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Human T-Cell Leukemia Virus Types I and II Exhibit Different DNase I Protection Patterns

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Human T-cell leukemia virus types I (HTLV-I) and II (HTLV-II) are human retroviruses which normally infect T-lymphoid cells. HTLV-I infection is associated with adult T-cell leukemia-lymphoma, and HTLV-II is associated with an indolent form of hairy-cell leukemia. To identify potential transcriptional regulatory elements of these two related human retroviruses, we performed DNase I footprinting of both the HTLV-I and HTLV-II long terminal repeats (LTRs) by using extracts prepared from uninfected T cells, HTLV-I and HTLV-II transformed T cells, and HeLa cells. Five regions of the HTLV-I LTR and three regions of the HTLV-II LTR showed protection by DNase I footprinting. All three of the 21-base-pair repeats previously shown to be important in HTLV transcriptional regulation were protected in the HTLV-I LTR, whereas only one of these repeats was protected in the HTLV-II LTR. Several regions exhibited altered protection in extracts prepared from lymphoid cells as compared with HeLa cells, but there were minimal differences in the protection patterns between HTLV-infected and uninfected lymphoid extracts. A number of HTLV-I and HTLV-II LTR fragments which contained regions showing protection in DNase I footprinting were able to function as inducible enhancer elements in transient CAT gene expression assays in the presence of the HTLV-II *tat* protein. The alterations in the pattern of the cellular proteins which bind to the HTLV-I and HTLV-II LTRs may in part be responsible for differences in the transcriptional regulation of these two related viruses.

The human T-cell leukemia virus types I (HTLV-I) and II (HTLV-II) are retroviruses which have been associated with human T-cell leukemias (18, 33, 35, 36, 53, 59, 60). HTLV-I is the etiologic agent of a highly aggressive leukemia-lymphoma (33, 35, 53), and HTLV-II has been associated with two cases of atypical hairy-cell leukemia (36, 59, 60). Both viruses have been shown to transform human T cells in vitro (6, 46, 54, 77); however, the reasons for the differences in the clinical diseases associated with these viruses are unknown.

The genetic organization of these two human retroviruses is similar (62, 65). In addition to the three genes normally found in retroviruses (*gag*, *pol*, and *env*), they contain at the 3′ portion of the genome additional open reading frames that encode three proteins (29, 37, 43, 69). One of these proteins, *tat*, is a nuclear protein (24, 68) important in both viral replication (7) and transcriptional activation (4, 14, 15, 21, 70–72). The HTLV-I and HTLV-II *tat* proteins differ in their molecular masses (43, 69) and transcriptional activating properties (64, 72). The molecular masses of the HTLV-I and HTLV-II *tat* proteins are 40 and 37 kilodaltons, respectively (43, 69). The HTLV-II *tat* protein is capable of transcriptional activation of both the HTLV-I and HTLV-II long terminal repeats (LTRs), whereas the HTLV-I *tat* protein is capable of activating only the HTLV-I LTR (64, 72). Differences in both the structure of the *tat* proteins and the regulatory sequences present in each LTR may be responsible for this altered transcriptional activation (4, 14, 15, 58, 63, 64, 71, 72).

Mutagenesis experiments indicate that several regions in the U3 region of the HTLV LTR are involved in transcriptional activation (3, 16, 48, 49, 56, 57, 63, 66). Both the HTLV-I and HTLV-II LTRs contain three 21-base-pair (bp) imperfect direct repeated sequences (62, 65) which are important in basal and *tat*-induced transcriptional regulation (3, 16, 48, 49, 56, 57, 63, 66). Two of these 21-bp repeats are contained in 51-bp imperfect repeated units in the HTLV-I LTR (62, 65). Several studies have indicated that a single 51- or 21-bp repeat was sufficient to give low levels of *tat*-induced *trans*activation either when present in the HTLV LTR (3, 16, 49, 56, 63) or when oligonucleotides complementary to the 21-bp repeat were inserted into an enhancerless early-region similar virus 40 (SV40) promoter (66). However, the level of *trans*-activation was higher when a 21-bp repeat was present in the HTLV LTR compared with heterologous promoters (3, 56, 66). Multiple copies of the 21-bp repeats also increased the level of *trans*-activation when present both in the HTLV LTR and in heterologous promoters (3, 56, 66). These results suggest that the 21-bp repeats act as inducible enhancer elements in the presence of the *tat* protein and that both the number of these repeats and the surrounding LTR sequences influence the level of *trans*-activation. Other studies have shown that regions other than the 21-bp repeats may also be important in gene expression of the HTLV-I LTR. These include a sequence important in basal transcriptional regulation located between −306 and −242 (57), a sequence important in *tat*-induced transcriptional regulation located between −160 and −117 (3, 49), and sequences in the R and U5 regions of the LTR which may be important in the efficiency of mRNA utilization or stability (48, 56, 57).

In vitro DNA-binding assays have demonstrated that multiple binding sites are present in several retroviral LTRs including those of the Moloney leukemia virus (73), the Rous sarcoma virus (61), and the human immunodeficiency virus (76). These binding sites demonstrated in vitro most likely correlate with sites required for in vivo transcriptional

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regulation. We began an investigation of the cellular DNA-binding proteins involved in the transcriptional regulation of the HTLV-I and HTLV-II LTRs. DNase I footprinting (17) of both the HTLV-I and HTLV-II LTRs with extracts prepared from HeLa cells, uninfected T lymphoid cells, and HTLV-I- and HTLV-II-transformed lymphoid cells indicated that multiple regions of both LTRs serve as binding sites for cellular proteins. Several HTLV-I and HTLV-II LTR fragments which had been shown to contain protected regions by DNase I footprinting were fused to plasmids containing the CAT gene and found to be inducible by the HTLV-II *tat* protein in transient-expression assays. These results demonstrate that the alterations in the pattern of cellular proteins which bind to the HTLV-I and HTLV-II LTRs may in part be responsible for differences in the transcriptional regulation of these two related viruses.

MATERIALS AND METHODS

DNA constructions and fragments used in DNase I footprinting. To generate plasmids in which probes for DNase I footprinting could be isolated, a 342-bp *Hha*I-*Sma*I fragment (+22 to −322) from the HTLV-I LTR (62) and a 345-bp *Bam*HI-*Ava*I fragment (+52 to −293) from the HTLV-II LTR (66) were cloned into the *Sma*I site and the *Sma*I-*Bam*HI sites of pUC19, respectively.

To footprint the noncoding strand of the HTLV-I LTR a 576-bp *Eco*RI-*Pvu*II fragment labeled at the *Eco*RI site was isolated from the *Hha*I-*Sma*I-containing HTLV-I pUC19 construct, whereas a 455-bp *Bam*HI-*Pvu*II fragment from this clone labeled at the *Bam*HI site was used to footprint the coding strand. To footprint the noncoding strand of the HTLV-II LTR, a 451-bp or a 358-bp *Bam*HI-*Pvu*II fragment labeled at the *Bam*HI site was isolated from the *Ava*I-*Alu*I or the *Ava*I-*Bam*HI HTLV-II construct, respectively, whereas a 572-bp *Eco*RI-*Pvu*II fragment from the latter clone labeled at the *Eco*RI site was used to footprint the coding strand. All probes used for DNase I footprinting were end labeled by using γ-^{32}P with T4 kinase, and the fragments were gel isolated and electroeluted before being used in DNase I footprinting assays.

The HTLV-I fragments used for construction of HTLV-I LTR-CAT gene fusions included fragments restricted at *Hin*fI-*Sma*I (−33 to −322), *Hin*fI-*Alu*I (−33 to −167), *Mbo*II-*Sma*I (−169 to −322), and *Hin*fI-*Aha*II (−33 to −244). The HTLV-II fragments used for construction of HTLV-II LTR-CAT gene fusions were *Hin*fI-*Hin*fI (−27 to −271), *Alu*I-*Hin*fI (−144 to −271), and *Hin*fI-*Alu*I (−27 to −144). Each fragment was treated with Klenow fragment, gel isolated, and ligated into pUC19 which had been cut with *Xba*I and end filled with Klenow fragment. These clones were then cut at the *Bam*HI-*Sal*I sites in the pUC19 polylinker and ligated into *Bgl*II-*Sal*I pA10 CAT, which is an enhancerless SV40 CAT fusion (40). Clones which contained inserts in both orientations were selected for use in transfection assays.

Cell lines and preparation of cellular extracts. HeLa spinner cells were maintained in suspension culture in minimal essential medium with 5% newborn calf serum. H9 (an uninfected T-cell lymphoid cell line) (19), Mo (an HTLV-II infected T-cell lymphoid cell line) (60), and SLB (an HTLV-I infected T-cell lymphoid cell line) (38) were grown in Iscoves media with 10% fetal calf serum. HeLa plate cells were grown in Dulbecco modified Eagle medium with 5% newborn calf serum.

For all extracts, a minimum of 7 ml (packed cell volume) was used. Nuclear extracts were prepared as described previously (12), loaded onto a heparin-agarose column, washed with 5 column volumes of buffer containing 100 mM KCl, eluted with buffer containing 500 mM KCl, and dialyzed against a buffer containing 20 mM Tris (pH 7.9), 100 mM KCl, 0.2 mM EDTA, 0.5 mM phenylmethylsufonyl fluoride, 0.5 mM dithiothreitol, and 20% glycerol. This extract was then used in DNase I footprinting assays.

DNase I footprinting. γ-^{32}P end-labeled DNA probe (1 to 5 ng) was added to each 60-μl reaction mixture, along with extract (0 to 200 μg), poly(dI-dC) (3 μg), and final concentrations of 10 mM Tris (pH 7.4), 50 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, and 10% glycerol. The DNA and extract were allowed to bind for 30 min at room temperature before the addition of DNase I (2 to 20 μg/ml), 1 mM $MgCl_2$ and 0.5 mM $CaCl_2$ (final concentrations). The reaction was stopped after 30 s with phenol-chloroform, and the products were ethanol precipitated and loaded on a 10% polyacrylamide–8 M urea sequencing gel (20, 76). G+A and C+T Maxam-Gilbert sequencing reactions were performed for each probe. All gels were then subjected to autoradiography.

Transfection conditions. For the transfections of the HTLV-I LTR-CAT and HTLV-II LTR-CAT constructs, HeLa cell plates were split on the day prior to transfection so that cells were 50 to 75% confluent at the time of transfection. The constructs (10 μg each) in either the presence of an expression plasmid containing the *tat*-II gene (4) or a beta-globin control plasmid (27) were transfected by using calcium phosphate with glycerol shock at 4 h as described previously (76). Transfected cells were harvested at 48 h and used for assays of chloramphenicol acetyltransferase (CAT) activity as described previously (26, 27). Following autoradiography of each CAT assay, both the unacetylated and acetylated chloramphenicol were quantitated by scintillation counting.

RESULTS

DNase I footprinting of the HTLV-I LTR. DNase I footprinting of both the coding and noncoding strands of the HTLV-I LTR was performed to identify cellular DNA-binding proteins. Owing to the large distances between several of these binding sites and the difficulty in visualizing DNase I-protected regions near the top of the gels, some sites are visualized only on one of the two DNA strands. Partially purified cellular extracts prepared from HeLa, H9 (T-cell lymphoid cell line) (19), Mo (HTLV-II transformed T-cell line) (60), and SLB (HTLV-I transformed T-cell line) (38) were used in these studies.

DNase I footprinting of the HTLV-I LTR revealed five protected regions (Fig. 1A and B). Sites 1, 2, 3, and 4 are seen on the noncoding strand (Fig. 1A), whereas sites 4 and 5 are seen on the coding strand (Fig. 1B). Relative to the transcriptional start site, site 1 extends from −38 to −74, site 2 extends from −82 to −97, site 3 extends from −110 to −151, site 4 extends from −192 to −247, and site 5 extends from −258 to −273. On the noncoding strand (Fig. 1A) partial protection was seen over site 1, whereas sites 2, 3, and 4 gave relatively complete protection. On the coding strand there was extension of protection over site 4 in lymphoid extracts (Fig. 1B, lanes 1 to 5) as compared with HeLa cell extracts (Fig. 1B, lanes 6 and 7), such that the 21-bp repeat farthest from the transcriptional start site was completely protected only in lymphoid extracts. There was partial protection on the coding strand over site 5 with all cell lines tested (Fig. 1B).

A summary of the sequences protected in the HTLV-I

LTR is shown in Fig. 2. An examination of these sequences reveals that site 1 contains the DNA sequence encoding the polyadenylation recognition sequence which is located 10 nucleotides upstream of the TATA sequence. No protection was seen over the TATA sequence. Site 2 contains the 21-bp repeat closest to the transcriptional start site. Site 3 contains two direct repeats of a sequence GAAGCCACC, which has previously been shown to be important for HTLV-I transcriptional regulation (3, 49). Site 4 contains an extensive region of protection including two 21-bp repeats and the sequences between these repeats. Site 5 contains a sequence, GAAAGGTCA, which may be important in regulating the basal level of transcription in the HTLV-I LTR (57).

DNase I footprinting of the HTLV-II LTR. Three protected regions were seen by DNase I footprinting of the HTLV-II LTR (Fig. 3) Sites 1 and 2 are seen on the noncoding strand (Fig. 3A), whereas sites 1, 2, and 3 are seen on the coding strand (Fig. 3B). Relative to the transcriptional start site, site 1 extends from −35 to −51, site extends 2 from −180 to −221, and site 3 extends from −247 to −261.

On the noncoding strand, there was protection over sites 1 and 2. Extracts prepared from the HTLV-II-infected cell line (Mo) gave extended protection over site 1 as compared with extracts prepared from the other cell lines (Fig. 3A, lanes 1 and 2). On the coding strand, there was protection over sites 1 and 3 with extracts prepared from all cell lines, but extension of protection over a core viral enhancer sequence in extracts prepared from lymphoid cell lines (Fig. 3B, lanes 1 to 6) as compared with HeLa cell extracts (Fig. 3B, lanes 7 and 8). This extended protection over the enhancer sequence in site 2 with lymphoid extracts (SLB, H9, and Mo) was also shown by using a shorter HTLV-II fragment (*Ava*I-*Alu*I), which contains both binding sites 2 and 3 (Fig. 3C). There was also protection over site 3 with these extracts (Fig. 3C).

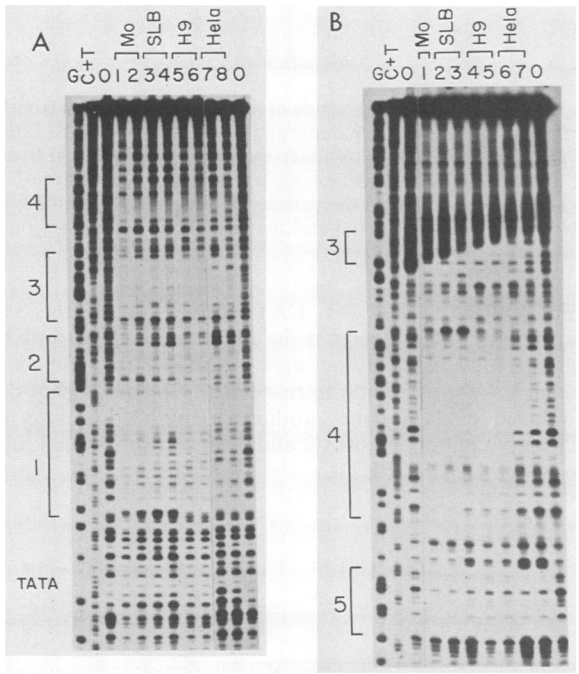

FIG. 1. DNase I footprinting of the HTLV-I LTR. Both the noncoding (panel A) and coding (panel B) strands of a fragment extending from +22 to −322 in the HTLV-I LTR were used in DNase I footprinting. (A) Partially purified extracts used on the noncoding strand were prepared from Mo (lanes 1 and 2), SLB (lanes 3 and 4), H9 (lanes 5 and 6), and HeLa (lanes 7 and 8) cells. Lanes 1, 3, 5, and 7 contain 100 μg of added extract, and lanes 2, 4, 6, and 8 contain 50 μg of extract. (B) For the coding strand, extracts from Mo (lane 1), SLB (lanes 2 and 3), H9 (lanes 4 and 5), and HeLa (lanes 6 and 7) cells were used. Lanes 1, 2, 4, and 6 contain 100 μg of added extract, and lanes 3, 5, and 7 contain 50 μg of extract. Lanes 0 contain no added extract. Lanes G and C+T are Maxam-Gilbert sequencing lanes. The position of the TATA sequence is indicated, as are the positions of the protected regions (sites 1 to 5).

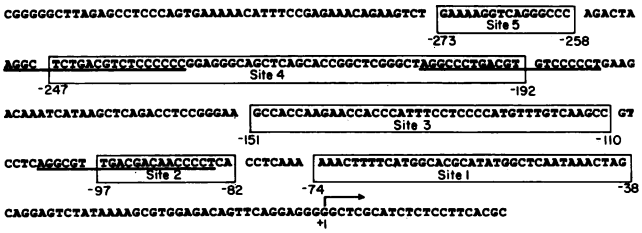

FIG. 2. Sequences protected in the HTLV-I LTR. The sequence of the HTLV-I LTR fragment used in DNase I footprinting extending from +22 to −322 relative to the start of transcription is shown. The regions of DNase I footprinting protection for sites 1, 2, 3, 4, and 5 are boxed, and the nucleotides protected for each site are also indicated. The three 21-bp repeats are underlined, and the start of transcription is indicated at +1 with an arrow.

A summary of the sequences protected in the HTLV-II LTR is shown in Fig. 4. An examination of these sequences reveals that site 1 contains the DNA sequence encoding the polyadenylation recognition sequence which is located 10 bp upstream of the TATA sequence. No protection was noted over the TATA sequence. Minimal protection was seen over the two 21-bp repeats closest to the transcriptional start site, in contrast to results seen in the HTLV-I LTR. Site 2 contains a viral enhancer sequence, TAGGAACTGAAA (10, 13, 25, 30–32), not present in the HTLV-I LTR and the 21-bp repeat farthest from the transcriptional start site. There was extension of protection over this viral enhancer sequence with lymphoid extracts. Site 3 contains a sequence, CAAAAGGTCAG, which has homology to the site 5 protected region in the HTLV-I LTR (Fig. 2).

Transcriptional regulation of the HTLV-I and HTLV-II LTRs. To determine the significance of each of the protected regions in the HTLV-I and HTLV-II LTRs in the transcriptional regulation of the viral LTR, a series of fragments which served as binding domains for cellular proteins were inserted into enhancerless SV40 plasmid pA10 CAT (40). DNase I footprinting of these fragments confirmed that binding activity for cellular proteins was retained by these fragments (data not shown). These HTLV-I and HTLV-II fragments were cloned in both orientations into pA10 CAT and tested after transfection into HeLa cells in both the presence and absence of the *tat*-II gene product. CAT assays were repeated three times, with similar results obtained in each experiment. S1 analysis following transfection of similar HTLV-I LTR CAT constructs has previously demonstrated that CAT activity correlated with steady-state mRNA levels (3, 49, 56).

The results of CAT assays following transfection of the HTLV-I LTR CAT constructs in both the presence and absence of the *tat*-II gene product are shown in Fig. 5A. The fragment containing all five HTLV-I binding domains (−31 to −322) was strongly induced by the *tat*-II protein when inserted in both orientations 5′ to the promoter of pA10 CAT. CAT fusion constructs containing deletions of HTLV-I LTR binding domains (−169 to −322, −33 to −244, and −33 to −167) inserted in both the sense and antisense orienta-

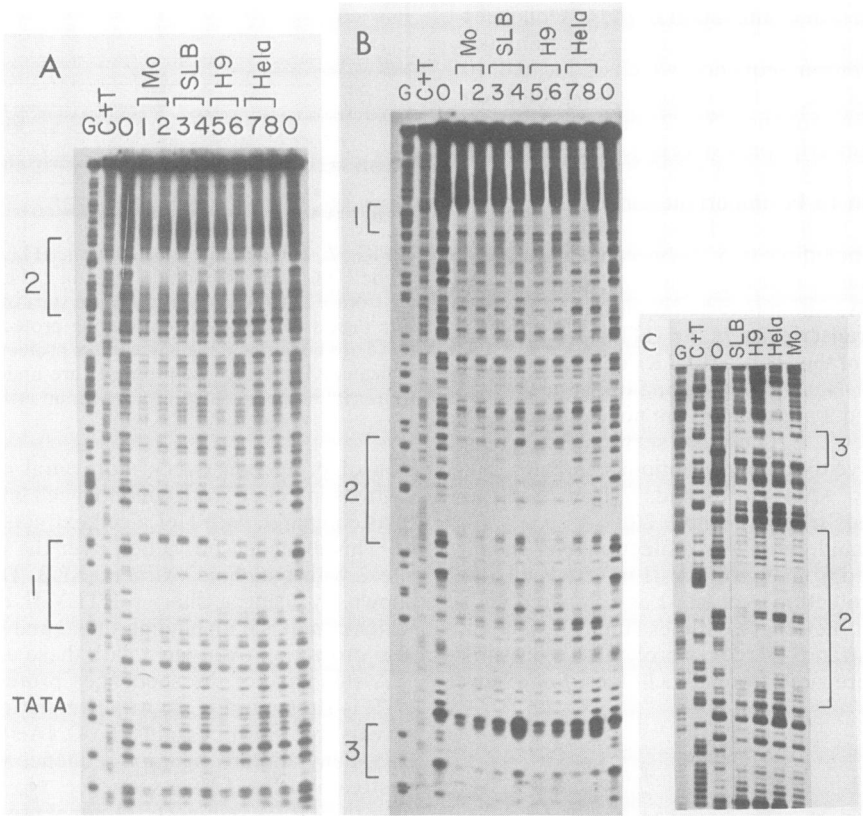

FIG. 3. DNase I footprinting of the HTLV-II LTR. Both the noncoding (A) and the coding (B) strands of a fragment extending from +52 to −293 in the HTLV-II LTR were used in DNase I footprinting. Partially purified extracts were prepared from Mo (lanes 1 and 2), SLB (lanes 3 and 4), H9 (lanes 5 and 6), and HeLa (lanes 7 and 8) cells. Lanes 1, 3, 5, and 7 contain 100 μg of added extract, and lanes 2, 4, 6, and 8 contain 50 μg of extract. Lanes 0 contain no added extract. Lanes G and C+T are Maxam-Gilbert sequencing lanes. The position of the TATA sequence is indicated, as are the positions of the protected regions (sites to 1 to 3). (C) The noncoding strand of a fragment extending from −144 to −271 was used in DNase I footprinting by using extracts prepared from Mo, SLB, H9, and HeLa cells. The protected regions for binding domains 2 and 3 are indicated.

tions gave lower levels of *tat*-II induced CAT activity than did the fragment which contained all five binding domains (Fig. 5A, lanes 5 to 16). The pA10 CAT vector was not induced by the *tat*-II protein (data not shown). However, all fragments remained inducible by the *tat*-II protein.

For the HTLV-II LTR CAT constructs, a fragment containing all three binding domains (−27 to −277) gave strong induction in response to *tat*-II (Fig. 5B, lanes 1 to 4). The fragment which contained only binding sites 2 and 3 (−144 to −271) gave full induction in the sense orientation, but decreased induction in the antisense orientation (Fig. 5B, lanes 5 to 8). The plasmid containing site 1 alone gave no induction in the sense orientation (Fig. 5B, lanes 9 and 10) or the antisense orientation (data not shown).

The induced levels of CAT activity in the presence of the *tat*-II protein for each of the HTLV-I (Fig. 6A) and the HTLV-II (Fig. 6B) constructs are shown, as are the DNA-binding domains present in each fragment. Basal levels of CAT conversion were between 0.17 and 0.42 for each construct tested. For the HTLV-I LTR CAT constructs (Fig. 6A), there was a 20 to 35% CAT conversion in the presence of *tat*-II when all five binding domains were present, but only a 1 to 10% CAT conversion in the presence of fragments which deleted portions of the HTLV-I binding domains. With the HTLV-II LTR CAT constructs, there was a 7% CAT conversion in the presence of *tat*-II when all three binding domains were present (Fig. 6B). When site 1 was deleted, there was a 9% CAT conversion in the sense orientation, but a decrease in induction in the antisense orientation. When binding sites 2 and 3 were deleted, the percent CAT conversion in response to *tat*-II did not differ from basal levels. Further studies are required to determine the relative effects of sites 2 and 3 on transcriptional induction of the HTLV-II LTR.

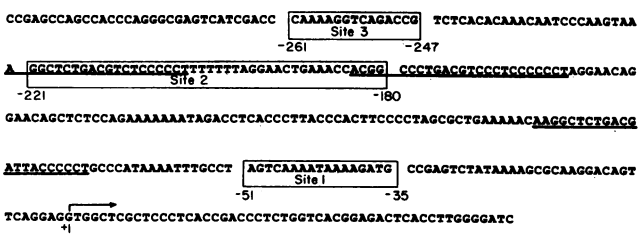

FIG. 4. Sequences protected in the HTLV-II LTR. The sequence of the HTLV-II LTR fragment used in DNase I footprinting extending from +52 to −293 relative to the start of transcription is shown. The regions of DNase I footprinting protection for sites 1, 2, and 3 are boxed, and the nucleotides protected for each site are also indicated. The three 21-bp repeats are underlined, and the start of transcription is indicated at +1 with an arrow.

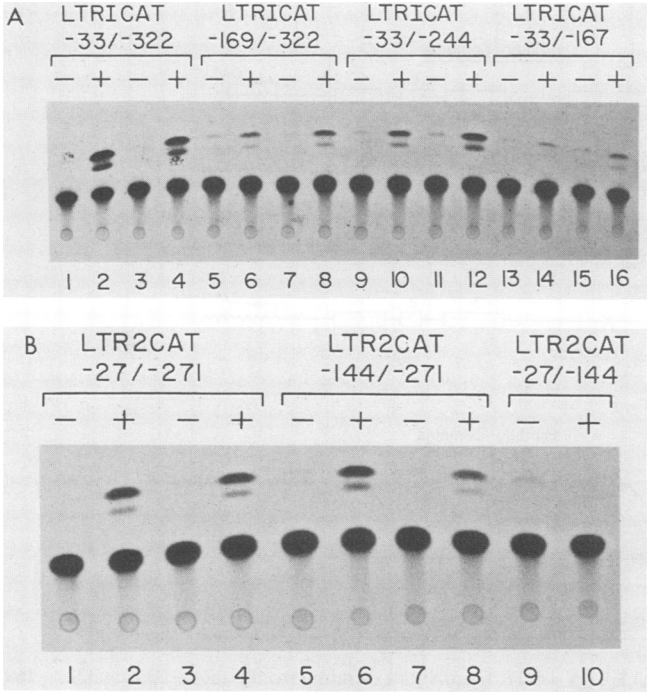

FIG. 5. CAT assays with different HTLV LTR constructs. (A) Fragments from the HTLV-I LTR from either −33 to −322, −169 to −322, −33 to −244, or −33 to −167 were inserted into pA10 CAT and cotransfected into HeLa cells either in the presence of a control plasmid (indicated by −) (odd-numbered lanes) or a *tat*-II expression plasmid (indicated by +) (even-numbered lanes). Fragments in their normal orientation (sense) relative to the transcriptional start site (lanes 1, 2, 5, 6, 9, 10, 13, and 14) and fragments in their opposite orientation (antisense) (lanes 3, 4, 7, 8, 11, 12, 15, and 16) were used in these transfections. (B) Fragments from the HTLV-II LTR from either −27 to −271, −144 to −271, or −27 to −144 were inserted into pA10 CAT and cotransfected into HeLa cells either in the presence of a control plasmid (indicated by −) (odd-numbered lanes) or a *tat*-II expression plasmid (indicated by +) (even-numbered lanes). Fragments in their normal orientation (sense) relative to the transcriptional start site (lanes 1, 2, 5, 6, 9, and 10) and fragments in the opposite orientation (antisense) (lanes 3, 4, 7, and 8) were used in these transfections.

DISCUSSION

Multiple sequences in the HTLV-I and HTLV-II LTRs serve as binding sites for cellular proteins. The ability of these sequences to function as *tat*-inducible enhancer elements in transfection experiments suggests that regions of binding demonstrated in vitro probably serve as functional elements in vivo. Studies on a number of other promoters including the SV40 and c-*fos* promoters have correlated in vitro binding data with in vivo functional data (23, 75, 79). Thus, the binding sites demonstrated by in vitro DNase I footprinting probably correlate with regions of the DNA required for in vivo transcriptional regulation.

Several previous mutagenesis studies of the HTLV-I LTR indicated that multiple regions of the LTR were required for transcriptional regulation (3, 16, 48, 49, 56, 57, 63, 66). The 21-bp imperfect direct repeats present in the HTLV-I LTR located between −84 and −104, −183 and −203, and −233 and −253 relative to the start of transcription have been demonstrated to be important for *tat*-induced transcriptional induction of the viral LTR (3, 16, 49, 56, 66). Data on the number of these 21-bp repeats critical for *trans*-activation have differed (3, 16, 49, 56, 66). However, it is clear that the level of *tat*-induced induction is low when only one 21-bp repeat is present and that this induction increases markedly when several of these repeats are present (3, 16, 49, 56, 66). In addition, when oligonucleotides complementary to the 21-bp repeats were tested with heterologous promoters, the level of *trans*-activation appeared lower than when these elements were present in the viral LTR (3, 66). Thus, sequences other than the 21-bp repeats are probably likely involved in transcriptional regulation of the HTLVI LTR, and the spacing between these sequences may be important (3, 48, 49, 56, 57).

Other studies on the HTLV-I LTR have demonstrated the importance of a region between −242 and −306 that is important in basal transcription (57), a region between −117 and −160 that is important in *tat*-induced activation (3, 49), and a region between +315 and −159 that is important in gene expression, perhaps by increasing mRNA stability or utilization (48, 56, 57). Our studies do not characterize the relative effects of these other HTLV regulatory regions with respect to those of the 21-bp repeats. Oligonucleotide-directed mutagenesis of the HTLV LTR will be required to address this point.

The DNase I footprinting results presented here are consistent with previous mutagenesis results indicating that multiple regions of the HTLV-I LTR are important in its transcriptional regulation. Five binding domains were noted in the HTLV-I LTR, but DNase I footprinting of even longer LTR fragments is required to definitively show the total number of binding sites present. Site 1 contains the DNA sequence encoding the polyadenylation recognition site and a region near position −55 previously suggested to be an important region in HTLV-I transcriptional regulation (57). Site 2 contains the first 21-bp repeat. Site 3 contains two direct repeats of a sequence, GAAGCCCACC, which has been shown to be located in a region important in *tat*-induced *trans*-activation (3, 49). Within this larger repeated sequence, a smaller sequence, CA(A/T)CC, is repeated four times in site 3. Site 4 contains a 58-bp DNase I protected region which is flanked by two 21-bp repeats. Site 5 contains a sequence, TAAAAGGTCAG, which may be important in regulating basal levels of transcription (57).

A detailed mutational analysis of the HTLV-II LTR has not been reported. However, oligonucleotides complementary to the HTLV-II 21-bp repeats have been shown to function as *tat*-inducible enhancer elements (66). DNase I footprinting of the HTLV-II LTR reveals three regions of DNase I protection. Site 1 includes the DNA sequence encoding the polyadenylation recognition sequence and corresponds to a similar protected region seen in the HTLV-I LTR (site 1). Site 2 contains the 21-bp repeat farthest from the transcriptional start site and a viral enhancer core sequence, TAGGAACTGAA, which is not present in the HTLV-I LTR. This enhancer sequence has been demonstrated for a number of other promoters including those of a number of viruses (adenovirus, polyomavirus, SV40, Moloney leukemia virus, Friend leukemia virus, avian sarcoma virus, and mouse mammary tumor virus [10, 13, 25, 30–32]) and the beta interferon gene (25). DNase I protection over site 2 differs in extracts prepared from lymphoid cells compared with HeLa cells. Similar alterations in binding over enhancer sequences by using lymphoid cell rather than HeLa cell extracts have been demonstrated for SV40 (11) and the human immunodeficiency virus (76). This altered binding may affect the regulation of certain genes in lymphoid cells (2, 22, 28). Site 3 contains a sequence which has

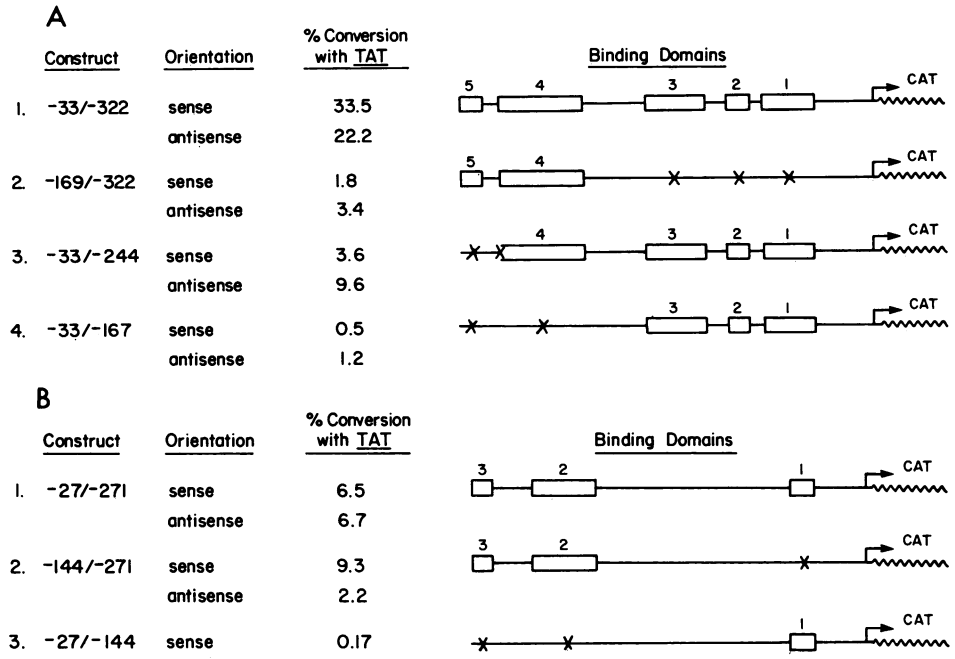

FIG. 6. CAT expression of HTLV-I and HTLV-II LTR constructs. (A) HTLV-I LTR fragments extending from either −33 to −322, −169 to −322, −33 to −244, or −33 to −167 in both the sense and antisense orientations were fused to the pA10 CAT transcriptional start site. (B) HTLV-II LTR fragments extending from either −27 to −271, −144 to −271, or −27 to −144 in both the sense and antisense orientations were fused to the pA10 CAT transcriptional start site. Symbols: ▭, binding domains encompassed by each fragment; ×, regions deleted; ——, pA10 CAT promoter region; ↱, transcriptional start site; ∿∿, CAT gene. The percent of CAT conversion in the presence of the *tat* protein was calculated by scintillation counting of unacetylated and acetylated [^{14}C]chloramphenicol.

homology to site 5 in the HTLV-I LTR and may be important in regulating basal levels of transcription.

The reason for our inability to detect complete protection in the HTLV-II LTR over the two 21-bp repeats closest to the transcriptional start site with the same cell extracts that resulted in protection over all three 21-bp repeats in the HTLV-I LTR is not known. This may be because not only the TGACG core sequence but also flanking DNA sequences affect the binding of cellular proteins to each 21-bp repeat. Thus, differences in the DNA sequences found in the 21-bp repeats may alter the affinity with which the same factor binds to each repeat or may allow for the binding of different cellular proteins to these sequences.

Binding of cellular proteins to other promoters containing the same core sequence as found in the 21-bp repeats, TGACG, has been demonstrated for the adenovirus early region 2, 3, and 4 promoters (20, 42, 67), the somatostatin gene (44), and the c-*fos* gene (23, 52). The CREB protein, a 43-kilodalton cellular protein, has been purified and found to bind to a regulatory region of the somatostatin promoter containing the TGACG core sequence (47). Thus, it is possible that this sequence found in the 21-bp repeats serves as a binding site for the CREB protein. In addition, a 47-kilodalton cellular protein, AP-1 (1, 44), has been purified and found to bind to a closely related core sequence, TGACT, in the SV40 and methallothionein promoters. This protein may also be involved in HTLV-I and HTLV-II transcriptional regulation.

Multiple sequences and cellular proteins are probably required for transcriptional regulation of HTLV-I and HTLV-II. At present it is not clear how many cellular proteins bind to each site, but it appears that some sites may bind multiple cellular proteins. It is likely that the interaction of proteins binding to these transcriptional regulatory elements is important in both basal and *tat*-induced transcriptional regulation. However, it is unlikely that the *tat* protein itself directly binds to the viral LTR or results in major alterations in DNase I footprinting of the viral LTR, since extracts prepared from HTLV-infected lymphoid cells do not differ markedly from uninfected lymphoid cell extracts. More likely, the *tat* proteins which can also stimulate a variety of cellular and viral genes (5, 9, 45) act indirectly in a manner similar to the adenovirus E1A protein (34, 39, 42, 67, 78). Differences in transcriptional regulatory sequences, the number and type of cellular DNA-binding proteins, and the specificity of the *tat* proteins may all be responsible for the differences observed in the genetic regulation between HTLV-I and HTLV-II.

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