Secondary Structure of the Human T-Cell Leukemia Virus Type 1 *rex*-Responsive Element Is Essential for *rex* Regulation of RNA Processing and Transport of Unspliced RNAs

HIDEO TOYOSHIMA,^{1,2} MICHIIYASU ITOH,¹ JUN-ICHIRO INOUE,¹ MOTOHARU SEIKI,^{1†}
FUMIMARO TAKAKU,² AND MITSUAKI YOSHIDA^{1*}

Department of Viral Oncology, Cancer Institute, Kami-Ikebukuro, Toshima-ku, Tokyo 170,¹ and Third Department of Internal Medicine, Medical School, University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113,² Japan

Received 2 January 1990/Accepted 28 February 1990

Rex protein of human T-cell leukemia virus type 1 (HTLV-1) induces cytoplasmic expression of unspliced *gag/pol* mRNA and singly spliced *env* mRNA and thus is essential for replication of the virus. This regulation requires a *cis*-acting *rex*-responsive element (RXE), located in the 3' region of the viral RNA. By external deletion, we have identified RXE composed of 205 nucleotides. The secondary structure of RXE was confirmed by studies on its susceptibility to nuclease digestions to consist of four stem-loops and a long stretch of stem structure. Substitution and deletion mutations revealed that two regions of the stem-loops and their secondary structures are essential for *rex* regulation. Similar secondary structures were found in the corresponding regions of HTLV-2, bovine leukemia virus, and human immunodeficiency virus. Furthermore, a sequence of 11 nucleotides in the RXE was found to be conserved in the secondary structures of HTLV-1, HTLV-2, and bovine leukemia virus. These observations suggest that the secondary structure as well as the conserved sequence may be important in expression of unspliced RNA even with diverged sequences as observed in these viruses.

Replication of human T-cell leukemia virus type 1 (HTLV-1) (16, 31) is controlled by two *trans*-acting proteins, Tax protein and Rex protein. Tax protein is a transcriptional activator of the HTLV-1 genome from the long terminal repeat (LTR) (2, 6, 7, 26, 29), whereas Rex protein modulates RNA processing or transport and induces cytoplasmic expression of unspliced *gag/pol* and singly spliced *env* mRNAs; otherwise, all of these mRNAs should be doubly spliced (8, 9, 25).

Rex protein is a nuclear protein (21) predominantly localized in nucleoli (28), suggesting that *rex* regulation is a nuclear event. The mechanism of this regulation is proposed to be by activation of nuclear transport or nuclear stabilization of unspliced RNA (10, 25). Our previous study showed that *rex* regulation requires a specific *cis*-acting element on RNA molecules (*rex*-responsive element [RXE]), which is located in sequences of the U3 and R segments of the 3' LTR (25). This region was previously proposed to form a stable secondary structure with large stems and loops and to be important in 3' RNA processing. As a result of this presumed secondary structure, the poly(A) signal located about 270 bases upstream becomes located close to the poly(A) site (22). A similar role of secondary structures in 3' RNA processing was proposed for HTLV-2 (27) and bovine leukemia virus (BLV) (21). Interestingly, these two viruses show regulation of RNA expression similar to that of HTLV-1 *rex* (4, 15). In BLV, the region responsive to *rex* regulation was also suggested to correspond to this region, with a potential secondary structure at the 3' RNA terminus (4).

Gene expression of human immunodeficiency virus (HIV) is regulated by the *rev* gene in a manner analogous to the

regulation of HTLV-1 by *rex* (5, 12, 18, 20). Furthermore, the Rex protein of HTLV-1 can also regulate HIV RNA expression (17), and the mechanisms of regulation by the Rev and Rex proteins were suggested to be similar (10a). The *cis*-acting responsive element for *rev* regulation (*rev*-responsive element [RRE]) does not show significant sequence homology to the HTLV-1 RXE sequence; however, the RRE was also proposed to form a stable secondary structure (13). Because of these observations, we were interested in the relationship between the secondary structure of RNA and *rex* regulation.

This report describes mutagenesis studies on the sequence containing the RXE. We identified two regions required for *rex* regulation. We also showed that the secondary structure of the RXE is essential for *rex* regulation.

MATERIALS AND METHODS

Construction of plasmids. pGANT is a derivative of pGAGdm, the construction of which was described previously (10). The whole sequence of the 3' LTR was removed from pGAGdm, and instead a *PvuII-SmaI* fragment of 733 bases of the neomycin phosphotransferase gene (1) was inserted at the *Bg/II* site, just upstream of the transcriptional terminator of the thymidine kinase (TK) gene of herpes simplex virus type 1. All mutations of the defective LTR (dL) fragment were inserted just upstream of the TK terminator of pGANT.

External deletions of dL were obtained with BAL 31, exonuclease III, and restriction enzymes, and mutants with internal deletions or linker substitutions were constructed by ligating appropriate fragments or by using appropriate linkers. All mutations constructed were confirmed by sequence analysis. Computer calculation of secondary structures was carried according to the program of Zuker and Stiegler (33).

The plasmids used for expression of *tax* and *rex* were pCM-TAX and pCM-REX, respectively (Itoh et al., in

* Corresponding author.

† Present address: Department of Virology, Cancer Research Institute, Kanazawa University, Takara-machi, Kanazawa 920, Japan.

FIG. 1. Construction of a reporter plasmid used for analyses. Abbreviations: SD and SA, sites for splicing donor and acceptor, respectively; NEO, *PvuII-SmaI* fragment of the neomycin resistance gene; TK, transcriptional terminator of the thymidine kinase gene. The site for insertion of various mutations of the dL fragment is indicated. Probe indicates a fragment used for detection of the transcripts of these constructs.

press); CM refers to the cytomegalovirus immediate early promoter.

Transfection and analysis of mRNA in cytoplasm. Plasmids were transfected into a human amnion cell line, FL, by the calcium phosphate method as described previously (7). Briefly, 6×10^5 cells were transfected with mixtures of various expression plasmids adjusted to the total amount of 15 μ g with *Escherichia coli* DNA. After culture for 40 h, the cells were harvested, cytoplasmic RNA was prepared by the nucleoside-vanadyl complex method, and poly(A)-containing RNA was analyzed by blot hybridization (24). The transfection efficiency in each experiment was monitored by determining RNA from pCM-TAX by rehybridization of the same filters with a *tax*-specific probe.

Synthesis of RNA substrates and structural analysis. A dL fragment from bases 302 to 620 of the 3' LTR (25) was inserted into pSP65 (14), and RNA was prepared by in vitro transcription followed by agarose gel electrophoresis. The RNA product was labeled with 32 P at the 5' end with T4 polynucleotide kinase. Full-length RNA was purified by electrophoresis in 5% acrylamide gel in 8 M urea. Nuclease digestions were performed as previously described (30). The RNA was preincubated in each digestion buffer at 37°C for 5 min. Digestion with RNase T₁ or A was carried out in 50 mM Tris hydrochloride (pH 7.5)–10 mM MgCl₂ for 5 to 10 min at 37°C with various concentrations of the enzymes. Digestion with cobra venom nuclease was carried out similarly in 25 mM Tris hydrochloride (pH 7.5)–10 mM MgCl₂–200 mM NaCl. The enzyme reaction was terminated by adding an equal volume of sample buffer (80% formamide, 16 mM EDTA, 0.05% xylene cyanol, 0.05% bromophenol blue).

RESULTS

External deletion of a previously defined *rex*-responsive fragment. Rex protein of HTLV-1 induces cytoplasmic expression of intron-containing viral RNAs, unspliced *gag/pol* mRNA, and singly spliced *env* mRNA (8–10, 25). We have reported that the *rex*-responsive sequence in the 3' LTR is within a region from nucleotides 302 to 560 (positions from the 5' end of the LTR), which contains 259 bases covering the 3' region of the U3 segment and almost all of the R segment (25). Since this sequence of 259 bases is so long, the whole sequence is unlikely to interact with a protein(s) as a unit. Therefore, we performed further external deletions

on the dL fragment from bases 302 to 620, which was previously shown to contain the RXE (25). We used a defective construct of HTLV-1, pGANT (LTR-*gag-neo-TK*; Fig. 1), to test the effects of the mutations. pGANT was derived from a defective viral construct, pGAGdm (10), and contains the original splice donor for *env* mRNA, the splice acceptor for *tax/rex* mRNA, a part of the neomycin resistance gene (*neo*), and a TK transcriptional terminator of herpes simplex virus type 1. Various mutants of the dL fragment were inserted just upstream of the TK terminator so that any alteration of transcriptional termination was canceled by the following TK terminator. Each construct was transfected into FL cells, a human amnion cell line, in the presence or absence of a *rex* expression plasmid, pCM-REX, and the transiently expressed cytoplasmic RNAs were analyzed by Northern (RNA) blotting. In all experiments, a *tax* expression plasmid, pCM-TAX, was also cotransfected to maintain activated transcription.

The construct pGANT, without any sequence of the dL fragment, expressed no detectable unspliced RNA even in the presence of *rex* (Fig. 2a, lanes 1 and 2). On the other hand, construct pGANLTR302-620, carrying the whole sequence of the dL fragment, expressed unspliced RNA efficiently in response to *rex* (Fig. 2a, lanes 3 and 4), confirming that this construct contained all essential elements and so was useful for analysis of the RXE.

External deletion of the dL fragment from the 5' side up to position 318 (LTR319-620) did not affect *rex* responsiveness. (Fig. 2a, lanes 5 and 6). Further deletion up to position 336 (LTR337-620) drastically reduced the activity, although the mutant still responded to *rex* (Fig. 2a, lanes 7 and 8). However, deletion up to position 350 (LTR351-620) completely abolished the responsiveness to *rex* (Fig. 2a, lanes 9 and 10). These results clearly indicated that AATAAA at bases 307 to 312, which was shown to be functional as the poly(A) signal (unpublished data), was not required for *rex* regulation, since LTR319-620 showed almost as high activity as did the wild-type dL fragment. Therefore, *rex* regulation to induce unspliced RNA expression in the cytoplasm was not associated with 3' RNA processing of the RNA. In some experiments shown here, the total amount of RNAs expressed was reduced in the presence of *rex*. This reduction was not due to variable efficiency of transfection because the level of RNA from cotransfected pCM-TAX was constant

FIG. 2. Effect of external deletion of the dL fragment on *rex* regulation. FL cells were transfected with each mutant with (+) or without (-) a *rex* expression plasmid. U and S indicate unspliced and spliced RNA, respectively. (a) External deletion from the 5' end. The mutants were pGANT carrying no RXE (lanes 1 and 2), pGANLTR302-620 (lanes 3 and 4), pGANLTR319-620 (lanes 5 and 6), pGANLTR337-620 (lanes 7 and 8), and pGANLTR351-620 (lanes 9 and 10). (b) External deletion from the 3' end. The mutants were pGANLTR319-560 (lanes 1 and 2), pGANLTR319-541 (lanes 3 and 4), and pGANLTR319-521 (lanes 5 and 6). (c) External deletion from both ends. The mutants were pGANLTR319-620 (lanes 1 and 2) and pGANLTR337-541 (lanes 3 and 4). (d) Diagram of sites of truncation of the dL. The relative activities of the mutants are also shown.

(data not shown). To avoid possible misjudgment due to these different levels of RNA, we determined the ratio of unspliced RNA to spliced RNA. The results confirmed the conclusion stated above.

For external deletion from the 3' side, we used a poly(A) signal-deficient mutant, LTR319-620, to avoid any influence of transcriptional termination. Deletion up to base 561 from the 3' side (LTR319-560) did not affect *rex*-responsive activity (Fig. 2b, lanes 1 and 2). Further deletion up to base 542 (LTR319-541) reduced but did not abolish this activity (Fig. 2b, lanes 3 and 4). However, deletion up to base 522 (LTR319-521) resulted in complete loss of *rex* responsiveness (Fig. 2b, lanes 5 and 6).

These results of deletion analyses from both sides strongly suggested that the essential RXE sequence is located within bases 337 to 541 in the LTR. To confirm this, we constructed a mutant containing the sequence from 337 to 541 (LTR337-541). Mutant pGANLTR337-541 was almost as active as the original construct (Fig. 2c, lanes 1 to 4). This result clearly indicated that all of the essential sequence of RXE is within bases 337 to 541 of the LTR.

Secondary structure of the RXE. The defined RXE corresponds to the region that can form a potential secondary structure proposed in our previous report (22). The requirement of such a long sequence of 205 bases as a target might be explained by the secondary structure of this sequence.

To study the secondary structure of the RXE, we synthe-

sized an RNA containing the whole dL fragment between bases 302 and 620 in an SP6 transcription system and labeled the 5' end with ^{32}P . The RNA was renatured in the presence of Mg^{2+} ion and was subjected to limited digestion with various nucleases as described by Tamm and Polisky (30): RNases T_1 and A, which preferentially cleave single-stranded regions, and cobra venom nuclease, which is specific for double-stranded regions. The digests were analyzed by polyacrylamide gel electrophoresis under denaturing conditions, and the sites cleaved were identified by comparing the bands with sequencing ladders. The sites that are sensitive to the nucleases are indicated on the secondary structure in Fig. 3, which was predicted with a computer program of Zuker and Stiegler (33). Most of the results on digestions with nucleases were consistent with this secondary structure, indicating that the RNA sequence of this region maintained the predicted secondary structure or a closely related one in solution. This secondary structure is very stable, with a free energy of -110.4 kcal (ca. -461.9 kJ) and is tighter than that predicted in the previous paper (22). For convenience in further analyses, we named the parts of the structure as shown in Fig. 3.

Internal mutagenesis of a *rex*-responsive fragment. The sequence containing the RXE determined by external deletion analyses corresponded to the upper part of the defined secondary structure, namely, stem I and stem-loops A, B, C, and D, suggesting that the secondary structure of this

FIG. 3. Secondary structure of the *rex*-responsive region. Sites sensitive to partial nuclease digestions are indicated on the RNA sequence. Arrows, dashed lines, and arrowheads denote sites of preferred cleavage by RNase A, RNase T₁, and cobra venom nuclease, respectively. The sequence is arranged in the secondary structure predicted with the computer program of Zuker and Stiegler (33). Nucleotide positions (n.p.) are numbered from the 5' end of the LTR. U3 and R indicate the U3 and R regions in the LTR, respectively; their border is indicated by a dotted line.

sequence may be important. To examine this possibility, we analyzed the RXE further by internal mutageneses of LTR19-620 based on the defined secondary structure.

(i) **Stem I.** Mutant LTR351-620, which could not form stem I, did not respond to *rex* (Fig. 2). This observation suggested that the sequence itself or the formation of stem I is required for *rex* regulation. To examine these two possibilities, we

introduced eight-base substitution mutations into the sequence of either or both strands of stem I: we replaced the sequence in stem I at bases 344 to 351 (TTCAGGAG) and/or at its complementary site, bases 447 to 454 (CTCCTGGAA) by an *EcoRI* linker (sub-344eco and/or sub-447eco). The two mutants with substitutions in either one of these two strands were both completely inactive in expression of unspliced

FIG. 4. Effect of mutagenesis in stem I on *rex* regulation. (a) The sequences of either or both strands of stem I of pGANLTR319-620 (lanes 1 and 2) were replaced by an *EcoRI* linker, constructing pGANsub-344eco (lanes 3 and 4), pGANsub-447eco (lanes 5 and 6), and pGANsub-344,477eco (lanes 7 and 8). Other conditions were as for Fig. 2. (b) Diagram of substitution mutations in the secondary structure.

FIG. 5. Effects of deletions of stem-loops A, B, and C on *rex* regulation. (a) The deletion mutants were pGANLTR319-620 (lanes 1 and 2), pGANdel-ABC (lanes 3 and 4), pGANdel-A (lanes 5 and 6), pGANdel-AB (lanes 7 and 8), and pGANdel-C (lanes 9 and 10). Other conditions were as for Fig. 2. (b) Diagram of positions of deletions of stem-loops A, B, and C.

RNA in response to *rex* regulation (Fig. 4, lanes 3 and 4 and lanes 5 and 6). However, the mutant with substitutions in both strands (sub-344,447eco), which should form a stem structure as in the original construct, showed almost the same activity in response to *rex* as the original construct (Fig. 4, lanes 1 and 2 and lanes 7 and 8). These results clearly demonstrate that the formation of stem I, but not the sequence of this region, is essential for *rex* regulation.

(ii) **Stem-loops A, B, and C.** Deletion of a sequence from bases 353 to 441 including the three stem-loops A, B, and C (del-ABC; Fig. 5b) resulted in complete inactivation (Fig. 5a, lanes 1 to 4), indicating the essential requirement of these three stem-loops. Computer analysis of the mutant showed that the secondary structure of the other parts remained unaltered, supporting this conclusion (data not shown). To determine which stem-loop is required, we next deleted one or two of these three stem-loops. A mutant with deletion of stem-loop A from bases 353 to 378 (del-A) showed reduced activity (Fig. 5a, lanes 5 and 6). Deletion of stem-loops A and B (del-AB) from bases 354 to 401 reduced the activity further (Fig. 5a, lanes 7 and 8), although the mutant still showed some response to *rex*. A mutant with a deletion of bases 401 to 422 including stem-loop C (del-C) also showed reduced activity (Fig. 5a, lanes 9 and 10). These results revealed that deletion of any one of stem-loops A, B, and C significantly reduced the activity but that any one stem-loop was dispensable for *rex* regulation. Apparently, the existence of all three stem-loops is necessary for full activity of RXE.

(iii) **Stem-loop D.** The observation that 3' external deletion of the sequence in stem-loop D (LTR319-521) completely inactivated the function of the RXE suggested the importance of this stem-loop. To test this, we first deleted almost all the sequence of stem-loop D from bases 460 to 544 (del-D). This mutant showed no response to *rex* (Fig. 6a, lanes 1 and 2 and lanes 7 and 8). To determine the essential sequence in stem-loop D, we introduced deletion mutations into the stem-loop as shown in Fig. 6c; deletion of 31 bases from 488 to 518, corresponding to the external portion of

stem-loop D (del-D.EX), resulted in complete loss of the *rex* response (Fig. 6a, lanes 5 and 6). Complementarily, deletions of the internal portion of stem-loop D (del-D.IN) at bases 460 to 484 and 522 to 541 resulted in maintenance of almost the same *rex* responsiveness as in the wild-type construct (Fig. 6b, lanes 1 to 4). Deletion of four bases from 502 to 505 in the external loop of stem-loop D (del-D.loop) did not affect the *rex* response significantly (Fig. 6a, lanes 3 and 4). These results indicated that the sequence of 37 bases at 485 to 521 in the external portion of stem-loop D excluding the external loop is essential for *rex* regulation. Mutant LTR319-521, with an external deletion from the 3' side, was completely inactive (Fig. 2b, lanes 5 and 6), although it had all of these 37 bases. These apparently contradictory observations suggest that the secondary structure of this region is essential for *rex* regulation.

From the results obtained in mutational analyses, we identified two essential regions, (i) a region of the three stem-loops A, B, and C and stem I and (ii) an external part of stem-loop D, and concluded that their secondary structures are required to form an active RXE.

DISCUSSION

HTLV-1 replication requires expression of unspliced mRNA in the cytoplasm, which is not usual in cellular mRNAs. This HTLV-1-specific regulation is accomplished by a viral Rex protein and a virus-specific RNA sequence, the RXE (8-10, 25). In this work, we showed that the RXE is composed of two separated regions in a sequence of bases 337 to 541 in the 3' LTR and also that the secondary structures of these two regions are required for activity. One of these regions included stem-loops A, B, and C and stem I, and the other was the external part of stem-loop D in the secondary structure shown in Fig. 3.

In the region consisting of stem-loops A, B, and C and stem I, destruction of stem I inactivated the RXE. Computer calculation of the secondary structures of the inactive mu-

FIG. 6. Mutageneses of stem-loop D and their effects on *rex* regulation. (a) Deletion of the external portion of stem-loop D (see panel c). The mutants were pGANLTR319-620 (lanes 1 and 2) and pGANDel-D (lanes 7 and 8). (b) Deletion of the internal portion of stem-loop D (see panel c). The mutants were pGANLTR319-620 (lanes 1 and 2) and pGANDel-D.IN (lanes 3 and 4). (c) Diagram of deletions of stem-loop D. Other deletions are as shown in Fig. 2.

tants indicated that the secondary structures of stem-loops A, B, and C were also modified by alteration of stem I. Moreover, deletion of all three stem-loops resulted in complete inactivation, indicating the importance of these stem-loops. These observations suggest that stem I might be required for maintaining the correct conformation of these three stem-loops and that the essential elements are within them. Any one of the three stem-loops was dispensable for activity, although there is no significant sequence homology among them. Therefore, it is conceivable that the requirement for a primary sequence is not strict or is for a very short sequence or that the requirement is not for any particular sequence. The requirement for the secondary structure of this region, which is supported by stem I, explains why the Rex protein requires the 3' end but not the 5' end of the genome: the 5' end of the viral RNA does not contain the U3 sequence, which is required to form stem I, and thus it cannot form the active conformation of RXE.

An element in stem-loop D is also an essential part of the secondary structure. The 37 nucleotides in the external part of stem-loop D are sufficient to maintain the activity in combination with the region of stem-loops A, B, and C. However, mutant LTR319-521, in which one strand of stem-loop D was truncated at the middle, was inactive, although it contained all 37 essential nucleotides. Therefore, the secondary structure of this part seems to be important. However, deletion of four nucleotides in loop D did not affect the activity significantly. Thus, the essential part in this region is located in 33 nucleotides in the external part of the stem structure. In this essential region, we identified a sequence of 11 to 14 nucleotides that is conserved in HTLV-1, HTLV-2, and BLV. The possible significance of this sequence is discussed later in this section.

rex function is thought to activate nuclear transport or enhance stabilization of intron-containing RNA (10, 25). Thus, these secondary structures of the RXE must be involved in these regulations through binding to a protein factor(s). There seem to be three possible mechanisms of

protein interaction: different proteins, possibly including Rex protein, may recognize the two regions independently, different parts of a single protein may interact with these two regions, or these two regions may form a single recognition site due to their tertiary structures, and this may interact with a protein. These possibilities can be distinguished by identification of the proteins that bind to the RXE. Recently, direct binding of the *rev* protein to the RRE has been reported (3, 32). Similarly, the *rex* protein of HTLV-1 might also directly bind to the RXE sequence that conforms the correct secondary structure.

The secondary structure of the RNA identified in this study was more compact than that proposed previously for the same region to explain 3' RNA processing (22). In both structures, a large stem in the secondary structure brings the poly(A) signal within consensus distance of the poly(A) site. We have evidence that the poly(A) signal is in fact functioning in polyadenylation at the right place (unpublished data). Therefore, the secondary structure of this region appears to have at least two functions in regulation of viral gene expression: 3' RNA processing and *rex* regulation. Since the fragment of the RXE defined previously contained the poly(A) signal, *rex* regulation was suspected to cooperate with 3' RNA processing at the same site, as proposed for BLV (4). However, the active RXE defined in this report did not contain both the poly(A) signal and the poly(A) site, thus excluding this possibility. The overlapping functional units, both of which require the secondary structure, could explain why HTLV-1 has such a long R sequence (229 bases) (23) compared with those (up to 80 bases) of most retroviruses of avian, mouse, or primate origin.

Regulation of RNA processing or transport similar to that of HTLV-1 *rex* was reported for HTLV-2 (15, 19), BLV (4), and HIV (5, 12, 18, 20). Possible secondary structures were also proposed for the corresponding regions of these viral RNAs: the 3' ends of HTLV-2 (27) and BLV (21) RNAs and the RRE in the *env* coding sequence of HIV (13, 18). Interestingly, these regions could be arranged into a second-

FIG. 7. Predicted secondary structures of 3' termini of BLV and HTLV-2 RNAs and the RRE of HIV. Sequences shown: (a) HTLV-2, bases 269 to 562; (b) BLV, bases 177 to 440 (positions from the 5' end of the LTR); (c) HIV, bases 7357 to 7568 from the 5' end of the viral genome. U3, R, the poly(A) signal, and the poly(A) site are indicated. Shaded areas denote the regions with sequence homology to the external region of stem-loop D of HTLV-1. (d) Comparison of the sequences of the external parts of stem-loop D of HTLV-1, HTLV-2, and BLV. Homologous sequences (●), different paired or unpaired bases in the same position (○), and sites of substitution mutations (↑) are marked.

any structure similar to that of the HTLV-1 RXE defined in this report: a long stretch of stem corresponding to stems I and II and three or four stem-loop structures corresponding to stem-loops A, B, C, and D (Fig. 7a to c). These observations suggest that these secondary structures may play similar roles to the secondary structures of the HTLV-1 RXE in regulation by *rex* or *rev*.

In these secondary structures, we identified a conserved sequence in the HTLV-related viruses HTLV-1, HTLV-2, and BLV, in the external region of stem-loop D (Fig. 7d), which was identified as an essential part of the RXE (Fig. 7a, b, and d). Substitution of two unpaired nucleotides in this sequence (Fig. 7d) reduced the activity drastically (data not shown), suggesting the importance of this sequence. This conserved sequence is of particular interest because it might be a responsive recognition site for a specific protein. This speculation is consistent with the observations that HTLV-1 *rex* can control RNA expression of HTLV-2 and also BLV (5a).

The HIV RRE can also be folded into a secondary structure similar to that of the HTLV-1 RXE (Fig. 7c), although this structure is somewhat different from that proposed previously (13). Moreover, HTLV-1 *rex* was reported to be able to control HIV RNA expression through the same region (17; Itoh et al., in press). However, no significant sequence homology like that observed among viruses of the HTLV subgroup was detected in the region of the RRE. Therefore, the secondary structure predicted for the RRE may play a role in cross-reactive regulation with

HTLV-1 *rex*. The involvement of such a secondary structure might be an advantage for conserving specificity and cross-reactivity even with diverged sequences.

ACKNOWLEDGMENT

This work was supported in part by a grant-in-aid for Special Project Research on Cancer Bio-Science from the Ministry of Education, Sciences and Culture of Japan.

LITERATURE CITED

1. Beck, E., G. Ludwig, E. A. Auerswald, B. Reiss, and H. Schaller. 1982. Nucleotide sequence and exact localization of the neomycin phosphotransferase gene from transposon Tn 5. *Gene* **19**: 327-336.
2. Chen, I. S. Y., D. J. Slamon, J. B. Rosenblatt, N. P. Shah, S. G. Quan, and W. Wachsman. 1985. The *x* gene is essential for HTLV replication. *Science* **228**:54-58.
3. Daly, T. J., K. S. Cook, G. S. Gray, T. E. Maione, and J. R. Rusche. 1989. Specific binding of HIV-1 recombinant *rev* protein to the *rev*-responsive element *in vitro*. *Nature (London)* **342**:816-819.
4. Derse, D. 1988. *trans*-Acting regulation of bovine leukemia virus mRNA processing. *J. Virol.* **62**:1115-1119.
5. Feinberg, M. B., R. F. Jarrett, A. Aldovini, R. C. Gallo, and F. Wong-Staal. 1986. HTLV-III expression and production involve complex regulation at the levels of splicing and translation of viral RNA. *Cell* **46**:807-817.
- 5a. Felber, B. K., D. Derse, A. Athanassopoulos, M. Campbell, and G. N. Pavlakis. 1989. Cross-activation of the *rex* proteins of HTLV-1 and BLV and of the *rev* protein of HIV-1 and nonreciprocal interactions with their RNA responsive elements. *New*

- Biol. 3:318-328.
6. Felber, B. K., H. Paskalis, C. Kleinman-Ewing, F. Wong-Staal, and G. N. Pavlakakis. 1985. The pX protein of HTLV-1 is a transcriptional activator of its long terminal repeats. *Science* 229:675-679.
 7. Fujisawa, J., M. Seiki, T. Kiyokawa, and M. Yoshida. 1985. Functional activation of long terminal repeat of human T-cell leukemia virus type I by trans-acting factor. *Proc. Natl. Acad. Sci. USA* 82:2277-2281.
 8. Hidaka, M., J. Inoue, M. Yoshida, and M. Seiki. 1988. Post-transcriptional regulator (rex) of HTLV-1 initiates expression of viral structural proteins but suppresses expression of regulatory proteins. *EMBO J.* 7:519-523.
 9. Inoue, J., M. Seiki, and M. Yoshida. 1986. The second pX product p27X-III of HTLV-1 is required for gag gene expression. *FEBS Lett.* 209:187-190.
 10. Inoue, J., M. Yoshida, and M. Seiki. 1987. Transcriptional (p40X) and post-transcriptional (p27X-III) regulators are required for the expression and replication of human T-cell leukemia virus type I genes. *Proc. Natl. Acad. Sci. USA* 84:3653-3657.
 - 10a. Itoh, M., J. Inoue, H. Toyoshima, T. Akizawa, M. Higashi, and M. Yoshida. 1989. HTLV-1 rex and HIV-1 rev act through similar mechanisms to relieve suppression of unspliced RNA expression. *Oncogene* 4:1275-1279.
 11. Kiyokawa, T., M. Seiki, S. Iwashita, K. Imagawa, K. Shimizu, and M. Yoshida. 1985. p27X-III and p21X-III proteins encoded by the pX sequence of human T-cell leukemia virus type I. *Proc. Natl. Acad. Sci. USA* 82:8359-8363.
 12. 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.
 13. Malim, M. H., J. Hauber, S.-Y. Le, J. V. Maizel, and B. R. Cullen. 1989. The HIV-1 rev trans-activator acts through a structured target sequence to activate nuclear export of unspliced viral mRNA. *Nature (London)* 338:254-257.
 14. Melton, D., P. Krieg, M. Rebagliati, T. Maniatis, K. Zinn, and M. Green. 1984. Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage Sp6 promoter. *Nucleic Acids Res.* 12:7035-7056.
 15. Ohta, M., H. Nyunoya, H. Tanaka, T. Okamoto, T. Akagi, and K. Shimotohno. 1988. Identification of a *cis*-regulatory element involved in accumulation of human T-cell leukemia virus type II genomic mRNA. *J. Virol.* 62:4445-4451.
 16. Poiesz, B. J., F. W. Ruscetti, A. F. Gazdar, O. A. Bunn, J. D. Minna, and R. C. Gallo. 1980. Detection and isolation of type C retrovirus particles from fresh and cultured lymphocytes of a patient with cutaneous T-cell lymphoma. *Proc. Natl. Acad. Sci. USA* 77:7415-7419.
 17. Rimsky, L., J. Hauber, M. Dukovich, M. H. Malim, A. Langlois, B. R. Cullen, and W. C. Greene. 1988. Functional replacement of the HIV-1 rev protein by the HTLV-1 rex protein. *Nature (London)* 335:738-740.
 18. Rosen, C. A., E. F. Tervilliger, A. I. Dayton, J. G. Sodroski, and W. A. Haseltine. 1988. Intragenic *cis*-acting art gene-responsive sequences of the human immunodeficiency virus. *Proc. Natl. Acad. Sci. USA* 85:2071-2075.
 19. Rosenblatt, J. D., A. J. Cann, D. J. Slamon, I. S. Smalberg, N. P. Shah, J. Fujii, W. Wachsmann, and I. S. Y. Chen. 1988. HTLV-II transactivation is regulated by the overlapping tax/rex constructional genes. *Science* 240:916-919.
 20. Sadaie, R. M., T. Benter, and F. Wong-Staal. 1988. Site-directed mutagenesis of two trans-regulatory genes (tat-III, trs) of HIV-1. *Science* 239:910-914.
 21. Sagata, N., T. Yasunaga, Y. Ogawa, J. Tsuzuku-Kawamura, and Y. Ikawa. 1984. Bovine leukemia virus: unique structural features of its long terminal repeats and its evolutionary relationship to human T-cell leukemia virus. *Proc. Natl. Acad. Sci. USA* 81:4741-4745.
 22. Seiki, M., S. Hattori, Y. Hirayama, and M. Yoshida. 1983. Human adult T-cell leukemia virus: complete nucleotide sequence of the provirus genome integrated in leukemia cell DNA. *Proc. Natl. Acad. Sci. USA* 80:3618-3622.
 23. Seiki, M., S. Hattori, and M. Yoshida. 1982. Human adult T-cell leukemia virus: molecular cloning of the provirus DNA and the unique terminal structure. *Proc. Natl. Acad. Sci. USA* 79:6899-6902.
 24. Seiki, M., A. Hikikoshi, T. Taniguchi, and M. Yoshida. 1985. Expression of the pX gene of HTLV-I: general splicing mechanism in the HTLV family. *Science* 228:1531-1534.
 25. Seiki, M., J. Inoue, M. Hidaka, and M. Yoshida. 1988. Two *cis*-acting elements responsible for post-transcriptional trans-regulation of gene expression of human T-cell leukemia virus type I. *Proc. Natl. Acad. Sci. USA* 85:7124-7128.
 26. Seiki, M., J. Inoue, T. Takeda, and M. Yoshida. 1986. Direct evidence that p40^x of human T-cell leukemia virus type I is a trans-acting transcriptional activator. *EMBO J.* 5:561-565.
 27. Shimotohno, K., D. W. Golde, M. Miwa, T. Sugimura, and I. S. Y. Chen. 1984. Nucleotide sequence analysis of the long terminal repeat of human T-cell leukemia virus type II. *Proc. Natl. Acad. Sci. USA* 81:1079-1083.
 28. Siomi, H., H. Shida, S. H. Nam, T. Nosaka, M. Maki, and M. Hatanaka. 1988. Sequence requirements for nucleolar localization of human T cell leukemia virus type 1 pX protein, which regulates viral RNA processing. *Cell* 55:197-209.
 29. Sodroski, J. G., C. A. Rosen, and W. A. Haseltine. 1984. Trans-acting transcriptional activation of the long terminal repeat of human T lymphotropic viruses in infected cells. *Science* 225:381-385.
 30. Tamm, J., and B. Polisky. 1983. Structural analysis of RNA molecules involved in plasmid copy number control. *Nucleic Acids Res.* 18:6381-6397.
 31. Yoshida, M., I. Miyoshi, and Y. Hinuma. 1982. Isolation and characterization of retrovirus from cell lines of human adult T-cell leukemia and its implication in the disease. *Proc. Natl. Acad. Sci. USA* 79:2031-2035.
 32. Zapp, M. L., and M. R. Green. 1989. Sequence-specific RNA binding by the HIV-1 rev protein. *Nature* 342:714-716.
 33. Zuker, M., and P. Stiegler. 1981. Optimal computer folding of large RNA sequences using thermodynamic and auxiliary information. *Nucleic Acids Res.* 9:133-148.