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Human T-Cell Leukemia Virus (HTLV) Type II Rex Protein Binds Specifically to RNA Sequences of the HTLV Long Terminal Repeat but Poorly to the Human Immunodeficiency Virus Type 1 Rev-Responsive Element

MICHELE T. YIP,[1,2] WILLIAM S. DYNAN,[3] PATRICK L. GREEN,[1,2] ALEXANDER C. BLACK,[2] SALVATORE J. ARRIGO,[1,2] ARIAN TORBATI,[1,2] SHAUN HEAPHY,[4] CRISTINA RULAND,[2] JOSEPH D. ROSENBLATT,[2] AND IRVIN S. Y. CHEN[1,2]*

Department of Microbiology and Immunology[1] and Department of Medicine,[2] University of California, Los Angeles, School of Medicine and Jonsson Comprehensive Cancer Center, Los Angeles, California 90024-1678; Department of Chemistry and Biochemistry, University of Colorado at Boulder, Boulder, Colorado 80309-0215[3]; and Medical Research Council Laboratory of Molecular Biology, Cambridge CB2 2QH, England[4]

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The human T-cell leukemia viruses (HTLVs) encode a *trans*-regulatory protein, Rex, which differentially regulates viral gene expression by controlling the cytoplasmic accumulation of viral mRNAs. Because of insufficient amounts of purified protein, biochemical characterization of Rex activity has not previously been performed. Here, utilizing the baculovirus expression system, we purified HTLV type II (HTLV-II) Rex from the cytoplasmic fraction of recombinant baculovirus-infected insect cells by heparin-agarose chromatography. We directly demonstrated that Rex specifically bound HTLV-II 5′ long terminal repeat RNA in both gel mobility shift and immunobinding assays. Sequences sufficient for Rex binding were localized to the R-U5 region of the HTLV-II 5′ long terminal repeat and correlate with the region required for Rex function. The human immunodeficiency virus type 1 (HIV-1), has an analogous regulatory protein, Rev, which directly binds to and mediates its action through the Rev-responsive element located within the HIV-1 *env* gene. We demonstrated that HTLV-II Rex rescued an HIV-1_{JR-CSF} Rev-deficient mutant, although inefficiently. This result is consistent with a weak binding activity to the HIV-1 Rev-responsive element under conditions in which it efficiently bound the HTLV-II long terminal repeat RNA.

The human T-cell leukemia virus types I (HTLV-I) and II (HTLV-II) are oncogenic retroviruses that have been associated with specific T-cell malignancies in humans. HTLV-I is the etiologic agent of adult T-cell leukemia (37, 54), and has also been linked with a chronic progressive myelopathy (13, 36). The related HTLV-II has been rarely associated with forms of leukemia related to hairy-cell leukemia (23, 42) and has been reported to be prevalent among a significant proportion of intravenous drug abusers in the United States and Europe (27, 40, 50).

In addition to the essential viral structural proteins, HTLV-I and HTLV-II encode two regulatory proteins, designated Tax and Rex, which are translated from the same double-spliced message by utilizing separate partially overlapping reading frames. p40 and p37 Tax proteins of HTLV-I and HTLV-II, respectively, act in *trans* to activate gene expression from the viral long terminal repeat (LTR) (5, 7, 11, 12, 18, 41, 46, 47, 52). The Rex gene encodes two proteins of 21 and 27 kDa for HTLV-I and 24 and 26 kDa for HTLV-II (24, 44). Like Tax, Rex is localized to the nucleus. Rex is thought to differentially and temporally regulate viral gene expression posttranscriptionally. The current theory is that Rex is required for transport of unspliced and singly spliced messages from the nucleus to the cytoplasm, facilitating expression of the structural genes *gag* and *env* (16). In HTLV-I, this action is mediated by Rex via a *cis*-acting element in the U3-R region of the 3′ LTR (1, 20, 21, 34). In HTLV-II, these sequences have been localized to the R-U5 region of the 5′ LTR (3, 33, 41).

Human immunodeficiency virus type 1 (HIV-1) also exhibits regulation of viral gene expression by an analogous regulatory protein, termed Rev, which is a phosphoprotein targeted to the nucleus by a nuclear-nucleolar localization signal. Rev has also been found to promote transport of unspliced and singly spliced RNAs that encode *gag/pol* and *env*, respectively, from the nucleus to the cytoplasm (10, 14, 16, 31); however, recent investigations in our laboratory suggest that regulation may also occur independently of nuclear export (1a). Rev has been shown to mediate its action through and directly bind to a highly structured region within the HIV-1 *env* gene, referred to as the Rev-responsive element (RRE) (9, 19, 32, 56).

Although Rev and Rex have many functional similarities, they are encoded by evolutionarily divergent viruses and, accordingly, have no homology at the nucleotide or amino acid level. Their respective response elements also show no sequence homology, although both possess a high degree of secondary structure. Thus, the initial finding that HTLV-I and HTLV-II Rex could functionally replace HIV-1 Rev (28) and that HTLV-I Rex could rescue a replication-defective Rev-deficient HIV-1 mutant was surprising (39). The ability of HTLV-I Rex to substitute for Rev was subsequently shown to be dependent on the RRE plus additional surrounding sequences (48); however, the analogous Rev protein is unable to act on the HTLV-I LTR (17).

In the present study, we describe the purification of HTLV-II Rex from recombinant baculovirus-infected insect

* Corresponding author.

cells. We also directly demonstrate the ability of HTLV-II Rex to bind RNA within the HTLV-II 5′ LTR. Furthermore, we compare the ability of HIV-1 Rev and HTLV-II Rex to bind to the HIV-1 RRE.

MATERIALS AND METHODS

Plasmid constructs. (i) Baculovirus expression vectors. Plasmid pUC20.2 contains the cDNA for HTLV-II *tax* and *rex* (Fig. 1). To construct pAcC4*rex*, pUC20.2 was digested with *Sph*I and treated with T4 polymerase to generate blunt ends and then digested with *Bam*HI. The resulting 1.43-kb *Sph*I-*Bam*HI fragment was cloned into transfer vector pAcC4 (which had been digested with *Nco*I), treated with the Klenow fragment to repair the ends, and then digested with *Bam*HI. Restoration of the *rex* ATG was confirmed by dideoxy sequencing (42). Plasmid pAc373*rex* was generated by digesting pUC20.2 with *Bam*HI and cloning the resulting 1.4-kb *Bam*HI fragment into transfer vector pAc373(*), which had been linearized with *Bam*HI. Transfer vectors pAcC4 and pAc373 were obtained from Cetus Corp. and from M. Summers, Texas A&M University, respectively.

(ii) Plasmids for RNA binding studies. Plasmid pG312-765 was constructed by amplifying the region between nucleotides (nt) 312 through 765 of the 5′ LTR in the HTLV-II proviral clone pH6neo (6, 45) by the polymerase chain reaction with 5′ and 3′ oligonucleotide primers containing flanking *Hin*dIII and *Eco*RI restriction sites, respectively. The polymerase chain reaction was performed as described previously (55), with the following modifications. Plasmid DNA (25 ng) and 100 ng of each oligonucleotide primer in a final volume of 25 μl were amplified for 40 cycles of denaturation at 96°C for 1 min and polymerization at 55°C for 20 s, 72°C for 1 min, and 75°C for 45 s. The amplified fragment was digested with *Hin*dIII and *Eco*RI and cloned into the pGEM-1 vector (Promega) linearized with *Hin*dIII and *Eco*RI.

Similarly, pGHIVRRE was constructed by amplifying the RRE (nt 7755 through 7988) of HIV-$1_{JR\text{-}CSF}$ (25) with 5′ and 3′ oligonucleotide primers that contain flanking *Hin*dIII and *Eco*RI sites, respectively. pKSHIVRREII was constructed by amplifying the RRE and surrounding sequences (nt 7706 through 8035) shown to be critical for Rex activity (48). The amplified fragment was digested with *Hin*dIII and *Eco*RI and cloned into pGEM-1 linearized with *Hin*dIII and *Eco*RI. The *Hin*dIII-*Eco*RI insert was also cloned into the vector pBluescript (Stratagene) to confirm its sequence by dideoxy sequencing (43). Constructs containing deletions of the HTLV-II 5′ LTR employed as templates for RNA synthesis are described elsewhere (3).

(iii) Plasmids for transfections. HIV-1 constructs and expression vectors are described in detail elsewhere (2). Briefly, pYKJRCSF (25) is an HIV-1 proviral clone. pYKJRCSFΔSal (2) is an HIV-1 Rev-deficient mutant generated by a frameshift mutation. BC12 (8) and PC-Rev (30) were obtained from Bryan Cullen, Duke University. BC*rex*II expresses only HTLV-II Rex from the cytomegalovirus promoter-enhancer elements in BC12.

Cells and viruses. *Spodoptera frugiperda* Sf9 cells were propagated as a monolayer culture in Grace medium (GIBCO) supplemented with yeastolate (4 g/liter) (Difco Laboratories) and 10% fetal calf serum (GIBCO) at 28°C. The baculovirus expression vectors containing HTLV-II *tax* and *rex* sequences, pAcC4*rex* and pAC373*rex*, were each cotransfected with wild-type baculovirus (*Autographa californica* nuclear polyhedrosis virus [AcNPV]) DNA into Sf9 cells, allowing homologous recombination to occur in vivo as described by Summers and Smith (49). At 5 days posttransfection, supernatants were harvested for subsequent infection of Sf9 cells. To isolate recombinant baculovirus, three or four rounds of plaque hybridization and visual selection for occlusion-negative plaques were performed. Plaque hybridization was performed with the 1.1-kb *Cla*I-*Bam*HI fragment from pUC20.2 containing *tax*/*rex* sequences labeled with [^{32}P]dCTP by random priming. For protein production, Sf9 cells were infected with the purified recombinants at a multiplicity of infection of 10. Extracts were prepared 3 days postinfection for optimal protein production.

Labeling of cells and immunoprecipitations. At 3 days postinfection, 2×10^5 cells were pelleted and suspended in 1 ml of methionine-free Grace medium supplemented with 20% fetal calf serum. Cells were labeled with 100 μCi of [^{35}S]methionine-cysteine (Trans ^{35}S-label; specific activity, 1,091 Ci/mmol; ICN Radiochemicals) for 4 h. Cells were pelleted and washed with Grace medium, lysed in 2 ml of RIPA lysis buffer (0.05 M Tris [pH 8.0], 0.1% sodium lauryl sulfate, 1% Triton X-100, 2 mM phenylmethylsulfonyl fluoride, 0.15 M NaCl, 1% deoxycholate), and incubated on ice for 15 min. Lysates were spun at 36,000 rpm for 1 h in an SW55 rotor (Beckman). A 1-ml sample of lysate was roll mixed with 7 μl of antiserum specific for the Rex carboxy-terminal tridecapeptide (41, 44) for 2 h at 4°C; 100 μl of 10% protein A-Sepharose CL-4B (Pharmacia) was then added and roll mixed for 1 h at 4°C. The Sepharose beads were washed with RIPA buffer and boiled for 3 min in sample buffer. The immunoprecipitated proteins were resolved by 10% sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) at 50 mA in 0.192 M glycine, 0.025 M Tris, and 0.1% SDS. The gel was fixed, treated with En^{3}Hance (Dupont/NEN), dried, and visualized by autoradiography.

Transfection. COS cells (5×10^6) were transfected by electroporation, as described previously (4), with a total of 25 μg of plasmid DNA, including 10 μg of the Rex expression vector 91023-p26 (41) and 15 μg of carrier DNA from BC12 (6). Cells were harvested and labeled with Trans ^{35}S-label 48 h after transfection for subsequent immunoprecipitation.

729-6 B cells or Jurkat T cells (10^7) were transfected by electroporation with 50 μg of total plasmid DNA. Supernatants were harvested 48 h posttransfection and assayed for HIV-1 p24 Gag protein by using the Abbott p24 antigen capture enzyme-linked immunosorbent assay.

Cell fractionation. (i) Small scale. Nuclear and cytoplasmic fractions were prepared by lysing baculovirus-infected insect cells in 0.65% Nonidet P-40–0.15 M NaCl–0.01 M Tris (pH 7.8)–1.5 mM $MgCl_2$ on ice for 3 min. Nuclei were pelleted by low-speed centrifugation, and the nuclear pellet was washed with the same lysis buffer and pelleted. The cytoplasmic fraction was spun at high speed to remove contaminating nuclei.

(ii) Large scale. To prepare cytoplasmic extracts for protein purification 3 days after infection, baculovirus-infected Sf9 cells were pelleted and washed with Grace medium. Cells were suspended in one packed cell volume of hypotonic lysis buffer (10 mM KCl, 10 mM Tris [pH 7.9], 1 mM dithiothreitol, 1 μg of phenylmethylsulfonyl fluoride per ml, leupeptin, aprotinin) and incubated for 10 min at 4°C. The suspension was homogenized with 25 strokes in a Dounce homogenizer with a B pestle. After spinning at low speed to pellet the nuclei, the cytoplasmic fraction was spun at high

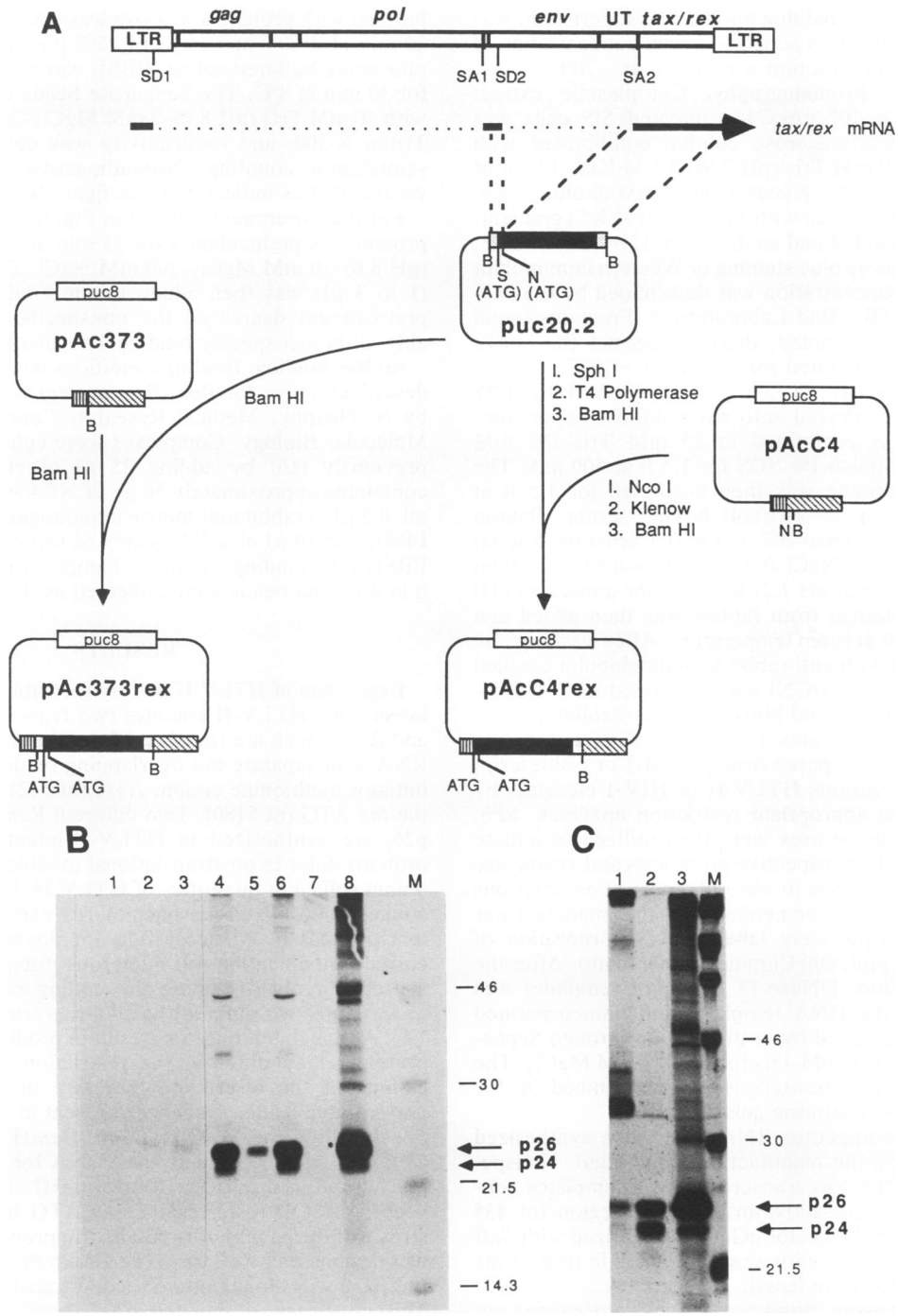

FIG. 1. (A) Construction of pAc373*rex* and pAcC4*rex*. (B and C) Expression of HTLV-II Rex in insect cells with baculovirus expression vectors. (B) HTLV-II Rex production in Sf9 cells infected with purified recombinant baculoviruses. Three independent recombinant baculoviruses were isolated for each construct, pAcC4*rex* and pAc373*rex*. After infection of Sf9 cells with the purified recombinants vAc373*rex* 1 through 3 and vAcC4*rex* 1 through 3, cells were labeled with [^{35}S]methionine-cysteine and total cell lysates were prepared. Immunoprecipitations were performed with Rex-specific antiserum and analyzed by 10% SDS–PAGE. Extracts prepared from uninfected Sf9 cells and COS cells transfected with the Rex expression vector 91023-p26 (41) were immunoprecipitated as controls. Lanes: 1, vAc373*rex* 1-infected Sf9 cells; 2, vAc373*rex* 2-infected Sf9 cells; 3, vAc373*rex* 3-infected Sf9 cells; 4, vAcC4*rex* 1-infected Sf9 cells; 5, vAcC4*rex* 2-infected Sf9 cells; 6, vAcC4*rex* 3-infected Sf9 cells; 7, uninfected control; 8, 91023-p26 transfected into COS cells; M, ^{14}C-labeled molecular size markers (kilodaltons; Amersham). The positions of p24 and p26 Rex proteins are indicated by the arrows. (C) Nuclear and cytoplasmic distribution of Rex in baculovirus-infected insect cells. vAcC4*rex*-infected Sf9 cells were labeled with [^{35}S]methionine-cysteine and lysed with Nonidet P-40. Nuclear and cytoplasmic extracts were prepared, immunoprecipitated with Rex-specific antiserum, and analyzed by 10% SDS–PAGE. As a control, a lysate prepared from COS cells transfected with the Rex expression vector 91023-p26 was immunoprecipitated in parallel. Lanes: 1, nuclear fraction of vAcC4*rex*-infected cells; 2, cytoplasmic fraction; 3, COS cells transfected with 91023-p26; M, ^{14}C-labeled molecular size markers. The positions of p24 and p26 Rex proteins are indicated by the arrows.

speed to remove any remaining nuclei. The supernatant was obtained, and glycerol was added to a final concentration of 30%. The cytoplasmic fraction was stored at −70°C.

Heparin-agarose chromatography. Cytoplasmic extract prepared from 3×10^8 vAcC4*rex*-infected Sf9 cells was loaded onto a heparin-agarose column equilibrated with buffer containing 50 mM Tris (pH 7.9), 0.1 M KCl, 12.5 mM $MgCl_2$, 1 mM EDTA, 20% glycerol, and 1 mM dithiothreitol. Bound proteins were eluted with a 0.1 to 1.0 M KCl gradient. Fractions were collected and analyzed by 12% SDS–PAGE, followed by Coomassie blue staining or Western immunoblot analysis. Protein concentration was determined by the Bio-Rad protein assay (Bio-Rad Laboratories). Fractions found to contain Rex were pooled, dialyzed against the above column buffer, and aliquoted for storage at −70°C.

Western blot analysis. Proteins were resolved by 12% SDS–PAGE and transferred onto nitrocellulose paper (Bio-Rad). Transfer was performed in 25 mM Tris–192 mM glycine–20% methanol–0.1% SDS for 1.5 h at 500 mA. The nitrocellulose membrane was then incubated for 1.5 h at room temperature in 1% (wt/vol) bovine serum albumin (BSA; grade VIII; Sigma)–3% (wt/vol) Carnation low-fat powdered milk–0.9% NaCl–0.01 M Tris–0.02% sodium azide–0.1% Tween 20 at pH 7.2. Rex-specific antiserum (41) (1:100 dilution) obtained from rabbits was then added and incubated overnight at room temperature. After washing, the blot was incubated with anti-rabbit immunoglobulin coupled to alkaline phosphatase (ICN) and developed with bromochloroindolyl phosphate and Nitro Blue Tetrazolium.

RNA synthesis. Templates for synthesis of sense and antisense RNA were prepared from pGEM-1 or pBluescript vectors containing various HTLV-II or HIV-1 elements by linearizing with the appropriate restriction enzymes. SP6, T3, or T7 RNA polymerases were then utilized to initiate transcription from their respective promoters and synthesize high-specific-activity RNA in vitro. Transcription reactions were performed as recommended by the manufacturer (Promega). Transcripts were labeled by incorporation of [α-^{32}P]CTP (20 mCi/ml, 800 Ci/mmol; Amersham). After the transcription reaction, DNase (3 mg/μg of template) was added to destroy the DNA template, and unincorporated nucleotides were removed by centrifugation through Sephadex G-50 columns in 10 mM Tris (pH 7.4)–2 mM $MgCl_2$. The integrity of the labeled transcripts was determined on 5% polyacrylamide nondenaturing gels.

Nonradioactive competitor RNAs were also synthesized as recommended by the manufacturer (Promega). Nonspecific competitor RNA was transcribed from templates containing the HIV-1 *trans*-activator response region (nt 435 through 508) or from the vector pGEM-1 linearized with *Sal*I or pBluescript linearized with *Sca*I, resulting in transcripts 120, 2,800, and 1,200 nt in length, respectively.

Gel mobility shift assay. Binding reactions were carried out with 50 pg of uniformly labeled RNA and 0 to 5.0 nM Rex in 20 μl of binding buffer (40 mM Tris [pH 8.0], 2 mM $MgCl_2$, 200 mM KCl, 8 μg of tRNA per ml, 20 μg of BSA per ml, 0.05% Triton X-100, 40 U of RNasin per ml). After incubation at 4°C for 15 min, the complexes were resolved on 6% polyacrylamide gels in 43 mM Tris (pH 8.0)–50 mM KCl at 4°C for 24 h with buffer recirculation. Nonradioactive competitor RNAs were added as indicated in the figure legends.

Immunobinding assays. (i) Rex binding. Binding conditions were identical to those described above, with 50 pg of uniformly labeled RNA (approximately 15,000 cpm) and 0 to 10 nM Rex in a reaction volume of 50 μl. After incubation at 4°C for 15 min, Rex-specific antiserum (0.5 μl) affinity purified with protein A-agarose was added and incubated for 20 min at 4°C. Then 15 μl of 25% protein A-Sepharose (in phosphate-buffered saline [PBS]) was added and roll mixed for 30 min at 4°C. The Sepharose beads were washed twice with 40 mM Tris (pH 8.0)–2 mM $MgCl_2$–200 mM KCl–0.05% Triton X-100, and radioactivity was determined by liquid scintillation counting. Nonradioactive competitor RNAs were added as indicated in the figure legends.

For the experiments shown in Fig. 6 and Table 2, the Rex protein was preincubated for 15 min at 37°C in 50 mM Tris (pH 8.0)–10 mM $MgCl_2$–100 mM NaCl. This pretreated Rex (1 to 3 μl) was then added to the binding reaction. This pretreatment decreased the nonspecific binding considerably, although specific binding was also decreased slightly.

(ii) Rev binding. Binding conditions were identical to those described above for Rex. Rev protein was kindly provided by S. Heaphy, Medical Research Council Laboratory of Molecular Biology. Complexes were collected as described previously (16) by adding 25 μl of culture supernatant containing approximately 50 μg of NR4/3C4.22 antibody per ml, 0.5 μl of rabbit anti-mouse immunoglobulin G (5 mg/ml in PBS), and 10 μl of a 25% slurry of protein A-Sepharose (in PBS) to the binding reactions. Samples were roll mixed for 1 h at 4°C, and beads were collected as described above.

RESULTS

Expression of HTLV-II Rex by recombinant baculovirus in insect cells. HTLV-II encodes two regulatory proteins, Tax and Rex, which are translated from the same doubly spliced RNA with separate but overlapping reading frames; the *rex* initiator methionine codon, ATG (nt 5121), lies upstream of the *tax* ATG (nt 5180). Two different Rex proteins, p24 and p26, are synthesized in HTLV-II-infected cells; the two proteins differ in posttranslational modification (15, 15a). To obtain sufficient quantities of HTLV-II Rex for biochemical studies, we utilized the baculovirus expression vector system that has been successfully employed in synthesis and correct modification of numerous foreign proteins. Our strategy involved cloning the coding region of HTLV-II *tax*/*rex* into two different baculovirus transfer vectors (Fig. 1A). Although both vectors result in production of nonfusion proteins, they differ in the restriction site employed for cloning of the insert and therefore in the amount of 5′ untranslated leader sequences present in the final construct. Specifically, the 1.43-kb *Sph*I-*Bam*HI fragment from pUC20.2, which contains the cDNA for HTLV-II *rex* and *tax*, was cloned into the *Nco*I-*Bam*HI sites of the transfer vector pAcC4, restoring the *rex* ATG immediately downstream of the baculovirus polyhedrin promoter. This plasmid was designated pAcC4*rex*. The 1.4-kb *Bam*HI fragment from pUC20.2 was cloned into a second transfer vector, pAc373. This construct, designated pAc373*rex*, contains 30 bp of untranslated leader sequences upstream of the *rex* ATG.

The constructs pAc373*rex* and pAcC4*rex* were each cotransfected with wild-type AcNPV DNA into insect cells, and recombinant baculoviruses were plaque purified as described in Materials and Methods. To analyze relative levels of HTLV-II Rex produced by the purified recombinant viruses, total cell extracts were prepared by infection of Sf9 cells, labeled with [^{35}S]methionine-cysteine, immunoprecipitated with antiserum specific for the Rex COOH-terminal tridecapeptide, and analyzed by SDS–PAGE. Three independent recombinant viruses for each vector, designated vAc373*rex* 1 through 3 and vAcC4*rex* 1 through 3, were purified and analyzed (Fig. 1B, lanes 1 through 3 and 4

through 6, respectively). Extracts prepared from uninfected Sf9 cells (lane 7) or COS cells transfected with an HTLV-II *rex* expression vector, 91023-p26 (41) (lane 8), were treated similarly. Bands that comigrate with p24 and p26 Rex expressed by 91023-p26 were prominent in vAcC4*rex*-infected cells (lanes 4 through 6) but were only barely detectable in the vAc373*rex*-infected cells (lanes 1 through 3) and were absent in the uninfected control (lane 7). Thus, more Rex protein was synthesized upon infection with vAcC4*rex* than upon infection with vAc373*rex*. This observation may be explained by the presence of the 30-bp leader sequences upstream of the *rex* ATG in the pAc373*rex* construct, which could conceivably interfere with optimal Rex expression. We also examined Tax expression by Sf9 cells infected with the vAcC4*rex* recombinant. HTLV-II Tax p37 was detectable in total cell extracts, indicating that both Rex and Tax are expressed in baculovirus-infected insect cells (data not shown).

Purification of baculovirus-expressed HTLV-II Rex from insect cells. The relative level of Rex production was a small fraction of the total protein, barely detectable by Coomassie blue staining (data not shown). To enrich for Rex in subsequent purification steps, we determined the localization of Rex in the baculovirus-infected insect cells by nonionic detergent fractionation (see Materials and Methods). Nuclear and cytoplasmic extracts were prepared and immunoprecipitated with Rex-specific antiserum (Fig. 1C, lanes 1 and 2). Although p24 and p26 Rex were present in both the nuclear and cytoplasmic fractions, approximately fourfold more Rex was detected in the cytoplasm of the Sf9 cells. In vAcC4*rex*-infected insect cells, Tax was predominantly found in the nuclear fraction (data not shown). This observation correlates with the previous report that HTLV-I Tax is localized to the nucleus of baculovirus-infected insect cells (22).

Since Rex was predominantly localized to the cytoplasm, we purified Rex from the cytoplasmic fraction of baculovirus-infected insect cells. The cytoplasmic extract was fractionated by heparin-agarose chromatography (see Materials and Methods). Proteins bound to heparin-agarose were eluted with a 0.1 to 1.0 M KCl gradient (Fig. 2A). Fractions were collected and assayed for the presence of Rex by Coomassie blue staining (Fig. 2B) or by Western blot analysis (Fig. 2C). The heparin-agarose column profile shows that the bulk of the cytoplasmic proteins that bound to heparin-agarose eluted between 0.1 and 0.3 M KCl. In the Western blot analysis, Rex was detected in the crude cytoplasmic extract, was absent in the flow-through fraction, and eluted with a peak at fraction 48; this fraction corresponds to elution at 0.6 M KCl and therefore separation from most other cytoplasmic proteins. The band migrating ahead of p24 Rex in the crude cytoplasmic extract is not typically seen and may represent a degradation product. Interestingly, p24 and p26 Rex copurified in this fractionation. These results were confirmed by analysis of the same fractions by SDS–PAGE followed by Coomassie blue staining. Fractions 44 through 52 contain relatively pure Rex; the estimated amount of purified Rex is low and represents approximately 0.1 to 0.2% of the total protein loaded onto the column. Fractions 46 through 50 were subsequently pooled, dialyzed, and stored at −70°C. This purified Rex-containing fraction was employed for the functional studies described below.

Rex binds RNA containing the RxRE. Previous studies by our laboratory, in which the HTLV-II 5′ LTR was linked to the reporter gene, chloramphenicol acetyltransferase, and cotransfected with HTLV-II *tax/rex* or *tax* expression vectors, demonstrated that Rex mediates a 5- to 10-fold increase in LTR-linked gene expression (41). Sequences important for Rex function, designated the Rex-responsive element (RxRE), must therefore lie within the 5′ LTR; since the Rex effect is posttranscriptional, it should be within the transcribed portion of the LTR. This specifies nucleotides between the beginning of R, where viral transcription initiates, and the 3′ boundary of the U5 region of the LTR (nt 315 through 765). Indeed, in transient expression assays utilizing various deletions of the LTR linked to chloramphenicol acetyltransferase, we recently localized the RxRE within this region (3). Furthermore, the reported functional similarities between Rev and Rex (16) and the affinity with which Rex bound to heparin-agarose, suggested that Rex might directly bind RNA. We first qualitatively assessed the ability of baculovirus-expressed Rex to bind HTLV-II LTR RNA sequences in a gel mobility shift assay (see Materials and Methods). Increasing amounts of Rex purified by heparin-agarose chromatography were incubated with uniformly labeled RNA synthesized in vitro, corresponding to nt 361 through 786 of the HTLV-II 5′ LTR (Fig. 3). In the presence of Rex, formation of a major RNA-protein complex was observed, which was specifically competed by nonradioactive sense RNA from nt 361 through 786 (herein referred to as 361–786 RNA) but not by RNA from the HIV-1 *trans*-activator response region or nonspecific pGEM-1 vector RNA. A minor complex of faster mobility was also seen in some experiments; its significance is unclear. These results suggested that Rex bound to HTLV-II LTR sequences.

To more quantitatively measure binding, we developed conditions for an immunobinding assay. The binding reactions included approximately 50 pg (≈10 pmol) of labeled RNA corresponding to nt 361 through 786 of the HTLV-II 5′ LTR and various amounts of purified Rex. Complexes were collected by immunoprecipitation with Rex-specific antiserum, followed by incubation with protein A-Sepharose. A dose response in which increasing amounts of Rex were incubated with labeled sense and antisense 361–786 RNA is depicted in Fig. 4A. Rex specifically bound the sense 361–786 RNA. At the higher concentrations of Rex, binding to antisense RNA was also observed.

To demonstrate the specificity of Rex binding to the 361–786 RNA, competition with unlabeled sense 361–786 RNA and unlabeled nonspecific Bluescript vector transcripts was performed (Fig. 4B). Molar ratios of 1:1, 1:50, and 1:1,000 labeled RNA to nonradioactive competitor were tested. Competition for binding to labeled sense and antisense 361–786 RNA was performed with 20 and 40 nM Rex, respectively. Only specific competitor RNA efficiently competed for binding with the labeled sense probe. In contrast, both the specific and nonspecific competitor RNAs efficiently competed for binding of Rex to the labeled antisense probe.

Since viral transcription initiates at nt 315 within the HTLV-II 5′ LTR, we tested whether sequences upstream of nt 361 are required for maximal Rex binding. The region between nt 312 and 765 (end of the LTR) was amplified by the polymerase chain reaction and cloned into the pGEM-1 vector (see Materials and Methods). This plasmid, designated pG312-765, was employed to synthesize labeled RNA. With 0.5 and 1.5 nM Rex, equivalent fractions of either 361–786 or 312–765 RNA were bound (data not shown). Thus, sequences necessary and sufficient for Rex binding are located between nt 361 and 765. This correlates with the region found to be essential for mediating Rex action on

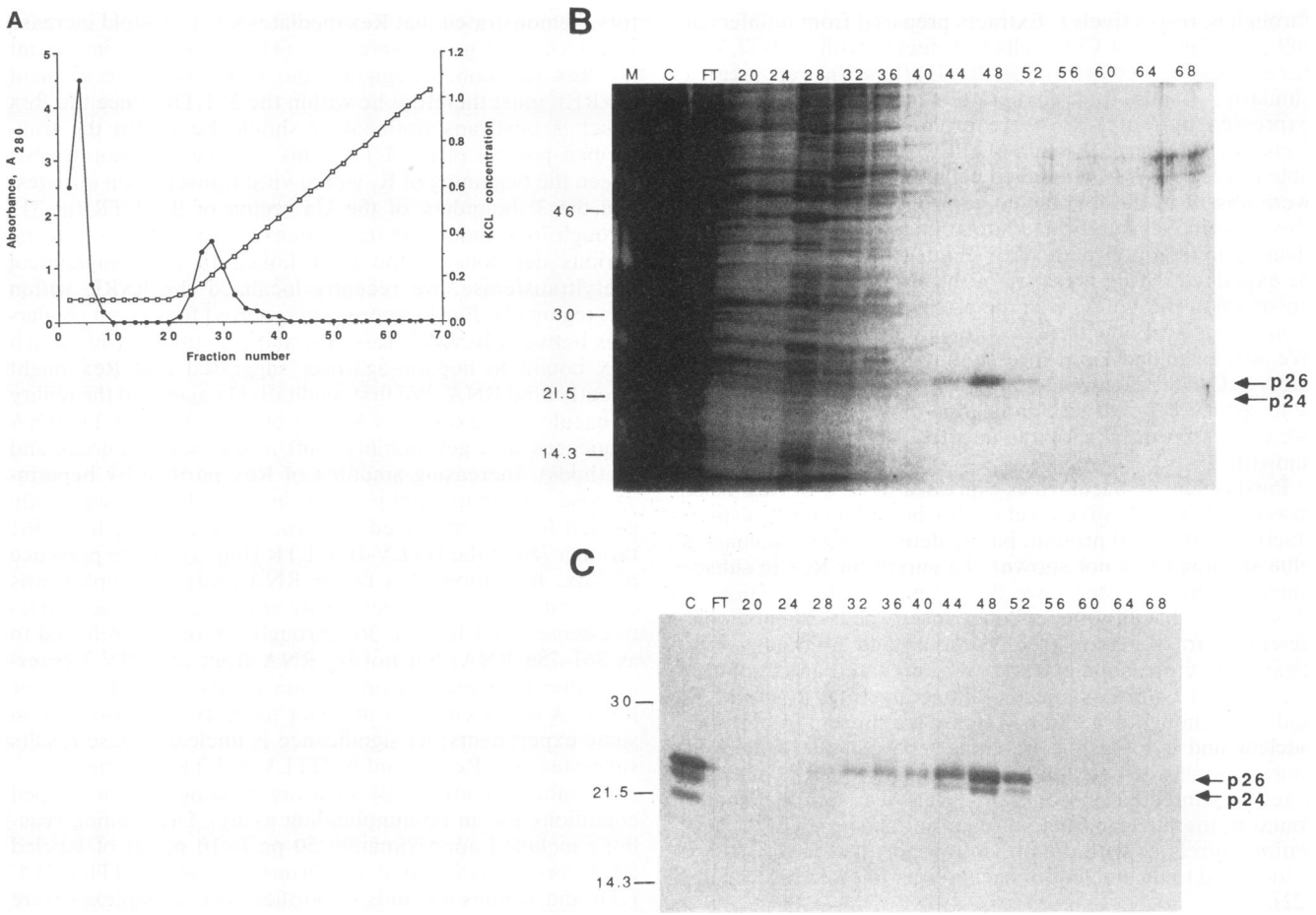

FIG. 2. Purification of Rex by heparin-agarose chromatography. (A) Heparin-agarose column profile. Cytoplasmic extract was prepared by Dounce homogenizing vAcC4*rex*-infected Sf9 cells and fractionated by heparin-agarose chromatography. Bound proteins were eluted with a 0.1 to 1.0 M KCl gradient. Fractions were collected, and absorbance and conductivity readings were recorded and plotted to generate the column profile. Symbols: ●, A_{280}; □, KCl concentration. (B) Analysis of fractions by SDS–PAGE. An aliquot of every fourth fraction between fractions 20 and 68 was analyzed by 12% SDS–PAGE, followed by Coomassie blue staining. The crude cytoplasmic extract (C) loaded onto the column and the flow-through fraction (FT) were run in parallel. p24 and p26 Rex proteins are indicated by the arrows. Sizes of molecular size markers (M) are indicated in kilodaltons. (C) Western blot analysis. Corresponding fractions analyzed directly by SDS–PAGE were assayed by Western blot analysis. Proteins were resolved by 12% SDS–PAGE and electrophoretically transferred to a nitrocellulose membrane that was then incubated with Rex-specific antiserum. The membrane was incubated with goat anti-rabbit immunoglobulin G coupled to alkaline phosphatase and subsequently developed. Crude cytoplasmic extract (C) and the flow-through fraction (FT) are indicated. p24 and p26 Rex proteins are indicated by the arrows. Molecular size markers (M) are shown.

LTR-linked chloramphenicol acetyltransferase constructs (3).

To further localize the region required for binding to Rex, smaller regions of the HTLV-II 5′ LTR were tested in the immunobinding assay. Regions tested that demonstrated negligible binding to Rex included nt 0 through 367 (U3 region of the LTR), nt 361 through 483, nt 460 through 520, and nt 645 through 750 (Fig. 5A and B). These results are summarized in Fig. 5C. Maximal binding to Rex occurred with transcripts from nt 361 through 765. The region between nt 361 and 520 consistently displayed approximately 50% of the binding seen with the 361–786 RNA probe (Fig. 5A), indicating that this region is able to bind Rex but is not sufficient for maximal Rex binding. Lack of Rex binding to the 361–483 and 460–520 RNAs suggests that transcripts between nt 361 and 460 and between nt 483 and 520 are crucial for binding.

Interaction of HIV-1 Rev and HTLV-II Rex with the HIV-1 RRE. HIV-1 Rev has previously been shown to bind to and mediate its action through the RRE, located within *env* gene sequences of HIV-1 (19, 32). It has also been shown that HTLV-I and HTLV-II Rex can functionally replace Rev in transient transfection assays with subgenomic HIV-1 constructs (14, 28) and that HTLV-I Rex can rescue a Rev-deficient HIV-1 mutant (39); these effects are mediated via the RRE (17). We tested whether HTLV-II Rex might also rescue a Rev-deficient HIV-1 mutant. Upon cotransfection of a Rev-deficient HIV-1_{JR-CSF} mutant and either a *rev* or HTLV-II *rex* expression vector into either 729-6 B cells or Jurkat T cells, rescue of the HIV-1 Rev-deficient mutant (2) was achieved (Table 1). However, rescue by Rex was consistently lower than rescue by Rev. These results, together with those previously published, suggested that Rex might bind directly to the HIV-1 RRE, analogous to Rev. We compared the ability of Rev and HTLV-II Rex to bind to the HIV-1 RRE. The RRE region (nt 7756 through 7990 of

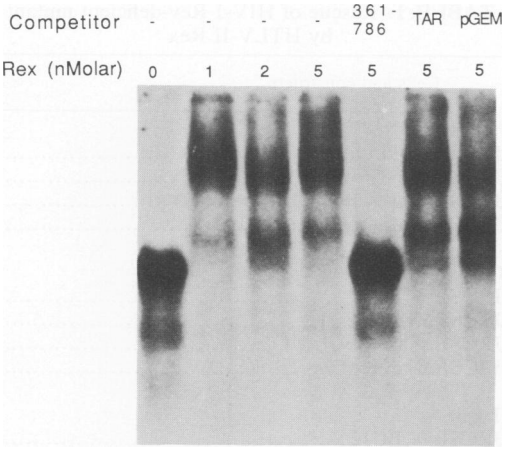

FIG. 3. Complexes between Rex and HTLV-II LTR RNA detected with the gel mobility shift assay. Binding reactions consisted of 50 pg of uniformly labeled HTLV-II LTR RNA corresponding to nt 361 through 786, 0 to 5 nM Rex (as indicated above the lanes) in 20 μl of buffer containing 40 mM Tris (pH 8.0), 2 mM $MgCl_2$, 200 mM KCl–8 μg of tRNA per ml, 20 μg of BSA per ml, 0.05% Triton X-100, and 40 U of RNasin per ml. After incubation for 15 min at 4°C, the complexes were resolved on 6% acrylamide gels in 43 mM Tris (pH 8.0)–50 mM KCl at 4°C for 24 h. Complexes were competed with 64 ng of nonradioactive specific and nonspecific competitor RNAs corresponding to sense 361–786 RNA, the *trans*-activator response (TAR) region, and pGEM-1 vector RNA (as indicated above the lanes).

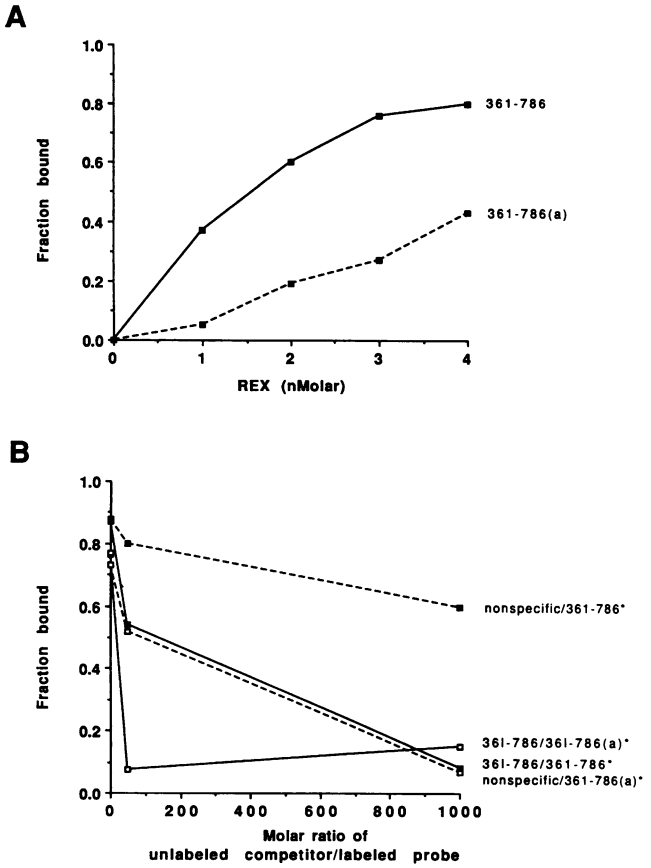

FIG. 4. Complex formation detected with the immunobinding assay. (A) Dose response of Rex binding to 361–786 RNA. Labeled sense and antisense RNA corresponding to nt 361 through 786 within the HTLV-II 5′ LTR was synthesized. The binding reactions consisted of approximately 50 pg of labeled RNA and 0 to 4.0 nM Rex in 50 μl of buffer containing 40 mM Tris (pH 8.0), 2 mM $MgCl_2$, 200 mM KCl, 16 μg of tRNA per ml, 20 μg of BSA per ml, 0.05% Triton X-100, and 40 U of RNasin per ml. Reactions were incubated at 4°C for 15 min. Complexes were detected by immunoprecipitation with Rex-specific antiserum and incubation with protein A-Sepharose as described in Materials and Methods. Sepharose beads were collected and counted by liquid scintillation. Symbols: ■——■, sense 361–786 RNA; ■----■, antisense 361–786 RNA. (B) Specificity of Rex binding to the 361–786 RNA. Binding of Rex to labeled sense and antisense 361–786 RNAs competed with nonradioactive sense 361–786 RNA or nonspecific Bluescript vector RNA. The molar ratios of labeled probe to nonradioactive competitor was 1:1, 1:50, and 1:1,000. Symbols: ■----■ and ■——■, labeled sense 361–786 RNA competing with unlabeled nonspecific Bluescript vector RNA and unlabeled sense 361–786 RNA, respectively (2.0 nM Rex); □----□ and □——□, labeled antisense 361–786 RNA competing with unlabeled nonspecific RNA and unlabeled sense 361–786 RNA, respectively (4.0 nM Rex); *, labeled probe; (a), antisense RNA.

HIV-1_{JR-CSF}) was amplified by the polymerase chain reaction and subcloned into the pGEM-1 vector to generate pGHIVRRE (see Materials and Methods). Labeled sense and antisense RNAs were synthesized from pGHIVRRE and used as probes. Under the binding conditions described above, Rev at 4 and 8 nM efficiently bound the sense RRE (Fig. 6A). In contrast, at concentrations of Rex (0.5 and 1.5 nM) capable of efficiently binding the 361–786 HTLV-II LTR RNA, no binding to the RRE was observed. Higher concentrations of Rex (5 and 10 nM) were also assayed, but no specific binding to the RRE was observed. At these concentrations, Rex exhibits significant binding to both sense and antisense RRE-containing RNA. We also tested binding conditions previously reported for Rev-RRE binding (19), and we were also unable to demonstrate specific binding of Rex to the RRE (data not shown).

In an attempt to assess whether any specific binding of the HIV RRE occurred, we utilized a larger region of the RRE that had previously been shown to be necessary for Rex responsiveness (48). In addition, we altered the conditions of the binding reaction to significantly reduce the nonspecific binding activity. Under these new conditions, we found, similar to the above results, that no binding to the RRE sequence of pGHIVRRE was observed. However, we observed weak binding to the larger RRE region of pKSHIVRREII (HIV-1 RRE II) relative to that of antisense RNA (Fig. 6B). Replicate binding assays were performed to determine the significance of the weak binding activity (Table 2), which was about 5- to 10-fold less than that observed with Rev. We conclude that there is very weak binding of Rex to the larger RRE sequence relative to the antisense sequence, but it is unclear how specific this binding is.

DISCUSSION

We purified HTLV-II Rex from the cytoplasmic fraction of baculovirus-infected insect cells by heparin-agarose chromatography. Baculovirus-infected insect cells expressed both species of Rex identical in size to Rex (p24 and p26), produced in mammalian cells. To determine whether Rex could bind RNA, analogous to Rev, we performed binding assays with purified baculovirus-expressed Rex and RNA corresponding the R-U5 region of the HTLV-II 5′ LTR. The

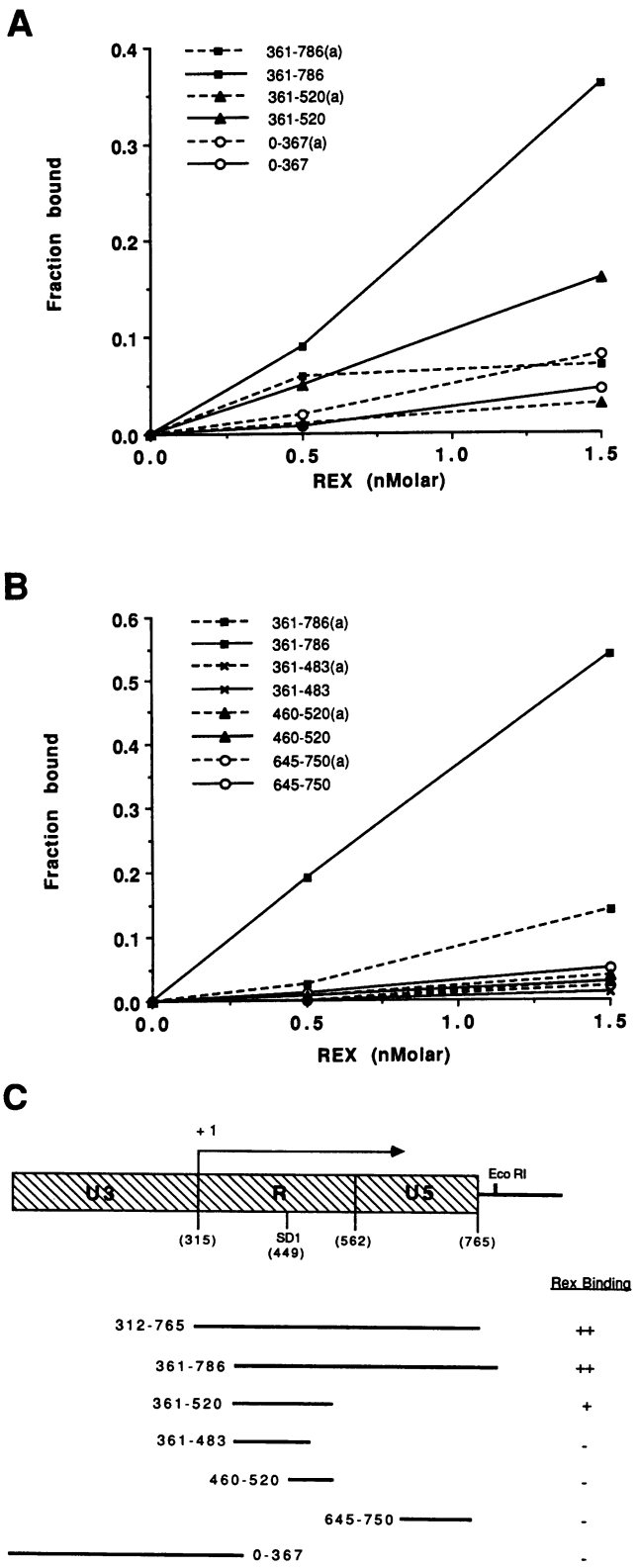

FIG. 5. Localization of Rex binding within the HTLV-II LTR. (A and B) Binding reactions were performed as described in Materials and Methods. Sense and antisense RNAs were prepared from various regions of the HTLV-II 5′ LTR and used as probes for binding with 0.5 and 1.5 nM Rex. Symbols for RNAs are indicated by nt numbers. (a), Antisense RNA. (C) Schematic diagram indicating location of RNA probes within the HTLV-II 5′ LTR and Rex binding ability. Nucleotide boundaries of the U3, R, and U5 regions and position of the splice donor site (SD) are indicated. The arrow indicates the initiation of transcription.

TABLE 1. Rescue of HIV-1 Rev-deficient mutant by HTLV-II Rex

Cells and constructs	HIV p24 (pg/ml)
729-6 B cells[a]	
pYKJRCSF (wild type) + BC12	>2,900
pYKJRCSFΔSal (Rev$^-$) + BC12	<6
pYKJRCSFΔSal + BC*rex*II (Rex only)	120
pYKJRCSFΔSal + pC*rev* (Rev only)	>2,900
729-6 B cells[b]	
pYKJRCSF + BC12	>2,900
pYKJRCSFΔSal + BC12	<50
pYKJRCSFΔSal + BC*rex*II	480
pYKJRCSFΔSal + pC*rev*	>2,900
Jurkat T cells[c]	
pYKJRCSF + BC12	>2,900
pYKJRCSFΔSal + BC12	<50
pYKJRCSFΔSal + BC*rex*II	210
pYKJRCSFΔSal + pC*rev*	1,360

[a] A total of 50 μg of plasmid DNA (20 μg of mutant or wild-type proviral construct and 30 μg of *rex*/*rev* expression vector or carrier DNA [BC12]) was transfected by electroporation into 10^7 729-6 B cells. Approximately 48 h after transfection, supernatants were harvested and assayed directly for HIV-1 Gag p24 by using the Abbott p24 antigen capture enzyme-linked immunosorbent assay. For lower concentrations of p24, the supernatants were concentrated by precipitation overnight with 0.5 volume of 20% polyethylene glycol–1.5 M NaCl and suspended in 200 μl of 25 mM Tris (pH 7.5)–5 mM dithiothreitol–50 mM KCl–0.25 mM EDTA–0.025% Triton X-100 before the assay for p24.

[b] Each plasmid (25 μg) was transfected by electroporation into 10^7 729-6 B cells. At 48 h posttransfection, supernatants were concentrated 16-fold by precipitation with polyethylene glycol as described in footnote *a* and assayed for HIV-1 p24 antigen.

[c] A total of 50 μg plasmid DNA (40 μg of mutant or wild-type proviral construct and 10 μg of *rex*/*rev* expression vector or carrier DNA [BC12]) was transfected by electroporation into 10^7 Jurkat T cells. At 48 h posttransfection, supernatants were harvested and assayed directly for HIV-1 p24 as described in footnote *a*.

formation of RNA-protein complexes was detected in both gel mobility shift assays and in immunobinding assays with Rex-specific antiserum, demonstrating the specificity of binding. Rex was found to specifically bind HTLV-II LTR RNA between nt 361 and 765. This region corresponds with that identified by functional assays to be required for Rex response RxRE. Thus, Rex, like Rev, is capable of binding RNA containing its respective response element.

Sequences required for Rex response, as determined by transient expression assays utilizing HTLV-II 5′ LTR-linked reporter gene constructs, lie within the R-U5 region (nt 315 through 765) of the HTLV-II LTR (41). Employing a different assay system, another group measured levels of spliced and unspliced RNAs in the presence of Rex and determined that a deletion downstream of nt 520 results in approximately 50% of the Rex response (33). Thus, sequences downstream of nt 520 are required for maximal Rex response in functional assays. In agreement, we demonstrate that the 361–520 RNA displays approximately 50% maximal binding, but that sequences between nt 361 and 765 within R-U5 are essential for maximal binding by Rex. These findings suggest either that there is more than one binding

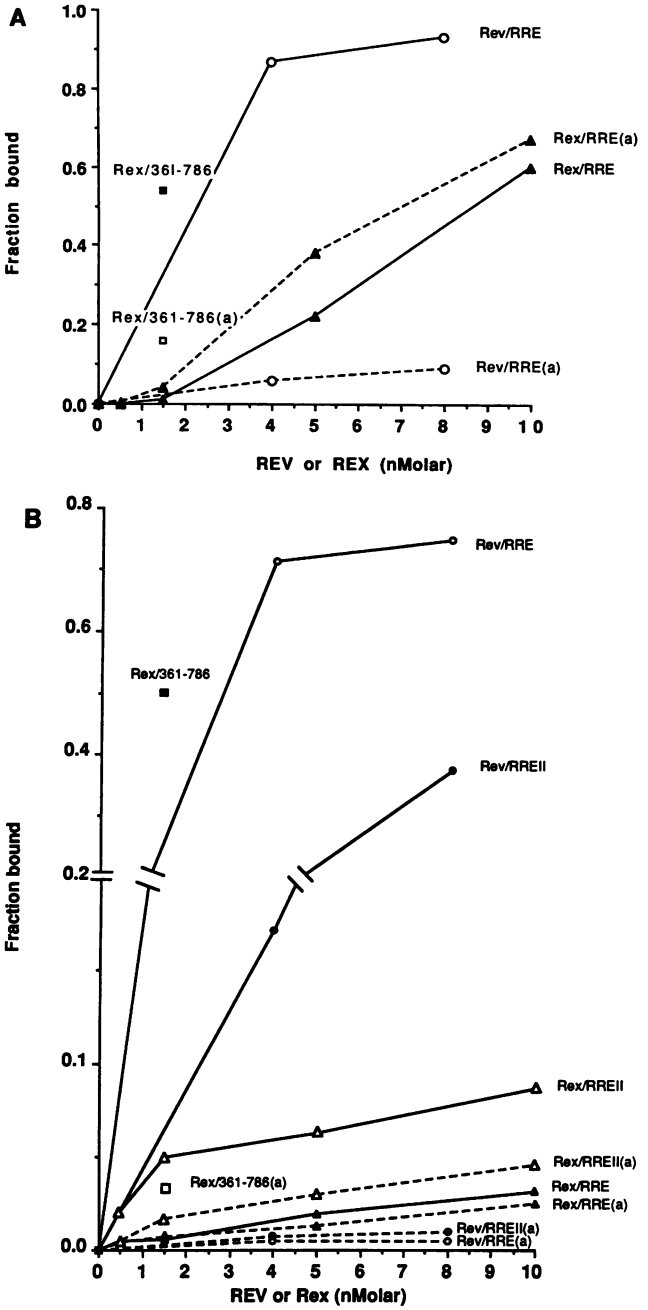

FIG. 6. Interaction of HIV-1 Rev and HTLV-II Rex with the HIV-1 RRE. (A) Binding reactions were performed as described in the Materials and Methods. Sense and antisense HIV-1 RRE RNA corresponding to nt 7756 through 7990 (HIV-1_{JR-CSF}) were used as probes. Either 4 and 8 nM Rev or 0 to 10 nM Rex was added to the binding reactions, followed by incubation for 15 min at 4°C. Complexes were collected by incubation with either Rev- or Rex-specific antiserum. As a control, 1.5 nM Rex was used in a binding reaction with sense and antisense 361–786 RNA. Symbols: ○----○ and ○——○, Rev binding to antisense and sense RRE, respectively; ▲----▲ and ▲——▲, Rex binding to antisense and sense RRE, respectively; □ and ■, squares is Rex binding to antisense and sense 361–786 RNA, respectively; (a), antisense RNA. (B) Binding reactions were performed as described above, with the exception that both Rex and Rev were preincubated at 37°C for 15 min in 100 mM NaCl–10 mM $MgCl_2$–10 mM Tris (pH 8.0). Sense and antisense HIV-1 RRE RNA and HIV-1 RRE II RNA corresponding to nt 7756 through 7790 and 7706 through 8036, respectively (HIV-1_{JR-CSF}), were used as probes. Rev and Rex concentrations and binding reactions were as described above. As a control, 1.5 nM Rex was used in a binding assay with sense and antisense 361–786 RNA. Symbols are as described above and labeled in the figure. Other symbols: ●----● and ●——●, Rev binding to antisense and sense RRE II RNA, respectively; △----△ and △——△, Rex binding to antisense and sense RRE II RNA, respectively. Note the break in the *y*-axis scale to better present lower values of binding.

TABLE 2. Binding of HTLV-II Rex to the HIV-1 RRE

Protein (nM)	RNA	Fraction bound (%)[a]
Rev (2.0)	RRE II	21.7
	RRE II	1.4
Rex (1.5)	361–786	34.7
	361–786	1.3
Rex (1.5)	RRE II	2.1 ± 0.95[b]
	RRE II	2.7 ± 2.2[b]
Rex (5.0)	RRE II	11.1 ± 1.5[b]
	RRE II	6.0 ± 3.6[b]

[a] Binding reactions were performed as described in the legend to Figure 6B.
[b] Mean ± standard deviation of binding performed in quadruplicate. *t* tests were performed, and the difference between binding of sense and antisense (a) RNAs gave *P* values of 0.48 (1.5 nM Rex) and 0.07 (5 nM Rex).

site within the 361–765 RNA or that Rex binds to the 361–520 RNA with lower affinity because of differences in stability of the secondary structure. The size of this region is similar to that identified for the HTLV-I RxRE region (51), and for the HIV-1 RRE, suggesting that formation of a complex secondary structure is necessary.

Since in HTLV-II the region required for Rex binding and the RxRE extend downstream of the first splice donor site (nt 449) and will therefore be removed by splicing upon generation of singly spliced (Fig. 7) and doubly spliced RNAs, the RxRE would only be present at the 5′ end of full-length RNA and partially or completely intact at the 3′ end of all viral RNAs. A differential Rex effect on *gag/pol* mRNA would be expected, since only *gag/pol* mRNA would contain the RxRE in its 5′ LTR sequences. To account for singly spliced *env* mRNA regulation by HTLV-II Rex, regulatory sequences within other regions of the HTLV-II genome may be required. In contrast to HTLV-II function, HTLV-I Rex function requires sequences within the 3′ LTR (specifically, within the U3-R region) to mediate its effects (51). Localization of the HTLV-I RxRE within the 3′ LTR, which would be present in all viral RNA species, implies the existence of additional regulatory elements outside the LTR in order for Rex to differentially regulate unspliced and singly-spliced RNAs.

The RRE and RxRE identified for HIV-1, HTLV-I, and HTLV-II are predicted by sequence analysis to form complex stable RNA secondary structures. Mutational analysis of the RRE suggests that maintenance of secondary structure rather than primary sequence is crucial for recognition and subsequent binding by Rev (35). We have directly demonstrated that Rex can bind RNA as well, presumably by recognizing the secondary structure formed by the RxRE region. Functional domains have also been identified within Rev and HTLV-I Rex (29, 38). Interestingly, both Rev and Rex possess an arginine-binding motif that has been identified in several RNA binding proteins (26). This region corresponds to the nuclear-nucleolar localization signal of Rev and Rex and, for Rev, is involved in binding to the RRE

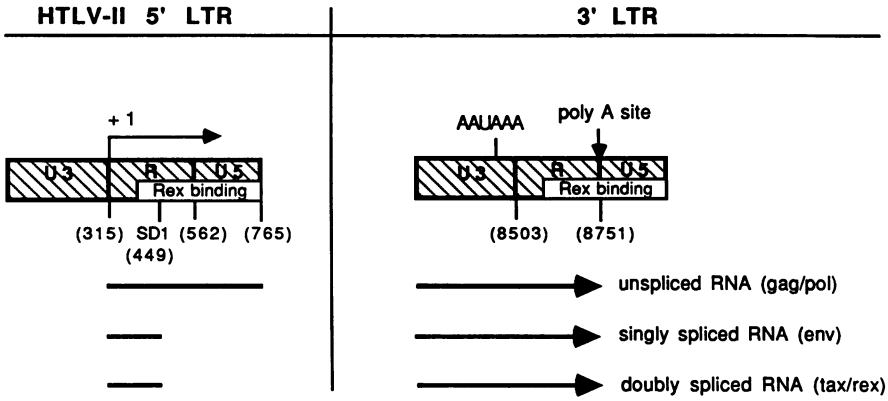

FIG. 7. Location of the HTLV-II Rex binding region within the 5' and 3' LTRs. HTLV-II 5' and 3' LTRs are depicted, including the nucleotide boundaries of the U3, R, and U5 regions. The positions of the transcription initiation site (+1), splice donor (SD), and the polyadenylation signal and site are indicated. Location of the Rex binding site within the LTRs is shown. Sequences contained in the unspliced, singly spliced, and doubly spliced RNA species are represented below the LTRs.

(29). The arginine- and lysine-rich peptide of another HIV-1 *trans*-regulatory protein, Tat, was recently found to bind specifically to its RNA response element, the *trans*-activator response region, which forms a stable hairpin (53). By analogy, the arginine-rich region at the HTLV-II Rex amino terminus may similarly be involved in binding to the highly structured RxRE region.

The sequence-specific HTLV-II RNA binding by Rex was not unexpected, based upon previously reported similarities between Rev and Rex. Not only are both phosphoproteins and localized to the nucleus, but they also have functional properties in common (16). Both Rev and Rex are involved in the posttranscriptional regulation and differential expression of the viral regulatory and structural genes. Despite these similarities, Rev and Rex and their respective response elements share little homology at either the nucleotide or amino acid level; nevertheless, Rex can activate the RRE and functionally substitute for Rev (32, 39). We have also demonstrated that HTLV-II Rex can rescue an HIV-1 Rev-deficient mutant. We therefore predicted that HTLV-II Rex might be able to bind the RRE. Consistent with previous functional data (48), Rex was unable to bind the minimal RRE, even in the presence of 10 times the concentration of Rex capable of binding HTLV-II RNA. The larger RRE region shown by Solomin et al. (48) to respond to Rex functionally appeared to bind Rex weakly relative to the antisense RNA; however, it is difficult to conclude from this weak binding whether it is specific. We propose several explanations for our observations. Rex may directly bind the larger RRE region specifically, albeit at markedly lower affinity than Rev. This would be consistent with our result that Rex is able to rescue the HIV-1 Rev-deficient mutant, although much less efficiently than Rev. It is also a possibility that Rex binds the RRE fortuitously, due to the high degree of secondary structure within the RNA. Once bound to the RRE, Rex could then mediate subsequent events in concert with other cellular proteins in a fashion similar to that of Rev. Alternatively, Rex may bind the RRE specifically but indirectly, requiring interaction with other cellular proteins to recognize the RRE. Rex may therefore recognize its homologous response element specifically but may nonspecifically interact with other RNA elements or may indirectly bind these elements. Further studies will clarify these issues and enable us to better understand how Rex intricately regulates viral gene expression.

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