 to 165) and the location of deletions or alanine substitutions introduced into wild-type GFP-Rev mutants. (B) Subcellular location of GFP-Rev deletion mutants (GFP-RDM13 and GFP-RDM14) and GFP-Rev containing alanine substitutions in the C terminus. Cf2Th cells were transfected with the specified Rev-GFP cDNAs in the presence or absence of 5 nM leptomycin B (LMB). Images of fixed and stained Cf2Th cells were obtained by confocal laser microscopy. Brightness and contrast were adjusted with Adobe Photoshop 4.0.

with C and D); however, this may be due to variability between experiments rather than differences in Rev binding. The RNA-binding activity of MRD8, together with the lack of RNA binding in MRD5, MRD6, and MRD7, indicate that two, non-contiguous regions of EIAV are required for RNA-binding activity. One region encompasses aa 57 to 130, and the second region is located in the C terminus of Rev, encompassing aa 144 to 165.

Two noncontiguous ARMs are required for RNA binding and nuclear export activity. Arginine-rich motifs are among the most common and well-characterized RNA-binding motifs (9) and are essential for RNA-binding activities of HIV Rev and Tat (24, 29, 41, 45). In HIV-1 Rev, a single 12-aa-long ARM located within the N-terminal domain functions in both RNA binding and nuclear localization (24). EIAV Rev contains two short Arg-rich motifs: RRDRW (aa 76 to 80) within the central region and KRRRK (aa 159 to 163) in the C-terminal region. Another motif within the central domain, ERLE (aa 93 to 96), was proposed as an RNA-binding motif based on studies showing that replacement of the motif with alanines abolished nuclear export and alternative splicing activity (23) and abrogated RNA binding in gel mobility shift assays (14). All three motifs are located within the regions we identified as essential for RNA-binding activity of EIAV Rev (Fig. 4). To directly test their contribution to RRE binding, we introduced alanine substitutions into each motif in the background of MBP Rev construct MRD1. The amount of RNA-protein complexes was greatly reduced with either MRD1-

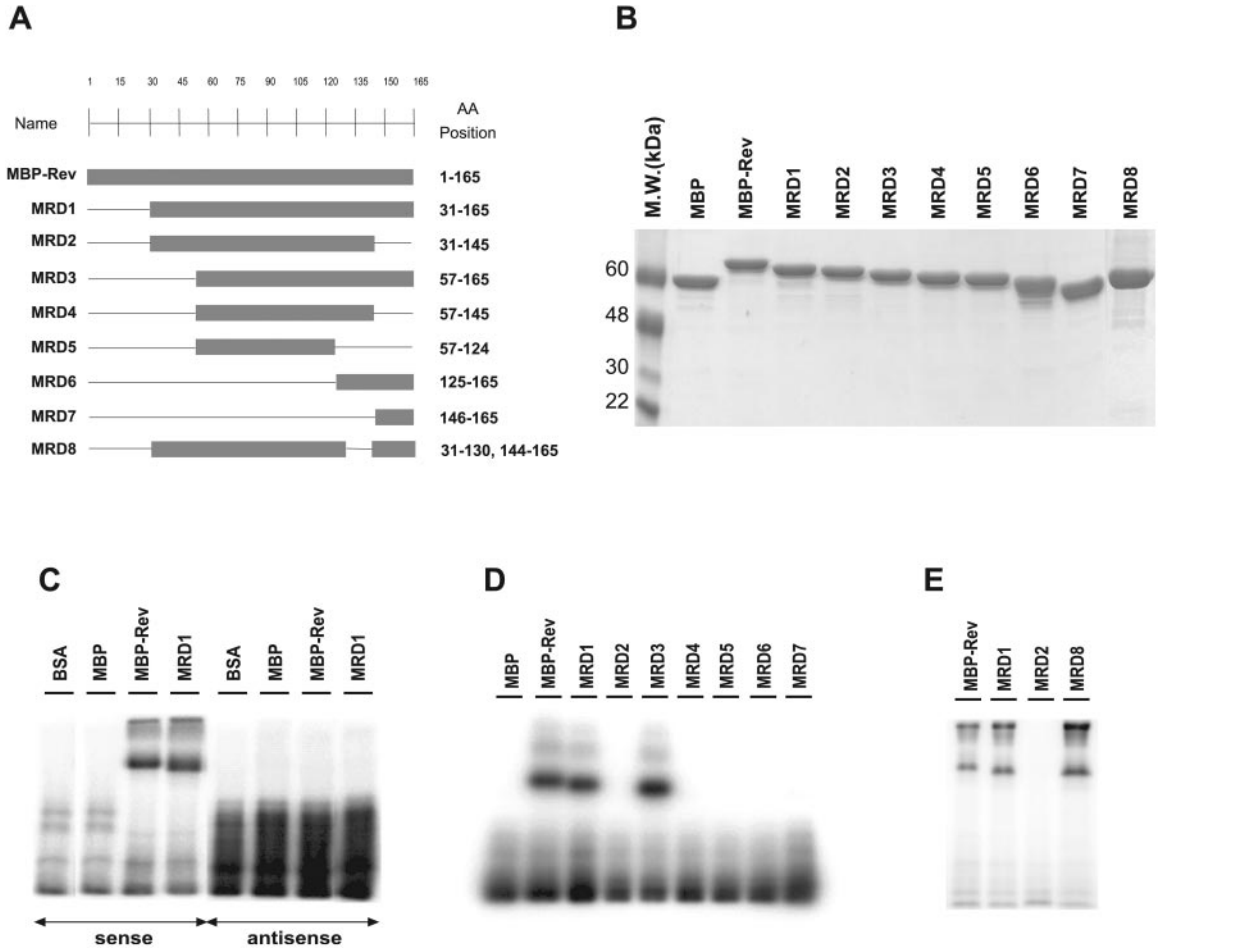

FIG. 4. Identification of sequences critical for RNA-binding activity of EIAV Rev. (A) MBP-Rev deletion mutants (MRD) containing 5′ or 3′ deletions used to map regions of Rev required for RNA binding. The Rev amino acids retained in each construct are indicated. (B) Expression and purity of MRD mutants were assessed by Coomassie staining of SDS-PAGE gels. The molecular size of each deletion mutant was confirmed using molecular size (MW) markers. (C) RNA-binding activity of MBP-Rev fusion proteins was determined by UV cross-linking and SDS-PAGE. Rev fusion proteins were incubated with radiolabeled EIAV RRE (nucleotides 5443 to 5565), cross-linked with UV irradiation, and treated with RNase. RNA-protein complexes were separated by SDS-PAGE and quantified using a PersonalFX scanner and Quantity One software (Bio-Rad). Negative controls included bovine serum albumin (BSA), MBP, and antisense RRE. (D and E) RRE-binding activity of Rev deletion mutants as described for panel C.

AADAA, where all of the residues except the Asp in the RRDRW motif were replaced by Ala, or MRD1-KAAAK, in which all Arg residues in the KRRRK motif were replaced by Ala (Fig. 5A). However, near wild-type levels of RNA-protein complexes were observed with MRD1-AALA, where the charged residues (Arg and Glu) in the ERLE motif were replaced by Ala. Thus, Arg residues in both the RRDRW and the KRRRK motif are essential for RNA binding. The finding that two separate, noncontiguous Arg-rich motifs are required for RNA binding indicates that EIAV Rev contains a bipartite RNA-binding domain, one portion of which overlaps the nuclear localization signal. This RNA-binding arrangement of EIAV Rev differs markedly from that of HIV-1 Rev.

Although others have reported a loss of RNA-binding activity due to alanine substitutions for the ERLE motif in the central domain of EIAV Rev (14), we observed no decrease in RNA binding when Ala was substituted for only the charged residues in the ERLE motif (MRD1-AALA). This suggested

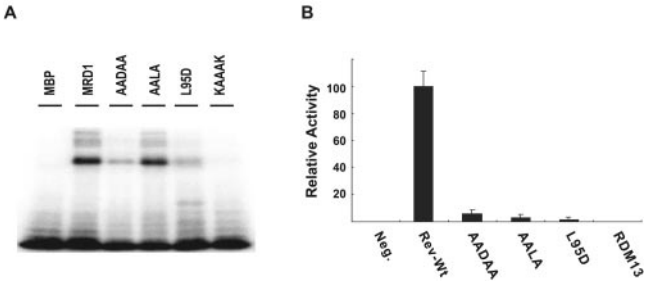

FIG. 5. RRE-binding activity and functional analyses of Rev mutants. A. Identification of specific motifs essential for RRE-binding activity of EIAV Rev. Point mutations introduced into the RRDRW, ERLE, and KRRRK motifs include alanine substitution of charged and/or Arg residues (AADAA, AALA, and KAAAK) and substitution of Asp for Leu in the ERLE motif (L95D). Binding activity was assessed as described in the legend to Fig. 4C. B. Activity of Rev point mutation mutants (AADAA, AALA, KAAAK, and L95D) and deletion mutant (RDM13) were assayed as described in the legend to Fig. 2B. Neg., negative control. Wt, wild type.

that the Leu residue at position 95 was critical for the RNA-binding activity. The predicted secondary structure of EIAV Rev, based on consensus results using five different prediction programs (16, 28, 29, 39, 42), places the ERLE motif in the middle of an α-helix, and it was previously suggested that the Ala substitutions may disrupt the α-helical structure (14). To explore this, we substituted an Asp for the Leu residue at position 95 (L95D). The Asp can act not only as a helix breaker but also can disrupt hydrophobic interactions between helices. The L95D mutation resulted in an 80% reduction of the maximum binding of Rev to RRE (Fig. 5A). Therefore, the ERLE may play a role in stabilizing the protein structure required for RNA-binding activity.

To extend our analyses of the RRDRW and ERLE motifs, we introduced the Ala or Asp substitutions in R1A cDNA and analyzed the effect of mutations on Rev nuclear export activity in transient transfection assays (Fig. 5B). All of the RRDRW and ERLE mutants had significantly reduced Rev nuclear export activity. In addition, deletion of the KRRRK motif (RDM13), which is required for both RNA binding and nuclear export, eliminated Rev nuclear export activity in cell-based transfection assays. The results of these functional studies support our RNA-binding studies and establish a critical role for these motifs in Rev nuclear export activity. Interestingly, the AALA mutant was able to bind to viral RNA but was defective for nuclear export activity. Although its exact role is not known, this confirms previous findings that ERLE is critical for Rev activity (14, 23). More detailed analyses of Rev-RRE interactions will further enhance the results of our in vitro RNA-binding assays.

DISCUSSION

The Rev/Rex proteins of complex retroviruses are essential regulatory proteins that mediate nuclear export of incompletely spliced viral mRNAs via discrete functional domains that interact with cellular proteins and viral RNA. This family of proteins contains an interchangeable nuclear export signal that interacts with the cellular protein Crm1 and an ARM that binds specifically to homologous viral mRNA. Compared to most lentiviral Rev proteins, EIAV Rev is atypical with respect to the organization of functional domains (20, 23), the spacing of critical residues in the NES (23, 33), the use of a purine-rich, exonic splicing element as an RRE (8, 34), and the regulation of alternative splicing of the bicistronic *tat*/*rev* mRNA (34). In addition, EIAV Rev is highly variable in vivo, and we have shown that changes in Rev phenotype correlate with changes in clinical stages of EIAV infection (4, 6, 7). To better understand the significance of Rev variation in vivo, we undertook more detailed analyses of the functional domains of EIAV Rev. Previous studies identified point mutations in EIAV Rev that reduced nuclear export (7, 20, 23, 33, 35), alternative splicing (7, 23), RNA binding (14), and nuclear localization activities (23). Here, we extend those studies to map functional domains and identify specific motifs that are essential for EIAV Rev activity. All domains essential for Rev activity are contained within the second exon of EIAV Rev (20, 23). The overall functional organization of EIAV Rev exon 2 includes the N-terminal nuclear export signal, a large central region that contains amino acids required for RNA binding, a nonessential region, and a C-terminal region required for both nuclear localization and RNA binding. Two short, noncontiguous ARMs are necessary for RNA binding: a central RRDRW motif and a C-terminal KRRRK motif that is also essential for nuclear localization. The bipartite ARM is unique among lentivirus Rev proteins and provides another example of how EIAV uses an unusual protein arrangement to carry out a common lentiviral function.

In HIV-1 Rev, the prototypical lentiviral Rev, a 17-aa ARM is located in the N-terminal half of the protein and serves as both a sequence-specific RNA-binding domain and the NLS (32, 37, 40). No typical ARM is found within EIAV Rev, and prior studies have not directly examined the RNA-binding domain of EIAV Rev. However, previous studies have identified several mutants defective in either nuclear localization or RNA binding (14, 23). Mutants containing alanine substitutions of 157DSKR160 (M15) or 161RR162 (M1) were defective in nuclear entry and showed reduced alternative splicing activity (23). Alanine substitutions of 76RRDR79 (M11) and 93ERLE96 (M27) resulted in loss of nuclear export activity (23), and the latter was defective in RNA binding and alternative splicing (14). Based on these results, it appeared that there was no overlap between the NLS and RNA-binding domain and that the EIAV Rev RNA-binding domain was distinct from the ARMs characteristic of other complex retroviruses.

The present study is the first to directly characterize the RNA-binding activity of EIAV Rev and provides further insight into the specific motifs essential for EIAV Rev activity. The EIAV Rev NLS is relatively compact and requires the KRRRK motif contained within the C terminus of Rev. Mutants that retained four or five basic residues at amino acids 159 to 163 were able to translocate to the cell nucleus (C3 and C6), while mutants with less than four basic residues in this motif remained in the cytoplasm (C1, C2, C4, and C5). The KRRRK motif is similar to the arginine-rich NLSs of HIV-1 Rev and HTLV-1 Rex (32, 36, 40) and is nearly identical to the KRRR nuclear localization motifs found in the *Drosophila melanogaster gcm* gene product (1) and the RNA-binding human DEDD caspase protein (44). The ERLE and RRDR motifs identified in previous mutants M11 and M27 (23) were both found to be important in the RNA-binding activity of EIAV Rev, but they act through different mechanisms. Mutations of the charged residues in the ERLE motif had no effect on RNA binding, whereas the L95D mutation reduced binding by more than 80%. Therefore, the ERLE motif may play a role in stabilizing the protein structure required for binding of Rev to the viral RNA. In contrast, the arginine residues in both the RRDRW and KRRRK motifs were required for RNA binding, suggesting these short ARMs directly contact the RRE. Based on the results of our deletion analyses, other residues in the central domain likely contribute to RNA binding through direct contact with RNA and/or stabilization of protein structure.

Arginine rich motifs are short, arginine-containing regions of 10 to 20 amino acids that mediate RNA binding of a number of viral and ribosomal proteins (9). The arginine residues are thought to play two general roles in RNA binding: first, as a probe to search for a high-affinity binding site, and second, to form a network of specific hydrogen bonds with the RNA backbone and specific bases (9). In the present study, we found

that RNA binding of EIAV Rev required two short ARMs separated by 79 amino acids in the primary protein sequence. One motif is located in the central region, and the second overlaps with the NLS in the C terminus. The results of UV cross-linking experiments showed that the C-terminal 20 amino acids of EIAV Rev are required, but not sufficient, for binding to the RRE. Importantly, site-specific mutation of arginine residues in either the central ARM or the C-terminal ARM abolished RRE binding. These results strongly suggest that the two ARMs interact with the EIAV RRE in concert and thus comprise a bipartite RNA-binding domain. Coordinated action of two, noncontiguous ARMs was also found to be necessary for binding of hepatitis delta antigen to viral RNA (13). It is not clear how two ARMs interact to bind viral RNA. The two domains may be in close proximity within the three-dimensional structure of the folded protein, where they could form a single RNA-binding domain containing at least seven arginine residues. Alternately, the two ARMs could interact with different regions of the viral RNA. Further analyses of EIAV Rev-RRE interactions will provide insight into this potentially novel class of RNA-binding motifs.

In addition to promoting export of incompletely spliced viral RNA, EIAV Rev regulates alternative splicing of exon 3 in the bicistronic *tat*/*rev* mRNA (34). Exon 3 contains a purine-rich region that binds both Rev and the SR protein SF2/ASF and thus functions as both the EIAV RRE and an exonic splicing enhancer (7, 21, 31). Alternative splicing, or exon 3 skipping, likely results from competition between these two functions at either the RNA binding step or at a downstream step involved in spliceosomal assembly or activation (7, 14, 21, 31). Previous studies reported a loss in alternative splicing activity in Rev mutants containing alanine substitution of the ERLE motif or in both the RRDR and KRRRK motifs (14). Mutational analyses of *cis*-acting sequences required for exon 3 skipping show a close correlation between alternative splicing activity and RNA-binding activity (8, 14). Together, these data suggest that RNA binding plays an essential role in alternative splicing. Studies to date, however, have been unable to identify an alternative splicing domain distinct from other Rev functional domains (23). Therefore, it has been difficult to assess the biological significance of alternative splicing in vivo. Exon 3 skipping was originally proposed as a novel mechanism for autoregulation of Rev (34); however, mRNAs lacking exon 3 comprise only a small percentage of viral mRNA in Rev-expressing cells (31). At present, the primary function of EIAV Rev appears similar to other lentiviral Revs in regulating the shift from production of multiply spliced to incompletely spliced viral mRNAs.

There is a relatively high rate of genetic variation in the region of the EIAV genome where the second exon of Rev overlaps the cytoplasmic portion of the transmembrane protein gp45 (2, 6, 7, 30). The NLS and RNA-binding motifs are highly conserved, while the region identified here as nonessential for Rev function is the site of a number of amino acid changes that significantly altered Rev phenotypes (6). The nonessential region spans a predicted α-helix, and our results indicate that deletion of this region does not alter the structure of the Rev in a way that compromises Rev function. Similarly, the overlapping region of TM is found within the cytoplasmic domain, which may also be able to accommodate some degree of genetic variation without loss of function. As such, this region of the viral genome may be able to tolerate genetic mutations in Rev and/or TM that confer a selective advantage in vivo. Future studies may reveal how genetic variation in Rev contributes to viral persistence while preserving the function of this critical lentiviral protein.

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