Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 5704–5708, July 1991
Biochemistry

The type I human T-cell leukemia virus (HTLV-I) Rex trans-activator binds directly to the HTLV-I Rex and the type 1 human immunodeficiency virus Rev RNA response elements

(human retroviruses/RNA–protein interactions/AIDS/adult T-cell leukemia/posttranscriptional regulation)

HAL P. BOGERD, GARY L. HUCKABY, YASMATH F. AHMED, SARAH M. HANLY, AND WARNER C. GREENE

Department of Medicine and Department of Microbiology and Immunology, Howard Hughes Medical Institute, Duke University Medical Center, Durham, NC 27710

Communicated by Robert C. Gallo, March 11, 1991 (received for review January 11, 1991)

ABSTRACT **The Rex protein of the type I human T-cell leukemia virus (HTLV-I) is essential for the replication of this pathogenic retrovirus and, surprisingly, can also replace the function of the structurally distinct Rev protein of the type 1 human immunodeficiency virus (HIV-1). Rex action requires a 255-nucleotide viral RNA stem–loop structure termed the Rex RNA response element (RexRE) located in the 3′ retroviral long terminal repeat. Rex function leads to the induced cytoplasmic expression of the incompletely spliced family of viral mRNAs that uniquely encode the HTLV-I structural and enzymatic proteins (Gag, Pol, and Env). Our studies now demonstrate that Rex acts by binding directly to the RexRE in a sequence-specific manner. These effects of Rex require the presence of a 10-nucleotide subregion of the RexRE that is essential for Rex function *in vivo*. Dominant-negative mutants of Rex also bind to the RexRE with high affinity, while a recessive-negative Rex mutant altered within its arginine-rich, positively charged domain fails to engage the RexRE. Analogously, both the wild-type and dominant-negative Rex proteins specifically bind to the structurally distinct HIV-1 Rev response element, a finding that likely underlies the respective stimulatory and inhibitory effects of these HTLV-I proteins in the heterologous HIV-1 system. However, consistent with their lack of amino acid homology, the binding sites for Rex and Rev within the HIV-1 Rev response element are distinct.**

The type I human T-cell leukemia virus (HTLV-I) is etiologically associated with adult T-cell leukemia (1–3) and tropical spastic paraparesis/HTLV-associated myelopathy (4, 5), whereas the type 1 human immunodeficiency virus (HIV-1) is the primary cause of AIDS (6, 7). For completion of their replicative life cycles, both of these pathogenic human retroviruses must balance the production of the completely and incompletely spliced classes of viral mRNA that broadly encode the regulatory and structural/enzymatic proteins of these viruses, respectively. In HTLV-I, this process is post-transcriptionally governed by the trans-acting 27-kDa Rex protein (8, 9) and the cis-regulatory Rex RNA response element (RexRE; refs. 10–12). Rex function through the RexRE serves to activate the cytoplasmic expression of the unspliced (*gag–pol*) or singly spliced (*env*) HTLV-I mRNAs (11, 12). In the absence of Rex, these incompletely spliced viral mRNAs remain sequestered in the nucleus (11). In the HIV-1 viral system, the 19-kDa Rev protein, acting through the Rev RNA response element (RevRE), similarly induces cytoplasmic expression of the *gag–pol* and *env* mRNAs of this virus (13–17). Though Rex and Rev are quite dissimilar in primary amino acid sequence, the Rex protein is capable of functionally replacing Rev, which leads to the rescue of replication of Rev-deficient mutants of HIV-1 (18). In the present study, we have explored the biochemical basis for these actions of Rex in the HTLV-I and HIV-1 retroviral systems. Specifically, we have investigated the potential RNA binding properties of both wild-type and mutated versions of the Rex trans-activator interacting with both the HTLV-I RexRE and HIV-1 RevRE.

MATERIALS AND METHODS

Expression and Partial Purification of Wild-Type and Mutant Rex Fusion Proteins in *Escherichia coli*. The coding regions of the wild-type *rex* gene and the recessive-negative M1 and dominant-negative M13 *rex* mutants (19) were amplified by polymerase chain reaction and inserted in-frame into the *Bam*HI and *Eco*RI sites of the pGEX2T vector (Pharmacia), which permits the expression of each in *E. coli* as a fusion protein with glutathione *S*-transferase (GST) (see Fig. 1). Codons for the arginine residues at positions 6 and 7 in the wild-type *rex* cDNA were modified by site-directed mutagenesis to conform with the codon preference in *E. coli*, which allowed a 2-fold overall increase in Rex production. The Rex constructs were used for transformation of the lon and ompT protease-deficient BL21 strain of *E. coli* from which the Rex proteins were partially purified by glutathione affinity chromatography on glutathione-agarose columns (20).

Logarithmic phase cultures (10 liters) of each Rex transformant were induced for 90 min at 37°C with isopropyl β-D-thiogalactopyranoside (0.1 mM), and cell pellets were suspended in 25 ml of TSE buffer (50 mM Tris·HCl, pH 8.0/25% sucrose/1 mM EDTA). Cells were lysed by the addition of lysozyme (60 mg) for 5 min at 25°C followed by digestion with DNase I (10 μg/ml) in the presence of 10 mM $MgCl_2$ and 1 mM $MnCl_2$. The samples were then supplemented with concentrated solutions of phosphate-buffered saline (1× final), Triton X-100 (1% final), Tween 20 (1% final), and dithiothreitol (10 mM final) followed by removal of cell debris by centrifugation. The supernatants were then applied to 1-ml glutathione-agarose columns previously washed in the same phosphate buffer/detergent mixture. For thrombin cleavage, the gel matrix was washed with 50 ml of cleavage buffer (50 mM Tris·HCl, pH 7.5/150 mM NaCl/2.5 mM $CaCl_2$/and 0.5% 3-[(3-cholamidopropyl)dimethylamino]-1-propanesulfonate), resuspended in 1 ml of cleavage buffer, and incubated with thrombin (20 units for 30 min at 30°C). The cleaved Rex protein was eluted, dialyzed against storage buffer (10 mM Hepes, pH 7.5/50 mM NaCl/10 mM

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: HTLV-I, type I human T-cell leukemia virus; HIV-1, type 1 human immunodeficiency virus; RexRE, Rex RNA response element; RevRE, Rev RNA response element; GST, glutathione *S*-transferase; TAR, trans-acting responsive region.

KCl/10% glycerol/2 mM dithiothreitol), and concentrated if necessary. For isolation of the GST–Rex fusion protein, the glutathione-agarose columns were directly eluted in buffer containing 10 mM glutathione.

RNA–Protein Binding Assays. Rex–RexRE binding reactions were performed with ^{32}P-labeled RexRE (^{32}P-RexRE) RNA templates [nucleotides 343–585; map positions refer to the 5′ long terminal repeat of HTLV-I (ref. 21)] prepared by *in vitro* transcription using *Acc* I-linearized pGEM-3Zf(+) RexRE plasmid, T7 RNA polymerase, and [α-^{32}P]GTP (NEN, 3000 Ci/mmol; 1 Ci = 37 GBq) as described by the manufacturer (Promega). These RexRE sequences were subcloned from pgΔTAT/R′ (12) and were originally obtained from the HTLV-I proviral clone CR-1 (a gift from Flossie Wong-Staal, University of California, San Diego). ^{32}P-labeled RevRE RNA templates (nucleotides 7359–7566) were similarly prepared by using an *Xba* I-linearized pGEM-3Zf(+) RevRE plasmid and T7 RNA polymerase (22). The pGEM plasmid, used as one template for the generation of unlabeled control RNA, was linearized with *Nae* I and transcribed with the SP6 polymerase. Similarly, HIV-1 transacting responsive region (TAR) RNA was generated by using SP6 polymerase and a pGEM-3Zf(+)-based TAR plasmid linearized with *Hin*dIII. This vector produces a 78-nucleotide run-off transcript spanning the entire HIV-1 TAR element initiated precisely at the normal HIV-1 start site (22). The transcribed RNA was separated from free nucleotides on a Sephadex G-50 Nick column (Pharmacia). Probes were denatured with heat (80°C for 3 min), renatured by cooling at room temperature for 3 min, and then stored on ice prior to use in the binding assay. Binding reactions were performed as described (13), but they were modified by the inclusion of 16S and 23S *E. coli* rRNA as nonspecific competitors in the Rex–RexRE binding studies. Briefly, the Rex fusion protein, 1 μg of tRNA, 4 μg of 16S and 23S rRNAs, and specific RNA competitor (when desired) were suspended in 9 μl of binding buffer (150 mM KCl/10 mM Hepes, pH 7.6/0.5 mM EGTA, 2 mM $MgCl_2$/1 mM dithiothreitol/10% glycerol/200 units of RNasin per ml) and incubated on ice for 10 min. Radiolabeled RexRE probe (≈2.5 ng, or ≈40,000 cpm) was then added in a 1-μl volume, and the reaction mixture was incubated for an additional 10 min on ice. Binding reactions were analyzed on 5% nondenaturing polyacrylamide gels containing 3% glycerol that were electrophoresed in a low ionic strength buffer containing 50 mM Tris·HCl and 50 mM glycine at pH 8.8.

RESULTS

Prokaryotic Expression of Recombinant Rex. *In vitro* analyses of Rex binding were facilitated by bacterial expression of both wild-type Rex protein and two classes of mutant Rex proteins. The Rex-M1 protein, containing a substitution mutation at residues 5–7 (Arg-Arg-Arg → Asp-Leu), represents a class of mutants that lacks biological activity but does not interfere with the function of the wild-type Rex protein and thus has been termed recessive negative (19). This mutation alters a positively charged peptide domain previously shown to function as a nuclear/nucleolar localization signal (19, 23). In contrast to the recessive-negative M1 mutation, the Rex-M13 mutant protein contains a substitution at residues 119–121 (Thr-Phe-His → Asp-Leu) that results in not only a complete loss of biological activity but also a protein that inhibits wild-type Rex function (19). Thus, this class of mutation has been termed dominant negative and presumably is altered within the activation or effector domain of this viral protein.

For bacterial expression, each of these *rex* cDNAs was inserted into the pGEX-2T vector (20) (Fig. 1*A*), and the resultant plasmids were used for transformation of the *E. coli* strain BL21 (lon and ompT protease deficient). Logarithmic

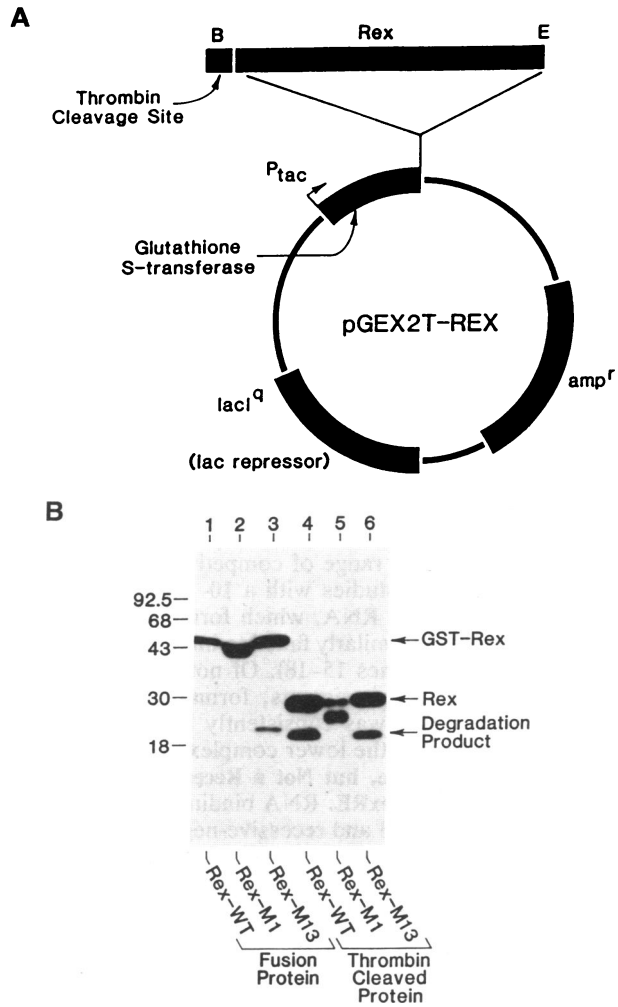

FIG. 1. Prokaryotic expression of HTLV-I Rex. (*A*) Schematic of the pGEX2T-REX expression plasmid. B, *Bam*HI; E, *Eco*RI. (*B*) Western blotting of wild-type, M1, and M13 Rex expressed as GST fusion (lanes 1–3) or thrombin-cleaved (lanes 4–6) proteins. Approximately 5 μg of each partially purified Rex protein preparation was electrophoresed through SDS/10% polyacrylamide gels, transferred to nitrocellulose, and incubated serially with rabbit anti-Rex antisera (1:1000 dilution, 4°C for 12–16 hr) (19) and 5 μCi of ^{125}I-labeled protein A (NEN; 1 hr at 20°C). The migration of known molecular size markers (in kDa) is also shown. WT, wild type.

phase cultures of each transformant were induced with isopropyl β-D-thiogalactopyranoside, and bacterial extracts containing the resultant 54- to 55-kDa GST–Rex fusion proteins were partially purified by affinity chromatography on glutathione-agarose columns. The wild-type, M1, and M13 *rex* expression vectors each directed the synthesis of appropriately sized fusion proteins as assessed by Western blotting with anti-Rex antibodies (19) (Fig. 1*B*, lanes 1–3). For use in selected experiments, the wild-type and mutant Rex polypeptides were specifically cleaved from the glutathione-bound GST fusion protein by the addition of thrombin (Fig. 1*B*, lanes 4–6). The resultant wild-type Rex protein and the Rex-M13 mutant protein migrated between 26 and 28 kDa while the Rex-M1 protein yielded an electrophoretic doublet indistinguishable from that observed when this cDNA was expressed in mammalian cells (19). This doublet is presumably the result of aberrant posttranslational processing of the M1 protein, because DNA sequencing has revealed no coding region changes other than the specific M1 mutation. The smaller immunoreactive species detected in lanes 3, 4, and 6

of Fig. 1*B* probably correspond to partial degradation products of the full-length Rex proteins.

Wild-Type Rex Binds to the HTLV-I Rex in a Sequence-Specific Manner. To investigate whether the HTLV-I Rex protein directly interacts with the HTLV-I RexRE, the wild-type Rex fusion protein was incubated with ^{32}P-RexRE RNA prepared by *in vitro* transcription (Fig. 2). When electrophoresed through low ionic strength polyacrylamide gels in the absence of added protein, the radiolabeled RexRE was detected as a single, rapidly migrating band (Fig. 2, lane 1). However, in the presence of the wild-type Rex fusion protein, two RNA–protein complexes with retarded mobility were produced with a concomitant marked reduction in free probe (Fig. 2, lane 2). These RNA–protein complexes reflected specific binding of Rex to the RexRE as the addition of graded amounts of unlabeled RexRE RNA competitor produced dose-dependent inhibition of their formation and reappearance of free probe (Fig. 2, lanes 3–6). In contrast, parental pGEM vector-derived RNA transcripts produced no detectable inhibition of Rex binding (Fig. 2, lanes 9–12) when titrated over the same range of competitor concentrations. Further, competition studies with a 10- to 1000-fold molar excess of HIV-1 TAR RNA, which forms a distinct RNA secondary structure, similarly failed to inhibit Rex binding to the RexRE (Fig. 2, lanes 15–18). Of note, in all unlabeled RexRE competition experiments, formation of the upper Rex–RexRE complex was consistently blocked in a more effective manner than the lower complex (Fig. 2, lane 4).

A Dominant-Negative, but Not a Recessive-Negative, Rex Mutant Binds to the RexRE. RNA binding properties of the dominant-negative M13 and recessive-negative Rex-M1 mutants were next evaluated (Fig. 3). When incubated with the radiolabeled RexRE probe, the Rex-M13 fusion protein yielded two complexes of retarded mobility that were indistinguishable from those produced by the wild-type Rex protein (Fig. 3, lanes 2 and 4). In contrast, the Rex-M1 mutant protein failed to produce any detectable complex (Fig. 3, lane 3). Thrombin-cleaved preparations of the wild-type Rex protein and the Rex-M13 protein also mediated RexRE binding, although less marked electrophoretic shifts were obtained, which reflects the smaller size (and charge) of these cleaved proteins (Fig. 3, lanes 6 and 8). However, even with removal of the GST moiety, no RNA binding activity was discerned with the cleaved Rex-M1 protein (Fig. 3, lane 7). These findings thus clearly demonstrate a marked difference

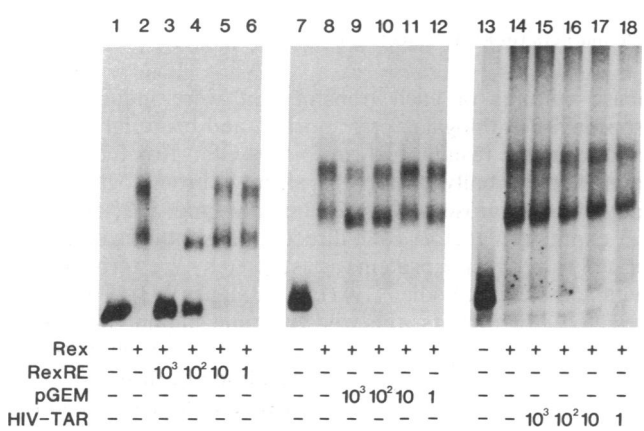

FIG. 2. Analysis of Rex binding to the HTLV-I RexRE. ^{32}P-RexRE RNA templates were incubated with medium alone (lanes 1, 7, and 13) or with GST–Rex fusion protein (≈500 ng of partially purified material; lanes 2, 8, and 14) and in the presence of graded amounts of unlabeled RexRE RNA (lanes 3–6), pGEM RNA (lanes 9–12), or HIV TAR RNA (lanes 15–18) competitors. The constituents of each reaction, including the molar excess of each unlabeled RNA competitor, are indicated below each lane.

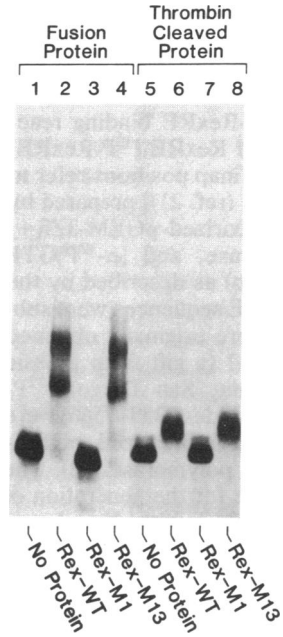

FIG. 3. Comparison of RNA binding properties of the wild-type Rex protein and the recessive-negative M1 and dominant-negative M13 mutant Rex proteins. Rex, Rex-M1, and Rex-M13 fusion proteins (lanes 2–4) or their thrombin-cleaved counterparts (lanes 6–8) were incubated with ^{32}P-RexRE RNA templates as described in Fig. 2. The migration of free ^{32}P-RexRE probe in the absence of added protein is shown in lanes 1 and 5. WT, wild type.

in the RNA binding phenotype of these dominant-negative and recessive-negative classes of Rex mutants and further confirm RNA binding activity by the cleaved Rex protein.

A 10-Base-Pair Subregion of the RexRE Is Specifically Required for Rex Binding. Sequences critical for Rex binding within the large RexRE secondary structure were next assessed by using a series of deletion and substitution mutants of this element (REM 3, 4, 5, 7, 8, 11, 12, and 12/13; ref. 12) as RNA templates in the *in vitro* Rex binding assay. The predicted secondary structure of the RexRE and the location of each mutation tested are presented in ref. 12. Rex bound effectively to the sense RexRE but not to the antisense version of this RNA element (Fig. 4, lanes 1–4). Further, Rex bound to RexRE templates containing the REM 3, 4, 5, and 7 deletion mutations (Fig. 4, lanes 5–12). However, Rex failed to bind to the REM 8 deletion, suggesting that the Rex binding site was removed or altered by this mutation (Fig. 4, lanes 13 and 14). To further delineate the critical RexRE sequences required for Rex binding, a substitution mutation was introduced into the nonoverlapping 10-base imperfect stem between bases 506 and 515 that was deleted in REM 8 but was present in REM 7, thus forming the REM 12 mutation (see ref. 12). Like REM 8, this REM 12 mutant template failed to support Rex binding (Fig. 4, lanes 17 and 18). In contrast, Rex binding persisted in the presence of the REM 11 substitution mutation, which altered six adjacent bases (see ref. 12) predicted to form a loop structure (Fig. 4, lanes 15 and 16).

These studies, however, did not distinguish between the possibilities that the inhibition of Rex binding produced by the REM 12 mutation reflected a loss of secondary structure (stem) versus a change in the primary nucleotide sequence of this RexRE subregion. To address this question, a compensatory substitution mutation was introduced between nucleotides 537 and 546 in the REM 12 plasmid, thus reconstituting a predicted perfect secondary stem structure in this region, albeit of a completely different sequence (REM 12/13). Analysis of the resultant REM 12/13 RNA template revealed no evidence for Rex binding (Fig. 4, lanes 19 and 20), suggesting that the primary sequences between nucleotides 506 and 515 and perhaps 537 and 546 are required for Rex recognition of the RexRE. Further, this pattern of *in vitro* binding by Rex to these different RNA templates proved precisely identical to the capacity of these mutant RexRE RNAs to mediate Rex function *in vivo* (12).

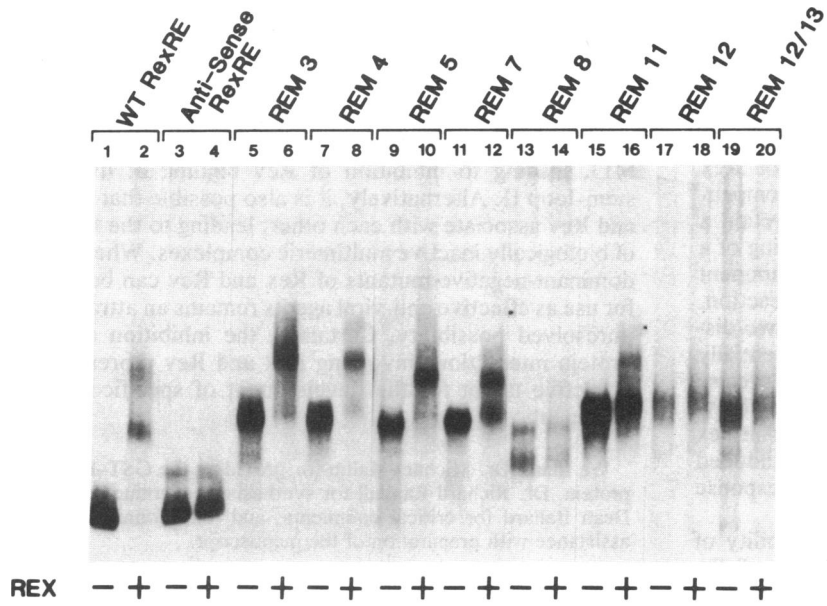

FIG. 4. Localization of sequences essential for Rex binding within the HTLV-I RexRE. A complete schematic summary of the predicted secondary structure of the HTLV-I RexRE and site of the various mutants of this response element is presented in ref. 12. The RexRE deletion mutants removed the following bases: REM 3, 377–399 (stem–loop 1); REM 4, 403–420 (stem–loop 2); REM 5, 422–467 (stem–loop 3); REM 7, 516–536 (part of stem–loop 4); REM 8, 506–546 (stem–loop 4). The REM 11 substitution mutation replaced the UUUAAA sequence between bases 499 and 504 with AGAUCU. The REM 12 mutation replaced the CUCAGGUCGA sequence between bases 506 and 515 with AGAUCUAUAA, whereas the REM 13 mutation replaced the UCCCUUGGAG sequence between bases 537 and 546 with UUAUAGAUCU; the REM 12/13 double mutation thus reconstitutes a predicted completely complementary stem structure between bases 506 and 515 and bases 537 and 546. The migration of each indicated radiolabeled RNA template was analyzed in the absence (−) or presence (+) of the wild-type (WT) Rex fusion protein. All RexRE probes tested encompassed sequences between nucleotides 303 and 635.

HTLV-I Rex Specifically Binds to the HIV-1 RevRE but at a Site Distinct from HIV-1 Rev. Despite the absence of amino acid sequence homology, the HTLV-I Rex protein can functionally replace the Rev protein of HIV-1 (18). Like Rex, HIV-1 Rev is an RNA binding protein that specifically interacts with a subregion domain of a highly structured HIV-1 RNA regulatory sequence termed the Rev RNA response element (RevRE) (nucleotides 7359–7566) (22, 24–27). In view of these unexpected functional properties of Rex in the heterologous HIV-1 system, we analyzed the ability of Rex to specifically bind to the RevRE (Fig. 5). In the absence of added protein, the radiolabeled RevRE probe migrated as a single major high mobility band (Fig. 5, lane 1). However, addition of the wild-type Rex fusion protein produced a retarded complex (Fig. 5, lane 2) that was blocked by competition by both unlabeled RevRE (Fig. 5, lane 3) and RexRE (Fig. 5, lane 4) but not by control pGEM-derived RNA transcripts (Fig. 5, lane 5). Prior studies have demonstrated that a hammerhead RNA domain within the RevRE, termed stem–loop II, is sufficient for the high-affinity Rev binding site (22, 27). To investigate whether Rex and Rev interact with the same or different binding sites within the RevRE, studies were performed with a RevRE mutant deleted of stem–loop II (ΔSL-II, lacking nucleotides 41–105 as shown in ref. 22). In agreement with previous results, the GST–Rev fusion protein bound specifically to the wild-type RevRE (Fig. 5, lanes 7 and 8) but failed to bind to the ΔSL-II RNA templates (Fig. 5, lanes 9 and 10). However, the Rex protein readily bound to the ΔSL-II RNA template (Fig. 5, lanes 11 and 12), suggesting that Rev and Rex interact at different sites on the RevRE. Further, like the wild-type Rex protein, the dominant-negative M13 Rex mutant bound to the RevRE (Fig. 5, lane 16) independent of stem–loop II (data not shown), whereas the recessive-negative Rex-M1 protein failed to bind to either the wild-type (Fig. 5, lane 15) or ΔSL-II RevRE (data not shown).

Although Rex is capable of binding to the HIV-1 RevRE, the reciprocal interaction of the Rev transactivator with the HTLV-I RexRE does not appear to occur. Specifically, under conditions where Rex binding to the RexRE is readily detectable, no evidence was obtained for Rev binding to this heterologous viral element (Fig. 5, lane 18).

DISCUSSION

The HTLV-I *rex* gene product is essential for the replication of this pathogenic retrovirus; it serves to activate the cytoplasmic expression of the unspliced or singly spliced viral mRNAs that encode the HTLV-I Gag, Pol, and Env proteins (8–11). In the absence of Rex, these incompletely processed viral mRNAs remain sequestered in the nucleus where they are either completely spliced or degraded (11). Rex may exert its posttranscriptional effects either by promoting the disassembly of spliceosomes engaging these long viral mRNAs

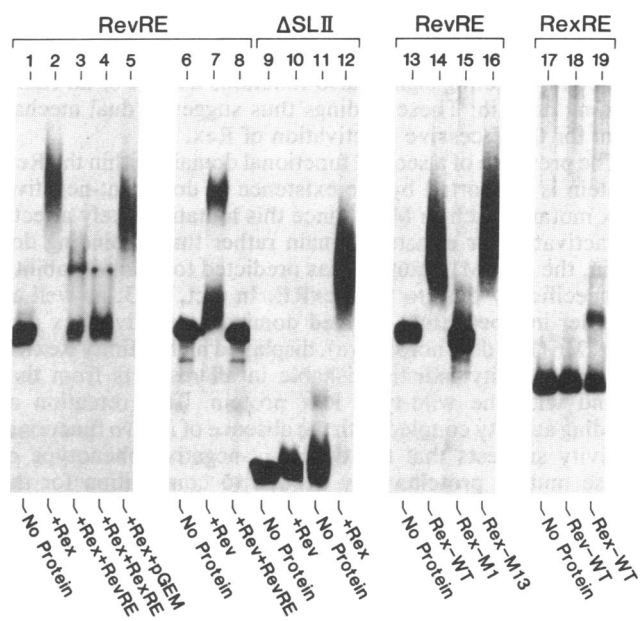

FIG. 5. Rex binds to the HIV-1 RevRE but at a site distinct from Rev. Radiolabeled HIV-1 RevRE probes were incubated with either no protein (lane 1) or the HTLV-I Rex fusion protein (lane 2) in the presence of a 1000-fold molar excess of unlabeled RevRE competitor (lane 3), RexRE competitor (lane 4), or control pGEM RNA (lane 5) as described in Fig. 2 except that no 16S or 23S rRNA was included in the reaction. Similarly, HIV-1 GST–Rev protein (a gift from Michael Malim, Duke University) was incubated with the radiolabeled RevRE probe in absence (lane 7) or presence (lane 8) of unlabeled RevRE competitor. Rev and Rex binding to the ΔSL-II deletion mutant of the RevRE were similarly studied (lanes 9–12) as were binding of Rex-M13 and Rex-M1 proteins to the wild-type RevRE (lanes 15 and 16). To examine the reciprocal ability of HIV-1 Rev to bind to the HTLV-I RexRE, Rev and Rex fusion proteins were incubated with ^{32}P-RexRE (lanes 18 and 19, respectively) under the normal Rex binding conditions.

(28) or alternatively by activating a specific nuclear export system that selectively delivers these viral transcripts to the cytoplasm (11). We now demonstrate that Rex action involves the direct sequence-specific binding of this viral transactivator to the RexRE. Further, Rex binding requires the presence of a specific 10-nucleotide subregion domain within this RNA element that is also essential for Rex function *in vivo* (12). The failure of Rex to bind to compensatory substitution mutants in this subregion that retain a perfect secondary stem structure, albeit via the folding of a distinct primary sequence, argues for a significant component of primary nucleotide recognition in this binding reaction. Interestingly, Rex binding to the RexRE produces two discrete complexes of retarded mobility that are differentially blocked by competition by unlabeled RexRE. The precise biochemical basis for this finding remains unknown; however, this result may reflect the binding of more than one Rex molecule to the RexRE, or alternatively, Rex-induced changes in the intrinsic conformation of this RNA response element.

We have also found a sharp difference in the ability of dominant-negative and recessive-negative mutants of the HTLV-I Rex protein to bind to the RexRE. These differences provide further support for a model of the Rex trans-activator that is comprised of at least two functional domains. The three arginine residues substituted in the recessive-negative M1 mutant have been shown to form an essential part of the nuclear/nucleolar localization signal of this viral transactivator (19, 23). As well, these arginine residues form a critical part of a sequence closely resembling the arginine-rich RNA binding motif recently identified in various bacteriophage antiterminator proteins and in the HIV-1 Rev and Tat proteins (29). Our present *in vitro* studies now demonstrate that this arginine-rich segment, in addition to being a nuclear/nucleolar targeting signal, also functions as part of an RNA binding domain. These findings thus suggest a dual mechanism for the recessive inactivation of Rex.

The presence of a second functional domain within the Rex protein is supported by the existence of dominant-negative Rex mutants such as M13. Since this mutation likely affects an activation or effector domain rather than a binding domain, the Rex-M13 mutant was predicted to retain its ability to specifically bind to the RexRE. In fact, M13, as well as another independently derived dominant-negative Rex mutant, M6 (19) (data not shown), displayed high-affinity RexRE binding activity indistinguishable in all respects from that found with the wild-type Rex protein. This retention of binding activity coupled with the absence of *in vivo* functional activity suggests that the dominant-negative phenotype of these mutant proteins may be due to competition for the RexRE binding site. Alternatively, should multimerization be required for the biological activity of Rex, these dominant-negative mutants may inhibit Rex function via the formation of biologically inactive mixed multimers (12).

Finally, consistent with the capacity of the HTLV-I Rex protein to rescue in trans the replication of Rev-deficient proviral mutants of HIV-1 (18), we now demonstrate that Rex can directly bind to the HIV-1 RevRE. In contrast, HIV-1 Rev appears unable to bind to the HTLV-I RexRE and also fails to function through this element (12). Although both Rex and Rev function through the RevRE (18), these trans-activators appear to bind to distinct subregions of the HIV-1 RNA response element. Specifically, the stem–loop II subregion is essential for Rev binding; however, this RevRE subregion appears entirely dispensable for Rex binding. This finding is consistent with the general lack of amino acid sequence similarity in these two viral trans-activators and fully complements prior functional studies implicating the involvement of different RevRE subregions in Rev and Rex responsiveness *in vivo* (12). At present, the precise location of the Rex binding site(s) in the RevRE remains undefined. However, we have found that the dominant-negative Rex-M13 mutant also binds to RevRE independent of stem–loop II, yet Rex-M13 is functionally transdominant over Rev. These findings suggest that the Rex and Rev binding sites may be interdependent with occupancy of the Rex site by M13, leading to inhibition of Rev binding at its site on stem–loop II. Alternatively, it is also possible that Rex-M13 and Rev associate with each other, leading to the formation of biologically inactive multimeric complexes. Whether such dominant-negative mutants of Rex and Rev can be adapted for use as effective anti-viral agents remains an attractive but unresolved possibility. Certainly, the inhibition of RNA–protein interactions involving Rex and Rev represents one attractive target for the development of specific anti-viral therapeutics.

We thank Dr. Michael Malim for providing the GST–Rev fusion protein, Dr. Richard Randall for synthesis of oligonucleotides, Dr. Dean Ballard for critical comments, and Ms. Bonnie Kissell for assistance with preparation of the manuscript.

1. Poiesz, B. J., Ruscetti, F. W., Gazdar, A. F., Bunn, P. A., Minna, J. D. & Gallo, R. C. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 7415–7419.
2. Poiesz, B. J., Ruscetti, F. W., Reitz, M. S., Kalyanaraman, V. S. & Gallo, R. C. (1981) *Nature (London)* **294**, 268–271.
3. Yoshida, M., Miyoshi, I. & Hinuma, Y. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 2031–2035.
4. Gessain, A., Barin, F., Vernant, J. C., Gout, O., Maurs, L., Calender, A. & de The, G. (1985) *Lancet* **ii**, 407–410.
5. Osame, M., Usuku, K., Ijicki, N., Amitani, H., Igata, A., Matsumoto, M. & Tara, H. (1986) *Lancet* **i**, 1031–1032.
6. Barre-Sinoussi, R., Chermann, J. C., Rey, F., Nugeyre, M. T., Chamaret, S., Gruest, J., Dauguet, C., Axler-Blin, C., Vezinet-Brun, F., Rouzioux, C., Rozenbaum, W. & Montagnier, L. (1983) *Science* **220**, 868–871.
7. Gallo, R. C., Salahuddin, S. Z., Popovic, M., Shearer, G. M., Kaplan, M., Haynes, B. F., Palker, T. J., Redfield, R., Oleske, J., Safai, B., White, G., Foster, P. & Markham, P. D. (1984) *Science* **224**, 500–503.
8. Inoue, J.-I., Yoshida, M. & Seiki, M. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 3653–3657.
9. Hidaka, M., Inoue, J., Yoshida, M. & Seiki, M. (1988) *EMBO J.* **7**, 519–523.
10. Seiki, J., Inoue, J.-I., Hidaka, M. & Yoshida, M. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 7124–7128.
11. Hanly, S. M., Rimsky, L. T., Malim, M. H., Hauber, J. H., Duc Dodon, M., Le, S.-Y., Maizel, J. V., Cullen, B. R. & Greene, W. C. (1989) *Genes Dev.* **3**, 1534–1544.
12. Ahmed, Y. F., Hanly, S. M., Malim, M. H., Cullen, B. R. & Greene, W. C. (1990) *Genes Dev.* **4**, 1014–1022.
13. Sodroski, J., Goh, W. C., Rosen, C., Dayton, A., Terwilliger, E. & Haseltine, W. (1986) *Nature (London)* **321**, 412–417.
14. Feinberg, M. B., Jarrett, R. F., Aldovini, A., Gallo, R. C. & Wong-Staal, F. (1986) *Cell* **46**, 807–817.
15. Sadaie, M. R., Benter, T. & Wong-Staal, F. (1988) *Science* **239**, 910–914.
16. Malim, M. H., Hauber, J., Fenrick, R. & Cullen, B. R. (1988) *Nature (London)* **335**, 181–183.
17. Malim, M. H., Hauber, J., Le, S.-Y., Maizel, J. V. & Cullen, B. R. (1989) *Nature (London)* **338**, 254–257.
18. Rimsky, L. T., Hauber, J., Dukovich, M., Malim, M. H., Langlois, A., Cullen, B. R. & Greene, W. C. (1988) *Nature (London)* **335**, 738–740.
19. Rimsky, L. T., Duc Dodon, M., Dixon, E. P. & Greene, W. C. (1989) *Nature (London)* **341**, 453–456.
20. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K., eds. (1989) *Current Protocols in Molecular Biology* (Greene, Brooklyn, NY; Wiley, New York), Suppl. 10, 16,7,i.
21. Seiki, M., Hattori, S., Hirayama, Y. & Yoshida, M. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 3618–3622.
22. Malim, M. H., Tiley, L. S., McCarn, D. F., Rusche, J. R., Hauber, J. & Cullen, B. R. (1990) *Cell* **60**, 675–683.
23. Siomi, H., Shida, H., Hyun Nam, S., Nosaka, T., Maki, M. & Hatanaka, M. (1988) *Cell* **55**, 197–209.
24. Zapp, M. L. & Green, M. R. (1989) *Nature (London)* **342**, 714–716.
25. Daly, T. J., Cook, K. S., Gray, G. S., Maione, T. E. & Rusche, J. R. (1989) *Nature (London)* **342**, 816–819.
26. Dayton, E. T., Powell, D. M. & Dayton, A. I. (1989) *Science* **246**, 1625–1628.
27. Heaphy, S., Dingwall, C., Ernberg, I., Gart, M. J., Green, S. M., Karn, J., Lowe, A. D., Singh, M. & Skinner, M. A. (1990) *Cell* **60**, 685–693.
28. Chang, D. & Sharp, P. (1989) *Cell* **59**, 789–795.
29. Lazinski, D., Grzadzielska, E. & Das, A. (1989) *Cell* **59**, 207–218.