Multiple sequence elements are required for regulation of human T-cell leukemia virus gene expression

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ABSTRACT The U3 region of the long terminal repeat (LTR) of human T-cell leukemia virus type I (HTLV-I) contains sequences that respond to the trans-activating transcription (tat) factor encoded by the pX region of the provirus. Results presented here show that there are multiple tat-responsive sequences within the LTR and that a single 21-nucleotide sequence, which is repeated three times within the U3 region, is sufficient to determine the response to the trans-activator. This sequence is capable of conferring a tat-responsive phenotype upon the HTLV-I and simian virus 40 promoters, independent of orientation. Sequences required for efficient HTLV-I LTR-directed gene expression are also located 3' to the site of RNA initiation, within the R and U5 regions of the LTR.

Human T-cell leukemia virus type I (HTLV-I) is the etiologic agent of adult T-cell leukemia/lymphoma (1–4). The genome of HTLV-I contains long terminal repeat (LTR) and *gag*, *pol*, and *env* gene sequences characteristic of all retroviruses. However, unlike the avian and murine retroviruses, HTLV-I contains additional overlapping open reading frames between the *env* gene and the 3' LTR (5). Expression of one of these regions results in the production of a 42-kDa nuclear protein (6–8), designated *tat*_I (9), that mediates transcriptional trans-activation of HTLV-I LTR-directed gene expression (9–13). The phenomenon of trans-activation is also observed with HTLV-II (9, 14) and bovine leukemia virus (BLV) (15, 16), both of which have a genome structure similar to that of HTLV-I (17, 18). In all these viruses, the tat-responsive sequences are located within the U3 region of the respective LTRs (19–23).

In this study a transient gene expression assay was used to localize those regions within the HTLV-I LTR that are essential for promoter activity and trans-activation. We report that a 21-base-pair (bp) sequence that is repeated three times within the U3 region contains the tat-responsive element. Our results show that the R and U5 regions contain sequences that are required for optimum levels of HTLV-I LTR-directed gene expression.

MATERIALS AND METHODS

Plasmid Constructions. Plasmids pU3R-I (9), pC–55 (pC55 in ref. 19), and pRSVcat (24) have been described. The scheme for construction of the HTLV-I *tat*-expression vector pHtat_I is shown in Fig. 1a. Deletion mutants were constructed by limited digestion with BAL-31 exonuclease (29). Recombinant DNA techniques were performed as described in ref. 30. Synthetic oligonucleotides were synthesized using an automated DNA synthesizer. Oligonucleotides were purified from 20% acrylamide gels, annealed, and inserted into the appropriate expression vector. The DNA sequence of LTR deletion mutants and hybrid oligonucleotide/promoter

FIG. 1. (a) Construction of a biologically active *tat*_I-expression vector, pHtat_I. To construct plasmid pHtat_I, the polylinker of plasmid pSP64 (Promega Biotec) was first inserted 3' to the HTLV-I LTR sequence –350 to +325 present on plasmid pU3R-I. A 610-bp proviral fragment encompassing nucleotides 5168–5778 (first black box), which contained the first coding exon of *tat*_I followed by the splice donor (SD) sequence, was cloned into the corresponding restriction sites in the polylinker. A 2522-bp fragment encompassing nucleotides 6498–9020 (second black box), which lacked the *env* coding sequences and contained the nonconserved region (NCR) (5), the second exon of *tat*_I [preceded by the splice acceptor (SA) sequence] and an intact 3' LTR, was inserted into the respective restriction sites present 3' to the 5168–5778 fragment. Nucleotide positions were calculated using the numbering system reported by Seiki *et al.* (5). The predicted splicing pattern to produce a functional *tat* transcript is shown. This plasmid lacks the ATG codon used to express the pp27 product (25) that overlaps the *tat*_I gene in an alternative reading frame. (b) Biological activity of plasmid pHtat_I. To test the activity of pHtat_I, HeLa cells that constitutively express CAT under control of a HTLV-I LTR were established by cotransfection of plasmids pU3R-I (9) and PSV2gpt (26). Mycophenolic acid-resistant clones were pooled and expanded. Positive clones were then cotransfected with plasmids pHtat_I and PSV2neo (27), and neomycin-resistant clones were selected and pooled. CAT assays and RNA analysis were performed using equal numbers of HTLV-I LTR-directed CAT-constitutive cells either lacking (–) or containing (+) plasmid pHtat_I. (Left) CAT assays were performed for 30 min (lane –) and 5 min (lane +). [¹⁴C]Chloramphenicol (lowest spot) and its acetylated products (upper spots) were separated by thin-layer chromatography and visualized by autoradiography. (Right) Total RNA was fractionated by electrophoresis and transferred to nitrocellulose membrane (28) and then was hybridized with an 800-bp CAT gene fragment that lacked the polyadenylation sequence. Only the relevant region of the autoradiogram is shown.

clones was confirmed by the Maxam and Gilbert sequencing method (31).

Transfection Procedures and Chloramphenicol Acetyltransferase (CAT) Assays. HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. The human T-cell line Jurkat was grown in RPMI

1640 plus 10% fetal bovine serum. In general, lymphoid cells (10^6 per ml) were transfected with a total of 10 μ g of plasmid DNA by the DEAE-dextran transfection method described by Queen and Baltimore (32). After a 48-hr incubation, cells were washed with phosphate-buffered saline and resuspended in 150 μ l of 250 mM Tris Cl (pH 8.0), and cell extracts were prepared by three freeze (-70°C)/thaw (37°C) cycles followed by centrifugation to remove cellular debris. CAT assays were performed as described (9). The amount of [^{14}C]chloramphenicol converted to acetylated forms was determined by liquid scintillation counting of the spots cut from the thin-layer chromatography plate.

RESULTS

Expression of the HTLV-I Trans-Activator Product. The *tat_I*, *tat_{II}*, and BLV *tat* messages are produced via a double-splicing mechanism whereby the initiator methionine codon of the *env* gene is joined in-frame with the *tat* coding region (9, 13, 18, 33, 34). In previous studies (9), expression of the *tat_{II}* protein was achieved via fusion of the amino-terminal seven amino acid codons of the CAT gene in-frame with the *tat_{II}* coding region. For the studies presented here, a new vector designed to express an authentic *tat_I* protein was constructed (Fig. 1*a*). This vector expressed a *tat* protein that was capable of trans-activation of the HTLV-I LTR in transient-expression assays (see below; Fig. 3). This vector was also capable of producing an active *tat_I* protein upon integration into a HeLa cell line, as demonstrated by the markedly increased levels of HTLV-I LTR-directed CAT mRNA and protein produced from an integrated copy of a HTLV-I LTR CAT plasmid (Fig. 1*b*). This observation extends previous results obtained with transient-expression assays (9), by showing that the *tat_I* gene product can induce the expression of genes stably integrated into the genome, with a concomitant increase in the steady-state level of HTLV-I LTR-directed mRNA.

Multiple *tat*-Responsive Elements Are Present Within the U3 Region. Earlier reports (19–21) demonstrated that the *tat*-responsive elements are present within the U3 region of the

LTR. For example, our previous studies (19) showed that HTLV-I LTR deletion mutants that retain nucleotides -159 to $+325$ (numbering relative to the translation initiation site) are responsive to the *tat* proteins, whereas a mutant with a more extensive deletion that leaves only nucleotides -55 to $+325$ is not. Further definition of the 5' boundary of the *tat*-responsive region was obtained by analysis of a deletion mutant that lacks nucleotides -161 to -55 (Fig. 2). Deletion of sequences present between nucleotides -159 and -89 eliminated the trans-activation response (Table 1). Note that the -89 deletion removes the 5' portion of the 21-bp repeat that is located proximal to the promoter sequences.

To examine the potential activity of LTR sequences 5' to nucleotide -159 , a series of internal deletions were created within the LTR that have as their 3' border nucleotide -55 (Fig. 2). As shown in Fig. 3*a*, plasmids lacking those sequences between nucleotides -162 and -55 were responsive to the *tat* gene product. This result shows that sequences outside the -161 to -55 region can confer the trans-activation phenotype. Furthermore, plasmid pC-215/-55, which lacks nucleotides -215 to -55 , retains a trans-activation phenotype, albeit at a reduced level. These results indicate that there are multiple sequences within the U3 region that determine the response to the *tat_I* product. Note that deletion -215 to -55 removes both of the 3' 21-bp repeats but leaves intact the 5' repeat sequence. As expected, no increase in CAT activity was observed after simultaneous transfection with plasmids pRSVcat and pHtat_I.

Results similar to those described above were obtained with plasmids in which the HTLV-I promoter elements (nucleotides -55 to $+325$) were replaced by the early-region promoter of simian virus 40 (Fig. 3*b*). These findings demonstrate that neither the promoter nor the R-region sequences of HTLV-I are required for trans-activation.

The dependence on the orientation of the *tat_I*-responsive sequences with respect to the promoter was also investigated. As shown in Table 1, the level of CAT gene expression directed by plasmid pC-55/+300 (not shown), which contains an inversion of the U3 sequences relative to the promoter, was regulated by *tat_I*. This observation confirms

FIG. 2. Deletion analysis of the HTLV-I LTR. A schematic illustration of LTR sequences present on plasmid pU3R-I is shown at the top. Limited digestion with BAL-31 exonuclease starting at position -159 or $+325$, respectively, generated 5' to 3' and 3' to 5' deletion mutants. Each 5' or 3' deletion mutant contains the identical pBR322 (5' deletion) or CAT gene (3' deletion) border. Internal deletions of the U3 region were generated by BAL-31 exonuclease digestion at the *Nde* I (nucleotide -55) site followed by ligation to *Cla* I linkers. Identical 3' borders were created by subcloning the U3 deletion fragments back into the original vector plasmid. Plasmids are named with respect to the endpoint of each deletion. Deletion endpoints were determined by Maxam and Gilbert DNA sequence analysis (31).

Table 1. Activity of LTR deletion and enhancer mutants

Plasmid	Relative CAT activity		Induction
	– pHtat _i	+ pHtat _i	
pU3R-I	1.00	21.0	21
pC-159	0.12	1.32	11
pC-89	0.03	0.03	1
pC-55/+325	0.15	2.40	16

Jurkat cells were transfected with the indicated plasmid DNA in the presence or absence of plasmid pHtat_i. The percent conversion of chloramphenicol to its acetylated forms was determined and normalized to the activity obtained with plasmid pU3R-I in the absence of pHtat_i. Induction was calculated as the ratio of CAT activity in the presence of pHtat_i to that in its absence.

reports (20, 21) that the HTLV-I LTR contains conditional enhancer sequences that are active in the presence of the trans-activator gene.

The tat-Responsive Sequences Are Present Within a 21-bp Sequence. The results described above suggest an involvement of the 21-bp sequence in the trans-activation response. To test this possibility, two 21-bp-long oligonucleotides were synthesized, one that corresponds to the repeat sequence located closest to the promoter, nucleotides –104 to –83 (TAR-21), and a variant, TAR-21A, that contains a single base-pair change at nucleotide –93, 13 nucleotides from the 5' end of the repeat sequence (Fig. 4a). These oligonucleotides were placed 5' to the HTLV-I promoter sequence (nucleotides –55 to +325) present on plasmid pC-55G (Fig. 4a). The structure of each plasmid was confirmed by direct nucleotide sequence analysis (31).

The level of CAT activity directed by these plasmids is shown in Fig. 4b. The level of CAT activity directed by plasmid pH21B, which contains the TAR-21 sequence, was markedly increased in the presence of the trans-activator as compared to similar transfections in the absence of the trans-activator gene. The level of CAT activity directed by plasmid pH21A, which contains a single point mutation in the TAR-21 sequence, was elevated by cotransfection with the tat_i-expression plasmid, albeit to a lesser extent than that observed with plasmid pH21B. Similarly, CAT activity directed by plasmid pH21AB, which contains one copy of

each oligonucleotide, was higher than that observed with the individual 21-bp sequences. Although a significant increase in the level of CAT activity was observed with the plasmids that contain the synthetic 21-nucleotide sequence, the induced level observed for these plasmids was well below that observed for the intact LTR, and also below that observed for a single 21-nucleotide sequence present in the natural context, as in pC-159 and pC-215/–55.

We observed that the sequence AGGCTC was present within many of the regions that were responsive to the tat gene product. To examine the potential role of this element in trans-activation, this sequence was synthesized and inserted in duplicate 5' to the HTLV-I promoter (Fig. 4A, plasmid pH7). The level of CAT activity directed by plasmid pH7 was not responsive to the tat_i gene product, indicating that this element does not confer a trans-activation phenotype.

R-Region Sequences Are Required for Optimal Levels of Gene Expression. The promoter activity of a LTR derived from another HTLV-I isolate (strain CH) was examined. Relative to the CAT activity directed by plasmid pU3R-I (strain CR), the activity directed by plasmid pU3R-I_{CH} was markedly reduced (Fig. 5a). Plasmid pU3R-I_{CH} is similar to pU3R-I except for several point mutations within the U3 region and deletion of sequences 3' to nucleotide +131 (unpublished observations). To localize the defect in the pU3R-I_{CH} LTR, chimeric LTRs were formed between both LTRs (Fig. 2). The chimeric construct pCH/CR exhibited similar activity to that of pU3R-I, whereas the activity of pCR/CH was much reduced (Fig. 5a). This observation suggested that absence of R-region sequences 3' to nucleotide +131 might explain the low activity obtained with plasmid pU3R-I_{CH}.

To examine the role of the R- and U5-region sequences in regulation of gene expression, a series of mutants with deletions extending from nucleotide +325 toward the site of RNA initiation were constructed within the HTLV-I_{CR} LTR present on plasmid pU3R-I (Fig. 2). The results (Fig. 5b) show that there was a progressive decrease in the level of CAT activity corresponding to progressive deletion of sequences within the R and U5 regions. Despite the low level of promoter activity observed with these plasmids, the level

FIG. 3. CAT assays of Jurkat cells cotransfected with the indicated plasmid DNA in the presence (+) or absence (–) of plasmid pHtat_i. CAT assays were performed 48 hr posttransfection. Shown are autoradiograms obtained from the linear portion of a time-course assay.

FIG. 4. Activity of plasmids bearing hybrid oligonucleotide-promoter elements. (a) The 21-nucleotide sequences shown were synthesized with 5' and 3' overhangs that correspond to either *Xba* I, *Hind* III, or *Cla* I restriction sites. Oligonucleotides were cloned into the polylinker present in plasmid pGM55G. Plasmid pGM55G contains the HTLV-I promoter-CAT gene sequences present on plasmid pC-55 inserted into plasmid pGEM-3 (Promega Biotec). (b) CAT assays of Jurkat cells transfected with the indicated plasmid DNAs in the presence (+) or absence (-) of plasmid pHtat₁.

of CAT activity increased significantly in the presence of the *tat*₁ product.

DISCUSSION

The results show that a 21-nucleotide-long sequence, TAR-21, within the LTR contains sequences that confer in part the response to the *tat*₁ gene product. This conclusion is based on the observation that placement of a synthetic copy of the TAR-21 sequence 5' to the HTLV-I promoter renders the promoter responsive to the trans-activator. In the natural promoter, the TAR-21 sequence is repeated three times. However, a single TAR-21 sequence is sufficient to encode the trans-activating response, as evidenced by the increased activity of promoters that contain only the 5' TAR-21 or 3' TAR-21 sequence. The TAR-21 sequence is repeated almost identically three times in the U3 region of the LTR of the closely related virus HTLV-II (35, 36); thus it is likely that these sequences serve a similar function for that virus.

Shimotohno *et al.* (37) have reported similar findings. However, they concluded that multiple TAR-21 sequences are required for the trans-activator effect. This result was somewhat surprising, as LTR deletion mutants that contain a single TAR-21 sequence are *tat*-responsive (19, 21). It is unlikely that the difference in the results reported by Shimotohno *et al.* can be attributed to the difference in the sequence of the synthetic TAR-21 used. The TAR-21 sequence that we used corresponds to the repeat located proximal to the promoter, whereas that used by Shimotohno *et al.* corresponds to the repeat located furthest from the promoter. However, we note that the 5' 21-bp element in the absence of the two 3' 21-bp repeats is responsive to *tat*₁ (plasmids pC-215/-55 and pC-186/-55). One possible explanation for the disagreement in results may simply lie in the efficiency of transfection. For example, Shimotohno *et*

al. reported that the activity of an intact HTLV-I LTR can only be detected in the presence of the *tat*₁ product. However, we obtained high levels of CAT gene expression with a similar plasmid (pU3R-I) both in the presence and in the absence of *tat*₁. Thus, the low activation obtained with a single 21-bp element could easily be missed.

The low level of induction observed for the promoters that contain the synthetic TAR-21 sequence as compared to the natural LTR constructs that contain a single 21-nucleotide sequence with additional LTR flanking sequences is notable. This observation may indicate that there are other sequences, absent from plasmids that contain the synthetic TAR-21 sequences, that also respond to the trans-activator. Alternatively, sequences nearby the TAR-21 repeats may contribute to the overall induction level, by increasing the strength of the promoter. Sequences nearby the TAR-21 sequences may also contribute to the overall response by binding transcription factors that facilitate interaction with the trans-activator product.

The results reported here also show that the TAR-21 sequences can function in an orientation-independent manner, in agreement with reports of Paskalis *et al.* (21) and Fujisawa *et al.* (20). Similar results have been obtained with HTLV-II (22) and BLV (22, 23). We attribute the failure to observe an orientation-independent trans-activation response in plasmid pHX1CAT, reported previously (19), to inhibition of the response by specific bacterial DNA sequences that are interposed between the TAR sequences and the promoter region and to the high constitutive enhancer activity obtained in certain cell lines (19).

Mutations within the R region demonstrate that a deletion of sequences 3' to the RNA start site results in a stepwise decline of the level of gene expression directed by the HTLV-I LTR. Results similar to these have been obtained by deletion analysis of R-region sequences within the BLV LTR (23). The R region of HTLV-I and of BLV is rich in GC sequences, some of which may resemble binding sites for the transcription factor Sp1 (38). Sequences may also affect the efficiency of mRNA utilization or mRNA half-life.

The mechanism of HTLV-I-induced leukemogenesis is unknown. It has been suggested that the *tat* products modulate expression of cellular genes involved in T-cell proliferation. Therefore, it is of interest to search for sequences similar to the TAR-21 sequences within the regulatory regions of cellular genes, the expression of which may be altered by HTLV-I infection.

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FIG. 5. CAT activity of Jurkat cells transfected with the chimeric LTR (a) or 3' deletion (b) mutants in the presence of plasmid pHtat₁. pU3R-I_{CR} is pU3R-I (10).

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