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In Vitro Mutagenesis of the Human T-Cell Leukemia Virus Types I and II *tax* Genes

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The *tax* gene of the human T-cell leukemia virus types I and II (HTLV-I and HTLV-II) is essential for viral replication and acts by increasing the level of RNA transcription. The *tax* genes of HTLV-I and HTLV-II encode proteins of 40 and 37 kilodaltons, respectively. By in vitro mutagenesis of the *tax* gene, we have investigated those regions of the protein which are essential for its function. Mutation of either the amino- or carboxy-terminal domain of the protein resulted in loss of *trans*-activation ability. In addition, specificity of its activity with regard to *trans*-activation of either the HTLV-I or HTLV-II long terminal repeats was conferred by the first 59 amino acids.

The human T-cell leukemia viruses types I and II (HTLV-I and HTLV-II) are associated with specific human T-cell malignancies (8, 12, 24). Both viruses will immortalize peripheral blood T lymphocytes in vitro, as defined by their continuous proliferation in the absence of exogenous interleukin-2 (3, 19, 21). The presence of a unique region at the 3′ end of the genome, originally known as the X region, is common to HTLV-I, HTLV-II (11, 16, 28, 31), and the related bovine leukemia virus (BLV) (26). There are two genes located within this region of the genome, the *tax* gene (previously referred to as *x*, *x-lor*, or *tat*), and the *rex* gene, which encodes a protein responsible for regulation of expression of viral proteins. We have previously demonstrated that the products of both are essential for the replication of HTLV-II; mutants of *tax* and *rex* transcribe very low levels of mRNA (4).

The proteins encoded by the *tax* gene ($p40^{taxI}$ for HTLV-I, $p37^{taxII}$ for HTLV-II [16, 33], and p34 for bovine leukemia virus [17, 25]) are necessary for *trans*-acting transcriptional activation (*trans*-activation) of the homologous viral long terminal repeat (LTR) (1, 6, 22, 34). We have analyzed the function of the *tax* protein by in vitro mutagenesis. Two groups of mutations were investigated: (i) those which abolish the activity of the *tax* protein, and (ii) those which alter the specificity of the *tax* protein with regard to activation of either HTLV-I or HTLV-II LTRs. The results of these experiments indicate that sequences at both the amino terminus and the carboxy terminus of the *tax* protein are required for its activity and that the specificity of the protein is determined by the amino terminus.

The expression vector 91023B is designed for high-efficiency expression in COS cells (37), and the *tax*I and *tax*II constructions in 91023B (called 91023-*tax*I and 91023-*tax*II) have been previously described (29). Briefly, a 62-nucleotide (nt) synthetic oligonucleotide encoding the first 17 amino acids of the *tax* gene was ligated to a restriction enzyme fragment encoding the remainder of the gene. These constructions, therefore, expressed only the *tax* gene, since they did not contain the first coding exon of the overlapping *rex* gene; therefore, all experiments reported here were performed in the absence of the *rex* products. This insert was ligated into the 91023B vector under transcriptional control of the adenovirus major late promoter. Recombinants between the *tax*I and *tax*II constructions were made by using a conserved *Cla*I restriction enzyme site located at a position equivalent to 59 amino acids from the NH_2 terminus of the protein (nt 7473 in the HTLV-I genome and 7385 in the HTLV-II genome) (Fig. 1). Site-directed mutations at amino acid 13 were made by codon substitutions in the synthetic linker encoding the first 17 amino acids of the protein. *tax*Ityr[13] was made by a TTC→TAT codon substitution in the *tax*I linker at position 50, and *tax*IIphe[13] was made by a TAT→TTC codon substitution at position 50 in the *tax*II linker sequence (29). Recombinants between these mutants and the wild-type proteins (*tax*Ityr[13]-*tax*II and *tax*IIphe[13]-*tax*I) were made by using the conserved *Cla*I site as described above (Fig. 1). *tax*II283Δ was made by a BAL 31 nuclease deletion of the 3′ end of the wild-type *tax*II construct and insertion of a synthetic oligonucleotide which contains a termination codon in all three reading frames (5′-GATCTTAAATAAGTAATCTAG) and encodes 283 amino acids compared with 331 amino acids of $p37^{taxII}$ (Fig. 1). Transcription of this construct is driven by the cytomegalovirus immediate-early promoter from plasmid BC12/CMV/IL-2 (5). The identity of each of the above constructions was confirmed by DNA sequencing, using the method of Maxam and Gilbert (18). The recombinant LTR-I-CAT and LTR-II-CAT constructions each contain the entire HTLV-I and -II LTRs, respectively, linked to the bacterial chloramphenicol acetyltransferase (CAT) gene and have been previously described (2, 4).

COS cells (9) were grown in Dulbecco modified Eagle medium supplemented with 6% calf serum and 3% fetal calf serum. Cotransfections of LTR-CAT plus *tax* gene expression constructs were performed by the calcium phosphate coprecipitation method (10) with modifications described elsewhere (29). Cells were harvested 40 to 48 h after transfection. CAT assays were performed on cell extracts prepared by three cycles of freezing and thawing and were analyzed by thin-layer chromatography (10). Quantification of CAT activity was determined by liquid scintillation counting of unmodified and acetylated forms of chloramphenicol.

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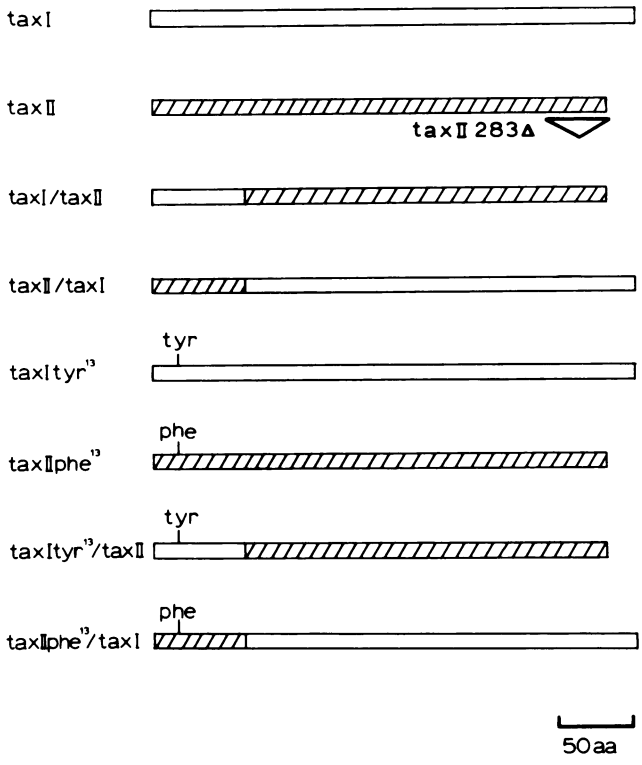

FIG. 1. Recombinant *tax* gene constructions. Schematic representation and mutant *tax* proteins. Names of the recombinant constructions are shown on the left. ▭, HTLV-I sequences; ▨, HTLV-II sequences. The carboxy-terminus region of the wild-type *tax*II protein deleted in *tax*II 238Δ is indicated beneath the *tax*II construct.

Cytoplasmic RNA was isolated from transfected cells 40 to 48 h after transfection by Nonidet P-40 lysis of cells (14). Total RNA was prepared from HTLV-infected cells by urea lysis and sedimentation through CsCl (13). In each nuclease protection experiment, a total of 25 μg of RNA was coprecipitated with 1.5×10^5 cpm of hybridization probe. Synthetic oligonucleotides complementary to nt 297 to 422 of the HTLV-I provirus genome (28) or 294 to 383 of the HTLV-II genome (31) were used as hybridization probes. These oligonucleotides were end labeled with ^{32}P to high specific activity (approximately 5×10^8 cpm/μg), a very sensitive means of detecting RNA transcripts. For confirmation, restriction endonuclease fragments derived from cloned HTLV proviruses were also used (see below). These probes had a specific activity of 2×10^6 and 5×10^6 cpm/μg. Hybridization, S_1 nuclease digestion, and gel electrophoresis were performed as described previously (36).

We initially tested the regions of the HTLV-II *tax* gene which are necessary for its activity. The HTLV-II *tax* gene encodes a protein of 331 amino acids. Previous studies by our laboratory have shown that the 17 NH_2-terminal amino acids are required for activation by the HTLV-II *tax* gene (35). Alteration of the amino acid at position 5 or deletion of amino acids 2 to 17 resulted in *tax* proteins which are not functional in regard to activation of the HTLV-II LTR (Table 1). A frameshift mutation at a position corresponding to amino acid position 59, resulting in a loss of downstream sequences, also abolishes activity (1). To investigate the role of the carboxy terminus further, we tested a deletion mutant generated by BAL 31 mutagenesis of the HTLV-II *tax* gene

TABLE 1. *trans*-Activation of the HTLV-I and -II LTRs by *tax* proteins[a]

Protein	% Acetylation of [^{14}C]chloramphenicol after *trans*-activation of[b]:	
	LTR-I-CAT	LTR-II-CAT
91023B	–	–
*tax*I	14	–
*tax*II	100	100
*tax*I-*tax*II	17	–
*tax*II-*tax*I	55	77
*tax*Ityr^{13}	24	34
*tax*IIphe^{13}	97	55
*tax*Ityr^{13}-*tax*II	100	53
*tax*IIphe^{13}-*tax*I	55	51
*tax*IIleu^{5c}	8	–
*tax*II (Δ2-17)[c]	–	–
*tax*II283Δ	–	–

[a] COS cells were cotransfected with the constructions indicated, and CAT activity was determined as described in the text.

[b] Values for percent acetylation of [^{14}C]chloramphenicol have been normalized to the percentage obtained with the *tax*II construction, since this was the highest value with both LTRs. The numbers represent the average of at least three assays performed with more than one preparation of each plasmid. All results shown were obtained within the linear range of the CAT assay. –, Failure of a construction to *trans*-activate the LTR above the basal level of expression (usually <1% of the value for *tax*II).

[c] Data for *tax*IIleu^5 and *tax*II (Δ2-17) are taken from reference 35.

followed by introduction of a stop codon, resulting in a protein which is 48 amino acids shorter at the carboxy terminus (*tax*II 283Δ, Fig. 1). This mutation resulted in loss of the ability to *trans*-activate the HTLV-II LTR (Table 1). These results indicate that both the carboxy- and amino-terminal domains of the *tax* protein are required for *trans*-activation.

The HTLV-I and HTLV-II *tax* genes can be distinguished by their phenotype with regard to activation of HTLV-I and HTLV-II LTRs (1, 29). The HTLV-II *tax* gene *trans*-activates the HTLV-I and HTLV-II LTRs, whereas the HTLV-I *tax* gene only *trans*-activates the HTLV-I LTR. We have used the different phenotypes of *tax*I and *tax*II to investigate the region of the *tax* protein which is important for their differential activities.

In vitro reciprocal recombinants between the *tax*I and *tax*II constructions were made in order to identify these regions of the proteins which are responsible for their distinct phenotypes. Table 1 shows the results obtained with the wild-type *tax* genes. As previously shown, *tax*II activated both the HTLV-I and HTLV-II LTRs, whereas *tax*I *trans*-activated the HTLV-I LTR to a lesser extent than *tax*II and did not detectably activate the HTLV-II LTR. The *tax* genes are sufficiently conserved between HTLV-I and -II (31) to allow construction of recombinants which maintain a colinear primary amino acid sequence. These recombinants were made by using a *Cla*I restriction endonuclease site conserved in HTLV-I and -II, generating hybrid genes encoding 59 amino acids at the NH_2 terminus derived from one protein and 294 or 272 amino acids at the COOH terminus derived from p40$^{tax\text{I}}$ or p37$^{tax\text{II}}$, respectively (Fig. 1). Both of these hybrid *tax* proteins were functional in *trans*-activation and resulted in significant production of CAT activity when transfected with the LTR-CAT constructions. The *tax*I-*tax*II hybrid with 59 amino acids derived from the NH_2 terminus of *tax*I had a similar phenotype to *tax*I, since it activated the HTLV-I LTR but not the HTLV-II LTR (Table 1). The *tax*II-*tax*I hybrid with 59 amino acids from the NH_2 terminus of *tax*II was similar in activity to the

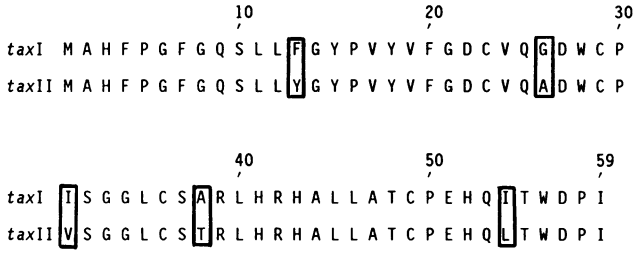

FIG. 2. Comparison of the NH_2-terminal 59 amino acids of *tax*I and *tax*II. The predicted amino acid sequences of *tax*I (28) and *tax*II (31) are shown. Single-amino-acid differences in this region are indicated. Note that *tax*II contains a proline residue at amino acid position 5, as previously corrected (29).

wild-type *tax*II protein, although with slightly reduced activity on both the HTLV-I and -II LTRs (55 and 77%, respectively). These results demonstrate that the amino-terminal 59 amino acids of the *tax* protein predominate in determining the specificity of *trans*-activation of the protein.

There are a total of five amino acid substitutions between $p40^{taxI}$ and $p37^{taxII}$ in the NH_2-terminal 59 residues of the protein (Fig. 2). The first difference is at amino acid position 13, where $p40^{taxI}$ has a phenylalanine residue and $p37^{taxII}$ has a tyrosine residue. Since amino acids 2 to 17 are critical for *tax* protein activity, we tested reciprocal amino acid substitutions at position 13. The results obtained with the position 13 mutants can be summarized as follows (Table 1). Substitution of tyrosine for phenylalanine or vice versa at position 13 quantitatively altered the pattern of *trans*-activation. Wild-type *tax*I did not activate the HTLV-II LTR, but this substitution enabled the resulting protein to activate the HTLV-II LTR. Thus, a single amino acid change was able to confer upon the *tax*I gene an ability to activate the HTLV-II LTR, although the level of activation was not as great as that with wild-type *tax*II or the *tax*II-*tax*I recombinant shown above. The reciprocal substitution of phenylalanine for tyrosine in *tax*II had minimal effects upon *trans*-activation, the mutant being similar to wild-type *tax*II in its activity. These data indicate that the amino acid residue at position 13 of the *tax* protein is a critical factor in controlling the pattern of *trans*-activation by the *tax* protein. However, the identity of this single amino acid is insufficient to determine the complete *trans*-activation phenotype observed with the 59-amino-acid hybrid proteins.

The above results were confirmed by analysis of the *trans*-activation behavior of further genetic recombinants between the amino acid position 13 mutants and the wild-type genes (Table 1). Recombinants between the wild-type *tax* genes and the position 13 mutants (*tax*Ityr^{13}-*tax*II and *tax*IIphe^{13}-*tax*I) were constructed about the conserved *Cla*I site as described above (Fig. 1). In each case, the double recombinants showed the phenotype determined by the amino-terminal 59 amino acids, confirming that this region is critical for the specificity of the protein. The *tax*IIphe^{13}-*tax*I recombinant had a similar specificity to both *tax*IIphe^{13} and wild-type *tax*II. The reciprocal *tax*Ityr^{13}-*tax*II recombinant had a phenotype similar to *tax*Ityr^{13} and was able to activate the HTLV-II LTR.

These results demonstrate that the amino acid at position 13 is important in determining the phenotype of the *tax* protein. However, the other four amino acid substitutions in the NH_2-terminal 59 amino acids must also contribute to the overall phenotype of the protein and the observed differences between the HTLV-I and -II *tax* proteins.

The amount of *trans*-activation induced by the *tax* gene (Table 1) generally corresponds to the relative level of RNA transcribed from the LTR (4, 6). We confirmed that the CAT activity correlated with RNA transcription in these experiments by analyzing RNA transcripts by S1 nuclease protection in parallel with the assays for CAT activity. RNA from cells cotransfected with the HTLV-I LTR-CAT construction (LTR-I-CAT) plus 91023-*tax* constructions was used in S1 nuclease protection studies (Fig. 3). With LTR-I-CAT plus 91023B vector alone, no protected fragment was seen, whereas cotransfection with the *tax* expression constructions resulted in protection of a fragment of 70 nt, corresponding to initiation of transcription at nt 353 (cap site) (28). The number of RNA transcripts which initiated at the cap site of the LTR correlated with the overall amount of CAT activity measured in these experiments (Table 1). Similar results were obtained with a 412-nt *Sma*I-*Bam*HI fragment of the HTLV-I LTR as the probe (positions 31 to 442) (data not shown).

Figure 4 shows the results obtained with the *tax*I and *tax*II constructions to activate transcription in *trans* from LTR-II-CAT. As expected, no nuclease-protected band was seen with the vector 91023B alone or with *tax*I, since $p40^{taxI}$ does not detectably activate the HTLV-II LTR. A protected fragment of 70 nt was seen with *tax*II, which corresponds to initiation of transcription at the U3-R boundary of the LTR as defined by S1 nuclease analysis of viral RNA (30). The *tax*I-*tax*II recombinant failed to activate transcription of the HTLV-II LTR, as did the *tax*I construction, while the *tax*II-*tax*I recombinant behaved similarly to *tax*II (Fig. 4). Therefore, as with *trans*-activation of the HTLV-I LTR, the relative abundance of RNA transcribed from the HTLV-II LTR corresponded to the level of activation measured by CAT activity.

The studies presented here demonstrate that HTLV *tax* proteins containing site-directed mutations within the first 17 amino acids at the NH_2 terminus have *trans*-activation phenotypes distinct from those of the wild-type molecules. The ability to discriminate between the *trans*-activation phenotype of $p40^{taxI}$ and $p37^{taxII}$ from HTLV-I and HTLV-II, respectively (29), has allowed further mapping of functional domains within the *tax* protein which are involved in *trans*-activation. Our results show that the amino-terminal part of the *tax* protein encodes functions necessary for *trans*-activation. This region of the protein is not only critical for the overall *trans*-activation capability of the protein, but the identity of the first 59 amino acids is sufficient to determine the specificity of the protein with regard to *trans*-activation of HTLV-I and HTLV-II LTRs.

Among the five amino acid substitutions in the first 59 amino acids of $p40^{taxI}$ and $p37^{taxII}$, alteration of just one of these residues at position 13 resulted in *tax* proteins with altered specificity for *trans*-activation. A single-amino-acid substitution at position 13 was sufficient to allow a mutant HTLV-I *tax* protein to *trans*-activate the HTLV-II LTR, but the reciprocal substitution in the HTLV-II *tax* protein did not substantially alter its phenotype. Thus, the other four amino acid substitutions must also contribute to the overall *trans*-activation capability of the protein, but the effects of these other substitutions were not individually examined. None of the five substitutions involved a radical change in the chemical nature of the residues involved (Fig. 2); for example, at position 13, phenylalanine and tyrosine both contain aromatic side groups. Although the specificity of the protein is conferred by the amino-terminal domain, the carboxy terminus is also required, since a small deletion of

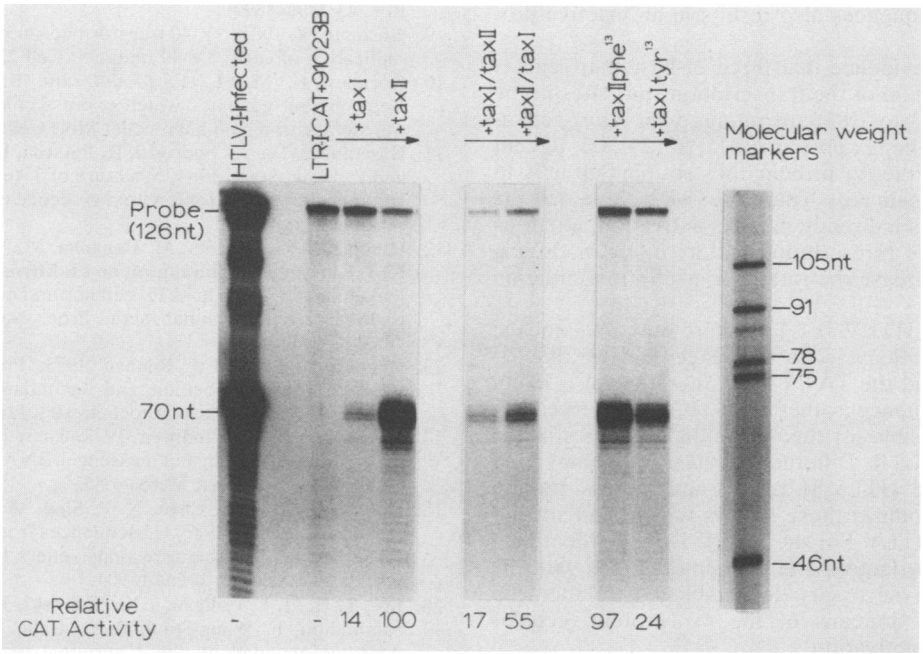

FIG. 3. Analysis of transcription from the HTLV-I LTR. S1 nuclease analysis of RNA from HTLV-I-infected cells (SLB-I) (33) (lane 1), COS cells cotransfected with either LTR-I-CAT plus 91023B vector control (lane 2), or recombinant *tax* expression constructs as indicated above the lanes (lanes 3 to 8). Shown is an autoradiograph of a typical representative experiment. The position of the 126-nt synthetic oligonucleotide hybridization probe which spans the cap site of the HTLV-I LTR is indicated. A protected fragment of 70 nt corresponds to RNA transcripts initiated at the cap site. Molecular weight markers are ^{32}P-end-labeled *Sau*3A digestion fragments of pBR322. Values for relative CAT activity are taken from Table 1.

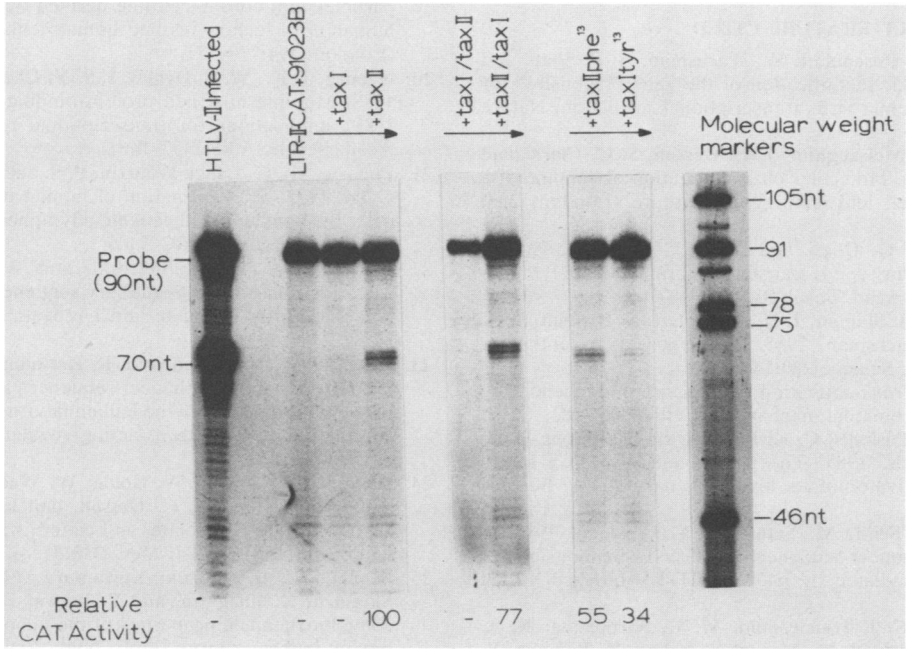

FIG. 4. Analysis of transcription from the HTLV-II LTR. S1 nuclease analysis of RNA from HTLV-II-infected cells (Mo-T) (27) (lane 1), COS cells cotransfected with either LTR-II-CAT plus 91023B vector control (lane 2), or recombinant *tax* expression constructs as indicated above the lanes (lanes 3 to 8). Shown is an autoradiograph of a typical representative experiment. The position of the 90-nt synthetic oligonucleotide hybridization probe which spans the cap site of the HTLV-II LTR is indicated. A protected fragment of 70 nt corresponds to RNA transcripts initiated at the cap site. Molecular weight markers are ^{32}P-end-labeled *Sau*3A digestion fragments of pBR 322. Values for relative CAT activity are taken from Table 1.

carboxy-terminal sequences also results in an inactive protein.

There is genetic evidence that three 21-base-pair repeats located in U3 upstream of the transcription start sites in the HTLV-I LTR (−253 to −83) are required for *trans*-activation of the LTR by the *tax* protein (7, 15, 22, 23, 32). Recent results indicate that the *tax* protein does not bind directly to the 21-base-pair repeats (20). Therefore, we believe that the *tax* protein functions indirectly in *trans*-activation, either as part of a complex of transcription factors or by modifying these factors, to increase the rate of transcription initiation (20).

The HTLV-I and HTLV-II LTRs share only 30% nucleic acid sequence homology, with the exception of the 21-base-pair repeats and the TATA box. Therefore, it is likely that nucleotide sequences other than these conserved sequences are responsible for the differential activities of the *tax* proteins on the LTR. Different cellular factors may bind to the HTLV-I and HTLV-II LTRs, and the *tax* protein interacts with or modifies those factors which bind specifically to either the HTLV-I or the HTLV-II LTR. Identification of these cellular factors and their interactions with the *tax* protein will be necessary to establish a relationship between functional domains of the *tax* protein, cellular proteins, and *trans*-activation.

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