JOURNAL OF VIROLOGY, Aug. 1996, p. 5511–5518 Vol. 70, No. 8
0022-538X/96/$04.00+0

Human T-Cell Leukemia Virus Type 2 Rex Inhibits Pre-mRNA Splicing In Vitro at an Early Stage of Spliceosome Formation

ANDREAS BAKKER,[1,2] XINQIANG LI,[1] CRISTINA T. RULAND,[3] DENNIS W. STEPHENS,[1] ALEXANDER C. BLACK,[1] AND JOSEPH D. ROSENBLATT[3]*

Division of Hematology-Oncology, Department of Medicine, UCLA School of Medicine,[1] and Department of Microbiology and Immunology, UCLA AIDS Institute,[2] Los Angeles, California 90024-1678, and University of Rochester Cancer Center, Rochester, New York 14642[3]

Received 29 March 1996/Accepted 29 April 1996

The Rex protein is an essential regulator of RNA expression in human T-cell leukemia virus types 1 and 2 (HTLV-1 and HTLV-2) that promotes the accumulation of full-length and partially spliced viral transcripts in the cytoplasm. Rex-mediated regulation correlates with specific binding to a cognate RNA recognition element which overlaps the 5′ splice site in the viral long terminal repeat. It has been unclear whether Rex directly affects splicing or only nuclear-to-cytoplasmic transport of viral mRNA. We demonstrate that HTLV-2 Rex is a potent inhibitor of splicing in vitro at an early step in spliceosome assembly. Inhibition requires phosphorylation of Rex and the ability of Rex to bind to the Rex response element. Direct inhibition of early spliceosome assembly by Rex may account for differential accumulation of unspliced transcripts and represents a novel mechanism of retroviral gene regulation.

Several human retroviruses have evolved similar strategies for viral gene expression. Structural Gag and Env proteins are expressed from unspliced *gag* or singly spliced *env* RNAs. Cytoplasmic expression of *gag* and *env* mRNA in human T-cell leukemia virus types 1 and 2 (HTLV-1 and HTLV-2) requires the nuclear posttranscriptional viral regulatory protein, Rex, while an analogous function is performed by Rev in human immunodeficiency virus type 1 (HIV-1) (2–4, 12, 19–21, 23). In HTLV-1 and -2, the Rex protein is expressed early in infection from a doubly spliced mRNA which also encodes the transcriptional activator, Tax (20, 23). In the absence of Rex, little or no *gag*/*env* mRNA is detected in the cytoplasm (4, 13, 21). The HTLV-2 Rex protein binds with specificity to an RNA sequence element, termed the RxRE (Rex response element), which forms a stem-bulge-loop secondary structure (4, 27). HTLV-1/2 RNAs are generated by using two splice donor sites located within the 5′ long terminal repeat (LTR) and upstream from *env* coding sequences (Fig. 1A). Mapping of the HTLV-2 RxRE to the R region of the LTR revealed that a consensus site for U1 small nuclear ribonucleoprotein (snRNP) (5′ splice site) is contained within the genetically defined Rex-binding element (4). Mutations which disable the 5′ HTLV-2 splice site significantly reduce Rex binding (4). Immediately downstream from the Rex binding domain are two *cis*-acting repressive elements, the cis-acting repressive sequence (CRS) and U5 inhibitory element (UIE), which appear to decrease RNA export from the nucleus to the cytoplasm through interaction with host proteins (2, 3). Rex has been shown to relieve constraints on RNA export from nucleus to cytoplasm conferred by the CRS and UIE (2, 3). It remains unclear whether the Rex-mediated cytoplasmic accumulation of incompletely spliced RNAs is due to effects on RNA transport from the nucleus, on RNA splicing, or on both. The location of the 5′ splice site within the RxRE suggested a direct role for Rex in the regulation of mRNA splicing. We therefore decided to determine whether in vitro splicing of viral pre-mRNAs is affected by Rex.

* Corresponding author. Mailing address: URCC, 601 Elmwood Ave., Box 704, Rochester, NY 14642. Phone: (716) 275-0842. Fax: (716) 273-1042. Electronic mail address: JRosenblat@CC.URMC.rochester.edu.

MATERIALS AND METHODS

Construction of splicing vectors. (i) HTLV-2 splicing constructs. All HTLV-2 splice donors were subcloned into recipient plasmid pBSA (a gift from J. D. Reilly and Mary Edmonds, University of Pittsburgh) restricted with *Bam*HI and *Eco*RI or *Eco*RV. Inserts were derived as follows: LTR SD (nucleotides [nt] 361 to 786) was derived from pGEM 361-786 (4) cut with *Bam*HI and *Eco*RI, LTR ΔSD (nt 361 to 786, s449,450) was derived from pM13 LII 361-786 s449,450 (2) restricted with *Bam*HI and *Eco*RI, LTR ΔCRS (nt 361 to 520) was derived from plasmid pM13 LII 361-520 (4) digested with *Bam*HI and *Sma*I, and LTR SDΔRxRE (nt 361 to 786Δ465–501 [Δ indicates deletion]) was derived from plasmid pM13 LII 361-786Δ465-501 (4) digested with *Bam*HI and *Eco*RI. The LTR U1 (nt 361 to 786 s447,448,456) construct was derived by site-directed mutagenesis (Amersham) using a 28-nt oligonucleotide (CTGAGAGGATACT TACCTGGGGAGGAGC) and pM13 LII 361-786 as the substrate, confirmed by dideoxy nucleotide sequencing, and subcloned as a *Bam*HI-*Eco*RI fragment into pBSA. The env SD construct (nt 5090 to 5810, splice donor at nt 5183) was derived from the HTLV-2 subclone pH6B 3.5 digested with *Bam*HI and *Pvu*II. Splicing vectors are outlined in Fig. 1A.

(ii) Multimerization of stem-bulge-loop sequences. The minimal RxRE was synthesized by annealing complementary oligonucleotides comprising nt 458 to 507 of the 5′ LTR of HTLV-2 (5′-GGGCCTCTCAGGTCGAGCTCGGCTGC CCCTTAGGTAGTCGCTCCCCGAGGGTCTTT-3′) adapted with restriction half sites of *Sma*I at the 5′ end and *Dra*I at the 3′ end. The annealed product was purified by electrophoresis on 2% low-melting-point NuSieve agarose in 1× Tris-acetate-EDTA and then subjected to excision and DNA extraction. The precipitated DNA was resuspended in 1× ligation buffer and ligated overnight at 16°C in the presence of 0.5 mM ATP, 3 U of T4 DNA ligase, and 10 U each of *Sma*I and *Dra*I. Ligation products were phenol extracted, ethanol precipitated, and resolved on a 2% low-melting-point agarose gel in 1× Tris-agarose-EDTA. Discrete bands, corresponding to products ranging from one to eight monomers, were gel purified and incubated in 1× ligation buffer together with dephosphorylated pGem 1 vector DNA restricted with *Sma*I and 2 U of T4 DNA ligase for 18 h at 16°C to form plasmid pAB410. The ligation mixture was transformed into *Escherichia coli* DH5α competent cells (Gibco-BRL), and transformants were selected on YT medium with ampicillin selection. Positive clones were selected by colony hybridization according to standard procedures (24), using the sense oligonucleotide labeled with T4 kinase and [γ-^{32}P]ATP to a specific activity of 5×10^9 cpm/μg. DNA sequences of isolated clones were confirmed by dideoxy sequencing.

(iii) Rx4 splicing constructs. Head-to-tail concatemers containing four copies of the minimal RxRE were cloned into splicing vector pBSAd I as follows. To generate an insertion at the 5′ end of exon I, pBSAd I was cut with *Pst*I, blunt ended, and dephosphorylated; to generate an insertion at the 3′ end of exon II, pBSAd I was cut with *Hin*cII and dephosphorylated; to generate an insertion at the center of the intron of pBSAd I, the splicing cassette was removed from pBSAd I by digestion with *Pst*I and *Hin*cII, and the resulting fragment was ligated to dephosphorylated pBluescript KS deleted for the *Hin*dIII site and cut with *Pst*I and *Hin*cII; the resulting plasmid was digested with *Hin*dIII. All vector DNAs were then blunt ended, dephosphorylated, and ligated with a blunt-ended *Eco*RI-*Xba*I fragment of pAB410 containing the reiterated minimal RxRE to form

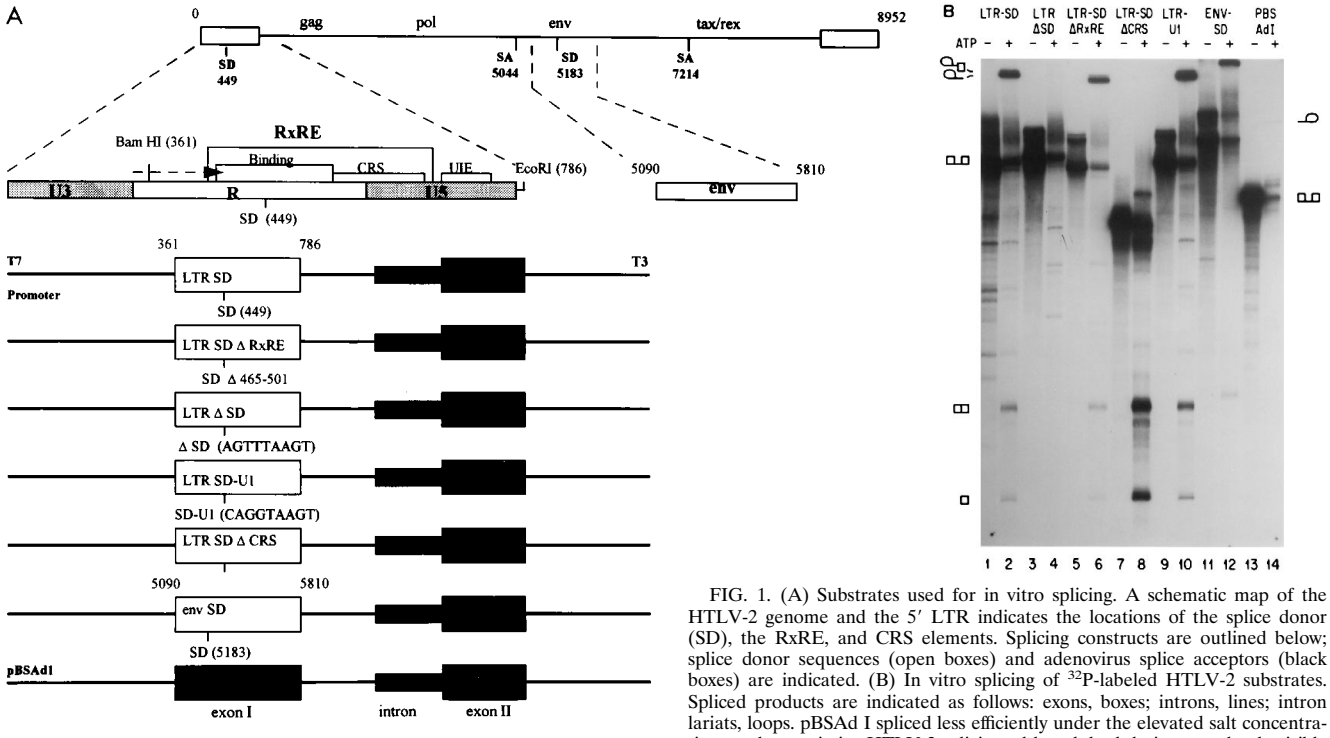

FIG. 1. (A) Substrates used for in vitro splicing. A schematic map of the HTLV-2 genome and the 5′ LTR indicates the locations of the splice donor (SD), the RxRE, and CRS elements. Splicing constructs are outlined below; splice donor sequences (open boxes) and adenovirus splice acceptors (black boxes) are indicated. (B) In vitro splicing of ^{32}P-labeled HTLV-2 substrates. Spliced products are indicated as follows: exons, boxes; introns, lines; intron lariats, loops. pBSAd I spliced less efficiently under the elevated salt concentration used to optimize HTLV-2 splicing, although both lariats are clearly visible on longer exposures (lanes 13 and 14).

pBSAd1-I (sense Rx4) and pBSAd1-Iα (antisense Rx4). Sense and antisense orientations of inserts were determined by dideoxy sequencing.

Expression and purification of Rex. HTLV-2 Rex was expressed and purified to near homogeneity as previously described (27). Briefly, 5×10^8 SF9 insect cells (ATCC 1711-CRL) were infected with the baculovirus clone vAcC4rex (27) for 3 days. The harvested cells were disrupted by Dounce homogenization in a Wheaton homogenizer in lysis buffer (10 mM KCl, 10 mM Tris-HCl [pH 7.9], 1 mM dithiothreitol, 500 μM phenylmethylsulfonyl fluoride); the postnuclear supernatant was adjusted to 100 mM KCl–1 mM $MgCl_2$–20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.4)–5% glycerol and then applied to a 5-ml heparin-agarose column (HiTrap; Pharmacia) equilibrated in the same buffer. The column was developed with 10 column volumes of a linear 100 to 1,000 mM KCl gradient; the peak fraction was identified by silver staining material of column fractions run on sodium dodecyl sulfate (SDS)–10% acrylamide gels and verified by Western blotting (immunoblotting). Pooled peak fractions were dialyzed against 60 mM KCl–20 mM HEPES (pH 7.4)–1 mM $MgCl_2$–0.5 mM EDTA–1 mM dithiothreitol–300 μM phenylmethylsulfonyl fluoride–10% glycerol and stored as aliquots at −80°C.

Immunobinding analysis. [^{32}P]CTP-labeled RNAs used in RNA binding reactions were synthesized according to standard protocols and purified on 3.5% polyacrylamide–8 M urea gels. Binding reactions were carried out with 50 pg of uniformly labeled RNA and 5 nM Rex in 50 μl of binding buffer (40 mM Tris-HCl [pH 8.0], 200 mM KCl, 2 mM $MgCl_2$, 0.05% Triton X-100, 20 μg of acetylated bovine serum albumin [BSA] per ml, 8 μg of tRNA per ml) in the presence of a 2,000-fold excess of specific (unlabeled RNA) or nonspecific (HIV-1 transactivating region [TAR] RNA) competitor RNA. After incubation at 4°C for 20 min, Rex-specific antiserum, affinity purified with protein A-agarose (Sigma), was added, and the mixture was incubated at 4°C for 20 min. Then 15 μl of protein A-Sepharose CL4B (Pharmacia) in binding buffer was added, and the mixture was incubated at 4°C with rotation for 30 min. The Sepharose beads were washed three times with 400 μl of binding buffer, and radioactivity was determined by liquid scintillation counting.

Transient transfection by electroporation for CAT assays. Transient transfections into Jurkat T cells were performed with a total of 25 μg of mixed plasmid and 5×10^6 cells in RPMI 1640 medium containing 10% fetal calf serum at a concentration of 2×10^7 cells per ml by electroporation as previously described (5). Following transfection, cells were resuspended in Iscove's medium containing 10% fetal calf serum at 10^6 cells per ml and incubated at 37°C with 5% CO_2. The incubated cells were harvested at 24 h posttransfection, cell viability was determined by trypan blue exclusion, and cells were lysed by three successive freeze-thaw cycles. Protein concentrations of cellular extracts were determined by the Bio-Rad protein microassay procedure. Equal amounts of protein from each sample were then used to assay for chloramphenicol acetyltransferase (CAT) activity, using the xylene extraction procedure (25).

In vitro splicing. HeLa nuclear extracts were prepared as previously described (6) except that nuclei were resuspended at a concentration of 10^9 cells per 1.5 ml. Splicing substrates were in vitro transcribed by using T7 or T3 RNA polymerase (Promega), 1 μg of linearized template, and α-^{32}P-labeled CTP (20 mCi/ml; 800 Ci/mmol; Amersham) as a precursor as previously described (27) and purified on a 3.5% polyacrylamide–8 M urea gel. Splicing reactions were performed for 2 h under standard conditions in a final volume of 30 μl with 17 μl of extract, unlabeled nonspecific competitor (yeast tRNA or HIV-1 TAR RNA) in 400-fold excess, 20,000 cpm (≈1 pmol) of labeled pre-mRNA substrate, purified Rex where applicable, and a final salt concentration of 90 mM KCl. Products of each reaction were proteinase K digested, phenol extracted, and ethanol precipitated. The precipitate was resuspended in 10 μl of 50% formamide–5 M urea and resolved on 6% acrylamide (19:1)–7 M urea gels in 75 mM Tris-borate (pH 8.3). Gels were dried and autoradiographed at −80°C. Molecular sizes of splicing products were verified by using either a radiolabeled 123-bp ladder (Bethesda Research Laboratories) or *Sau*3A1-restricted pBS (Stratagene) as a marker.

Phosphatase treatment of purified Rex. Three hundred nanograms of purified Rex and 40 μg of BSA were added to a 50-μl packed bead volume of calf intestine phosphatase (CIP)-agarose (100 U of CIP; Sigma P-0762), prewashed three times in 10 bead volumes of CIP buffer containing 100 μg of BSA per ml, and incubated for 60 min at 37°C as recommended by the manufacturer. An equivalent amount of protein unrelated to splicing, BSA, was processed in a parallel reaction to control for the contamination of splicing extracts by phosphatase after the bead treatment and removal of calf intestine alkaline phosphatase (CIAP) by centrifugation. Fifty microliters of Rex or control supernatant was equally divided for assay in the in vitro splicing reaction or for immunobinding as described below, and the structural protein integrity and extent of dephosphorylation were confirmed by Western blot analysis.

Western blot analysis. CIAP-treated and untreated Rex was resolved by electrophoresis on an SDS–12% polyacrylamide gel and transferred onto nitrocellulose paper (Hybond; Amersham). The membrane was blocked for 1 h in 1% (wt/vol) BSA (Sigma)–100 mM NaCl–10 mM Tris (pH 7.5)–0.1% Tween 20 at room temperature. Polyclonal Rex-specific antiserum (1:1,000) was then added, and the mixture was incubated for an additional 3 h. After washing, the blot was incubated with anti-rabbit immunoglobulin coupled to alkaline phosphatase and developed with the Vectastain ABC system (Vector Laboratories Inc., Burlingame, Calif.).

Gel retardation analysis of splicing complexes. Splicing reactions were terminated by addition of 5× stop buffer (5 mg of heparin per ml, 1× Tris-borate-EDTA, 50% glycerol, 0.025% bromphenol blue), and 20 μl of each reaction mixture was resolved at room temperature on a 3% acrylamide (60:1)–0.5% agarose composite gel in running buffer (50 mM Tris-borate, 1 mM EDTA [pH

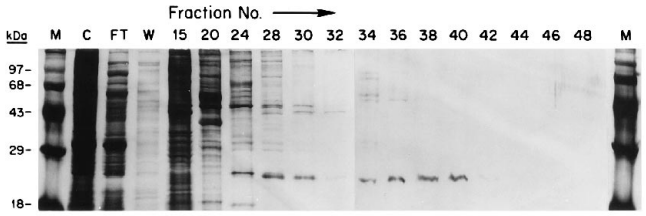

FIG. 2. Affinity chromatography of cytoplasmic extracts derived from SF9 cells infected with a baculovirus clone of HTLV-2 Rex. Aliquots of fractions from a heparin-agarose column were electrophoresed on SDS–10% acrylamide minigels and subjected to silver staining. Rex protein was identified by its molecular mass; Rex-containing fractions 36 through 40 were pooled, dialyzed, and stored in aliquots at −80°C. The identity and quality of the pooled preparation were confirmed by Western blot analysis (data not shown). M, size markers; C, control, FT, flowthrough; W, wash.

8.3]) for 5 h at 250 V. Gels were dried and autoradiographed at −80°C. The identities of splicing complexes (16) were verified by electroblotting of complexes to Zeta-Probe GT membranes (Bio-Rad) followed by hybridization with radiolabeled antisense RNA probes to U2, U4, U5, and U6 RNAs and autoradiography at −80°C according to the manufacturer's recommendations (data not shown).

RESULTS

In vitro splicing of HTLV-2 splice donors. To assay for direct Rex effects on splicing from viral splice sites in vitro, sequences containing wild-type and mutant HTLV-2 LTR splice donors were cloned upstream of the first intron and L2 exon of the major late transcription unit of adenovirus type 2 in the splicing vector pBSA (Fig. 1A). All viral splice donors were active in vitro and gave rise to intermediate and fully spliced products (Fig. 1B) which were identified by their predicted gel mobilities. A dinucleotide substitution at the viral LTR exon-intron border (LTR ΔSD) abolished splicing in vitro (Fig. 1B, lanes 3 and 4), indicating the obligatory role of the native 5′ LTR splice site. Deletions within the stem-bulge-loop structure (nt 465 to 501) of the RxRE (LTR SDΔRxRE) (2) or the removal of the CRS elements (LTR SDΔCRS) did not adversely affect splicing (lanes 5 to 8). In the case of LTR SDΔCRS, deletion of the *cis*-acting CRS element within the intron appeared to enhance splicing in vitro (lane 8). Because of the smaller intron size, LTR SDΔCRS branched intermediates migrate below the precursor RNA in our gel system. Similarly, Env SD 5′ exon and intron sequences are larger than their LTR SD counterparts and therefore migrate more slowly in denaturing gels (lanes 11 and 12).

Affinity purification of HTLV-2 Rex. HTLV-2-infected cells express two forms of Rex protein, p24 and p26, which differ in the extent of serine phosphorylation (10). Green et al. (10) have demonstrated that serine phosphorylation at multiple sites significantly enhances the binding of Rex to HTLV-2 target RNAs. To obtain the phosphorylated p26 form of Rex, we expressed HTLV-2 Rex in a baculovirus expression vector system as previously described (27). Cytoplasmic extracts of infected SF9 cells were fractionated by heparin-agarose chromatography using a linear 0.1 to 1.0 M KCl gradient. Fractions were collected and analyzed by SDS-gel electrophoresis. The presence of Rex was determined by silver staining (Fig. 2) and confirmed by Western blotting (data not shown). Rex eluted near homogeneity at 600 mM KCl, and peak fraction numbers 36 through 40 were pooled and dialyzed prior to storage at −80°C. The biological activity of purified Rex was confirmed by using a previously described (27) sensitive immunobinding assay (data not shown).

Inhibition of pre-mRNA splicing by Rex. LTR SD pre-mRNA contains the splice donor within the 5′ LTR and RNA-binding elements for Rex (RxRE) and heterogeneous nuclear ribonucleoproteins I (CRS) and A1 (UIE). When splicing reaction mixtures with the LTR SD substrate were incubated with increasing amounts of purified Rex, formation of intermediate and product RNAs was markedly decreased (Fig. 3, lanes 6 to 8). Inhibition of splicing was observed at levels as low as 19 nM (lane 7). At a concentration of 38 nM, splicing from LTR SD was effectively abolished and accumulation of unspliced substrate was observed (lane 8). To rule out nonspecific degradation of the pre-mRNA by Rex, we incubated pre-mRNA with Rex under splicing conditions without the addition of nuclear extract (lane 4). Comparison with reaction mixtures which contained nuclear extract (lane 3) or nuclear extract and Rex (lane 5) reveals that the limited RNA degradation observed is a property of the nuclear extract and occurs when splicing is prevented by omission of ATP from the reaction (lanes 3 and 5) or inhibited by Rex (lanes 7 and 8).

Deletion of nt 465 to 501 within the RxRE results in only residual RNA binding of Rex in vitro and greatly diminishes expression of a CAT reporter gene linked to the HTLV-2 LTR (4). We have failed to detect Rex binding to RNA containing the splice donor within *env*. We therefore compared the abilities of Rex to inhibit splicing of substrates to which Rex binds with high affinity (LTR SD) or binds poorly (LTR ΔRxRE, env SD, and pBSAd I). In comparison with wild-type LTR SD, LTR SDΔRxRE mutated within Rex binding sequences was markedly less inhibited (Fig. 4A, lanes 3 to 5 and 7 to 9). The env SD and pBSAd I substrates (lanes 11 to 13 and 15 to 17) showed little or no inhibition by Rex. Quantitative estimates indicated that the extent of inhibition was greater than 90% for

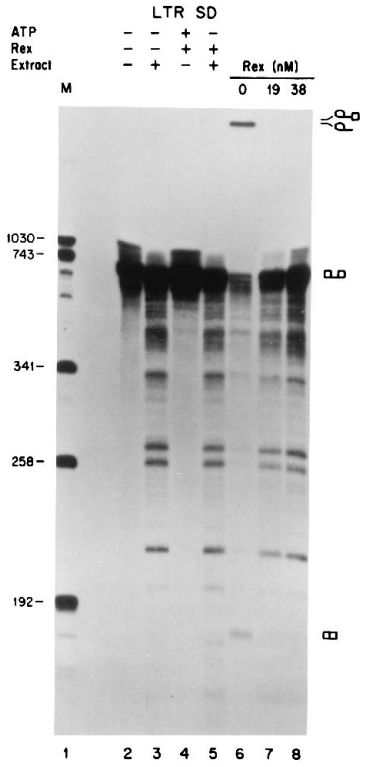

FIG. 3. Inhibition of HTLV-2 pre-mRNA splicing in vitro. Where indicated, Rex was added to a final concentrations of 38 nM (lanes 4 and 5) or 19 and 38 nM (lanes 7 and 8). Lane 1, radiolabeled, *Sau*3A1-restricted pBS plasmid as a molecular weight marker (M); lane 2, substrate pre-mRNA only; lane 3, addition of HeLa nuclear extract; lane 4, addition of Rex protein and ATP; lane 5, addition of Rex protein and HeLa nuclear extract; lanes 6 to 8, dose-dependent inhibition of splicing by Rex. Sizes are indicated in base pairs.

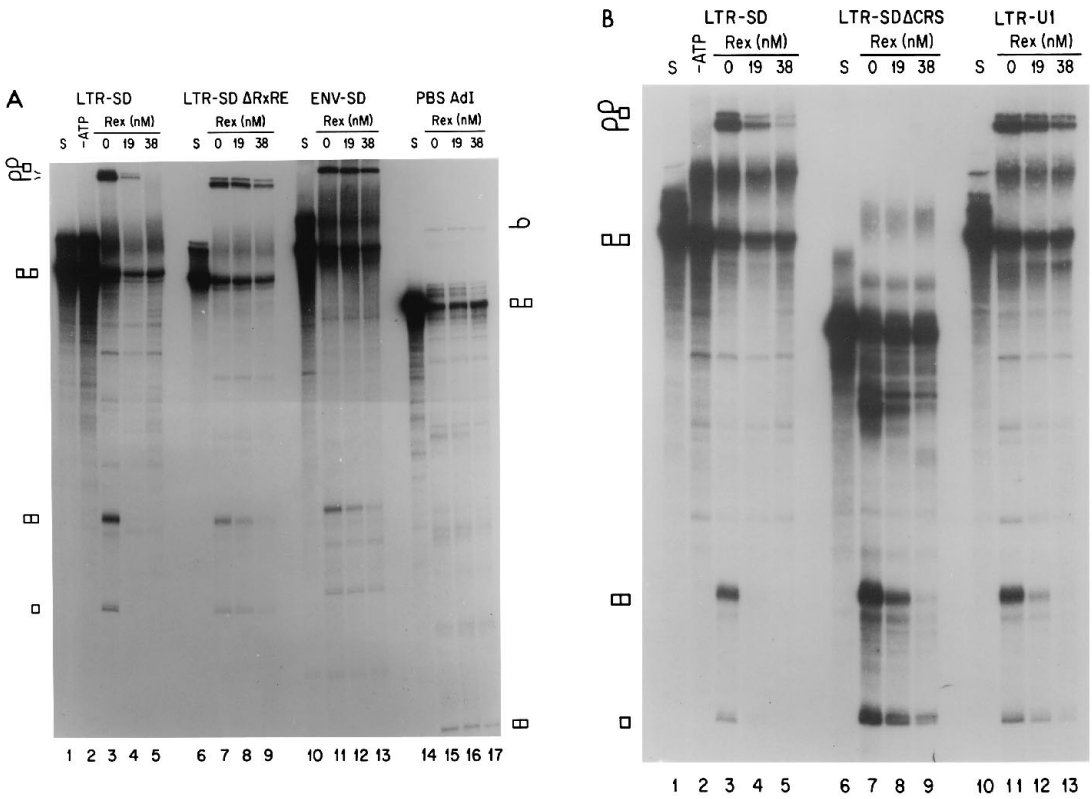

FIG. 4. (A) Specific inhibition of HTLV-2 pre-mRNA splicing by Rex. S, substrate RNA only. The relative levels of RNA substrate binding of Rex derived in parallel immunobinding reactions were as follows: LTR SD, 100.0%; LTR ΔRxRE, 38.6%; and env SD, 21.0%. Numbers represent values corrected for nonspecific binding due to Rex antiserum alone and the negative control, pBSAd I. (B) Rex-mediated inhibition of splicing from substrates with altered *cis*-acting elements. In the text that follows, percent RNA substrate binding to Rex relative to that of the wild type is indicated in brackets. LTR SDΔCRS [57] is missing nt 520 to 786 of the LTR, deleting both the CRS (nt 520 to 630) and UIE (nt 645 to 750). This deletion causes the branched lariat RNAs to migrate below the substrate RNA. LTR U1 [110] has a perfect 12-of-12-bp U1 consensus match at the LTR splice donor. Details are as for Fig. 1B.

LTR SD and substantially diminished for LTR SDΔRxRE (19%), env SD (7%), and pBSAd I (8%) substrates (Table 1).

Constructs in which CRS elements were deleted (LTR SDΔCRS) or in which a perfect U1 snRNP consensus (12-of-12-nt match to U1 snRNP, compared with the 9-of-12-nt match in wild-type HTLV-2) was introduced bound Rex with affinity similar to that of LTR SD and demonstrated similar inhibition in the presence of 38 nM Rex (Fig. 4B; Table 1). Hence, Rex-mediated inhibition of splicing in vitro is not dependent on the presence of CRS elements or on a suboptimal splice site, as described for Rev (6). However, it is possible that a suboptimal splice site predisposes the LTR splice donor to Rex inhibition in vivo. We therefore sought to create a Rex-binding element that mediates the Rex response but does not contain a splice donor within Rex binding sequences.

Rex binding to a reiterated minimal RxRE. Mutational analysis of the RxRE of HTLV-2 has shown that a stable stem-bulge-loop structure (nt 465 to 501) is critical for Rex protein binding to the RxRE (3, 4). To understand the functional role of the stem-bulge-loop within the RxRE, we investigated its ability to bind Rex protein in vitro. Prior immunobinding experiments in our laboratory had established that RNA encod-

TABLE 1. Relative levels of inhibition by Rex

Rex concn (nM)	% Inhibition[a]						Figure
	LTR SD	LTR SDΔRxRE	env SD	pBSAd I	LTR SDΔCRS	LTR U1	
0	0 (19)						1C
19	90 (2)						1C
38	100 (0)						1C
0	0 (64)	0 (53)	0 (30)	0 (12)			1D
19	55 (29)	4 (51)	0 (30)	0 (14)			1D
38	92 (5)	19 (43)	7 (29)	9 (11)			1D
0	0 (65)[b]				0 (117)[b]	0 (41)[b]	1E
19	94 (4)[b]				16 (98)[b]	24 (31)[b]	1E
38	98 (1)[b]				74 (31)[b]	85 (6)[b]	1E

[a] For each concentration of Rex, percent inhibition = 1 − (spliced lariat/unspliced substrate in the presence of Rex)/(spliced lariat RNA/unspliced substrate in the absence of Rex) × 100. The splicing efficiency measured as spliced lariat RNA/unspliced substrate RNA remaining is given in parentheses.

[b] Calculated on basis of spliced double-exon product RNA/unspliced substrate remaining. Values were derived by using densitometric scanning and the NIH Image 1.49 software for analysis.

ing nt 460 to 503 of the RxRE shows only residual binding activity in a Rex immunobinding assay (unpublished results). To enhance Rex binding to this sequence, we reiterated nt 458 to 501 to form a tetrameric minimal binding site (Rx4) which excluded the U1 consensus sequence (nt 447 to 455) of the 5′ LTR splice donor.

We used the Rex immunobinding assay to quantitatively measure Rex binding to radiolabeled Rx4 RNA. RNA from nt 361 to 786, which includes the full-length RxRE (nt 405 to 630), was used as the positive control. The specificity of the Rex-RNA interaction was determined by competition with a 2,000-fold excess of unlabeled probe RNA or with excess nonspecific HIV-1 TAR RNA, which also forms a stem-loop but does not bind Rex protein. As a negative control, we used RNA from nt 361 to 786Δ465 to 501 (361-786Δ465-501 RNA), which contains an internal deletion of the essential stem-bulge-loop sequence and shows minimal binding activity in vitro (4).

As shown in Fig. 5A, Rex binding to Rx4 RNA (37%) exceeds binding to the full-length RxRE (21%). In contrast, Rex affinity for Rx4 RNA in antisense orientation (2%) falls to the level of the negative control 361-786Δ465-501 RNA (2%), demonstrating that multimerization of the essential stem-bulge-loop structure of the RxRE specifically enhances Rex affinity to a level equivalent to or exceeding that of the native binding site.

Correlation of Rex-Rx4 binding with Rex function. Using a transient transfection assay, we have demonstrated previously that expression from an HTLV-2 LTR containing an intact RxRE and a CRS element (nt 520 to 630) linked to a CAT gene (pLII CAT) is low in the presence of Tax, and addition of Rex in *trans* rescues gene expression (3, 4). Expression of the CAT reporter linked to an LTR deleted for RxRE but not CRS elements, however, remains minimal even in the presence of Rex (3).

To test whether Rx4 can substitute for the RxRE in the context of the HTLV-2 LTR, we inserted Rx4 upstream of the CRS element of pLII 367, 520-630 CAT (3) to generate plasmid pAB420. Plasmid pLII 367, 520-630 CAT contains all of U3 and the 5′ 43 nt of the R region upstream of a CRS element (nt 520 to 630) linked to a CAT gene. It is therefore transactivated by Tax but not by Rex. The LTR-CAT constructs were cotransfected with HTLV-2 Tax or Tax/Rex expression vectors into Jurkat T cells. As previously shown, CAT expression from pLII 367, 520-630 CAT remained low in the presence of Rex (Fig. 5B) (3). In contrast, reporter gene expression from both pLII CAT and pAB420 is rescued when Rex is supplied in *trans* (Fig. 5B). Furthermore, pAB420 is activated to the same extent as pLII CAT, suggesting that Rx4 functions as efficiently as the wild-type RxRE in transient transfections. Therefore, Rex binding to a multimerized minimal binding element appears to correlate with Rex responsiveness in a functional assay.

Rx4 mediates inhibition of pre-mRNA splicing in vitro. Rex binding to RxRE RNA and Rex rescue of LTR-driven reporter gene expression are sensitive to mutations within the overlapping splice donor site in the RxRE (4). More importantly, we show that Rex inhibition of viral RNA splicing in vitro depends on the presence of an intact RxRE in the 5′ LTR. To determine whether Rx4 could mediate Rex inhibition of pre-mRNA splicing in a heterologous context, we inserted Rx4 DNA in sense and antisense orientations into the intron of an adenovirus splicing vector, pBSAd1, to yield plasmids pBSAd1-I and pBSAd1-Iα, respectively. Splicing reaction mixtures were incubated in the presence of 0, 19, and 38 nM purified Rex protein. In the absence of Rex, substantial amounts of LTR SD pre-mRNA are spliced to intermediate and product RNAs (Fig. 6, lane 2). Addition of Rex to reaction mixtures containing LTR SD RNA leads to complete inhibition of splicing

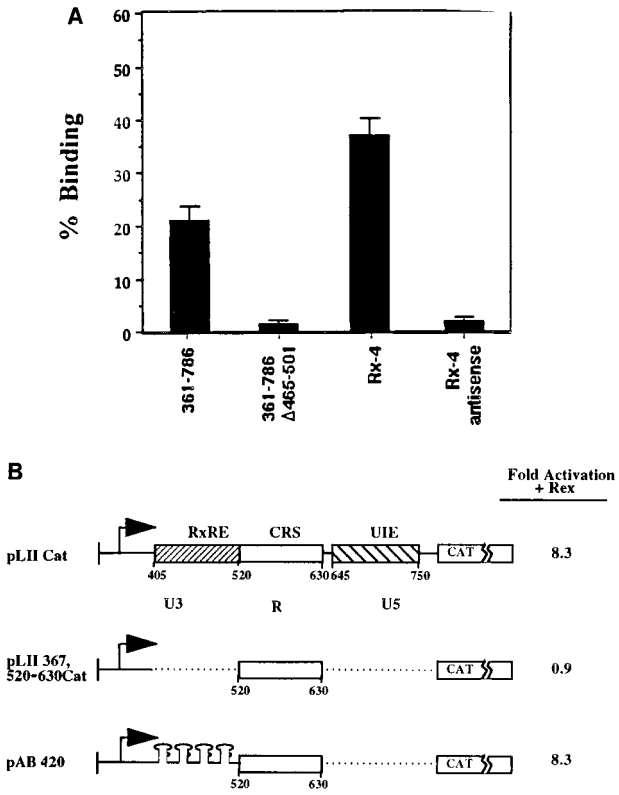

FIG. 5. (A) Specific Rex binding to RxRE and Rx4 RNA in vitro. RNA-Rex complexes were detected by immunoprecipitation with Rex-specific antiserum and incubation with protein A-Sepharose as described in Materials and Methods. Sepharose beads were collected and counted by liquid scintillation. 361-786 RNA contains the splice donor, full-length RxRE, and CRS elements of the 5′ LTR and serves as the positive control. 361-786Δ465-501 RNA has a deletion of the stem-bulge-loop motif essential for Rex binding and serves as a negative control. Rx4 RNA contains four stem-bulge-loop motifs in series. RNAs for Rx4 and Rx4 antisense were transcribed from pAB410 in both sense and antisense orientations, using T3 and T7 phage promoters, respectively. Results are the means of three independent assays. Standard errors are indicated. (B) Correlation of Rex binding to Rx4 RNA with function in a transient expression assay. Expression of CAT activity in Jurkat T cells transfected with CAT reporter constructs was measured 24 h posttransfection. CAT expression is driven by the HTLV-2 promoter of the 5′ LTR. All reporter constructs were cotransfected with the HTLV-2 *tax* expression plasmid BC20.2Sph or BC20.2, expressing both *tax* and *rex*. Fold activation was calculated by dividing the relative level of CAT activity in the presence of both Tax and Rex by the level of CAT activity in the presence of Tax alone. Results are the means of three independent transfections.

(lanes 3 and 4). Incubation of LTR SD RNA together with a 400-fold excess unlabeled Rx4 RNA in the presence of 38 nM Rex reverses the inhibition of splicing (lane 5). This finding suggests that Rx4 RNA can efficiently compete for Rex binding to the RxRE under conditions of RNA splicing. In contrast, little or no inhibition is observed for LTR SDΔRxRE RNA substrates, even at 38 nM Rex (lanes 7 to 9). Both pBSAd1-I and pBSAd1-Iα pre-mRNAs, containing Rx4 sequence in sense and antisense orientations, respectively, spliced efficiently in vitro (lanes 11 and 15). However, while pBSAd1-Iα RNA remains nonresponsive to Rex at both concentrations tested (lanes 16 and 17), splicing of pBSAd1-I RNA is substantially inhibited at 19 nM Rex and abrogated at 38 nM Rex (lanes 12 and 13). This finding suggests that the Rx4 sequence in the intron of pBSAd1-I RNA binds Rex protein and makes this RNA susceptible to Rex-mediated inhibition of RNA splicing in vitro.

Correlation of inhibition of splicing with Rex phosphorylation. Studies by Green et al. have shown that Rex must be

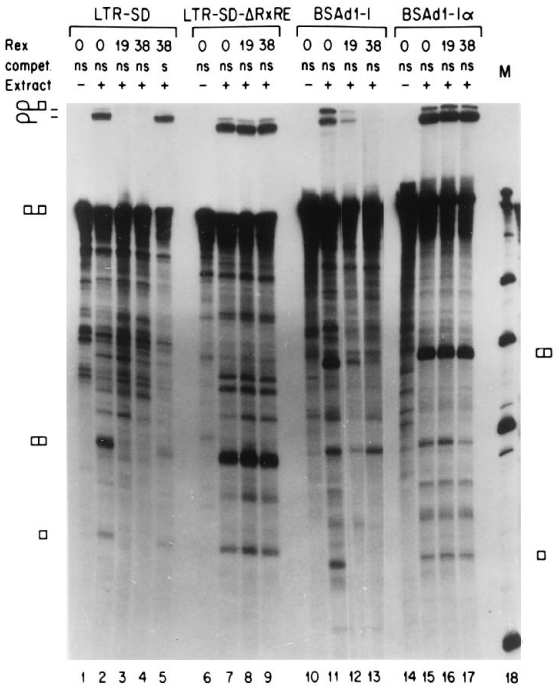

FIG. 6. Inhibition of splicing by Rex of RNA substrates containing the Rx4 sequence. RNA substrates were spliced in vitro under standard conditions. LTR SD contains nt 361 to 786 of the HTLV-2 5′ LTR, including the 5′ splice donor, full-length RxRE, and CRS elements; LTR SDΔRxRE contains a deletion from nt 465 to 501 of the LTR SD sequence, which removes the essential stem-bulge-loop motif of the RxRE; pBSAd1-I and pBSAd1-Iα contain the Rx4 sequence in the intron of pBSAd1 in the sense and antisense orientations, respectively. Rex protein concentrations (nanomolar) are indicated at the top. Unlabeled nonspecific (ns) competitor RNA is HIV-1 TAR RNA; unlabeled specific competitor RNA (s; lane 5) is Rx4 RNA. Competitor RNA was added in 400-fold excess. pBluescript KS (Stratagene) was digested with *Sau*3A1, end labeled with [γ-^{32}P] ATP, and used as a molecular weight marker (M; lane 19).

phosphorylated in order to bind efficiently and have demonstrated that incubation of p26 Rex with CIAP results in the formation of dephosphorylated p24 Rex (10, 11). We dephosphorylated purified Rex protein with CIAP coupled to agarose beads and removed CIAP by centrifugation prior to splicing. The extent of dephosphorylation and the integrity of the protein were monitored by Western blotting (Fig. 7A). We then compared the inhibition of splicing in the presence of phosphatase-treated and untreated Rex (10). As shown in Fig. 7B, dephosphorylated Rex showed a markedly attenuated effect on splicing in vitro relative to phosphorylated Rex. Immunobinding studies done in parallel demonstrated a decrease in the ability of dephosphorylated Rex to bind RNAs with specificity. This finding suggested that Rex-mediated inhibition of splicing in vitro is a function of its ability to specifically bind its RNA recognition element.

Inhibition of splicing at an early step in spliceosome assembly. The spliceosome assembly pathway can be separated into early ATP-independent events such as U1 snRNP binding and later ATP-dependent steps. Reaction mixtures from which ATP is omitted permit the stable binding of U1 snRNP and other splicing factors to form the commitment complex but block further addition of snRNPs to form a functional spliceosome (26). To determine the step at which Rex inhibited splicing, we preincubated LTR SD with nuclear extract in the absence of ATP for increasing amounts of time. At defined intervals, ATP together with Rex was added, the incubation continued, and products of the splicing reactions were assayed. Addition of ATP alone restored splicing (Fig. 8A, lane 3). Simultaneous addition of ATP and Rex resulted in marked inhibition of splicing (lanes 4 and 5). However, preincubation for 5 min or longer in the absence of ATP markedly attenuated the effect of Rex (lanes 6 to 9). Since U1 snRNP binding occurs within the first minutes of incubation, these results suggest that Rex effects on splicing are diminished if U1 snRNP is allowed to interact with the splicing substrate in the absence of Rex. Gel retardation analysis of splicing complex assembly on radiolabeled LTR SD substrate confirmed that Rex was acting early in spliceosome assembly. Generally, four ribonucleoprotein complexes can be resolved on nondenaturing gels (17). A nonspecific heterogeneous complex H forms in the absence of ATP and represents primarily heterogeneous nuclear ribonucleoprotein binding to the substrate (17). Following ATP addition, three splicing specific complexes, A, B, and B* (alternatively designated C) are formed in a time-dependent manner. The earliest complex A contains U2 and U1 snRNP and is subsequently joined by U4/U6 and U5 as a tri-snRNP particle to form complexes B and B* (17, 26). Figure 8B, lanes 3 to 6, shows the formation of three ATP-dependent complexes over time in the absence of Rex. In the presence of Rex, formation of all three complexes, including the earliest complex, A, is impaired (lanes 7 to 10). This finding suggests that Rex acts prior to the addition of U2 to the commitment complex to form complex A. To test whether Rex can affect the

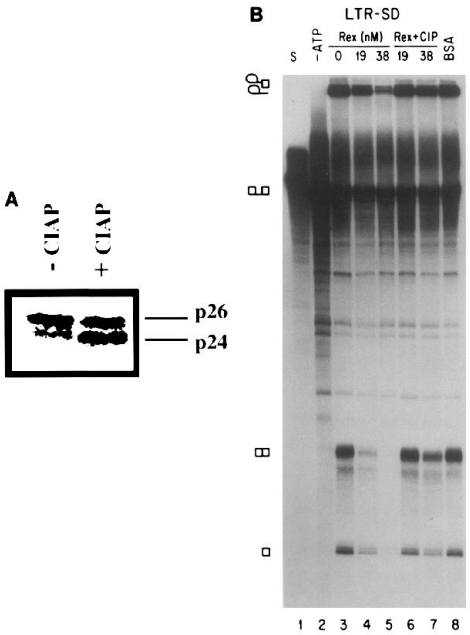

FIG. 7. (A) Dephosphorylation of Rex protein by CIAP. Rex was treated with CIAP as described in Materials and Methods and analyzed by Western blotting. The positions of dephosphorylated (p26) and dephosphorylated (p24) Rex are indicated. (B) Inhibition of splicing requires phosphorylated Rex. Splicing reactions were performed and analyzed as for Fig. 1B. Rex was dephosphorylated by treatment with CIP prior to addition to the splicing reaction mixture (lanes 6 and 7). Splicing reactions were performed as described above. Phosphatase treatment of an equivalent amount of BSA as a control did not adversely affect the splicing assay (lane 8). Immunobinding reactions done in parallel revealed that CIP treatment dramatically reduced Rex RNA binding activity. In the text that follows, numbers in boldface are percentages of wild-type binding, and the actual percentages of input RNA bound by Rex are given in brackets. Untreated Rex: wild-type 361-786 RNA (LTR SD) = 100 [65], control 361-786Δ465-501 RNA (LTR SD ΔRxRE) = 25 [16]. CIAP-treated Rex: wild-type 361-786 RNA (LTR SD) = 0, control 361-786 Δ465-501 RNA (LTR SDΔRxRE) = 3 [2]. Relative inhibition was calculated for the double-exon product/substrate remaining as described in the footnote to Table 1 and resulted in 0, 48, and 93% inhibition for 0, 19, and 38 nM Rex, respectively, and 0 and 10% inhibition for 19 and 38 nM CIAP-treated Rex.

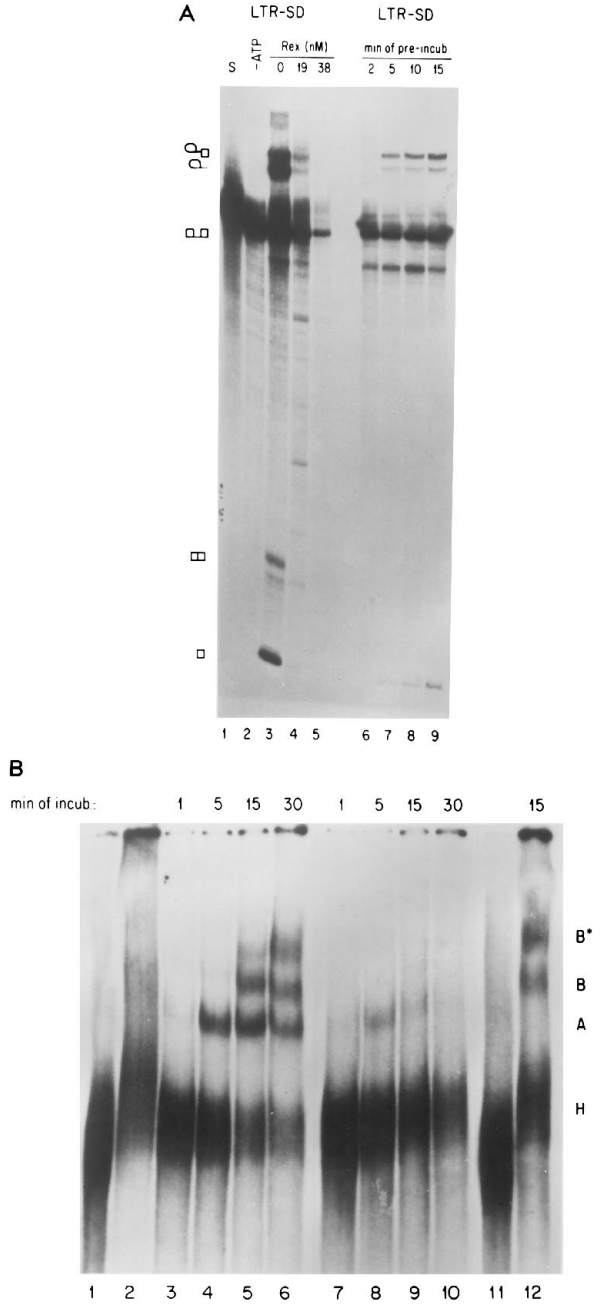

FIG. 8. (A) Preincubation of precursor RNA with splicing extract in the absence of ATP attenuates the Rex effect. Control splicing reactions were done in the presence of 0, 19, and 38 nM Rex (lane 3 to 5). Preincubation was performed as follows. Standard splicing reactions were set up, omitting ATP, creatine phosphate, and Rex (to 38 nM), which were added back to the reactions after 2, 5, 10, or 15 min (lanes 6 to 9). The incubation was then continued for 90 min, and reactions were processed as for Fig. 1B. (B) Gel retardation of splicing complexes with LTR SD. Incubation times are indicated at the top, and splicing complexes are indicated at the right. Lanes 1 and 11 contain LTR SD substrate RNA only; lane 2 contains a complete splicing reaction minus ATP and creatine phosphate. Lanes 3 to 6 show formation of ATP-dependent complexes after 1, 5, 15, and 30 min of incubation in the absence of Rex, and lanes 7 to 10 show formation in the presence of Rex (38 nM). Lane 12 represents the complex profile with addition of Rex at 10 min of incubation, followed by an additional incubation time of 5 min.

stability of existing complexes, we added Rex after a 15-min incubation of LTR SD, HeLa extract, and ATP and then continued the incubation. Lane 5 shows that 15 min are sufficient for the assembly of all three complexes. However, addition of Rex at this time led to the loss of only complex A (lane 12) during continued incubation. This finding suggests that Rex prevented de novo formation of complex A but allowed existing A complexes to progress to the B and B* configurations. This finding was consistent with a Rex effect early in spliceosome assembly. Alternatively, addition of Rex at 15 min may specifically destabilize complex A but not complexes B and B*.

DISCUSSION

We have shown that HTLV-2 Rex can effectively inhibit splicing of RNAs containing a Rex RNA-binding element in vitro. Inhibition is demonstrated on splicing substrates which contain the native RxRE or a multimerized minimal RxRE but not on RNAs carrying a deletion of a stem-bulge-loop structure necessary for RNA binding. Inhibition is not observed for substrates carrying the second HTLV-2 splice donor, located in the *env* region of the genome, or heterologous control substrates. This lack of inhibition suggests that Rex binding to the RxRE in the 5′ LTR has the potential of inhibiting viral RNA splicing in vivo. RNA substrates, with or without CRS elements, splice well in vitro, suggesting that CRS RNA makes no intrinsic contribution to the splicing regulation of HTLV-2 RNAs. If Rex inhibits splicing by merely binding to the RxRE to interfere with assembly of spliceosomes, other RNAs should become Rex responsive by providing the RxRE in *cis* and Rex protein in *trans*. To test this hypothesis, we first created a minimal Rex-binding element (Rx4) that does not include the splice donor site. When inserted into the intron of an adenovirus splicing substrate, Rx4 mediates inhibition of splicing by Rex at levels comparable to those observed for the HTLV-2 5′ LTR exon (LTR SD). Our finding that inhibition requires a functional Rex-binding element, phosphorylated Rex protein, and occurs with specificity indicates that Rex binding to RNA is essential for inhibition to occur.

Rex binding near the splice donor site in the LTR may interfere with early steps in splicing, such as commitment complex formation. This question was addressed by adding Rex to commitment complexes, formed for increasing amounts of time by preincubating RNA substrates together with HeLa nuclear extract in the absence of ATP. Commitment complexes are formed by the interaction of U1 snRNP with the 5′ splice site within the first 2 min of preincubation. Inhibition was attained only after very brief preincubations and lost after prolonged exposure to HeLa extract, suggesting that Rex cannot abrogate splicing of RNA substrates that are already committed to the splicing pathway. However, only a limited amount of splicing activity was recovered by prolonged preincubation of substrate RNAs in ATP-deficient HeLa extracts, suggesting residual effects of Rex on continuing rounds of RNA splicing. Gel retardation analysis shows that the formation of higher splicing complexes (A, B, and B*′) is inhibited in the presence of Rex protein. The loss of complex A after addition of Rex to assembled splicing complexes may reflect a block to the recruitment of new commitment complexes in the presence of Rex. In this case, the pool of A complex existing at the time of Rex addition may be depleted by maturation of assembled A complexes into complete spliceosomes (B and B*), and diminished formation of new A complexes may be due to Rex binding to pre-mRNAs. These observations are consistent with the inhibition of splicing at an early step of spliceosome formation. Since mutations within the 5′ splice site also reduce Rex affinity for the RxRE (4), Rex may inter-

fere with U1 snRNP interactions with the splice donor. However, results of gel retardation experiments suggest that Rex may affect the formation or stability of complex A. Further analysis is required to unambiguously identify the splicing component affected by Rex binding to pre-mRNA.

Inhibition of splicing at an early ATP-independent step by a retroviral protein is in contrast to HIV-1 Rev, which has been suggested to affect binding of U4/U6-U5 snRNP, a later step in spliceosome assembly (16). Inoue and coworkers have noted the accumulation of unspliced HTLV-1 transcripts in both the nucleus and the cytoplasm of transiently transfected cells in the presence of Rex (13). These observations are consistent with a model in which Rex binds its cognate RNA motif in proximity to the splice donor and may thus effectively exclude splicing factors from the early assembly of a commitment complex at the splice site.

The effect of Rex on splicing suggests a bimodal mechanism of action in which 5′ splicing may be inhibited, contributing to unspliced transcript accumulation, while nuclear-to-cytoplasmic transport of *gag* mRNA is facilitated through the RxRE-Rex interaction with the CRS. Such a bimodal mechanism has been postulated for HIV-1 Rev on the basis of RNA transport experiments with *Xenopus laevis* oocytes (8). Investigators had previously noted Rex effects on nuclear-to-cytoplasmic transport mediated through the RxRE in the reiterated 3′ LTR (15). The reported ability of HTLV-1/2 Rex to act as a substitute for HIV-1 Rev in Rev-deficient HIV-1 clones could conceivably be due to effects on splicing and/or transport, although we tend to favor the latter because of the distance between the HIV-1 splice donor and Rev binding sequences (18, 22, 28). Transport effects alone would not explain differential expression of HTLV-2 *gag*, *env*, and *tax*/*rex* mRNAs, all of which contain the reiterated 3′ RxRE. It is possible that there are present within *gag* or *env* sequences in HTLV-1 and -2 additional *cis*-acting regulatory elements that may also affect splicing, similar to those already described for Rous sarcoma virus and avian sarcoma virus (1, 9, 14, 28). As opposed to the observed mechanism for Rex, these elements are thought to act on the basis of RNA secondary structure, suboptimal 3′ splice sites, or other mechanisms which are poorly understood. Direct inhibition of early spliceosome assembly by Rex may therefore represent a novel mechanism of retroviral regulation in HTLV-1 and -2.

ACKNOWLEDGMENTS

We thank Judy Gasson for the use of fast protein liquid chromatography equipment, John Fraser for assistance in Rex purification, Paula Grabowski for technical advice regarding splicing and preparation of extracts, David Chang for critically reviewing the manuscript, and Elisa Weiss and Dotti Garces for administrative assistance. We are grateful to Douglas Black for helpful discussions, technical advice, and manuscript review.

A.B. is supported by UCLA AIDS Institute Amgen fellowship grant C920331 from the Center for AIDS Research. A.C.B. is supported by NIH grants R29CA 61545 and K11CA01566. J.D.R. is supported by NIH grants CA53632 and CA52410 and a Shannon Award.

REFERENCES

1. **Arrigo, S., and K. Beemon.** 1988. Regulation of Rous sarcoma virus RNA splicing and stability. Mol. Cell. Biol. **8:**4858–4867.
2. **Black, A. C., I. S. Y. Chen, S. Arrigo, C. T. Ruland, T. Allogiamento, E. Chin, and J. D. Rosenblatt.** 1991. Regulation of HTLV-II gene expression by Rex involves positive and negative cis-acting elements in the 5′ long terminal repeat. Virology **181:**433–444.
3. **Black, A. C., C. T. Ruland, J. Luo, A. Bakker, J. K. Fraser, and J. D. Rosenblatt.** 1994. Binding of nuclear proteins to HTLV-II cis-acting repressive sequence (CRS) RNA correlates with CRS function. Virology **200:**29–41.
4. **Black, A. C., C. T. Ruland, M. T. Yip, J. Luo, B. Tran, A. Kalsi, E. Quan, M. Aboud, I. S. Y. Chen, and J. D. Rosenblatt.** 1991. Human T-cell leukemia virus type II Rex binding and activity require an intact splice donor site and a specific RNA secondary structure. J. Virol. **65:**6645–6653.
5. **Cann, A. J., Y. Koyanagi, and I. S. Y. Chen.** 1988. High efficiency transfection of primary human lymphocytes and studies of gene expression. Oncogene **3:**123–128.
6. **Chang, D. D., and P. A. Sharp.** 1989. Regulation by HIV Rev depends upon recognition of splice sites. Cell **59:**789–795.
7. **Dignam, J. D., R. M. Lebovitz, and R. G. Roeder.** 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from mammalian nuclei. Nucleic Acids Res. **11:**1475–1489.
8. **Fisher, U., S. Meyer, M. Teufel, C. Heckel, R. Luhrman, and G. Rautman.** 1994. Evidence that HIV-1 Rev directly promotes the nuclear export of unspliced RNA. EMBO J. **13:**4105–4112.
9. **Gontarek, R. R., M. T. McNally, and K. Beemon.** 1993. Mutation of an RSV intronic element abolishes both U11/U12 snRNP binding and negative regulation of splicing. Genes Dev. **7:**1926–1936.
10. **Green, P. L., Y. Xie, and I. S. Y. Chen.** 1991. The Rex protein of human T-cell leukemia virus type II differ by serine phosphorylation. J. Virol. **65:** 546–550.
11. **Green, P. L., M. T. Yip, Y. Xie, and I. S. Y. Chen.** 1992. Phosphorylation regulates RNA binding by the human T-cell leukemia virus Rex protein. J. Virol. **66:**4325–4329.
12. **Hikada, M., J. Inoue, M. Yoshida, and M. Seiki.** 1988. Post-transcriptional regulator (rex) of HTLV-I initiates expression of viral structural proteins but suppresses expression of regulatory proteins. EMBO J. **7:**519–523.
13. **Inoue, J., M. Itoh, T. Akizawa, H. Toyoshima, and M. Yoshida.** 1991. HTLV-I rex protein accumulates unspliced RNA in the nucleus as well as in the cytoplasm. Oncogene **6:**1753–1757.
14. **Katz, R. A., and A. M. Skalka.** 1990. Control of retroviral RNA splicing through maintenance of suboptimal processing signals. Mol. Cell. Biol. **10:** 696–704.
15. **Kim, J. H., P. A. Kaufman, S. M. Hanly, L. T. Rimsky, and W. C. Greene.** 1991. Rex transregulation of human T-cell leukemia virus type II gene rexpression. J. Virol. **65:**405–414.
16. **Kjems, J., and P. A. Sharp.** 1993. The basic domain of Rev from the human immunodeficiency virus type 1 specifically blocks the entry of U4/U6 · U5 small nuclear ribonucleoprotein in spliceosome assembly. J. Virol. **67:**4769–4776.
17. **Konarska, M. M., and P. A. Sharp.** 1987. Interactions between small nuclear ribonucleoprotein particles in the formation of spliceosomes. Cell **49:**763–774.
18. **Lewis, N., J. Williams, D. Rekosh, and M.-L. Hammarskjold.** 1990. Identification of a cis-acting element in human immunodeficiency virus type 2 (HIV-2) that is responsive to the HIV-1 Rev and human T-cell leukemia virus types I and II Rex proteins. J. Virol. **64:**1690–1697.
19. **Malim, M. H., J. Hauber, R. Fenrick, and B. R. Cullen.** 1988. Immunodeficiency virus Rev trans-activator modulates the expression of the viral regulatory genes. Nature (London) **335:**181–183.
20. **Nagashima, K., M. Yoshida, and M. Seiki.** 1986. A single species of pX mRNA of human T-cell leukemia virus type I encodes *trans*-activator $p40^{x}$ and two other phosphoproteins. J. Virol. **60:**394–399.
21. **Nosaka, T., H. Siomi, Y. Adachi, M. Ishibashi, S. Kubota, M. Maki, and M. Hanataka.** 1989. Nucleolar targeting signal of human T-cell leukemia virus type I rex-encoded protein is essential for cytoplasmic accumulation of unspliced viral mRNA. Proc. Natl. Acad. Sci. USA **86:**9798–9802.
22. **Rimsky, L., J. Hauber, M. Dukovich, M. H. Malim, A. Langois, B. R. Cullen, and W. C. Greene.** 1988. Functional replacement of the HIV-1 rev protein by the HTLV-I rex protein. Nature (London) **335:**738–740.
23. **Rosenblatt, J. D., A. J. Cann, D. J. Slamon, I. S. Smalberg, N. P. Shah, J. Fuji, W. Wachsman, and I. S. Y. Chen.** 1988. HTLV-II transactivation is regulated by the overlapping tax/rex nonstructural genes. Science **240:**916–919.
24. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Press, Cold Spring Harbor, N.Y.
25. **Seed, B., and J. Y. Sheen.** 1988. A simple phase extraction assay for chloramphenicol acetyltransferase activity. Gene **67:**271–277.
26. **Sharp, P. A.** 1994. Split genes and RNA splicing. Cell **77:**805–815.
27. **Yip, M. T., W. S. Dynan, P. Green, A. C. Black, S. Arrigo, A. Torbati, S. Heaphy, C. T. Ruland, J. D. Rosenblatt, and I. S. Y. Chen.** 1991. 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. J. Virol. **65:**2261–2272.
28. **Zhang, L., and C. M. Stoltzfus.** 1995. A suboptimal *src* 3′ splice site is necessary for efficient replication of Rous sarcoma virus. Virology **206:**1099–1107.