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Comparative Analysis of Rev Function in Human Immunodeficiency Virus Types 1 and 2

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The Rev proteins of the related but distinct human immunodeficiency virus types 1 and 2 (HIV-1 and HIV-2) display incomplete functional reciprocity. One possible explanation for this observation is that HIV-2 Rev is unable to interact with the HIV-1 Rev-response element (RRE1). However, an analysis of the biological activity of chimeric proteins derived from HIV-1 and HIV-2 Rev reveals that this target specificity does not map to the Rev RNA binding domain but is instead primarily determined by sequences known to mediate Rev multimerization. Both HIV-1 and HIV-2 Rev are shown to bind the RRE1 in vitro with identical RNA sequence specificity. The observation that HIV-2 Rev can inhibit RRE1-dependent HIV-1 Rev function in *trans* indicates that the direct interaction of HIV-2 Rev with the RRE1 also occurs in vivo. These data suggest that HIV-2 Rev forms a protein-RNA complex with the RRE1 that leads to only minimal Rev activity. It is hypothesized that this low level of Rev function results from the incomplete and/or aberrant multimerization of HIV-2 Rev on this heterologous RNA target sequence.

Replication of human immunodeficiency virus type 1 (HIV-1) is critically dependent on the functional expression of the viral Rev *trans* activator (12, 34). The HIV-1 Rev (Rev1) protein is required for the cytoplasmic expression of the unspliced and singly spliced mRNA species that encode the viral structural proteins (11–14, 25). These HIV-1 transcripts bear the *cis*-acting RNA target sequence for Rev1, the highly structured HIV-1 Rev-response element (RRE1) (25). Rev1 binds to the 234-nucleotide RRE1 with high affinity in vitro and has also been shown to interact directly with the RRE1 in vivo (3, 6, 7, 15, 17, 27, 35, 38). Recently, a small ~13-nucleotide sequence within the RRE1 has been shown to form the primary RNA binding site for Rev1 (1, 8, 15, 36).

Mutational analysis of the Rev1 protein has demonstrated the existence of at least two essential domains (23). The more N terminal of these, extending from approximately amino acid (aa) 15 to aa 65, contains at its core an arginine-rich motif required for the nuclear and nucleolar localization of Rev1 (Fig. 1A) (16, 19, 23). This motif, which extends from aa 34 to aa 50 within Rev1, has also been shown to be both necessary and sufficient for sequence-specific binding to the RRE1 (2, 18, 24, 30, 39). Flanking this basic motif are essential sequences that have been shown to mediate the multimerization of Rev1 (24, 30, 39). As the basic motif of Rev1 is fully sufficient for RRE1 binding (2, 18), it appears unlikely that Rev1 multimerization is a prerequisite for this interaction. It has, instead, been proposed that Rev1 binds to the RRE1 primary binding site as a monomer (4, 24, 36). Rev1 multimerization on the RRE1 RNA substrate is hypothesized to occur subsequent to this initial interaction. It remains unclear whether secondary Rev binding sites on the RRE1 are specific, although footprinting analysis does indicate that they are discrete (17, 36).

A second domain in Rev1, located between approximately aa 75 and aa 84, plays no role in mediating the Rev1-RRE1 interaction but is critical for Rev1 function in vivo (Fig. 1A) (23, 24, 26, 28, 30, 37, 39). Indeed, Rev1 proteins bearing a defective form of this leucine-rich motif exhibit a dominant negative phenotype. It has therefore been proposed that this domain mediates the functional interaction of Rev1 with a cellular factor(s) involved in regulating RNA transport and/or splicing (23, 24, 26, 28, 30, 37, 39).

Several groups have confirmed the existence in HIV type 2 (HIV-2) of a regulatory protein functionally equivalent to Rev1 (9, 21, 22, 32). A *cis*-acting RNA target sequence for HIV-2 Rev (Rev2) has also been mutationally defined (9, 20–22). The RRE2 displays a predicted RNA secondary structure similar to that observed in the RRE1 and also exhibits a high (~64%) level of nucleotide sequence identity (20, 22). A comparison of the primary amino acid sequence of Rev1 and Rev2 strongly suggests that these related proteins have also retained a similar domain organization (Fig. 1A). A basic motif is required for the nuclear localization of Rev2 and is believed to mediate binding to the RRE2 (10). A Rev2 motif similar in both location and sequence to the essential leucine-rich domain of Rev1 is also observed (Fig. 1A), although it has not yet been shown to serve the same function (26).

While Rev1 and Rev2 are both functionally and mechanistically similar, differences do exist. In particular, while Rev1 can efficiently complement an HIV-2 provirus lacking a functional Rev2 protein, Rev2 is not able to effectively substitute for Rev1 (9, 21, 22, 32). It was therefore proposed that Rev2 may not be able to functionally interact with the RRE1 (22). Subsequently, it was reported that, while both Rev1 and Rev2 are able to specifically bind to the RRE2 in vitro, Rev2 lacks the ability to bind the RRE1 (9). Here, we report, in contradiction to this earlier work, that Rev2 can indeed efficiently and specifically interact with the RRE1. Our analysis is consistent with the hypothesis that the resultant weak Rev response is instead due to the incomplete and/or aberrant multimerization of Rev2 on the RRE1.

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MATERIALS AND METHODS

Plasmid constructions. A cDNA copy of the *rev* gene of HIV-2 ROD was isolated by using the polymerase chain reaction (29). The flanking primers used in this procedure were designed to introduce a unique *Nco*I site coincident with the initiation codon of Rev2 and a *Bgl*II site between the C-terminal threonine residue of Rev2 and the translation stop codon (Fig. 1A). The *rev2* gene was then cloned into the previously described expression plasmid pBC12/CMV (25). The final pcRev2N expression plasmid was derived by insertion of an epitope tag at this introduced C-terminal *Bgl*II site. The introduced peptide sequence includes aa 171 to 184 of the HIV-1 Nef protein, then a stop codon, and has the sequence DQCHGMDDPEREVLEWRDL.

We have previously described a deletion mutant of the *rev1* gene, termed pΔ12-14, that substitutes a unique *Bgl*II site in place of Rev1 aa 99 to 113 (23). This mutant, which retains full Rev1 activity, was further modified by insertion of the epitope tag described above at the introduced *Bgl*II site to give the expression plasmid pcRev1N. The indicator constructs pgTat1 and pgTat2 and the *trans*-dominant Rev1 mutant expression plasmid pM10 have been described previously (22, 23).

rev gene chimeras were made by using existing *Ava*I (A in Fig. 1A) and *Bam*HI (B in Fig. 1A) sites in *rev1* as well as a *Bgl*II site (C in Fig. 1A) introduced into *rev1* in a previously described mutant, termed M9, that we have shown is phenotypically wild type (23). These restriction enzyme sites were introduced into the corresponding locations in the *rev2* gene by using PCR. These separate mutations convert a short stretch of amino acids in Rev2 to the sequence seen in Rev1 (boxes A and B, Fig. 1A) or, in the case of C, introduce the dipeptide sequence Asp-Leu. These introduced sites, together with the flanking unique sites *Sac*I (5′) and *Xho*I (3′), were used to generate the Rev chimeras shown in Fig. 1B. All constructs were confirmed by DNA sequencing. The HIV-1 proviral clone pHIV1Δrev, which lacks a functional *rev* gene because of the presence of a frameshift mutation in the second *rev* coding exon, has been described previously (12, 23).

We have previously described the derivation of a vector that expresses the Rev1 protein fused to the C terminus of the enzyme glutathione S-transferase (GST) (24). A similar GST-Rev2 expression vector was constructed by introduction of *rev2* into the unique *Bam*HI site of the isopropyl-β-D-thiogalactopyranoside-inducible GST expression vector pGEX-2T (Pharmacia LKB Biotechnology), thus fusing the complete Rev2 open reading frame to the GST carboxy terminus. DNA sequencing confirmed that the GST and Rev2 coding sequences were in frame, while Western blot (immunoblot) analysis confirmed the integrity and identity of each fusion protein (data not shown). Plasmids used for in vitro synthesis of either the full-length RRE1 RNA or the stem-loop 2 (SLII) segment of the RRE1 were described previously (36). SLII is a 66-nucleotide subdomain of the RRE1 that has been shown to be both necessary and sufficient for Rev1 binding in vitro (27).

Cell culture and DNA transfection. The monkey cell line COS was maintained as described previously (22). Cells were transfected with DEAE-dextran and chloroquine (5).

Immunoprecipitations. COS cell cultures (35 mm) were transfected with 250 ng of the indicator construction pgTat1 or pgTat2 together with 250 ng of each Rev expression vector, unless stated otherwise in the figure legend. Cells were metabolically labeled at 48 h after transfection with ^{35}S-Translabel (ICN) at 100 μCi per well for 2 h (5). Tat or Rev proteins were immunoprecipitated (5) with anti-Tat or anti-Nef rabbit antipeptide antiserum, respectively, resolved by electrophoresis through sodium dodecyl sulfate (SDS)–14% polyacrylamide gels, and visualized by autoradiography (25). The rabbit anti-Nef antiserum was raised against a synthetic peptide spanning aa 171 to 184 of the HIV-1 Nef protein (HXB3 strain) conjugated to keyhole limpet hemocyanin. The sequence of this synthetic peptide is CHGMD DPEREVLEWRFDSR.

Analysis of HIV-1 provirus replication. COS cell cultures (35 mm) were transfected with 250 ng of pHIV1Δrev together with 250 ng of one of the *rev* expression plasmids. Supernatants were harvested after 5 days, and Rev function was monitored by an enzyme-linked immunoassay system for soluble $p24^{gag}$ expression (Dupont, NEN Research Products, Inc., Billerica, Mass.) (23). To assess the *trans* dominance of Rev2, we cotransfected COS cell cultures (35 mm) with 25 ng of pHIV1Δrev, 50 ng of pcRev1N or control vector, and 500 ng of pcRev2N or control vector. HIV-1 Gag protein expression was then assayed as above.

RNA binding assays. SLII and full-length RRE1 RNA were synthesized by using SP6 polymerase in the presence or absence of [^{32}P]UTP. Transcripts were gel purified as previously described (36). Rev1 and Rev2 were partially purified after expression in *Escherichia coli* as GST fusion proteins (33). RNA binding assays were performed as described previously (24), using the ^{32}P-labeled full-length RRE1 probe. Chemical interference assays were performed as previously described, using a 5′-end-labeled SLII RNA probe that had been subjected to diethyl pyrocarbonate (DEPC) modification (31, 36).

RESULTS

Our initial aim was to map sequences within the Rev2 protein that contribute to the inability of Rev2 to function effectively in the HIV-1 proviral context. For this purpose, we separately introduced three unique restriction enzyme sites into a cDNA clone encoding Rev2 (Fig. 1A). The A and B sites precisely matched the location of sites found in the HIV-1 *rev* gene, while C marks the location of an introduced restriction enzyme site previously shown to be phenotypically silent in the Rev1 context (23). These introduced sites facilitated the construction of the chimeric Rev expression plasmids diagramed in Fig. 1B. To permit the in vivo detection of each of these distinct Rev proteins, we also introduced a short epitope tag, derived from the C terminus of the HIV-1 Nef protein, at the C terminus of both the wild-type and chimeric Rev proteins. This introduced sequence had no detectable phenotypic effect on either the Rev1 or Rev2 protein (Fig. 2), as was predicted from the known dispensability of the highly variable C-terminal domain of Rev1 in vivo (23, 26).

We have previously described the use of genomic *tat* gene expression vectors derived from either HIV-1 (pgTat1) or HIV-2 (pgTat2) as indicators of Rev function (22, 25). In the absence of Rev, each indicator construct exclusively expresses a fully spliced *tat* mRNA that encodes the two-exon form of Tat. This protein comprises 86 aa in HIV-1 and 130 aa in HIV-2 (Fig. 2, lane 1). In the presence of Rev1, each of these constructs expresses an unspliced cytoplasmic *tat* mRNA species that encodes a truncated, one-exon form of Tat (72 aa in HIV-1, 99 aa in HIV-2; Fig. 2, lane 2). As previously reported (22), Rev2 functions effectively in the

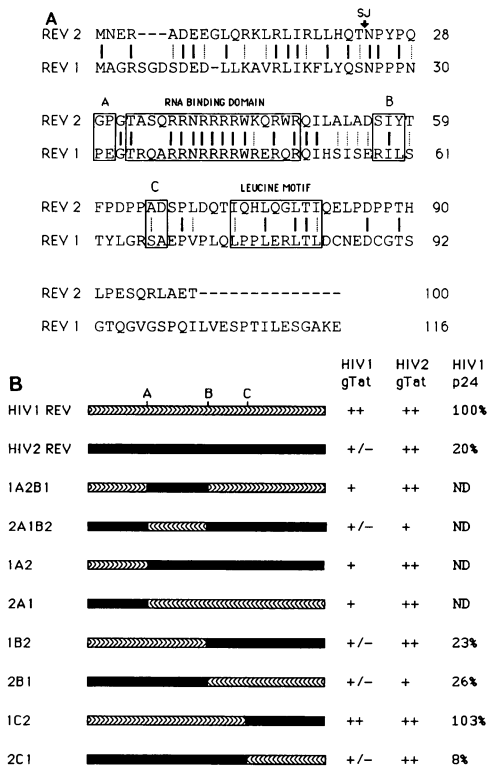

FIG. 1. Structure and activity of chimeric Rev proteins. (A) Protein sequence alignment of Rev2 and Rev1. Sites used to construct chimeric proteins are boxed and lettered. These coincide with restriction enzyme sites normally present in the *rev1* gene (A and B) or with an introduced, phenotypically silent site (C). The boxed Rev1 sequence labeled "RNA binding domain" has been shown to be necessary and sufficient for sequence-specific binding to the RRE1 both in vitro and in vivo (2, 18, 24). The Rev1 sequence labeled "leucine motif" is critical for in vivo Rev1 function but plays no detectable role in RRE1 binding or Rev1 multimerization (24, 26, 30, 39). (B) The structure of each Rev chimera is represented schematically. Chimeric clones are named for their derivation; thus, 1A2 contains Rev1 sequences from the N terminus to site A and Rev2 sequences from A to the C terminus. The relative activity of these chimeras on the pgTat1 and pgTat2 indicator constructs, as determined in Fig. 2 and in other experiments, is indicated. +/−, <20% of the activity of Rev1; +, 20 to 50% of the activity of Rev1; ++, >50% of Rev1 activity. The ability to rescue $p24^{gag}$ protein expression from a Rev-deficient HIV-1 provirus was also determined for selected Rev chimeras (23). These values are expressed as a percentage of the activity of Rev1 and represent an average of two experiments. ND, not determined.

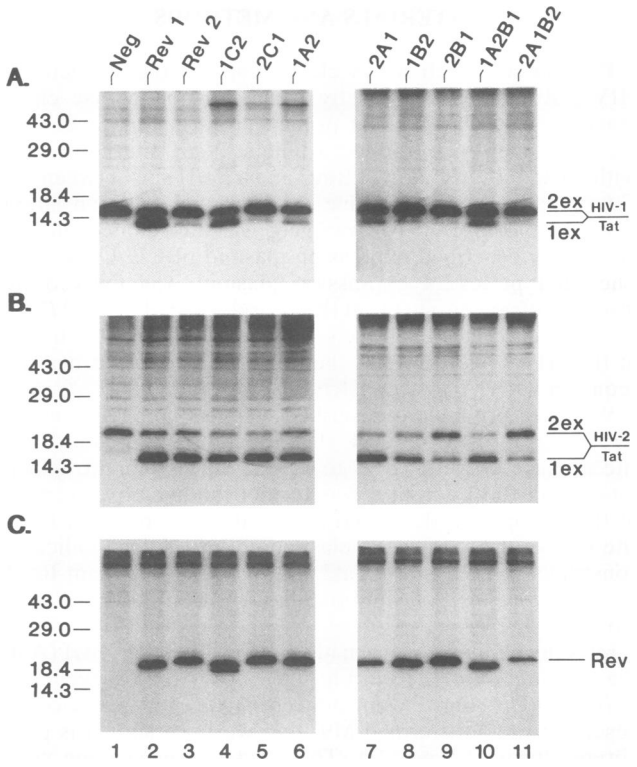

FIG. 2. Immunoprecipitation analysis of chimeric Rev protein activity and expression. COS cell cultures were transfected with pgTat1 (A) or pgTat2 (B) together with the negative control plasmid pBC12/CMV (25) (lane 1), the Rev1 expression vector pcRev1N (lane 2), the Rev2 expression vector pcRev2N (lane 3), or the indicated chimeric expression plasmid. At 48 h after transfection, cultures were labeled with [^{35}S]methionine and [^{35}S]cysteine for 2 h (5). Labeled cell extracts were subjected to immunoprecipitation with antibodies specific for Tat1 (A), Tat2 (B), or, in the case of Rev, for the introduced C-terminal epitope tag (C). Precipitated proteins were resolved by electrophoresis through an SDS-14% polyacrylamide gel and visualized by autoradiography. The relative migration of known protein molecular mass markers is indicated at the left of each panel (in kilodaltons). The two-exon (2ex) and one-exon (1ex) forms of Tat1 migrate at ~15.5 and ~14 kDa, respectively, while the two-exon and one-exon forms of Tat2 migrate at ~21 and ~17 kDa, respectively (22).

context of the pgTat2 indicator construct but is ~10-fold less active than Rev1 when assayed on pgTat1 (Fig. 2, lane 3).

All the chimeric constructs indicated in Fig. 1B functioned as effectively as Rev2 when assayed on the pgTat2 indicator construct, with the exception of 2B1 and 2A1B2, which displayed approximately twofold-lower activity (Fig. 2B). All Rev chimeras appeared to be synthesized normally, as assessed by immunoprecipitation analysis, with the exception of 2A1B2, which yielded a reduced signal (Fig. 2C). In addition, 1C2 reproducibly migrated as a dimer. The derivation of all chimeric Rev genes, including 1C2, was confirmed by DNA sequence analysis, and the origin of this phenomenon is therefore unclear.

If the inability of Rev2 to function effectively in the HIV-1 context reflects inefficient binding to the RRE1, then it is predicted that the source of the RNA binding domain would determine the efficiency of Rev function. The A and B sites indicated in Fig. 1A flank Rev1 sequences known to be necessary and sufficient for specific binding to the RRE1 both in vitro and in vivo (2, 18). However, the introduction of the Rev1 RNA binding domain into Rev2 failed to complement the low function of Rev2 in the HIV-1 context when introduced alone, in 2A1B2, or when introduced with flanking N-terminal sequences, in 1B2 (Fig. 2A, lanes 8 and 11). In contrast, insertion of the predicted RNA binding domain of Rev2 into Rev1, in 1A2B1, resulted in a protein significantly more active than Rev2 when assayed on the HIV-1 indicator construct pgTat1 (Fig. 2, lane 10). We therefore conclude that sequences outside of the known RNA binding domain of Rev must contribute to the target specificity of Rev2. However, these sequences must be within the N-terminal essential domain, as the 1C2 vector is fully active on the RRE1 (Fig. 2A, lane 4). This observation

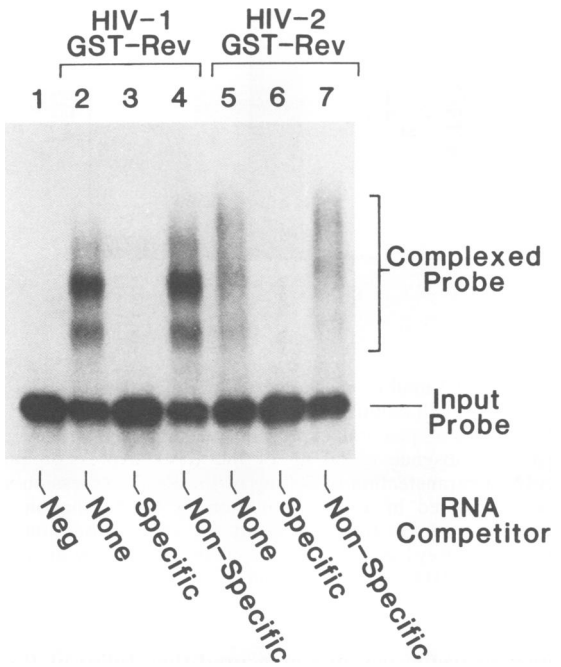

FIG. 3. Specific interaction of Rev2 with the HIV-1 RRE in vitro. A constant level of a radiolabeled RNA probe consisting of the entire RRE1 was incubated with 1 μg of either GST-Rev1 (lanes 2 to 4) or GST-Rev2 (lanes 5 to 7) in the absence of additional competitor (lanes 2 and 5), in the presence of 250 ng of the unlabeled RRE1 probe (lanes 3 and 6), or in the presence of 250 ng of *E. coli* tRNA (lanes 4 and 7). Neg, negative control.

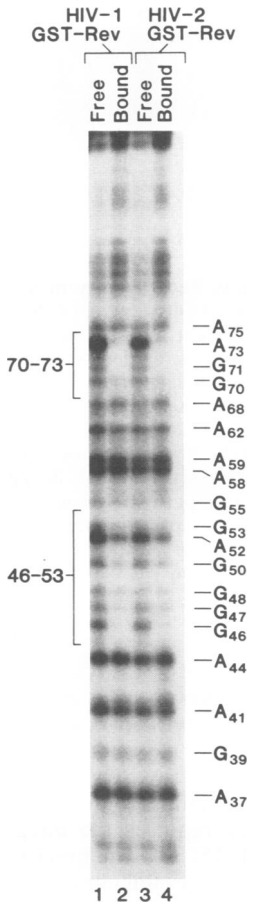

FIG. 4. Modification interference analysis of the Rev2-RRE1 interaction. A DEPC-modified, 5′-end-labeled SLII RRE1 probe was incubated with sufficient GST-Rev1 or GST-Rev2 protein to bind ~25% of the input RNA (36). Free probe (lanes 1 and 3) and probe bound by either GST-Rev1 (lane 2) or GST-Rev2 (lane 4) was isolated after native gel electrophoresis and cleaved at DEPC-modified purines by treatment with aniline (31). A total of 4×10^3 cpm derived from each RNA sample was subjected to electrophoresis through a denaturing 12% polyacrylamide gel, and the cleavage products were visualized by autoradiography. Nucleotides that displayed interference are indicated by brackets.

excludes the leucine-rich activation domain of Rev1 as a contributor to this phenomenon and demonstrates that Rev2 indeed contains a functionally equivalent C-terminal sequence (26) (Fig. 1A).

Analysis of additional Rev chimeras suggests that sequences both N and C terminal to the arginine-rich RNA binding motif contribute to the target specificity of Rev2. For example, the substitution of N-terminal sequences from Rev2 into Rev1, in 2A1, reduced activity on the RRE1 but not the RRE2. Similarly, the replacement of N-terminal Rev2 sequences with sequences from Rev1, in 1A2, resulted in a small but reproducible approximately twofold increase in activity on the RRE1 (Fig. 2A). The most significant distinction is, however, between 1B2, which behaves indistinguishably from Rev2, and 1C2, which has the same phenotype as Rev1 (Fig. 2A, compare lanes 4 and 8). These two Rev chimeras only differ by the 6 aa located between the B and C sites indicated in Fig. 1A. Yet, while both proteins are fully active on the RRE2, only 1C2 is effective when assayed on the RRE1. Interestingly, it has previously been observed that mutation of this 6-aa segment can result in a Rev protein with a recessive negative phenotype (30).

To further confirm this latter observation, we also assayed selected Rev chimeras for their ability to complement an HIV-1 provirus lacking a functional Rev gene (23) (Fig. 1B). As predicted from the data presented in Fig. 2, 1C2 was as effective as Rev1 in rescuing Gag protein expression from this proviral mutant. In contrast, the 1B2, 2B1, and 2C1 chimeras were no more effective than Rev2 when assayed in this HIV-1 proviral context.

Specific binding of Rev2 to the RRE1. Several groups have demonstrated that Rev1 binds the RRE1 with high specificity in vitro (3, 6, 7, 15, 17, 27, 38). It has also been demonstrated that a fusion protein consisting of Rev1 fused to the C terminus of the enzyme GST (GST-Rev1) binds the RRE1 with the same affinity and specificity as preparations of nonfusion recombinant Rev1 protein (24, 36). To assess the ability of Rev2 to specifically bind the RRE1, we therefore expressed Rev2 as a similar GST fusion protein (GST-Rev2).

We have previously used RNA gel retardation analysis to demonstrate the specific interaction of Rev1 with the full-length RRE1 (24, 27, 36). These data are reconfirmed in Fig. 3, where Gst-Rev1 is shown to form at least two discrete protein-RNA complexes with the RRE1. Formation of these complexes is effectively inhibited by a specific, but not by a nonspecific, competitor RNA. A similar level of the recombinant GST-Rev2 protein was also observed to form protein-RNA complexes with the RRE1 probe. Again, binding of the GST-Rev2 protein to the RRE1 probe was specific, as demonstrated by its resistance to addition of excess nonspecific competitor RNA and sensitivity to a specific RNA

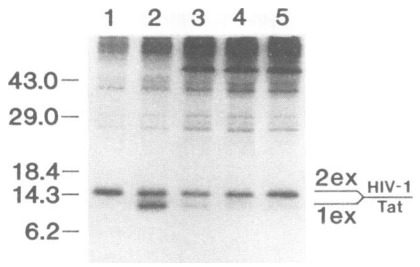

FIG. 5. Rev2 inhibits Rev1 activity in *trans*. COS cell cultures (35 mm) were transfected with 250 ng of the indicator construction pgTat1 together with various combinations of mutant or wild-type *rev* gene expression vectors, as detailed below. At 48 h posttransfection, cultures were labeled with [^{35}S]methionine and [^{35}S]cysteine and subjected to immunoprecipitation with a rabbit anti-Tat1 antiserum (23). Lane 1, pgTat1 plus 290 ng of pBC12/CMV (negative control); lane 2, pgTat1 plus 40 ng of pcRev1N plus 250 ng of pBC12/CMV (positive control); lane 3, pgTat1 plus 40 ng of pcRev1N plus 250 ng of pcRev2N; lane 4, pgTat1 plus 40 ng of pcRev1N plus 250 ng of pM10; lane 5, pgTat1 plus 40 ng of pBC12/CMV plus 250 ng of pcRev2N. 2ex, two-exon form; 1ex, one-exon form. Numbers on left show molecular mass in kilodaltons.

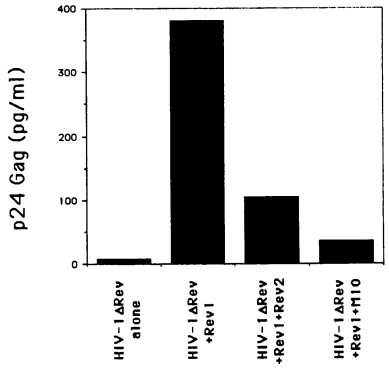

FIG. 6. Rev2 inhibits rescue of a Rev-defective HIV-1 provirus by Rev1. COS cell cultures (35 mm) were transfected with 25 ng of the Rev-deficient proviral expression plasmid pHIV1Δrev in the presence or absence of 50 ng of the Rev1 expression plasmid pcRev1N. Cotransfection of 500 ng of the Rev2 expression vector pcRev2N resulted in a 72% inhibition in Rev1 function, while cotransfection of the *trans*-dominant *rev* gene expression vector pM10 reduced Rev1 activity by 91%, as determined by quantitation of supernatant HIV-1 p24^{gag} protein.

competitor (Fig. 3, lanes 5 to 7). A purified preparation of nonfusion Rev2 protein was also observed to bind the RRE1 specifically under these experimental conditions (data not shown).

Formation of a specific protein-RNA complex between Rev1 and the RRE1 requires the integrity of the primary Rev1 binding site (1, 15, 36). The precise location of this site has been mapped by several techniques, including modification interference analysis, to two discontinuous sequences within the RRE1 that map to coordinates 46 to 53 and 70 to 73 (where the 5′ end of the 234-nt RRE1 is position 1) (1, 15, 36). These data are confirmed in Fig. 4, where it is shown that DEPC modification of purines within either of these two stretches inhibits Rev1 binding (Fig. 4, lanes 1 and 2). We therefore asked whether Rev2 binding to the RRE1 could also be shown to require the integrity of specific nucleotide residues. Surprisingly, these data revealed that Rev2 binding was indeed dependent on the recognition of specific purine residues and that these were both qualitatively and quantitatively indistinguishable from those mapped by using Rev1.

Rev2 can inhibit Rev1 activity in *trans*. The data presented in Fig. 3 and 4 argue that Rev2 binds to the RRE1 in vitro in a manner essentially indistinguishable from Rev1. Clearly, however, Rev2 is not fully functional on the RRE1. We therefore asked whether expression of Rev2 in *trans* might result in an inhibition of Rev1 function. Initially, these experiments were performed with the pgTat1 indicator construct. Remarkably, Rev2 was found to inhibit Rev1 function in this assay by approximately fivefold when present in excess (Fig. 5, compare lanes 2 and 3). Indeed, the level of activity seen when both Rev1 and Rev2 were present was indistinguishable from that seen with Rev2 alone (lane 5), thus arguing that Rev2 can compete with Rev1 for RRE1 binding and/or form mixed multimers with Rev1. Under these experimental conditions, Rev2 inhibited Rev1 function only slightly (approximately twofold) less effectively than an activation domain-negative Rev1 mutant, termed M10, previously shown to exhibit a *trans*-dominant negative phenotype (compare lanes 3 and 4) (23).

To further demonstrate that Rev2 can indeed inhibit Rev1 function in *trans*, we also measured the ability of Rev2 to interfere with the rescue by Rev1 of an HIV-1 provirus bearing a defective *rev* gene (Fig. 6). Again, Rev2 was observed to markedly inhibit Rev1 function, resulting in a level of viral rescue that was essentially indistinguishable from that seen with Rev2 alone (Fig. 1B).

DISCUSSION

In this study, we examined the molecular basis for the inability of the Rev2 protein to efficiently activate structural protein expression via the RRE1. Given the extensive mechanistic similarity between Rev1 and Rev2 and the observation that both these viral regulatory proteins are fully active on the RRE2, it was predicted that this phenomenon likely reflected a more fastidious RNA sequence requirement on the part of Rev2 (9, 21, 22). However, our analysis of the biological activity of Rev1/Rev2 chimeras supports this hypothesis only in part. Thus, the 1C2 chimera, consisting of the entire RNA binding and multimerization domain of Rev1 attached to the leucine-rich activation motif of Rev2, was indeed observed to display the same in vivo phenotype as the parental Rev1 protein (Fig. 1 and 2). However, the substitution into Rev2 of the arginine-rich Rev1 sequence known to be both necessary and sufficient for sequence-specific binding to the RRE1 (2, 18) (in, for example, the 1B2 chimera) was not sufficient to significantly enhance function on the RRE1 (Fig. 1 and 2). Trivial explanations for this negative result were eliminated by the demonstration that these Rev chimeras, including 1B2, remained active on the RRE2 and were synthesized at comparable levels in vivo (Fig. 2). Instead, our analysis indicates that Rev1 sequences that flank this basic motif and that are known to play a key role in the multimerization of Rev1 (24, 30, 39) are a major determinant of this in vivo target specificity (Fig. 1).

If the low level of Rev2 function on the RRE1 is indeed primarily a function of amino acid sequences located outside of the arginine-rich RNA binding domain, then Rev2 should be able to specifically interact with the RRE1 in vitro. Our analysis confirms that Rev2 can indeed bind the RRE1 (Fig. 3) and that it does so with a nucleotide sequence specificity

that is indistinguishable from that of Rev1 (Fig. 4). It therefore appears that the in vitro interaction between Rev2 and the RRE1 is comparable in terms of both affinity and specificity to that observed with Rev1. As these proteins differ markedly in their biological activity on the RRE1 target, either the in vivo interaction of Rev2 with the RRE1 must be significantly less efficient than that observed in vitro or this interaction does indeed occur but leads to only a minimal level of Rev activity. To distinguish between these two possibilities, we examined the effect of the Rev2 protein on the ability of Rev1 to activate HIV-1 structural protein expression (Fig. 5 and 6). These data revealed that Rev2 can markedly inhibit Rev1 function via the RRE1 when present in *trans*, resulting in a level of activity that is closely comparable to that seen with Rev2 alone. By analogy to previously reported *trans*-dominant negative mutants of Rev1 (23, 24, 28, 30, 37, 39), these data therefore suggest that Rev2 can compete with Rev1 for binding to the RRE1 and/or form mixed multimers with Rev1 on the RRE1 that are only minimally active.

If Rev2 can bind to the RRE1 in vivo, then why does this result in only a low level of biological activity? The observation that sequences known to be involved in Rev multimerization are a major determinant of this sequence specificity (Fig. 1) suggests that the multimerization of Rev2 on the RRE1, a process believed critical for Rev function in vivo (15, 24, 30, 39), may be incomplete or aberrant. It has, for example, been suggested that the interaction of Rev1 with the RRE1 primary binding site may nucleate assembly of an extensive ribonucleoprotein structure that blocks the interaction of HIV-1 mRNAs with cellular splicing factors (15). If Rev2 multimerization on the RRE1 is inefficient, then this coating of the viral mRNAs might occur too slowly to interdict spliceosomal assembly. Conversely, it has also been suggested that Rev1 assembles into an ordered, multimeric complex on the RRE1 that efficiently presents the leucine-rich activation domain to a currently unidentified cellular cofactor (26). It is therefore possible that Rev2 molecules bound to the RRE1 assemble into an aberrant structure that is unable to effectively interact with this cellular target. Whatever the actual basis for the low activity of Rev2 on the RRE1, our data do strongly suggest that there are specific steps subsequent to RRE1 binding that are important for the biological activity of Rev.

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