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Identification of a *cis*-Regulatory Element Involved in Accumulation of Human T-Cell Leukemia Virus Type II Genomic mRNA

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The X gene products of the human T-cell leukemia viruses type I and II are thought to be involved not only in viral replication but also in mediating the expression of certain cellular genes. These X gene products are known to be translated from doubly spliced viral mRNA, while viral structural proteins, such as the *gag*, *pol*, and *env* gene products, are translated from unspliced or singly spliced viral mRNA. One of the X gene products of human T-cell leukemia virus type II, tax_2 protein, has been shown to be responsible for transcriptional stimulation from the viral long terminal repeat. The other X gene product(s) of human T-cell leukemia virus type II, the rex_2 protein(s), is located in the nuclear fraction of virus-infected cells, but its function is not known. This article reports evidence that rex_2 protein(s) enhances the accumulation of unspliced viral RNA by interacting posttranscriptionally, either directly or indirectly, with a *cis*-regulatory element downstream from the first splice donor site in the long terminal repeat.

The human T-cell leukemia viruses type I and II (HTLV-I and HTLV-II) are closely associated with specific T-cell malignancies in humans. The former is the etiological agent of adult T-cell leukemia/lymphoma (20, 27, 48), while the latter has been linked to at least three cases of hairy-cell leukemia (5, 22, 29, 44). HTLVs are known to immortalize normal human peripheral blood T lymphocytes in vitro when cocultured with virus-producing cells (6, 25).

HTLVs and bovine luekemia virus (BLV) have in common unique nonstructural genes, called pX's or X's (18, 24, 31, 32, 34, 37, 41), between the *env* gene and the 3′ long terminal repeat (3′-LTR) sequence, in addition to *gag*, *pol*, and *env* genes that are common to most retroviruses. Human immunodeficiency viruses type I and II (HIV-I and -II) also contain similar regulatory genes, namely *tat* (7, 8, 12, 26, 47) and *rev* (also referred to as *art* or *trs*) (10, 30, 43). The 38-kilodalton (kDa) protein, tax_2 protein (also referred to as pXc, *x-lor*, or *tat* protein), which is encoded from the first open reading frame (ORF) of the X region in HTLV-II, is known to activate transcription from the viral LTR (3, 4, 11, 42), depending on the presence of the 21-base-pair (bp) enhancer core sequence within the LTR (38). Another ORF encodes proteins of 24 and 24 kDa, rex_2 proteins (also referred to as pXb's of *tel* proteins), which are localized in the nuclear fraction of virus-infected cells (35). Recently, evidence was obtained that the *rev* gene product of HIV, a counterpart of the rex_2 protein(s) of HTLV-II, might be involved in either repression of splicing or stabilization of viral unspliced or singly spliced mRNA (10, 30, 43). Moreover, rex_1 protein of HTLV-I, the gene product which corresponds to rex_2 protein(s) of HTLV-II, was suggested to regulate the expression of both genomic and subgenomic mRNA by a posttranscriptional mechanism (19, 21).

In genomic mRNA transcribed from HTLV-II provirus, there are two splice donor and acceptor sites, which generate three major mRNA species of 9.0, 4.5, and 2.0 kilobases (kb) (46). The 9.0-kb mRNA represents a genomic mRNA, mainly encoding the *gag* and *pol* proteins; the 4.5-kb subgenomic mRNA, spliced from the first splice donor and acceptor sites, encodes the *env* protein; and the 2.0-kb subgenomic mRNA, spliced at both the first and second splice sites, encodes X proteins (45, 46). To clarify the functions of these *rex* proteins, we examined the function of rex_2 protein(s) of HTLV-II in the expression of viral mRNA. We report evidence that rex_2 protein(s) regulates the production of both unspliced and singly spliced viral mRNA and that this regulation is mediated by a *cis*-regulatory element (CRE) within the LTR.

MATERIALS AND METHODS

Nomenclature. pC-Xb, pC-Xc, and pC-Xbc are plasmids which express rex_2, tax_2, or both, respectively. Xb and Xc were originally named after we determined the nucleotide sequence of HTLV-II provirus DNA (40) and correspond to the ORFs for rex_2 and tax_2, respectively. Nomenclature for HTLV and HIV genes is that followed by Gallo et al. (14).

Plasmid carrying the 5′ half of HTLV-II provirus. Plasmid construction was done by the methods described by Maniatis et al. (23). The 5′ half (nucleotides [nt] 312 to 5094; numbered from the first nucleotide of the 5′ end of the provirus) of the HTLV-II provirus (39), which spans the cap site (nt 315) and the first splice donor site (nt 449) and acceptor site (nt 5043), was subcloned into the *Hin*dIII-*Bam*HI sites of pCMVCAT (13), together with a simian virus 40 (SV40) sequence containing the late polyadenylylation site (nt 2533 to 2770 of SV40 DNA) and the pBR322 sequence (nt 375 to 651) in the 5′ to 3′ direction. The resultant construct, pC4.8, carries the cytomegalovirus (CMV) promoter, which is placed just upstream of the cap site of HTLV-II DNA, the 5′ half of HTLV-II DNA, SV40 DNA, and pBR322 DNA, in that order (Fig. 1A). When introduced into COS-1 cells, pC4.8 produced 5.1-kb RNA in the unspliced form and 0.5-kb RNA in the singly spliced form with a structure similar to that transcribed from the 5′ half of the proviral genome, as ascertained by Northern (RNA) blot analysis (data not shown). The 5.1-kb RNA would represent the 5′ half of the viral genomic RNA which encodes *gag* and *pol* mRNAs, and the 0.5-kb RNA would represent a structure of the spliced form of the primary transcript of HTLV-II. This splicing from the first splice

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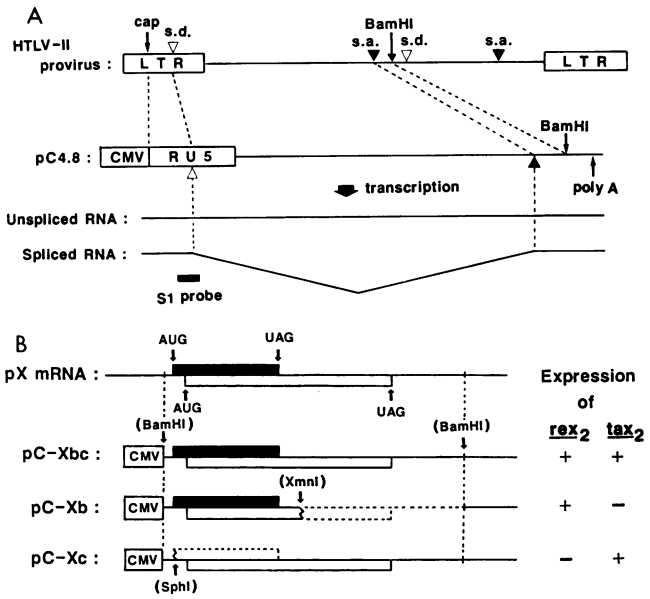

FIG. 1. (A) Schematic representation of the pC4.8 construct. Construction of pC4.8 is described in Materials and Methods. pC4.8 contains the CMV promoter, the 5′ half of HTLV-II DNA containing the cap site, and the first splice donor site and acceptor site. An SV40 sequence containing the late polyadenylation site is located downstream of the HTLV-II sequence. When introduced into COS-1 cells, pC4.8 produced both unspliced and spliced RNAs driven by the CMV promoter. Abbreviations: s.d., splice donor (▽): s.a., splice acceptor (▼): CMV, CMV promoter: poly A, SV40 polyadenylation site. A thick bar shows the RNA region (nt 361 to 474 of HTLV-II DNA) which is protected by a probe in S1 nuclease analysis to detect both spliced and unspliced RNAs as described in Materials and Methods. (B) Schematic representation of the pX mRNA and mRNAs from plasmids that express the pX gene or its mutants. Closed bar, rex_2 ORF; open bar, tax_2 ORF; open box, noncoding region of mRNA derived from CMV promoter. A detailed procedure for the construction of pC-Xbc, pC-Xb, and pC-Xc is described in Materials and Methods. The 3′ half of HTLV-II DNA (*Bam*HI fragment) was introduced into pCMVCAT in place of the *cat* gene. The resultant construct, pC-Xbc, carries both rex_2 and tax_2 ORFs. pC-Xb was constructed from pC-Xbc by eliminating the carboxyl-terminal half (*Xmn*I to *Bam*HI sites) of the coding sequence of tax_2 ORF. pC-Xc was constructed by eliminating the methionine initiation codon for rex_2 ORF without affecting the initiation codon of tax_2 ORF. These plasmids expressed rex_2 and/or tax_2 as described in Materials and Methods. pC-Xbc and pC-Xb are positive for rex_2 expression in COS-1 cells, but pC-Xc is negative. pC-Xbc and pC-Xc are positive for tax_2 expression in COS-1 cells, but pC-Xb is negative. Abbreviations are as in panel A.

donor and acceptor sites would be essential for producing *env* and pX mRNAs in the virus-infected cells. In COS-1 cells, expression of the CMV promoter-directed chloramphenicol acetyltransferase (*cat*) gene was not affected by either the presence or absence of tax_2 protein or rex_2 protein(s), introduced by transfection (data not shown).

Plasmids carrying the 3′ half of HTLV-II provirus. Three plasmid constructs were created to express either tax_2, rex_2, or both by placing the 3′ half of the HTLV-II DNA fragment containing the X ORFs under control of the CMV promoter (Fig. 1B). The pC-Xbc construct was created by introducing the *Bam*HI fragment (nt 5090 to 8554) of HTLV-II DNA (37) into the *Hin*dIII-*Bam*HI site of pCMVCAT (13) in place of the *cat* gene ORF. The resultant pC-Xbc construct has both intact tax_2 and rex_2 ORFs directed by the CMV promoter. pC-Xb was constructed from pC-Xbc by deleting the *Xmn*I-*Bam*HI fragment (nt 7788 to 8550), resulting in elimination of the carboxyl-terminal half of the coding sequence of the tax_2 ORF but not affecting the rex_2 ORF which is located upstream of the *Xmn*I site. pC-Xc was constructed by exonucleolytic digestion of the sequence around the *Sph*I site (nt 5124), which is located in the 5′ terminus of the rex_2 ORF, resulting in the elimination of the methionine initiation codon (nt 5121 to 5123) for rex_2 but not affecting the initiation codon (nt 5180 to 5182) of tax_2, which is located downstream of the *Sph*I site. Expression of rex_2 protein(s) was ascertained by Western blot (immunoblot) analysis with antibody against the carboxy-terminal peptide of rex_2 protein(s) (35), while expression of tax_2 protein was confirmed by assay of chloramphenicol acetyltransferase (CAT) (16) with the plasmid pILTR-CAT (38), which carries the HTLV-I LTR-directed *cat* gene. It is known that tax_2 protein enhances transcriptional activity from the HTLV-I LTR (38). HTLV-I LTR-specific *trans*-activation (more than 20-fold increase in relative CAT activities) was observed when either pC-Xbc or pC-Xc was cotransfected into COS-1 cells with pILTR-CAT, while pC-Xb did not (data not shown). By Western blot analysis, proteins of both 24 and 26 kDa were detected in COS-1 cells transfected with either pC-Xbc or pC-Xb, but not with pC-Xc (data not shown).

Quantitative S1 nuclease assay of RNA. COS-1 cells were transfected with plasmids by the calcium phosphate precipitation method as described previously (17). COS-1 cells (3×10^6) were seeded on 100-mm dishes. The next day, precipitated plasmid DNA (26 μg per dish) was added, and the cells were incubated at 37°C in 10 ml of Dulbecco modified Eagle medium supplemented with 5% fetal calf serum. After 5 h of incubation, the dishes were washed with 10 ml of Tris-buffered saline (pH 7.4) and incubated in the same growth medium for 43 h. Total cellular RNA was extracted, and S1 nuclease analysis was performed as described previously (1). The specific DNA probe of 272 bp consisted of the *Bam*HI-*Sac*I fragment (nt 361 to 474, including the splice donor site at nt 449) of HTLV-II DNA (36) and a flanking fragment from pUC19 (nt 245 to 402). This probe was ^{32}P-labeled at the *Bam*HI end, hybridized with 20-μg samples of RNA at 56°C for 6 h, incubated with S1 nuclease (200 U/ml) at 37°C for 30 min, and electorphoresed in a 6% polyacrylamide gel. With the protected probe, a band of 113 nt was obtained with unspliced RNA and one of 88 nt with the spliced RNA. As a control for quantitation of the amount of RNA expressed by the indicator plasmid, an additional plasmid, pSVL, was cotransfected as an internal standard, and its transcripts were assayed simultaneously. The transcripts from pSVL were detected as a band of 176 nt by hybridization with a 538-bp probe of the *Xho*I-*Sal*I fragment of pSVL (nt 1503 to 2040), which includes the polyadenylylation site (nt 1651 to 1656), ^{32}P-labeled at the *Xho*I end. The hybridized bands were traced with a densitometer (Shimadzu, CS-9000; transmission at 590 nm), and the area of each peak was measured. From experiments repeated several times, it was shown that the amount of transcripts from pSVL was not affected by cotransfection with either pBR322, pC-Xc, pC-Xb, or pC-Xbc in COS-1 cells (data not shown). The production of transcripts from pC4.8 was quantitated by calculating the ratio of the area of each peak to that of the peak of the internal standard in the same lane.

Assay of CAT activity. COS-1 cells were transfected with plasmids 48 h prior to cell harvest by the method described above, except that the amount of calcium phosphate-precipitated DNA was adjusted to 22 μg per dish. Cell extracts were prepared and CAT enzyme activities were assayed as

described previously (16). The indicated transfections were performed in parallel at least three times. The conversion rate was calculated by dividing the radioactivity of acetylated chloramphenicol by total radioactivity. Data within the linear range of the reaction are presented and are the averages for three independent experiments.

Introduction of a deletion in pC4.8. Various deletion mutants were created from pC4.8 without affecting the sequences near the splice donor site (nt 449) and splice acceptor site (nt 5043). Construct pC4.7 was created from pC4.8 by eliminating the sequence from the cap site (nt 314) to the *Bam*HI site (nt 360) of HTLV-II DNA (36) (see Fig. 3A). Construct pC1.7 was created from pC4.8 by eliminating the sequence between the *Bgl*II site (nt 1387) and *Xho*I site (nt 4436) (Fig. 3A). Construct pC4.8-S was created from pC4.8 by eliminating the sequence between the *Sma*I site at nt 521 and another *Sma*I site at nt 2935 of HTLV-II DNA (39) (Fig. 3A). Construct pC4.8-SB was created from pC4.8-S by eliminating the sequence around the *Sma*I site (nt 521 of HTLV-II DNA) by *Bal* 31 exonuclease digestion after digestion with *Sma*I, without loss of the *Sac*I site (nt 474) located 25 bp downstream of the first splice donor site (nt 449) (Fig. 3A).

Introduction of the R-U5 sequence into pCMVCAT. The *Bam*HI-*Eco*RI fragment (nt 360 to 790) of HTLV-II DNA (39) was inserted into the *Hin*dIII site of pCMVCAT (13) in the 5′ to 3′ direction. The resultant construct, pCMVRU 5CAT, carries a part of the R and all of the U5 sequence between the CMV promoter and coding region of the *cat* gene (see Fig. 4A).

Materials and cells. Enzymes and pBR322 were purchased from Takara Shuzo (Kyoto, Japan), pSVL was from Pharmacia (Uppsala, Sweden), radioactive nucleotides were from Amersham, and [^{14}C]chloramphenicol was from New England Nuclear Corp. pCMVCAT (13) was kindly supplied by H. Hofstetter at CIBA-GEIGY (Basel, Switzerland).

RESULTS

Regulation of production of viral RNAs by rex_2. When introduced into COS-1 cells, pC4.8 produced both spliced and unspliced viral RNAs driven by the CMV promoter. To determine whether rex_2 or tax_2 protein(s) regulates the production of these viral RNAs, pC4.8 was transfected into COS-1 cells either alone or in combination with pC-Xc, pC-Xb, or pC-Xbc, and the amounts of viral RNAs produced in the cotransfected cells were quantitated by S1 nuclease protection analysis.

Cotransfection of either pC-Xb (rex_2^+ tax_2^-) or pC-Xbc (rex_2^+ tax_2^+) increased the level of unspliced viral RNA and reduced that of spliced RNA compared with that of pC4.8 alone (Fig. 2A), while cotransfection of pC-Xc (rex_2^- tax_2^+) had no effect on level of the unspliced or spliced RNA. Quantitative analysis by densitometry indicated a more than fourfold increase in the level of unspliced RNA and a more than threefold reduction in that of spliced RNA in the presence of rex_2 expression compared with the levels without expression of rex_2. The dose-dependent effect of rex_2 protein(s) is shown in Fig. 2B, where the increase in unspliced viral RNA and decrease in spliced viral RNA depended on the amount of plasmid pC-Xb (rex_2^+ tax_2^-) which was cotransfected. These results indicate that rex_2 protein(s) enhances the amount of unspliced viral RNA and reduces that of spliced RNA in a dose-dependent manner irrespective of the presence of tax_2.

Presence of a CRE in the LTR. Since neither tax_2 nor rex_2 protein affects the transcriptional activity of the CMV promoter, we assumed that rex_2 protein(s) regulates the amounts of unspliced and spliced RNAs by a posttranscriptional mechanism. Accordingly, we thought that rex_2 protein(s) may interact, either directly or indirectly, with these RNA species and that these RNAs may contain a sequence that is recognized by rex_2 protein(s). To identify this rex_2-responsive CRE, we introduced various deletions into pC4.8 at various locations in the 5′ half of the HTLV-II sequence without affecting the sequences near the splice donor and acceptor sites. These plasmids were then transfected into COS-1 cells either alone or in combination with pC-Xb,

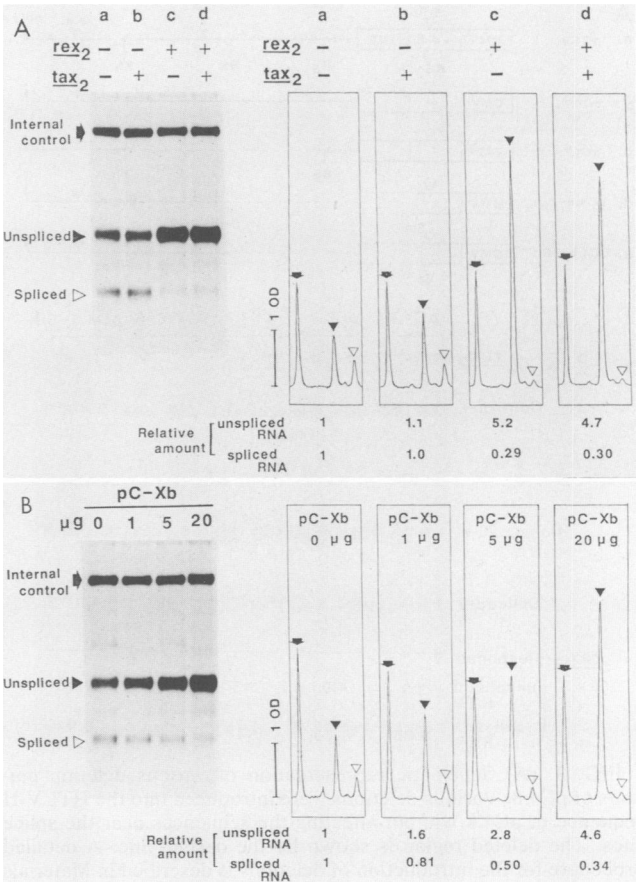

FIG. 2. (A) Analysis of RNA by the S1 nuclease protection method. COS-1 cells were cotransfected with 4 μg of pC-4.8, 20 μg of pBR322 (lane a), pC-Xc (lane b), pC-Xb (lane c), or pCXbc (lane d), and 2 μg of pSVL as an internal standard control. For RNA extracted from these transfected cells, S1 nuclease analysis was performed as described in Materials and Methods. A band of 113 nt (▶) was derived from the protected probe with unspliced RNA, one of 88 nt (▷) was from the spliced RNA, and one of 176 nt (→) was from the other protected probe with the transcripts from pSVL. Curves on the right are densitometric tracings aligned with the respective hybridized bands. The amount of transcripts from pC4.8 was standardized by dividing the area of each peak by that of the peak of the internal standard in the same lane. Values below the densitometric tracing represent the ratios of the standardized values of areas of peaks with those of the corresponding peak in lane a. (B) Dose-response effect of rex_2 protein(s) on production of spliced and unspliced viral RNAs. pC4.8 (4 μg) was cotransfected with pSVL (2 μg) and various amounts of pC-Xb, which expresses rex_2, as described in Materials and Methods. The total amount of transfected DNA was adjusted to 26 μg by adding various amounts of pBR322 as a carrier. S1 nuclease analysis was performed, and data are presented as described for panel A.

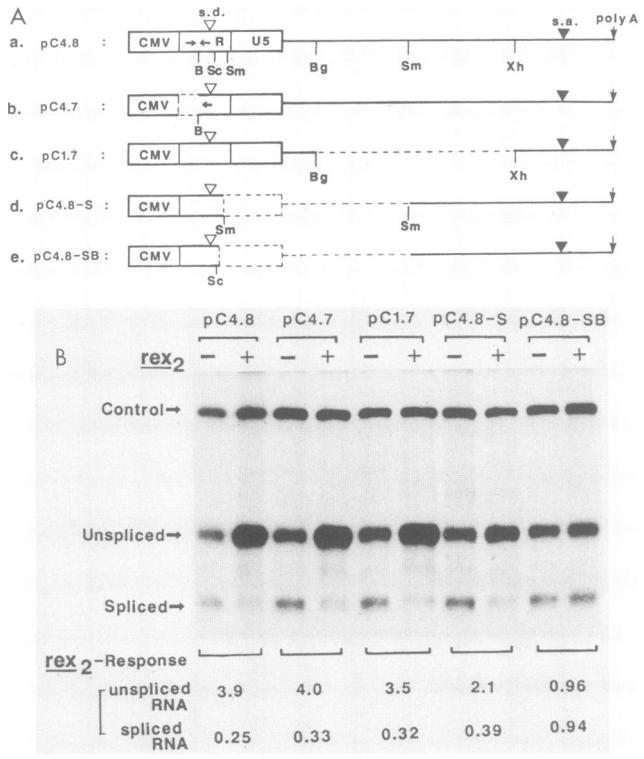

FIG. 3. (A) Schematic representation of various deletion mutants of pC4.8. Various deletions were introduced into the HTLV-II sequence in pC4.8 without affecting the sequences near the splice sites. The deleted region is shown by the dashed line. A detailed procedure for the introduction of deletions is described in Materials and Methods. pC4.7 lacks the HTLV-II sequence located 3′ to the *Bam*HI site which contains the upstream half of the 16-bp dyad element. pC1.7 lacks the sequence between the *Bgl*II site and the *Xho*I site. pC4.8-S lacks the sequence between two *Sma*I sites. pC4.8-SB lacks the sequence adjacent to the *Sma*I site of pC4.8-S but retains the *Sac*I site. Abbreviations: B, *Bam*HI site: Sc, *Sac*I site: Sm, *Sma*I site: Bg, *Bgl*II site: Xh, *Xho*I site. Other abbreviations are as in the legend to Fig. 1. Arrows in the R region represent 16-bp dyad elements. (B) S1 nuclease analysis of transcripts from deletion mutants. Mutant plasmids (4 μg) and pSVL (2 μg) were cotransfected into COS-1 cells with pC-Xb or pBR322 (20 μg) as a negative control. S1 nuclease analysis was performed as described in Materials and Methods. The values written below the densitometric tracings represent the extent of rex_2 response of unspliced and spliced RNAs. The area of each peak was measured by densitometry and standardized as described for Fig. 2B. The extent of rex_2 response was calculated by dividing the standardized value of the area of each peak in lane $rex_2(+)$ by that of the corresponding peak in lane $rex_2(-)$.

which expresses rex_2 protein(s), and the resulting changes of unspliced and spliced RNAs were analyzed quantitatively by the S1 nuclease protection assay. The extent of the response of both spliced and unspliced RNAs to rex_2 protein(s) was calculated as described in the legend to Fig. 3B. One of the mutants, pC4.7, lacks a sequence including the upstream half of the 16-bp dyad elements in the R region of the LTR (Fig. 3A). When introduced into COS-1 cells, pC4.7 produced both unspliced and spliced RNAs, whose responses to rex_2 protein(s) were the same as those of pC4.8 (Fig. 3B). This finding suggests that the possible hairpin structure in the R region of RNA (33) is not involved in recognition by rex_2. Another mutant, pC1.7, lacks the sequence between the *Bgl*II and *Xho*I sites and extends from the 3′ portion of the *gag* region to the 5′ portion of the *pol* region (Fig. 3A). When introduced into COS-1 cells, pC1.7 produced both spliced and unspliced RNAs, whose responses to rex_2 protein(s) were also the same as those of pC4.8 (Fig. 3B). These results suggest that the major part of the sequence (nt 1387 to 4436) located between the splice donor and acceptor sites is not involved in recognition by rex_2. pC4.8-S lacks the sequence between two *Sma*I sites, one located 71 bp downstream of the splice donor site (nt 449) and the other in the *pol* region (Fig. 3A). In COS-1 cells, pC4.8-S produced both spliced and unspliced RNAs and showed responses to rex_2 protein(s), but less than those of pC4.8 (Fig. 3B). These results suggest that the sequence around the *Sma*I site just downstream of the splice donor site might be responsible for rex_2 regulation. pC4.8-SB lacks the sequence adjacent to the *Sma*I site located 71 bp downstream of the splice donor site but retains the *Sac*I site located 25 bp downstream of the splice donor site (Fig. 3A). pC4.8-SB produced both spliced and unspliced RNAs in COS-1 cells, but expression of rex_2 had no effect on these RNAs. These results suggest that the CRE that is recognized by rex_2 protein(s) is present between the first splice donor and acceptor sites, specifically near the splice donor site in the R-U5 segment.

CRE responds to rex_2 in a heterologous gene. To test whether the CRE responded to rex_2 protein(s) and regulated gene expression in a foreign gene, the R-U5 sequence, which contains the CRE, was introduced into a noncoding region of pCMVCAT, and the effect of rex_2 protein(s) on expression of CAT activity was examined. pCMVRU5CAT was formed by the introduction of the R-U5 sequence of HTLV-II into pCMVCAT between the CMV promoter and the *cat* gene (Fig. 4A). In COS-1 cells, the coexpression of either rex_2 or tax_2 did not enhance expression of the *cat* gene from pCMVCAT, as described above. When pCMVRU5CAT was introduced into COS-1 cells by cotransfection with pC-Xbc (rex_2^+ tax_2^+) or pC-Xb (rex_2^+ tax_2^-), increased CAT activities were observed compared with those obtained with pCMVRU5CAT alone (Fig. 4A); however, expression was not enhanced by cotransfection of pC-Xc (rex_2^- tax_2^+) (Fig. 4A). The extent of increase in CAT activity was dependent on the amount of plasmid expressing rex_2 that was cotransfected (Fig. 4B). These data show that rex_2 protein(s) enhances expression of a heterologous gene when the R-U5 fragment is introduced between the cap site and the coding region of the heterologous gene.

DISCUSSION

The rex_2-dependent regulation of the production of viral RNAs seems to be due to a posttranscriptional mechanism for the following reasons. First, since CMV promoter activity is not affected by rex_2 protein(s), transcription from the CMV promoter, which directs the proviral sequence, is not likely to be regulated by rex_2 protein(s). Second, when the R-U5 segment was introduced into the nonpromoter region of a foreign gene, rex_2-dependent enhancement of the gene was observed. Since the transcriptional activity of the CMV promoter is not affected by rex_2 protein(s), the acquisition of a response to rex_2 protein(s) provides evidence against transcriptional control by rex_2 protein(s). Third, enhancement of the accumulation of unspliced RNA is dependent on the presence of the CRE, which is located outside the promoter region. Fourth, the dual effects of an increase and a decrease in the amounts of the two species of RNA transcribed from the same promoter are difficult to explain by regulation at the transcriptional level. It is possible that

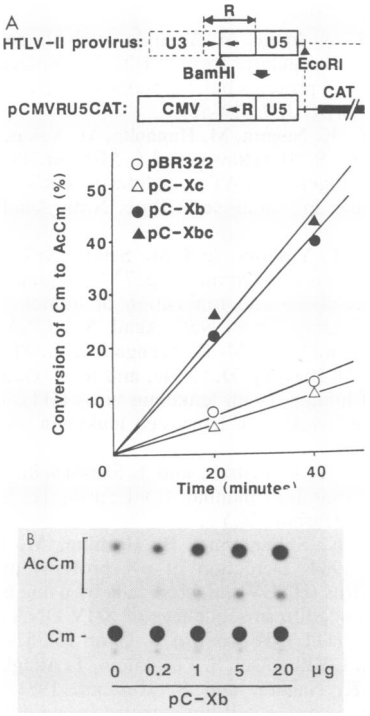

FIG. 4. (A) Enhancement of CAT activity expressed from pCMVRU5CAT. The upper part of the figure shows the construction of pCMVRU5CAT as described in Materials and Methods. pCMVRU5CAT was constructed by introducing the *Bam*HI-*Eco*RI fragment of HTLV-II DNA between the CMV promoter and *cat* coding region of pCMVCAT. Arrows in the R region represent 16-bp dyad elements. The *cat* ORF is shown by the thick line. CMV, CMV promoter. The lower part of the figure shows the results of the CAT assay. pCMVRU5CAT (2 μg) was cotransfected with 20 μg of pC-Xc (△), pC-Xb (●), pC-Xbc (▲), or pBR322 as a negative control (○) into COS-1 cells 48 h before cell harvest, as described in Materials and Methods. The *x* axis represents the reaction time, and the *y* axis represents the extent of conversion of [^{14}C]chloramphenicol (Cm) to its acetylated form (AcCm). (B) Dose-response effect of rex_2 protein(s) on enhancement of CAT activity expressed by pCMVRU5CAT. COS-1 cells were cotransfected with 2 μg of pCMVRU5CAT and various amounts (0 to 20 μg) or pC-Xb as described for panel A. The total amount of transfected DNA was adjusted to 22 μg per dish by adding an appropriate amount of pBR322 DNA as a carrier. Abbreviations are as for panel A.

the presence of the CRE of HTLV-II renders the transcriptional activity of the CMV promoter dependent on rex_2 protein(s), but this seems unlikely. We are currently examining the effect of rex_2 protein(s) on transcription in the presence of the CRE.

Since these results suggest that rex_2 protein(s) enhances the accumulation of unspliced RNA by a posttranscriptional mechanism, this accumulation may be caused either by inhibition of the splicing process or inhibition of the decay of unspliced RNA by rex_2 protein(s). The former possibility explains the dual effect of both an increase in the amount of unspliced RNA and a reduction in the amount of spliced RNA. However, it does not explain the observation that the increase in the amount of unspliced viral RNA is much greater than the decrease in that of spliced viral RNA. The accumulation of unspliced RNA by rex_2 protein(s) may be due partly to stabilization of unspliced viral RNA. This possibility is consistent with the observation that rex_2 protein(s) enhances *cat* gene expression from pCMVRU5CAT in COS-1 cells. However, a detailed study including analysis of the amount of the RNA in these cells will be necessary to confirm the function of rex_2 protein(s).

The presence of a rex_2-responsive element in unspliced RNA but not in spliced RNA suggests that the primary target of rex_2 protein(s) is unspliced RNA. An explanation for our results is that rex_2 protein(s) interacts with the primary transcripts before they are spliced, resulting in stabilization of unspliced RNA and interference with the splicing process. The location of the CRE near the splice donor site may explain why interaction of rex_2 protein(s) with the CRE inhibits splicing.

All three species of HTLV-II mRNA in virus-infected cells have the same U3 and R sequence in the 3′ noncoding region. Among these mRNA species of HTLV-II, only the genomic mRNA carries the CRE sequence of the 5′ LTR. Our results suggested that the CRE in the 5′ LTR is involved in the rex_2-dependent enhancement of accumulation of genomic mRNA. However, in BLV, a sequence within the 3′ LTR is shown to be required for regulation by p18, a protein corresponding to the rex_2 protein(s) of HTLV-II (9). We have not examined whether the U3-R region at the 3′ terminus of HTLV-II mRNA responds to rex_2 protein(s). There may be another rex_2-responsive element in the 3′ LTR of HTLV-II.

In the HTLV and BLV families, there are possible common stem-loop structures in both the 5′ and 3′ noncoding regions of mRNA (33). A stem-loop structure in the 3′ noncoding region of histone mRNA is known to be related to the stability of mRNA (2, 15). In HTLV-II, elimination of the possible stem-loop structure of the 5′ terminal region of the RNA did not alter the amount of unspliced viral RNA in either the presence or absence of rex_2, suggesting that this structure is not involved in rex_2 regulation. The effect of the other possible stem-loop structure in the 3′ LTR on accumulation of the mRNA remains to be analyzed. However, such a structure itself, if present, might not be involved in the selective accumulation of genomic mRNA because the sequence of the 3′ noncoding region is present in both the spliced and unspliced mRNA of virus-infected cells.

rev protein plays an important role in enhancing accumulation of higher-molecular-weight species of HIV mRNA, which code for the *gag-pol* and *env* gene products, and repressing the amounts of lower-molecular-weight species of mRNA, which code for nonstructural gene products (10, 30, 43). The rex_1 protein of HTLV-I has been suggested to play an important role in expression of the *gag* gene product in combination with tax_1 protein (21). Hidaka et al. suggested that the rex_1 protein regulates the expression of both genomic and subgenomic mRNA by a posttranscriptional mechanism (19). Our findings are consistent with their results, in that rex_2 protein(s) enhances the accumulation of unspliced viral mRNA, which could represent genomic viral mRNA. However, in HTLV-I, the rex_1 protein increases the levels of both genomic and singly spliced transcripts (19). In BLV, p18 is required for accumulation of viral mRNAs representing genomic and singly spliced transcripts (9). Contrary to these reports, our results suggest that the rex_2 protein(s) reduces the amount of singly spliced viral RNA transcribed from the 5′ half of HTLV-II DNA. Since our construct, pC4.8, does not carry the 3′ half of viral DNA, which encodes the *env* and pX products, it is possible that processing of *env* and pX mRNA in HTLV-II-infected cells is regulated by a more complex process through another *cis* element in the 3′ half of the viral transcripts which responds to rex_2 protein(s). This possibility is suggested by the pres-

ence of *rev*-responsive sequence in the *env* gene (28). Such interactions of the rex_2 protein(s) and the CRE(s) may regulate the production of genomic and subgenomic viral mRNAs of HTLV-II.

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