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cis-Acting Inhibitory Elements within the *pol-env* Region of Human T-Cell Leukemia Virus Type 1 Possibly Involved in Viral Persistence

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Human T-cell leukemia virus type 1 (HTLV-1) remains latent throughout the life of the carrier, with cells containing the provirus and viral gene expression efficiently down-regulated. On a molecular level, exactly how viruses are down-regulated in vivo remains unresolved. We described here the possibility that down-regulation results from the presence of inhibitory elements within the *gag-env* region of the provirus in fresh peripheral blood mononuclear cells from carriers. In vitro experiments then revealed that potent *cis*-acting inhibitory elements (CIEs) are indeed contained in two discrete fragments from the *pol* region and weaker ones in the *env* region. The effect of CIEs is relieved by the HTLV-1 posttranscriptional regulator Rex through binding to the Rex-responsive element (RxRE), suggesting that Rex might interfere with pre-mRNA degradation and/or activate the export of mRNA molecules harboring both of the inhibitory elements and RxRE on the same RNA molecule. Thus, we propose the hypothesis that such functions of CIEs may be involved in HTLV-1 persistence.

Human T-cell leukemia virus type 1 (HTLV-1) is an exogenous retrovirus that causes adult T-cell leukemia (ATL) as well as HTLV-1-associated myelopathy (HAM)/tropical spastic paraparesis (15, 21, 49, 52, 71). After infection in humans, the virus requires a long latent period until the onset of disease (29, 48, 65, 73). To date, little is known about the mechanism(s) by which the virus effects this process.

Expression of the viral genes is regulated at the transcriptional level by host nuclear factors, such as Sp1, TIF-1, Ets, Myb, and CREB, through the U3 region of the viral 5′ long terminal repeat (LTR) (3, 4, 11, 17, 37, 41, 64). Sequences downstream of the RNA initiation site, including the R and U5 regions, are also important for HTLV-1 expression at the transcriptional and posttranscriptional levels (26, 27, 42, 58). The HTLV-1 genome contains the pX region, which encodes Tax, Rex, and p21X proteins (31, 40, 58). Tax activates transcription of the virus through the LTR and several cellular genes (14, 18, 22, 34, 38, 39, 62, 63, 69). Rex recognizes the Rex-responsive element (RxRE) of the viral transcripts and enhances unspliced and singly spliced forms of viral transcripts, resulting in an increase in the production of Gag/Pol and Env proteins, respectively (20). Because Tax and Rex are produced from a doubly spliced viral transcript (designated *tax*/*rex* mRNA), the accumulation of Rex induces a decrease in the level of *tax*/*rex* mRNA, resulting in the down-regulation of viral gene expression. Thus, in viral latency, gene expression regulated at the posttranscriptional level by Rex has been suggested (20, 59). The function of the p21X protein is still unknown, but we recently reported the discovery of a singly spliced mRNA, responsible for producing the p21X protein, termed p21X mRNA, which is distinct from *tax*/*rex* mRNA (45).

Rapid induction of the *tax*/*rex* mRNA was observed in the cultured peripheral blood mononuclear cells (PBMCs) of patients with ATL or of asymptomatic HTLV-1 carriers, indicating that the viral gene expression was efficiently down-regulated in vivo (13, 30). On the contrary, we recently reported that the p21X mRNA is constitutively expressed in fresh PBMCs, suggesting that the expression mechanism of the p21X mRNA is quite different from that of *tax*/*rex* mRNA as well as genomic/*gag-pol* mRNA and *env* mRNA (47).

Studies of the expression mechanism of p21X mRNA in various HTLV-1-infected cell lines revealed that defective HTLV-1 genomes lacking the *gag-pol-env* region acquire the ability to express p21X mRNA but not *tax*/*rex* mRNA, which is expressed from the complete viral genomes (2, 43). In the present study, we examined whether similar defective viral genomes having a large deletion exist in specimens of fresh PBMCs from patients with HAM and ATL and from healthy carriers, in which the expression of p21X mRNA is observed. Our result strongly indicated that such a large deletion can explain the difference in the in vivo expression mechanisms between the p21X mRNA and *tax*/*rex* mRNA. Therefore, this evidence raised the possibility that gene expression from the complete viral genome but not from the defective genome is down-regulated in vivo.

Based on this assumption, we hypothesized that the HTLV-1 *gag-pol-env* region involves an element(s) which exerts its inhibitory effect on in vivo HTLV-1 gene expression. Finally, we newly identify and characterize the *cis*-acting inhibitory elements (CIEs) within the *pol* region. Thus, we propose the hypothesis that CIEs may play an important role in persistent HTLV-1 infection.

MATERIALS AND METHODS

Cells. Human T-cell lines MT-2 (HTLV-1 positive), TL-Su (positive), Molt-4 (negative), and Jurkat (negative) were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (45). The human cervical carcinoma cell line HeLa (ATCC CCL-2) was cultured in Eagle's minimum essential medium supplemented with 10% FBS. Heparinized peripheral blood samples were obtained from 10 patients with ATL and 15 patients with

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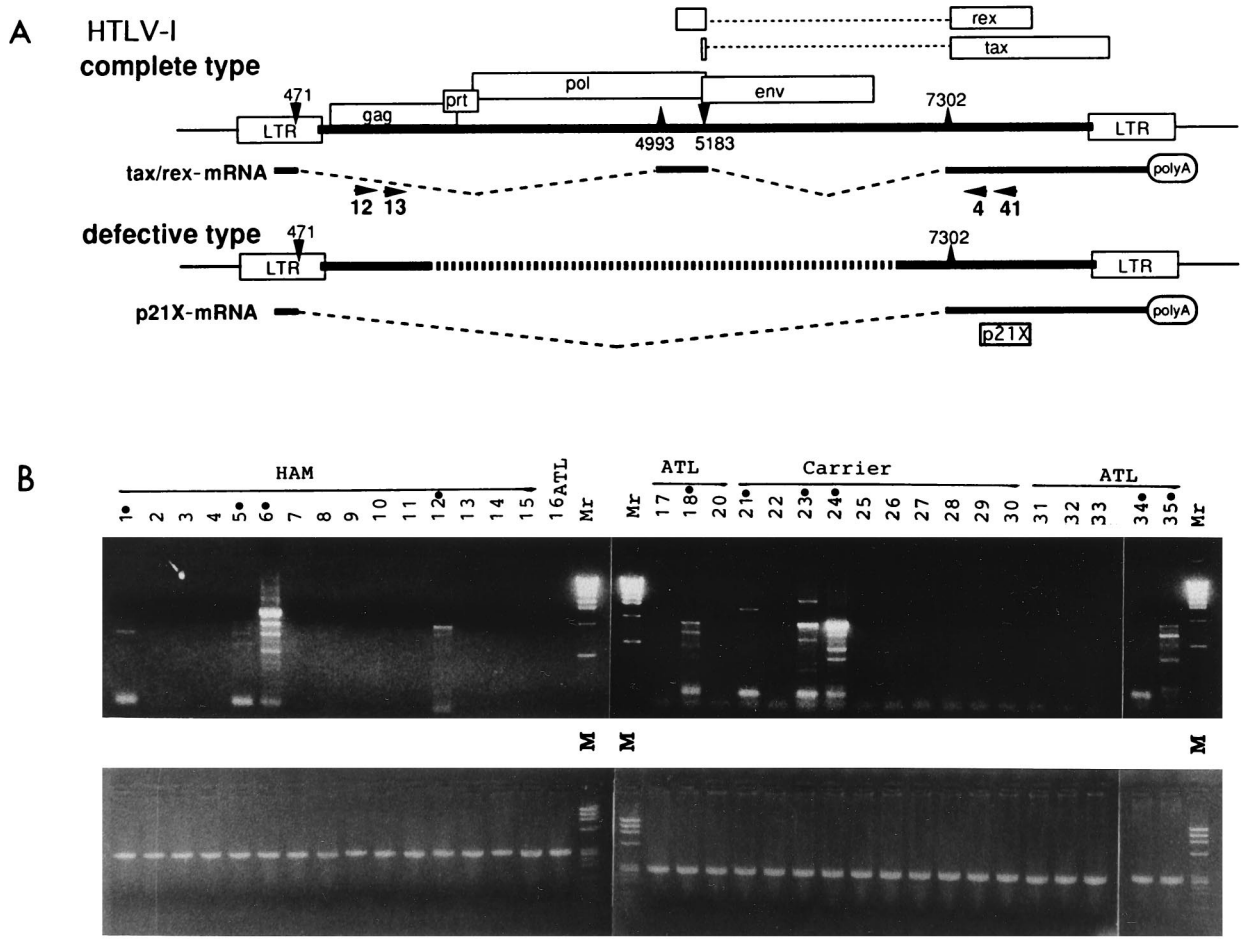

FIG. 1. Detection of defective proviruses in PBMCs from HTLV-1-infected individuals. (A) Schematic diagrams for complete and defective HTLV-1 proviral genomes which express *tax*/*rex* mRNA and p21X mRNA, respectively. The locations of seven viral genes, *gag*, *prt*, *pol*, *env*, *tax*, *rex*, and p21X are indicated by open boxes. The vertical arrowheads with numbers indicate sites of the splicing donor (downward) or acceptor (upward). PCR primers PX12 (nt 1184 to 1197), PX13 (nt 1219 to 1239), PX4 (nt 7337 to 7356), and PX41 (nt 7357 to 7376) are indicated by the horizontal arrowheads numbered 12, 13, 4, and 41, respectively. The broken line in the defective provirus genome indicates the deleted region as described previously (43). DNA sequence information was from the HTLV-1 prototype ATK sequence (57). (B) The genomic DNAs from 34 PBMC specimens (see Table 1) were analyzed by using TS-PCR with primers PX12 and PX41 for the first step and primers PX13 and PX4 for the second step as described in Materials and Methods. Specimen numbers are indicated above the lanes. Specimens positive for amplified bands are marked with dots. Molecular weight standards, λ-*Eco*T14I-digested DNA (Takara Shuzoh), are in lane Mr. In the lower panel, the same DNAs were analyzed by using PCR (30 cycles) with primers (5′-AGACAGCCGCATCTTCTTGTGC-3′ and 5′-CTCCTGGAAGATGGTGATGG-3′) for detection of the *GAPDH* gene as a control. Molecular weight standards, ϕX184 RF-*Hae*III-digested DNA (GIBCO BRL), are in the lane M.

HAM and from 10 HTLV-1 healthy carriers. All sera from the 35 clinical specimens used were seropositive for anti-HTLV-1 antibodies assayed by the particle agglutination method (Serodia-HTLV-I kit; Fujirebio Inc.). The proportions of flower cells (typical aberrant ATL cells) for the patients with ATL were as follows: patient 16, 22%; patient 17, 12%; patient 18, 8%; patient 19, 8%; patient 20, 44%; patient 31, 27%; patient 32, 24%; patient 33, 10%; patient 34, 95%; and patient 35, 83%. PBMCs were isolated by using Ficoll-Paque (Pharmacia) and stored at −80°C until use.

Synthetic oligonucleotides. Oligonucleotide primers for PCR or PCR coupled with reverse transcription (RT-PCR) were synthesized with a Cyclone DNA synthesizer (Biosearch Inc.) by the phosphoramidite method and purified with oligonucleotide purification cartridges (Applied Biosystems Inc.). DNA sequence information was used, and nucleotide numbers referred to positions in the HTLV-1 prototype ATK sequence (57).

Quantitative detection of HTLV-1 pX DNAs and qualitative detection of defective proviral genomes in PBMCs by TS-PCR. Genomic DNAs were isolated from the cells by the sodium dodecyl sulfate-phenol method (55), and copy numbers of the HTLV-1 genome DNA per 10^5 PBMCs were quantitatively estimated by two-step PCR (TS-PCR) as previously described (1). Briefly, to detect the HTLV-1 pX region, the genomic DNAs were titrated in 10-fold dilutions for TS-PCR with the following primers: for the first-step PCR, X1P (5′-CCCACTTCCCAGGGTTTGGACAGAGTCTTC-3′, nucleotides [nt] 7324 to 7353) and X4P (5′-GGGGAAGGAGGGGAGTCGAGGGATAAGGAA-3′, nt 7527 to 7556; for the second-step PCR, X2P (5′-CGGATACCCAGTCTAC GTGTTTGGAGACTGT-3′, nt 7358 to 7388) and X3P (5′-GAGCCGATAAC GCGTCCATCGATGGGGTCC-3′, nt 7487 to 7516). The reciprocal of the endpoint dilution that gave a positive reaction was considered the titer.

To analyze the defective proviral genomes in HTLV-1-infected cell lines, we successfully applied PCR as described previously (43). Here, the TS-PCR procedure was applied to improve the sensitivity for detection of the defective proviral genomes. Briefly, the genomic DNAs were analyzed by using TS-PCR with primers PX12 (5′-ACGGCCCCCCAAGTCCTTCCAGTC-3′, nt 1174 to 1197) and PX41 (5′-CGCCTTGTACACAGTCTCCA-3′, nt 7357 to 7376) for the first step and primers PX13 (5′-CCTAACCATCGCCCATGGCAA-3′, nt 1219 to 1239) and PX4 (5′-AACACGTAGACTGGGTATCC-3′, nt 7337 to 7356) for the second step as shown in Fig. 1. Under these conditions, the size of the expected product from the template DNA of the complete proviral genome was too long to be amplified, whereas the large deletion between the locations of primers PX13 and PX4 in the defective proviral genomes makes the template small enough to amplify.

Quantitative detection of viral mRNAs, *tax*/*rex* mRNA, and p21X mRNA in PBMCs by RT/TS-PCR. Total RNAs from cells were isolated by the guanidinium thiocyanate-phenol-chloroform method (9). The amounts of *tax*/*rex* mRNA and p21X mRNA were quantitatively estimated by RT/TS-PCR with primers as described previously (47). Briefly, total RNA was titrated in 10-fold dilutions for RT/TS-PCR, and the reciprocal of the endpoint dilution that gave a positive reaction was considered the titer. The series of RT/TS-PCR products, 144 bp, derived from *tax*/*rex* mRNA with primers PX1 (5′-ACGCCGGTTGAGTCGCG

TTCT-3′, nt 405 to 425) and PX2 (5′-TTAGAGGTTCTCTGGGTGGGGA-3′, nt 7530 to 7551) for the first-step amplification and with primers PX3 (5′-ATC CCGTGGAGACTCCTCAA-3′, nt 5095 to 5114) and PX4 for the second-step amplification have been described elsewhere (47). The other series of RT/TS-PCR products, 272 and 81 bp, derived from *tax*/*rex* mRNA and p21X mRNA, respectively, with primers PX1 and PX2 for the first-step amplification and with primers PX10 (5′-CCTCCTGAACTGCGTCCGCC-3′, nt 446 to 465) and PX4 for the second-step amplification have also been described elsewhere (47). RNA from HTLV-1-infected cell lines had been analyzed to evaluate the sensitivity and specificity of RT/TS-PCR as described in a previous report (47). The sensitivity of RT/TS-PCR is about 10^3 to 10^4 times that of the conventional RT-PCR procedure, and the specificity is high enough even if hybridization with radioisotope-labeled probes is not used. *tax*/*rex* mRNA or p21X mRNA in RNA from HTLV-1-infected MT-2 cells (positive control) could be detected in as much as a 10^7- or 10^8-fold dilution, respectively. Here, we applied this highly sensitive and quantitative RT/TS-PCR procedure to detect both *tax*/*rex* mRNA and p21X mRNA in the PBMCs of patients with ATL and HAM and of healthy carriers. For RT-PCR detection of the glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) gene transcript, primers (5′-AGACAGCCGCATCTTCTTGTGC-3′ and 5′-CTCCTGGAAGATGGTGATGG-3′) were used, and by 35 cycles of RT-PCR, the 294-bp band was amplified in all RNA specimens used.

Construction of plasmids. The *gag-pol-env*-pX region of the HTLV-1 proviral genome was separated into segments R1 (nt 1351 to 3182), R2 (nt 3165 to 4984), R3 (nt 4951 to 6635), R4 (nt 2268 to 4078), R5 (nt 4061 to 5782), R6 (nt 1351 to 2268), R7 (nt 2268 to 3182), R9 (nt 4061 to 4984), and R21 (nt 7302 to 8201) as shown in Fig. 2A. Each segment was amplified by PCR with each primer pair, all of which adjoin a tag sequence (indicated by underlining) containing a *Not*I restriction site at the 5′ end: R1, 1351FN (5′-CCAGCGGCCGCGACCTCCA AGACCTCCTG-3′) and 3182RN (5′-ACTGCGGCCGCCCAGGGGTTTGCT GGGTT-3′); R2, 3165FN (5′-TTTGCGGCCGCAACCCAGCAAACCCCTG G-3′) and 4984RN (5′-AAAGCGGCCGCAATACCAATGGGTTTGTT-3′); R3, 4951FN (5′-AAAGCGGCCGCCGTTCCCTCAGCAATAAA-3′) and 6635RN (5′-AAAGCGGCCGCACTCAGGTTTTATAAGAG-3′); R4, 2268FN (5′-AAAGCGGCCGCCCGATAGCCTTGTTCTCA-3′) and 4078RN (5′-TTA GCGGCCGCATATGTTGAGACAGCGCC-3′); R5, 4061FN (5′-TTAGCGGC CGCGGCGCTGTCTCAACATAT-3′) and 5782RN (5′-AATGCGGCCGCCT CGAGGATGTGGTCTAG-3′); R6, 1351FN and 2268RN (5′-AAAGCGGCC GCGAAGGACTGTCATGTCTG-3′); R7, 2268FN and 3182RN; R9, 4061FN and 4984RN; and R21, 7302FN (5′-ATAGCGGCCGCCCCACTTCCCAGGG TTTG-3′) and 8201RN (5′-TATGCGGCCGCAGGAAGAGTACTGTATGA-3′). Genome DNA from the HTLV-1-infected cell line TL-Su was used as a template. Each amplified fragment was inserted at a *Not*I site of the pRC/CMV-CAT vector (Invitrogen Inc.), which is under the control of the cytomegalovirus (CMV) promoter, in the proper orientation, and the clones obtained were called CMV-CAT-R1, -R2, -R3, -R4, -R5, -R6, -R7, -R9, and -R21. The parental plasmid pRC/CMV-CAT was used as a positive control, called CMV-CAT. From plasmid CMV-CAT-R2 cut with *Xba*I, the short fragment containing the 3′ half of the R2 segment was removed, and the large fragment was purified and religated to obtain a deletion plasmid, called CMV-CAT-R8, in which the R8 segment corresponds to the sequence from nt 3165 to 4080. From the CMV-CAT-R8 plasmid, CMV-CAT-d1R8 and -d2R8, containing the 5′-truncated R8 fragments *d*1R8 (nt 3368 to 4080) and *d*2R8 (nt 3444 to 4080), respectively, were created with a Kilo Sequence deletion kit (Takara Shuzoh).

To mutate two ATTTA pentamers within the R8 segment to AGGTA, CMV-CAT-R8M was generated from CMV-CAT-R8 by PCR-mediated mutagenesis as described previously (54). Using the proviral DNA from TL-Su cells, the RxRE fragment (nt 319 to 620) (67) was amplified by PCR with a forward primer (5′-tcctctagaGGAGTCTATAAAAGCGTG-3′ or 5′-cttgggcccGGAGTCTATA AAAGCGTG-3′) and a reverse primer (5′-ttctctagaGATCTGTAACGGCGCA GA-3′ or 5′-tttgggcccGATCTGTAACGGCGCAGA-3′), which adjoin a tag sequence containing an *Xba*I (the underlined tctaga sequence) or *Apa*I (the underlined gggccc sequence) restriction site at the 5′ terminus. Each RxRE fragment was inserted in an *Apa*I or *Xba*I site of CMV-CAT-R8 or -R7 and -R21, respectively, in the sense orientation, and the following plasmids were obtained: CMV-CAT-R8-RxRE(+), CMV-CAT-R7-RxRE(+), and CMV-CAT-R21-RxRE(+). Plasmids CMV-CAT-R7-RxRE(−), -R8-RxRE(−), and -R21-RxRE(−) were obtained by inserting the RxRE at the same location as in the RxRE(+) plasmids but in the antisense orientation.

The luciferase-coding gene (Luc) fragment from pGV-C (Toyo Inki) was inserted into the *Hin*dIII site of pRC/CMV, creating a Luc reporter plasmid, called CMV-Luc. The Rex expression plasmid, called SRα-rex, was described previously (44). Each construct was confirmed by DNA sequencing.

CAT assays. For each transfection experiment, the total amounts of plasmid DNA in each solution was adjusted to be equal by addition of a control plasmid, pBluescript (Stratagene), and the total volume of each solution was adjusted to be equal by addition of Tris-EDTA buffer. HeLa or Jurkat cells were transfected with a mixture of the chloramphenicol acetyltransferase (CAT) reporter plasmids indicated and 1 μg of the internal control Luc reporter plasmid CMV-Luc, using Lipofectin reagent (GIBCO BRL) in serum-free culture medium for 16 h and further cultured for 48 h in medium containing 10% FBS. Thereafter, whole-cell lysates in Reporter Lysis buffer (Promega Inc.) were prepared and subjected to luciferase and CAT assays as described previously (42). Transfection efficiency was normalized by luciferase activity.

Northern blot analysis. Forty hours posttransfection, the HeLa cells were harvested and total RNA was extracted with Isogen reagent (Nippon Gene Co. Ltd.). After DNase I (Pharmacia) treatment, 15 μg of RNA was subjected to Northern blot analysis as described previously (66). As probes, the CAT-encoding gene fragment (*Hin*dIII/*Hin*dIII fragment from pRC/CMV-CAT) or the *neo* gene fragment (760-bp PCR amplified DNA from the *neo* gene of pRC/CMV-CAT) was labeled by using a Multiprime kit (Amersham) with [α-^{32}P]dCTP (Amersham). CAT and *neo* mRNAs are independently expressed from pRC/CMV-CAT in the transfectants.

Runoff transcription. In vitro transcription-grade HeLaScribe nuclear extract (Promega Biotec) supplemented with 40 U of RNase inhibitor (Stratagene) was incubated with [α-^{32}P]GTP (Amersham) and a template plasmid DNA as specified by the manufacturer (Promega Biotec). The ^{32}P-labeled runoff products were electrophoresed on a 4% polyacrylamide–8 M urea gel and then exposed to X-ray film.

RESULTS

Analysis of in vivo HTLV-1 gene expression in PBMCs from patients with HAM and ATL and from healthy HTLV-1 carriers. Total RNAs from the fresh PBMCs of 35 HTLV-1-infected individuals were purified and used for detection of the viral transcripts by RT/TS-PCR. As summarized in Table 1, p21X mRNA was expressed (groups C and D) in the PBMCs from 4 of 13 HAM patients examined, 4 of 10 ATL patients examined, and 3 of 10 carriers examined. *tax*/*rex* mRNA was expressed (groups B and D) in the PBMCs from four HAM patients, three ATL patients, and six carriers. The data showed no significant correlation between expression of the two mRNAs, indicating that p21X mRNA expression is independent of *tax*/*rex* mRNA expression and vice versa. Expression of the mRNAs was found at similar frequencies in the three groups with HTLV-1 infection.

Using the genome DNAs isolated from the specimens described above, copy numbers of the viral genome DNA per 10^5 PBMCs were measured by detecting the pX region by the TS-PCR method, and the data are shown in Table 1. In all of the specimens, the HTLV-1 pX region was detected as expected. HTLV-1 genome copy numbers seemed to be highest in the ATL patient group, followed by the HAM patient group and then the carrier group. These results, consistent with the previous results (32, 72), suggested that viral mRNA expression is not correlated with the numbers of HTLV-1 genomic copies in the PBMCs.

Detection of a defective proviral genome with a large deletion between the *gag* and *env* genes in PBMCs which express p21X mRNA in vivo. We have clarified the expression mechanism of p21X mRNA by analyzing the organization of the proviral genomes present in representative HTLV-1-infected cell lines which are positive or negative for the expression of p21X mRNA (43). Specifically, the defective HTLV-1 genomes having a large deletion between the *gag* and pX genes including the second-exon region acquire the ability to express p21X mRNA but not *tax*/*rex* mRNA. To examine whether this mechanism is applicable in vivo, we analyzed the organization of the proviral genomes present in the specimens described above. The genome DNAs were analyzed by TS-PCR with primers PX12 and PX41 for the first step and with primers PX13 and PX4 for the second step (Fig. 1). After TS-PCR, a 6,138-bp band was expected to be amplified from the template DNA of the complete proviral genome, but it was not observed in any specimen (Fig. 1). This is because, under these conditions, the expected product is too long to be amplified. Instead, several smaller bands were clearly amplified in 10 specimens (samples 1, 5, 6, 12, 18, 21, 23, 24, 34, and 35) but not in the others (Fig. 1B and Table 1). The bands amplified from these 10 specimens are between 300 bp and 2 kbp in size, whereas

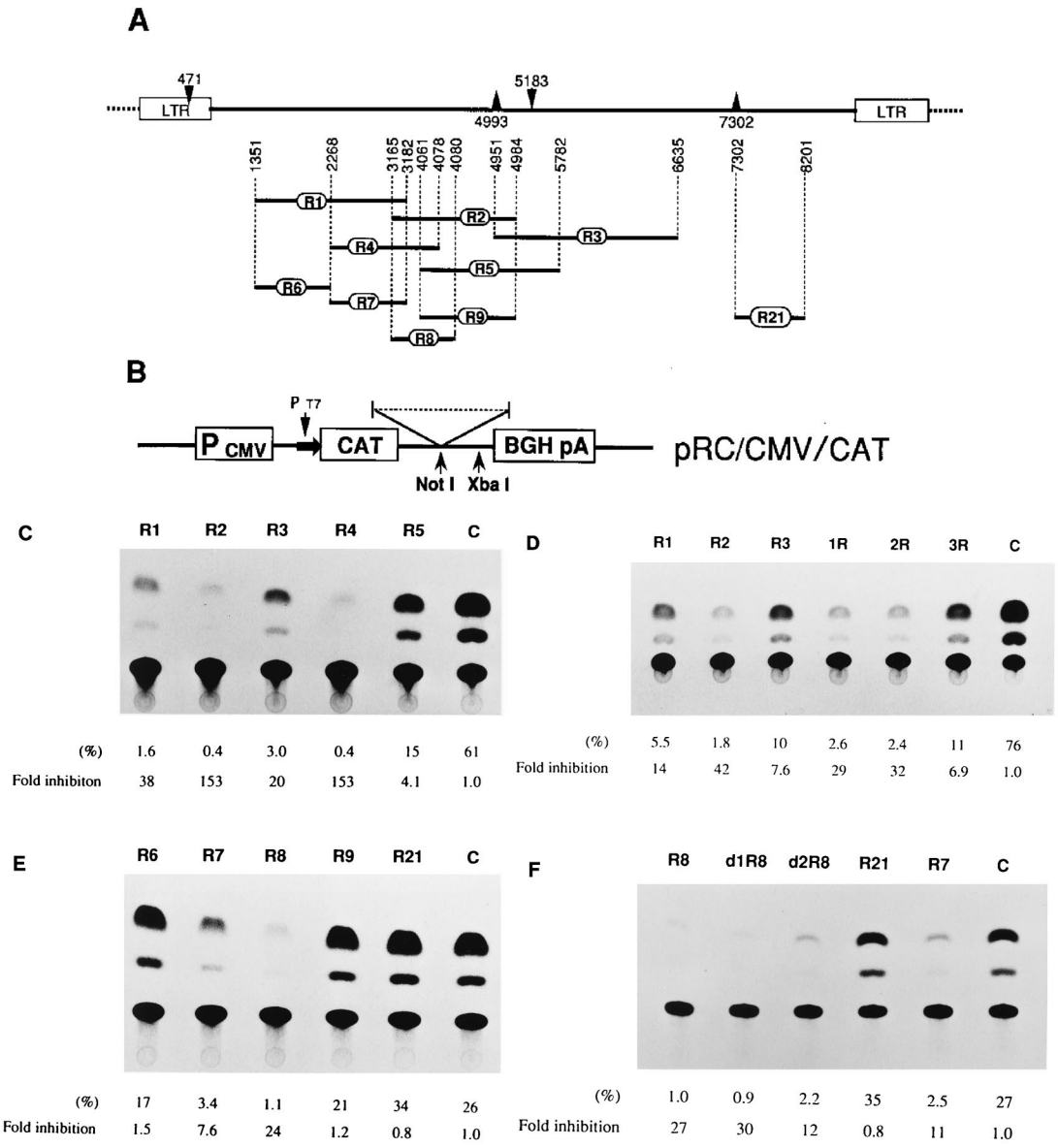

FIG. 2. (A) Locations of the fragments (R1 to R9 and R21) from HTLV-1. (B) Various CAT expression plasmids. A fragment shown in panel A was inserted in the *Not*I site of the basal CAT expression plasmid control with the CMV promoter (P_{CMV}), pRC/CMV-CAT (Invitrogen Inc.). The CAT coding and bovine growth hormone gene poly(A) signal regions are designated by CAT and BGH pA, respectively. P_{T7}, T7 RNA polymerase promoter. (C) HeLa cells were transfected with 2 μg of CMV-CAT control (lane C) or equivalent molar amounts of CMV-CAT-R1, -R2, -R3, -R4, and -R5 (lanes R1 to R5), and CAT activities were determined. (D) Plasmids CMV-CAT-1R, -2R, and -3R, which inserted R1, R2, and R3 in the antisense orientation into the *Not*I site of the pRC/CMV-CAT vector. These plasmids (lanes 1R to 3R) were compared to CMV-CAT-R1, -R2, and -R3 (lanes R1 to R3) by subjecting them to CAT assays. Lane C, control CMV-CAT. (E) Two micrograms of control CMV-CAT (lane C) or equivalent molar amounts of CMV-CAT-R6, -R7, -R8, -R9, and -R21 (lanes R6 to R9 and R21) was transfected into cells, and their CAT activities were determined. (F) HeLa cells were transfected with 2 μg of control CMV-CAT (lane C) or equivalent molar amounts of CMV-CAT-R8, -d1R8, -d2R8, -R21, and -R7 (lanes R8, d1R8, d2R8, R21, and R7), and their CAT activities were determined. (G) Jurkat cells were transfected with 8 μg of control CMV-CAT (lane C) or equivalent molar amounts of CMV-CAT-R1, -R2, -R3, -R4, -R5, -R6, -R7, -R8, -R9, -R21, and -R8M (lanes R1 to R9, R21, and R8M), and their CAT activities were determined.

those from specimen 34 were too faint to be detected in the photograph.

Two representative amplified bands from specimens 6 and 24 were cloned in pBluescript II (Stratagene). DNA sequencing revealed that the inserts comprise 1,183 and 868 bp, respectively, and have a large partial deletion within the *gag-pol-env* region (data not shown). The sequence data also indicated that the rearrangement between the *gag* and *env* regions occurs

TABLE 1. Detection of *tax*/*rex* and p21X mRNAs and of HTLV-1 genomes in fresh PBMCs from HTLV-1-infected individuals

Group	Sample no.[a]	*tax*/*rex* mRNA[b]	p21X mRNA[b]	HTLV-1 DNA copy no./10^5 cells[c]	Detection of defective HTLV-1[d]
A	3	$<10^0$	$<10^0$	10^4	−
	4	$<10^0$	$<10^0$	10^1	−
	8	$<10^0$	$<10^0$	10^2	−
	11	$<10^0$	$<10^0$	10^1	−
	13	$<10^0$	$<10^0$	10^2	−
	14	$<10^0$	$<10^0$	10^1	−
	15	$<10^0$	ND[e]	+[f]	−
	18	$<10^0$	$<10^0$	10^3	−
	20	$<10^0$	$<10^0$	10^3	−
	25	$<10^0$	$<10^0$	10^2	−
	26	$<10^0$	$<10^0$	10^1	−
	31	$<10^0$	$<10^0$	10^5	−
	32	$<10^0$	$<10^0$	10^4	−
	33	$<10^0$	$<10^0$	10^4	−
B	2	10^1	$<10^0$	10^1	−
	9	10^2	$<10^0$	10^3	−
	10	10^1	$<10^0$	10^1	−
	16	10^1	$<10^0$	10^1	−
	22	10^1	$<10^0$	10^2	−
	27	10^1	$<10^0$	10^1	−
	28	10^1	$<10^0$	10^0	−
	29	10^2	$<10^0$	10^0	−
	30	10^1	$<10^0$	10^0	−
C	1	$<10^0$	10^2	10^1	+
	6	$<10^0$	10^2	10^2	+
	12	$<10^0$	10^1	10^1	+
	23	$<10^0$	10^1	10^2	+
	24	$<10^0$	10^1	10^1	+
	34	$<10^0$	10^1	10^4	+
	35	$<10^0$	10^1	10^4	+
D	5	10^1	10^2	10^2	+
	18	10^1	10^1	10^1	+
	19	10^2	10^1	10^1	ND
	21	10^1	10^1	10^1	+

[a] Blood samples were obtained from 10 patients with ATL (samples 15 to 20 and 31 to 35), 15 patients with HAM (samples 1 to 15), and 10 asymptomatic carriers (samples 21 to 30).
[b] Quantitatively estimated by RT/TS-PCR as described in Materials and Methods.
[c] Quantitatively estimated by TS-PCR as described in Materials and Methods.
[d] Based on results in Fig. 1.
[e] ND, not done.
[f] Not tested quantitatively.

at nt 1555 and 6511 and at nt 1356 and 6627, respectively. Moreover, the viral mRNA transcripts specific for the defective genomes identified in specimens 6 and 24 were determined by the RT/TS-PCR technique (data not shown), suggesting that they are also actively transcribed in vivo. Given their similar sizes, the other PCR-amplified bands from the remaining specimens (samples 1, 5, 12, 18, 21, 34, and 35) may have derived from similar defective genomes with the large partial deletion within the *gag-pol-env* region. Furthermore, given the observation of the multiple PCR-amplified bands, most of these PCR-positive specimens contain cells infected with multiple defective proviral genomes with similar large deletions.

We observed that these PCR-positive specimens, samples 1, 5, 6, 12, 18, 21, 23, 24, 34, and 35, coincided with the specimens positive for p21X mRNA expression in fresh PBMCs. In contrast, all of the PCR-negative specimens tested were found to coincide with the specimens negative for p21X mRNA expression, in which some are positive for the *tax*/*rex* mRNA expression and the others are negative. This strong coincidence suggested that a cell which contains the defective genome(s) with the large deletion between the *gag* and *env* genes can express p21X mRNA in PBMCs from HTLV-1-infected individuals as well as in HTLV-1-infected cell lines as described previously.

Identification of CIEs within the HTLV-1 *gag-pol-env* region. We have found that p21X mRNA is constitutively expressed in fresh PBMCs infected with HTLV-1 (47). Additionally, we described above that the mechanism by which defective HTLV-1 genomes with the large deletion between the *gag* and *env* regions acquire the ability to express p21X mRNA is applicable to fresh PBMCs infected with HTLV-1. These findings led to the assumption that viral gene expression, namely, the expression of p21X mRNA, from such defective genomes is not down-regulated in vivo. On the contrary, in vivo expression from the complete virus is known to be generally strongly down-regulated. This assumption led to the working hypothesis that the region within the *gag-env* region plays an important role in down-regulation of HTLV-1 gene expression. In other words, it is possible that a DNA fragment within the *gag-env* region is able to repress viral gene expression.

To prove this hypothesis, we prepared the following CAT expression plasmids containing short DNA fragments from the HTLV-1 proviral genome. The *gag-pol-env* region (nt 1351 to 6635), which was present in the complete proviral genome but deleted in the defective proviral genomes as described previously, was divided into five overlapping segments, R1 to R5 (Fig. 2A). These segments were amplified by PCR as described in Materials and Methods, and each segment was inserted into the *Not*I site located between the downstream region of the CAT coding region and the upstream region of the bovine growth hormone poly(A) signal of the pRC/CMV-CAT vector as shown in Fig. 2B. The five plasmids obtained were called CMV-CAT-R1, -R2, -R3, -R4, and -R5. Because the *Not*I site is located upstream of the poly(A) signal, the sequences of the inserts are present in the mRNA molecules of their CAT transcripts. Their relative CAT activities in HeLa cells were compared with that of the insertless plasmid CMV-CAT (Fig. 2C), demonstrating that the R1, R2, and R4 segments exert a strong inhibitory effect on CMV promoter-directed CAT expression; however, the R3 segment exerts a weaker inhibitory effect. These results suggested the presence of potent inhibitory elements in a part of the *gag-pol* region (nt 1351 to 4984). In addition, lower inhibitory activities caused by R3 (Fig. 2C, lane R3) might indicate the existence of other inhibitory elements. The elements for the inhibitory activity might be located in the 3′ half of R3, which is present in the *env* coding region, because R5, which includes the 5′ half of R3, exerts almost no inhibitory effect.

When the R1, R2, and R3 segments were inserted in the antisense orientation into the same site of the pRC/CMV-CAT vector, the constructs showed inhibitory effects similar to those of CMV-CAT-R1, -R2, and -R3, respectively (Fig. 2D). This orientation independence for the inhibitory effects in these segments raised the possibility that the inhibitory elements act as transcriptional repressor sequences. To test this hypothesis, the R1, R2, and R3 segments were inserted upstream of the CMV promoter region of pRC/CMV-CAT in both orientations, and the CAT activities of these constructs were measured. Insertion of these segments upstream of the promoter region in the CAT expression plasmid showed no effect compared with the control CMV-CAT (data not shown). Therefore, these observations suggested that the inhibitory elements within the *gag-pol-env* region do not contain ordinary transcriptional repressor sequences. Taken together, these data sug-

gested that the elements are *cis* acting and exert an inhibitory effect only when present in the coding region. Therefore, they were called CIEs.

The CIEs within the 5′ part of the *pol* region are located at least in two discrete regions. To further analyze the potent inhibitory elements in detail, the region from nt 1351 to 4984 within the *gag-pol* region was divided into four overlapping segments, R6 to R9 (Fig. 2A), which were cloned into the pRC/CMV-CAT vector as described in Materials and Methods. For a control plasmid with an insert of comparable size, the R21 segment (7302 to 8201) within the HTLV-1 pX region was also cloned into the pRC/CMV-CAT vector, and the plasmid was called CMV-CAT-R21. The results of CAT assays in Fig. 2E show that the CAT activities were consistently 10- to 18-fold lower after transfection into HeLa cells with CMV-CAT-R7 and -R8 compared to either the control plasmid CMV-CAT or CMV-CAT-R21. This result indicated that R7 and R8 contain the potent inhibitory elements but R6, R9, and R21 do not. Because all of R7 and R8 are present in R1 and R2, respectively, the inhibitory activities caused by R1 and R2 appeared to be derived from those of R7 and R8, respectively.

Because R7 and R8 share a 20-nt sequence (from nt 3165 to 3182), possibly the overlapped sequence is responsible for their inhibitory effect. However, as shown in Fig. 2F, the 5′-truncated R8 fragment (from nt 3368 to 4080), deleting the overlapped sequence, still showed a potent inhibitory effect compared with the parental R8, indicating that R7 and R8 contain the independent inhibitory elements.

We next examined whether the inhibitory elements function in $CD4^+$ T cells, which are natural targets for HTLV-1 infection. Transfection of the plasmids used in the assays described above into Jurkat cells, a $CD4^+$ T-cell line, and subsequent CAT assays showed results essentially equal to those for HeLa cells (Fig. 2G). The results indicated that the inhibitory elements in the R7 and R8 segments also function in $CD4^+$ T cells.

Two ATTTA pentamer motifs in R8 are not responsible for the inhibition activity of R8. Recent studies have shown that AU-rich sequences within some of the short-lived mRNAs, including those encoding proto-oncogenes, cytokines, and lymphokines, play a role in their rapid degradation. In the most obvious sequence motif in the AU-rich sequences is a variable number of AUUUA pentamers (6, 60, 61, 70). In one of the segments containing the inhibitory elements, R8, two ATTTA motifs were found, suggesting the possibility that the transcript from CMV-CAT-R8 was rapidly degraded due to these ATTTA motifs in the transcript. To test this hypothesis, these two motifs in R8 were mutated to AGGTA pentamers, which have been shown to be nonfunctional (8). As shown in Fig. 2G, lanes 8 and 11, the mutation, R8M, in R8 did not affect the inhibitory effect of R8. Also, this result may support the results of a recent study (33).

Repression by the CIEs is relieved by Rex. For full replication of the virus, especially in the acute infectious phase, we assumed that the inhibition by the CIEs might be abrogated by Tax and/or Rex. First, we studied the relationship with Tax regulation. To test this, we exchanged the CMV promoter of CMV-CAT-R7, -8, and -21 for the HTLV-1 LTR and prepared the Tax-responsive CAT plasmids LTR-CAT-R7, -R8, and -R21, respectively. These plasmids were cotransfected into HeLa cells with or without the Tax expression plasmid pCD-SRα-tax. In the absence of Tax, the relative CAT activities of LTR-CAT-R7 and -R8 were compared with that of LTR-CAT-R21, demonstrating that the R7 and R8 segments exert a strong inhibitory effect on LTR promoter-directed CAT expression (data not shown). In the presence of Tax, moreover, their relative CAT activities were compared with that of LTR-CAT-R21, demonstrating that the R7 and R8 segments also exert the same level of inhibitory effect (data not shown). These results indicated that the inhibitory effect of R7 and R8 is not affected by Tax, suggesting that the inhibitory effect of the CIEs is independent of Tax. Moreover, these experiments using CMV and LTR promoters demonstrated promoter independence for the inhibitory effect of the CIEs.

Second, we studied the relationship with Rex regulation, because it may be more possible that the CIEs occur at a posttranscriptional level. To use the above-mentioned transcription units as model substrates for the investigation of Rex activity, we modified them by inserting an HTLV-1 subgenomic DNA fragment corresponding to the RxRE sequence between the R7, R8, or R21 insert and the poly(A) signal of CMV-CAT-R7, -R8, or -R21 plasmid, respectively, in either the sense or antisense orientation. The Rex-responsive or nonresponsive plasmids CMV-CAT-R7-RxRE(+) or -RxRE(−), CMV-CAT-R8-RxRE(+) or -RxRE(−), and CMV-CAT-R21-RxRE(+) or -RxRE(−), respectively, were prepared. These plasmids were cotransfected into HeLa cells with either a Rex expression plasmid (pCD-SRα-rex) or a control plasmid (pCD-SRα) (44), and their CAT activities were determined (Fig. 3A). In the absence of Rex, insertion of the R7 and R8 segments in these plasmids, irrespective of whether they contain RxRE in the sense or antisense orientation, decreased CAT expression compared with that of the R21 segment, a result is consistent with that observed in the case of the plasmids without RxRE. These results showed that the inhibitory effect of R7 and R8 is not influenced by the presence of RxRE on the same RNA molecule.

Compared with the results in the absence of Rex, the CAT activities of CMV-CAT-R7-RxRE(+) and -R8-RxRE(+) were markedly increased by cotransfection of a Rex expression plasmid (pCD-SRα-rex), whereas that of CMV-CAT-R21-RxRE(+) was increased relatively little. On the contrary, CMV-CAT-R7-RxRE(−), -R8-RxRE(−), or -R21-RxRE(−) showed no increase by Rex. These results suggested that the inhibition by R7 and R8 is extensively relieved by Rex through binding to the RxRE structure. Moreover, it is noteworthy that Rex almost completely rescued the CAT activities of CMV-CAT-R7-RxRE(+) but only partially rescued those of CMV-CAT-R8-RxRE(+), suggesting the presence of different inhibitory mechanisms for R7 and R8.

The CIEs act at the RNA level. Because Rex mediates the export of any RNA molecule that harbors an RxRE, irrespective of whether it contains intronic sequences, we assumed that the CAT activities are correlated with the level of the CAT RNA expression. To test this, RNAs isolated from these transfectants were analyzed by Northern blotting with the ^{32}P-labeled CAT gene probe. The results showed that the amounts of the CAT transcripts in the transfectants correlated well with the levels of CAT activity (Fig. 3B). Thus, the result suggested that Rex might interfere with the inhibition by R7 and R8 on the CAT RNA molecule by interacting with RxRE on the same molecule.

We next examined whether CIEs affect the level of transcription driven by the CMV promoter, using the HeLa nuclear extract in vitro transcription system (Fig. 4). Based on size, these runoff transcription products were found to be expectedly generated from the indicated template DNAs. Since products with similar sizes show similar band intensities, the similar intensities of those generated from CMV-CAT-R7, -R8, and -R21 cut with *Xba*I reveal no significant difference in the amount of the transcripts either with or without CIEs. This

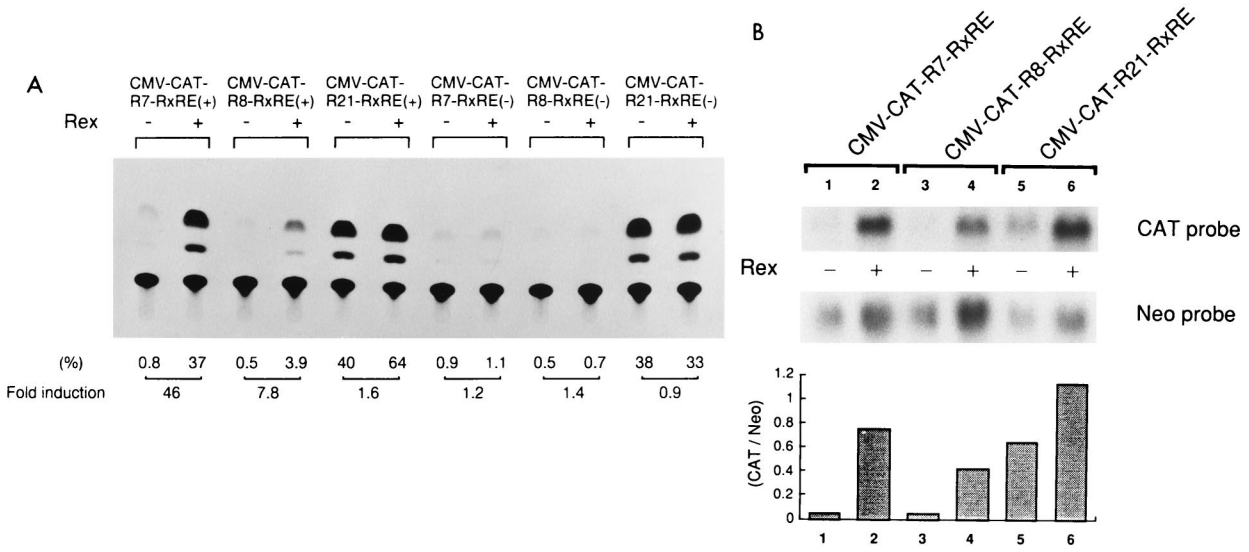

FIG. 3. (A) Two micrograms of each of the plasmids indicated at the top was cotransfected into HeLa cells with 3 μg of the control plasmid pCD-SRα or the Rex expression plasmid pCD-SRα-rex (Rex − or +, respectively) as described previously (44), and their CAT activities were determined. (B) Six micrograms of each of the CAT expression plasmids indicated at the top was cotransfected into HeLa cells with 9 μg of the control pCD-SRα (Rex −) or the Rex expression plasmid pCD-SRα-rex (Rex +). At 48 h posttransfection, total RNAs were extracted and subjected to Northern blot analysis with the ^{32}P-labeled CAT gene DNA or the ^{32}P-labeled *neo* gene DNA as a probe. Each lane shows the 2.4-kb band corresponding to CAT mRNA (top) and the 1.4-kb band corresponding to *neo* mRNA (bottom). Quantitation of CAT mRNA levels in the transfectants is shown at the bottom. Quantitation was performed by carrying out densitometric assays for the signal intensity of each Northern blotting band on the developed X-ray films; CAT mRNA values were normalized to the amount of the *neo* mRNA in the same lane, and the final CAT mRNA amounts are presented in arbitrary units (CAT/Neo).

result suggests that CIEs have no ability to act at the transcriptional level.

DISCUSSION

We originally found the singly spliced p21X mRNA, which is expressed from defective proviruses having a large deletion of the *gag-pol-env* region, in HTLV-1-infected cell lines (43, 45). Here, we show a high correlation between the expression of p21X mRNA and the existence of the defective proviruses with a large deletion of the *gag-pol-env* region in fresh PBMCs of HTLV-1-infected individuals, asymptomatic carriers, and patients with ATL and HAM. Thus, we concluded that p21X mRNA is mainly expressed from the defective provirus with a large deletion not only in HTLV-1-infected cell lines but also in PBMCs of HTLV-1-infected individuals.

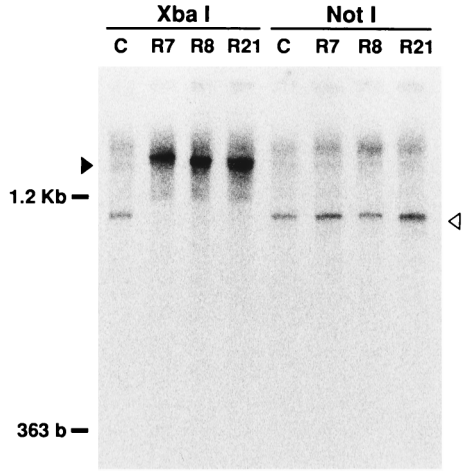

FIG. 4. Runoff transcription products in HeLa nuclear extract in vitro transcription assays. As template DNAs, plasmids CMV-CAT-R7, -R8, and -R21 and control CMV-CAT (lanes R7, R8, R21, and C, respectively) were digested with *Xba*I or *Not*I (Fig. 2B), and 0.1 μg of each digested DNA was subjected to in vitro transcription assays, followed by electrophoresis and autoradiography. Runoff products generated from CMV-CAT-R7, -R8, and -R21 digested with *Xba*I, which are expected to be approximately 1.8 kb in length, are indicated by the closed triangle (*Xba*I, lanes R7, R8, and R21). Runoff products generated from control CMV-CAT digested with *Xba*I or *Not*I and CMV-R7, -R8, and -R21 digested with *Not*I, which are expected to be approximately 0.9 kb in length, are indicated by the open triangle (*Xba*I, lane C; *Not*I, lanes C, R7, R8, and R21). Each of these products shows the expected size. The positions of the standard transcripts (363 bases (b) and 1.2 kb in length) generated from the HeLa nuclear extract positive control DNA (CMV) (Promega Biotec) are indicated at the left.

It is well known that *tax/rex* mRNA is rapidly induced after in vitro cultivation of fresh PBMCs from HTLV-1-infected individuals (13, 15, 30, 45). On the contrary, the same level of p21X mRNA expression was observed in fresh uncultured and cultured PBMCs of HTLV-1-infected individuals (47), indicating no induction of the p21X mRNA in the cultured PBMCs. Because the defective HTLV-1 genomes cannot express *tax/rex* mRNA, cells infected only with such defective viruses express neither Tax nor Rex. The absence of Tax in the cells leads to no transactivation of the LTR of the defective genomes, resulting in no induction of p21X mRNA expression in these cells. Thus, we speculated that full activation of p21X mRNA expression occurs in in vivo HTLV-1-infected PBMCs, although *tax/rex* mRNA expression in the cells infected with the complete proviral genome is down-regulated. We then considered that these differences in regulation mechanisms between p21X mRNA and *tax/rex* mRNA expression are explained by differences in viral genome organization. Based on this idea, we hypothesized the existence of an inhibitory element(s) somewhere in the *gag-pol-env* region which is contained in the complete virus but not in defective viruses.

We searched for such putative inhibitory elements in the *gag-pol-env* region. Two discrete fragments (R7 and R8) within the 5′ terminus of the *pol* region showed a strong inhibitory effect, and a fragment (R3) containing the *env* region further showed a weaker inhibitory effect. Northern analysis revealed that the inhibition of CAT activities by the inhibitory elements within the *pol* region is due to the decrease in the amounts of

CAT transcripts, indicating that the fragments may function at the transcriptional level. This inference is supported by the observation that the effects of the fragments showed no orientation dependence. However, when inserted upstream of the gene promoter instead of downstream of the CAT gene, the fragments showed no effect (data not shown). In addition, CAT assays and Northern analysis revealed that Rex, known as a posttranscriptional regulator, relieved the effect of the inhibitory elements through binding to the RxRE structure, whereas Tax, known as a transactivator, exerted no effect on this repression. With these CAT plasmids containing either R7, R8, or R21 used as reporter genes, infection with vaccinia virus/T7 RNA polymerase allowed equivalent levels of CAT expression among them in HeLa cells (data not shown). The gene expression system in the cytoplasm based on T7 RNA polymerase is known not to be necessary to localize DNA to the nucleus. Furthermore, a runoff transcription assay revealed that CIEs exert no effect on transcription driven by the CMV promoter in HeLa nuclear extract in the presence of an RNase inhibitor (Fig. 4). Taken together, these results suggest that CIEs act on the posttranscriptional level, such as by destabilizing RNA containing them in nuclei but not in the cytoplasm. The upregulation of the RNA level by Rex shown in Fig. 3B might be caused by stabilization of RNA demonstrated previously for Rex and Rev (19, 36, 56).

To transport to the cytoplasm balanced levels of spliced and unspliced viral RNAs, all classes of retrovirus have suboptimal splicing sites (7, 28). It has been shown that mutations which enhance the rate of viral RNA splicing are defective for reproduction. Because *tax*/*rex* mRNA expression from the complete HTLV-1 virus is down-regulated in vivo in spite of the absence of the CIEs in *tax*/*rex* mRNA, inhibition by the CIEs may act before or during the slow splicing reaction caused by the suboptimal splicing-site sequences. The full mechanism of the inhibition by the CIEs remains to be examined; however, it is likely that Rex effectively interferes with the degradation and/or promotes the export of mRNA containing RxRE and CIEs from the nucleus to the cytoplasm.

A number of *cis*-acting sequences derived from various regions of human immunodeficiency virus (HIV) have been shown to reduce the expression of chimeric reporter genes (5, 10, 35, 53). The inhibitory effects of such sequences mapped to *gag*, *pol*, and *env* (5, 10, 35) are caused by nuclear sequestration of the RNA containing them. On the other hand, the repressive effects of such sequences mapped to *gag* (56) are caused by the destabilization of the RNA containing them. Both repression effects are counteracted by the effect of Rev–Rev-responsive element interaction. These observations are very similar to ours. These inhibitory sequences of HIV have shown orientation dependence, although we showed here orientation independence for the inhibitory effects of the HTLV-1 CIEs. We cannot explain this difference, although no significant sequence homologies exist between the CIEs of HTLV-1 and the whole sequence of HIV. There are significant sequence homologies among HTLV-1 and the related retroviruses simian T-cell leukemia virus type 1, HTLV-2, and bovine leukemia virus (12). Like HTLV-1, the HTLV-1-related retroviruses are not produced at detectable levels in PBMCs of the infected host but are often expressed when these cells are cultured in vitro (24, 68, 74). We previously reported the identification of the singly spliced pX mRNA transcripts, corresponding to p21X mRNA in HTLV-1, common to all of these HTLV-1-related retroviruses (46). Thus, we speculate that CIEs are present in all of these retroviruses.

Because a frequent finding in HTLV-1-infected people is the presence of already activated $CD8^+$ T cells (cytotoxic T lymphocytes [CTL]) specific for viral proteins (23, 25, 50, 51), the infected cells, once expressing the viral proteins, are efficiently eliminated by such CTL. Thus, latent virus such as HTLV-1 may involve some mechanism like CIEs, which repress the expression of viral proteins, for avoiding the CTL response of the host.

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