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HTLV *x* Gene Mutants Exhibit Novel Transcriptional Regulatory Phenotypes

WILLIAM WACHSMAN, ALAN J. CANN, JANICE L. WILLIAMS, DENNIS J. SLAMON, LARRY SOUZA, NEIL P. SHAH, IRVIN S. Y. CHEN

The human T-cell leukemia viruses, HTLV-I and HTLV-II, contain a gene, termed *x*, with transcriptional regulatory function. The properties of the *x* proteins were analyzed by constructing mutant genes containing site-directed deletions and point mutations. The results demonstrate that the amino terminal 17 amino acids of the *x* protein constitute part of a functional domain that is critical for the transcriptional activating properties of the protein. Within this region, substitution of a leucine residue for a proline residue results in major changes in the *trans*-activation phenotype of the protein. The mutant HTLV-II *x* protein, though incapable of activating the HTLV-II long terminal repeat, will block *trans*-activation of the HTLV-II long terminal repeat by the wild-type protein. The altered phenotype of this mutant suggests a potential negative regulatory function of the *x* protein.

THE HUMAN T-CELL LEUKEMIA VIrus (HTLV), types I and II, and bovine leukemia virus (BLV) comprise a distinct group of oncogenic retroviruses with common structural and functional features. All are etiologically associated with lymphoid malignancies: HTLV-I with adult T-cell leukemia (*1*), HTLV-II with some cases of variant T-cell hairy-cell leukemia (*2*), and BLV with enzootic leukosis in cattle and sheep (*3*). Studies in vitro show that HTLV-I and HTLV-II immortalize normal T cells from humans, primates, and rodents (*4*). Because they lack sequences homologous to a known oncogene (*5*), and because they exhibit a random pattern of viral integration (*6*), HTLV-I, HTLV-II, and BLV appear to induce leukemogenesis by a mechanism distinct from that of other groups of oncogenic retroviruses.

Sequences unique to HTLV and BLV, termed the X region, are located at the 3′ end of the viral genome (*5*). We have shown through mutational studies that the HTLV-II X region is required for efficient replication of the virus, and that it acts in *trans* to increase the level of proviral transcription (*7*). At least two proteins appear to be encoded by different but overlapping X region open reading frames (*8*). One of these proteins is produced by the *x* gene (also variously termed the *x-lor* or *tat* gene) in HTLV-I-, HTLV-II-, and BLV-infected cells; its size is 40 kD ($p40^{xI}$), 37 kD ($p37^{xII}$), and 34 kD in these cells, respectively (*9–11*).

Transient cotransfection assays have been used to show that the *x* protein enhances transcription in *trans* (*trans*-activation) from the viral long terminal repeat (LTR) (*12–15*). In addition, the HTLV-II *x* protein, $p37^{xII}$, *trans*-activates heterologous promoters: the HTLV-I LTR and the E1A-inducible early region promoter, EIII, from adenovirus (*16*). The adenovirus EIII promoter is normally dependent on the adenovirus E1A protein for efficient transcription. The E1A gene is essential for efficient adenovirus replication (*17*) and is also necessary for the oncogenic properties of adenovirus in rodent cells (*18*). The functional analogies between *x* and E1A provide further support for a role of *x* in cellular transformation by HTLV. Together, these data support the hypothesis that the *x* gene participates in cellular immortalization by inducing abnormal expression of cellular genes.

The results of our present studies on the mutagenesis of $p40^{xI}$ and $p37^{xII}$ implicate the amino terminus of the molecule as a functional domain for *trans*-activation. Our data demonstrate that a single amino acid substitution at position 5 of the amino terminus in both $p37^{xII}$ and $p40^{xI}$ alters the phenotype of *trans*-activation for various promoters. In addition, the mutant HTLV-II *x* protein inhibits the action of the wild-type protein in *trans*-activation of the HTLV-II LTR.

The *x* gene messenger RNA (mRNA) is composed of three exons, of which the last two are used to encode the *x* protein (*19, 20*). To obtain efficient *x* protein expression, we previously developed constructs of the HTLV-I and HTLV-II *x* genes (termed *x*I and *x*II) that, after transfection, express the native *x* protein without the need for RNA splicing (*21*). These *x* constructs were generated by linking a synthetic DNA oligonucleotide, which encodes amino acids 1 through 17 of the HTLV-I or HTLV-II *x* protein, to the bulk of *x* gene sequences obtained from clones of the respective provirus. The expression vector p91023-B (*22*) was used to express these constructs, yielding high levels of *x* protein in transfected COS cells.

To test whether the amino terminus of $p37^{xII}$ was critical for *trans*-activation, we modified the wild-type *x*II construct by deleting codons 2 through 17 in the oligonucleotide linker (Fig. 1). Expression of this mutant construct, *x*IIΔ(2–17), resulted in production of an appropriately smaller protein of approximately 35 kD (Fig. 2). When cotransfected with the promoter/CAT

W. Wachsman, A. J. Cann, J. L. Williams, D. J. Slamon, N. P. Shah, I. S. Y. Chen, Division of Hematology-Oncology, Department of Medicine, Jonsson Comprehensive Cancer Center, UCLA School of Medicine, Los Angeles, CA 90024.
L. Souza, Amgen Biologicals, 1900 Oak Terrace Lane, Thousand Oaks, CA 91320.

(chloramphenicol acetyltransferase) gene recombinants, HTLV-I LTR/CAT, HTLV-II LTR/CAT, or adenovirus EIII/CAT, the *x*II construct acted in *trans* to induce transcription from each of the promoters, thereby expressing CAT protein (Table 1). In contrast, the *x*IIΔ(2–17) mutant did not induce expression from any of these promoters. These data indicate that the amino terminus of $p37^{xII}$ contains a functional domain required for *trans*-activation of the HTLV-I and HTLV-II LTRs, as well as for the adenovirus EIII promoter.

To define specific amino acids in the *x* protein amino terminus that are critical to the *trans*-activation domain, we generated site-specific mutants of the *x* gene constructs within the 17-codon oligonucleotide linker. Of the first 17 codons in $p40^{xI}$ and $p37^{xII}$, 16 are conserved. Both proteins have proline residues at positions 5 and 16, which might be expected to influence the conformation and hydrophobicity of this region. Substitution of a leucine for proline at position 5 ($Pro^5 \rightarrow Leu^5$) in both $p40^{xI}$ and $p37^{xII}$ (Fig. 1) led to different phenotypes of *trans*-activation. Expression of the mutant HTLV-II *x* protein after transfection is shown in Fig. 2.

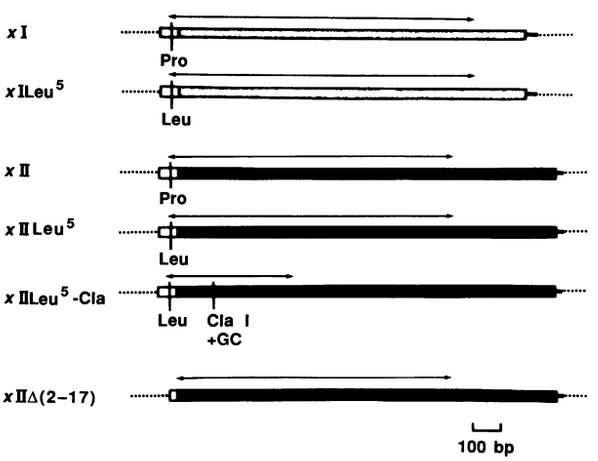
These mutant constructs, termed *x*I Leu^5 and *x*II Leu^5, were assayed for their ability to activate the HTLV-I LTR, the HTLV-II LTR, and the adenovirus EIII promoter. Table 1 shows data from the cotransfection assays of promoter/CAT recombinants and *x* constructs. The HTLV-I *x* protein mutant construct *x*I Leu^5 was unable to *trans*-activate the HTLV-I LTR. In comparison, the wild-type *x*I construct or SV 3.9 [a recombinant that expresses $p40^{xI}$ from a spliced message (*12*)] activated the HTLV-I LTR by more than 35 times above background. Consistent with previous studies (*12, 21*), neither the wild-type nor the mutant *x*I genes *trans*-activated HTLV-II LTR/CAT or EIII/CAT.

When the mutant HTLV-II construct *x*II Leu^5 was assayed, different results were obtained with the various promoters. The wild-type $p37^{xII}$ expressed from either the *x*II construct or SV-HTLV [a construct that produces spliced mRNA to encode $p37^{xII}$ (*12*)] enhanced expression from the HTLV-I LTR by about 150 times over baseline, the HTLV-II LTR about 20 times, and the EIII promoter about 20 times. In contrast, the *x*II Leu^5 mutant had reduced activity, but still induced expression from some of the promoter/CAT recombinants: The HTLV-I LTR was enhanced about 10 times above baseline levels and the EIII promoter was activated about 15 times. There was no detectable induction of the HTLV-II LTR. These results confirm the critical nature of the amino terminal sequences in the *x* protein for the *trans*-activation process. The data also demonstrate that the Leu^5 substitution has different effects on the phenotype of *trans*-activation for distinct promoters.

Since the *x*II Leu^5 construct has detectable but weakened activity on some promoters, it seems to have maintained some functions involved in *trans*-activation. Therefore, we determined whether these Leu^5 mutant proteins would influence *trans*-activation by the wild-type *x* protein when present together in the same cells. The wild-type and mutant *x* gene constructs were cotransfected together with an HTLV LTR/CAT gene recombinant. The results (Table 2) are normalized to *trans*-activation of the LTR by wild-type *x*, which is expressed from the HTLV-II recombinant construct SV-

Table 1. *Trans*-activation phenotypes of *x* gene and mutant derivatives. COS cells (2×10^6) were transfected with 5 μg of each of the indicated constructs by calcium phosphate–mediated transfection (*30*). Cells were harvested 40 to 48 hours after transfection, and CAT activity was determined by thin-layer chromatography (*31*). The values for percentage acetylation of [^{14}C]chloramphenicol are normalized to the percentage of conversion observed in the cotransfection of SV-HTLV with each promoter/CAT recombinant. The numbers represent the average of at least three assays. A less than threefold difference in CAT activity was not considered meaningful. The structure of HTLV-I LTR/CAT, HTLV-II LTR/CAT, and adenovirus EIII/CAT is as described (*7, 12, 16*). SV-HTLV and SV 3.9 are recombinants of proviral HTLV-II and HTLV-I DNA (*12*), respectively, expressed in the vector pSV2neo (*32*). SV-HTLV contains HTLV-II sequences from nucleotide position 362 (*5*) of the 5′ LTR to nucleotide position 8550 of the 3′ LTR (*12*). SV 3.9 contains HTLV-I sequences from nucleotide position 4990 (*5*) to about 8890 (*12*). Both of these recombinants express *x* protein from a spliced mRNA. The wild-type and mutant *x*I and *x*II gene constructs (*21*) (Fig. 1) express *x* protein from mRNA that does not require processing. p91023-B (*22*) is the vector base for *x*II, *x*II Leu^5, *x*II Leu^5/Cla, *x*IIΔ(2–17), *x*I, and *x*I Leu^5.

Construct	Acetylation (%)		
	HTLV-I LTR/CAT	HTLV-II LTR/CAT	Adenovirus E3/CAT
p91023-B	0.6	3.0	4.1
SV 3.9	29.9	2.9	10.2
*x*I	38.7	3.2	10.8
*x*I Leu^5	0.8	2.6	6.2
SV-HTLV	100	100	100
*x*II	93.4	76.7	96.5
*x*II Leu^5	8.3	3.1	63.7
*x*II Leu^5/Cla	0.4	5.0	5.2
*x*IIΔ(2–17)	0.6	3.3	4.8



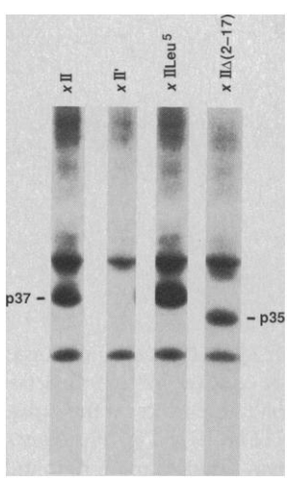


Fig. 1 (left). Schematic diagram of HTLV *x* gene constructs. The method of construction was as described (*21*). Briefly, a synthetic double-stranded oligonucleotide linker containing consensus translation initiation sequences and sequences encoding amino acids 1 through 17 of HTLV-I and HTLV-II *x* were joined to the remainder of the *x* gene sequences at nucleotide position 7347 for HTLV-I and 7259 for HTLV-II. The open and solid boxes represent HTLV-I and HTLV-II proviral sequences, respectively. The open box denotes the synthetic oligonucleotide linker in which the *x* protein residue 5 is indicated. The oligonucleotide linker for *x*IIΔ(2–17) is deleted for *x*II codons 2 through 17. The position of the Cla I site where two base pairs (+GC) have been inserted into the construct *x*II Leu^5/Cla is shown. The double-ended arrow above each construct denotes the extent of coding sequences. These *x* gene constructs were expressed by using the eukaryotic expression vector p91023-B (*22*). The previously published sequence of HTLV-II was incorrect at nucleotide 7223 (*5*), and should be "C" instead of "T." **Fig. 2 (right).** Radioimmunoprecipitation assay (RIPA) of *x* proteins from transfected cells. COS cells (2×10^6) were transfected with 10 μg of each of the indicated constructs by the calcium phosphate precipitation technique. Forty hours after transfection, cells were metabolically labeled with [^{35}S]methionine (100 μCi/ml) for 3 hours. Cells were harvested by lysis in RIPA buffer. Radioimmunoprecipitation of the *x* proteins was performed with specific antisera (α pX-IV-5 + α pX-IV-6) that recognizes the HTLV-II *x* protein (*10*). The plasmids used in the transfection are indicated above each lane. The $p37^{xII}$ and mutant $p35^{xII}$ protein bands are indicated. In the lane marked *x*II′, competition with the antisera was achieved by preliminary incubation with the synthetic peptides used to generate the *x*-specific antisera, thereby demonstrating specificity for $p37^{xII}$.

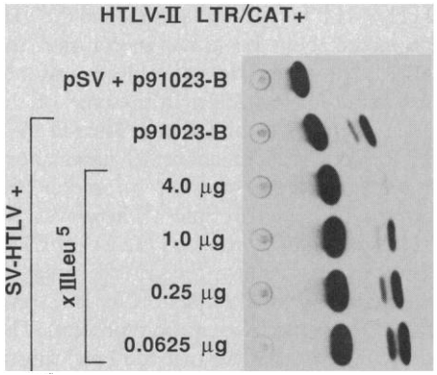


Fig. 3. Dose dependence of the inhibition of $p37^{xII}$-induced HTLV-II LTR *trans*-activation by $p37^{xII}$ Leu^5. COS cells (2×10^6) were transfected with each of the indicated constructs by the calcium phosphate precipitation technique. Each cotransfection included 3 μg of HTLV-II LTR/CAT, 3 μg of pSV2neo (pSV) or SV-HTLV, and various amounts of *x*II Leu^5. Where less than 4 μg of *x*II Leu^5 were used, the total amount of DNA was kept constant by adding the vector p91023-B. Determination of CAT levels was by thin-layer chromatography (*24*). Migration of [^{14}C]chloramphenicol is from left to right.

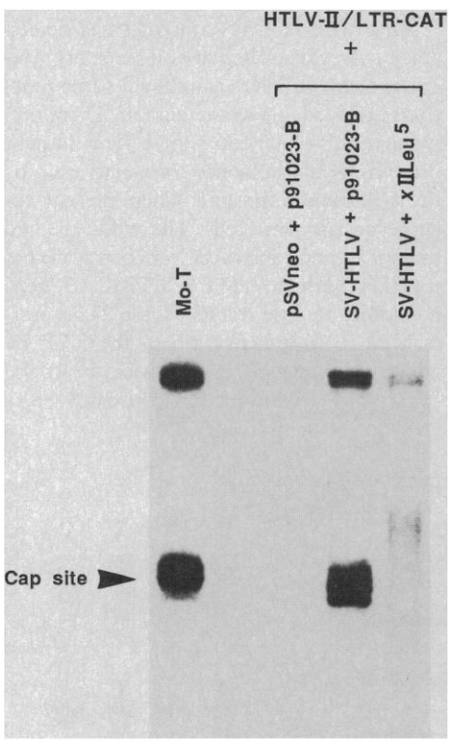


Fig. 4. Inhibition of transcriptional activation of the HTLV-II LTR by *x*II Leu^5. COS cells were transfected as described in the legend to Fig. 3. Forty-four hours after transfection, the cells were harvested and polyadenylated [poly(A)$^+$] RNA was prepared. This poly(A)$^+$ RNA was assayed by the hybridization/S_1 nuclease technique for RNA transcripts correctly initiated from the HTLV-II LTR. The hybridization probe was a 90-mer oligonucleotide labeled with ^{32}P at the 5′ end and complementary to sequences extending from position 294 to 383 in the HTLV-II proviral LTR. Hybridization of 25 μg of poly(A)$^+$ RNA from the transfected COS cells with the probe (10^6 cpm) was performed overnight at 46°C. The S_1 nuclease digestion (350 U of BRL S_1 nuclease) was conducted at room temperature for 1 hour. Lane Mo-T, presented for comparison, represents the results of an S_1 assay of 5 μg of whole-cell RNA, extracted from the HTLV-II–infected Mo-T cell line, with the same 90-mer probe under identical conditions. The protected fragment of 70 nucleotides, indicative of RNA transcribed from HTLV-II LTR cap site (position 314 in the proviral sequence) is denoted next to the autoradiogram.

HTLV. Concurrent transfection of the *x*II or *x*IIΔ(2–17) constructs and SV-HTLV had little effect on the *trans*-activation of the HTLV-II LTR. However, when *x*II Leu^5 was transfected, the induction of the HTLV-II LTR by SV-HTLV was reduced nearly 20-fold. When the amount of transfected *x*II Leu^5 was reduced, and the amount of transfected HTLV-II LTR/CAT + SV-HTLV (Fig. 3) was kept constant, HTLV-II LTR *trans*-activation increased to levels observed with SV-HTLV alone. Thus, this inhibitory phenomenon depends on the amount of the mutant gene present. In contrast with *x*II Leu^5, cotransfection of the HTLV-I mutant *x*I Leu^5 with SV-HTLV did not inhibit *trans*-activation of the HTLV-II LTR (Table 2). Thus, the substitution of Leu^5 for Pro^5 influences the normal *trans*-activation process of the HTLV-II LTR by the wild-type HTLV-II *x* protein.

Radioimmunoprecipitation of labeled cellular lysates indicated that the amount of $p37^{xII}$ expressed from the *x*II construct was at least ten times that from SV-HTLV (*21*). To ascertain the significance of relative levels of $p37^{xII}$ and $p37^{xII}$ Leu^5 on the inhibition of HTLV-II LTR *trans*-activation, we performed cotransfection experiments in which both the wild-type protein $p37^{xII}$ and the mutant protein $p37^{xII}$ Leu^5 were expressed by p91023-B (*x*II and *x*II Leu^5, respectively). No inhibition was observed with equal amounts of *x*II and *x*II Leu^5 cotransfected. When the amount of *x*II transfected was about one-third the amount of *x*II Leu^5, *trans*-activation was reduced to one-fifth. These results suggest that the inhibition may be due to competition between the wild-type and mutant gene products for a rate-limiting step in LTR *trans*-activation.

In previous studies, we demonstrated that a frameshift at the Cla I site (amino acid codon position 59) in the HTLV-II *x* gene resulted in a mutant protein that failed to *trans*-activate either the HTLV-I or HTLV-II LTR (*12*). The construct *x*II Leu^5/Cla introduces a frameshift mutation at this same Cla I site in *x*II Leu^5. The mRNA transcribed from this mutant is identical to *x*II Leu^5 mRNA, with the exception of two additional bases at the frameshift locus. The mutation does not alter the level of *x* mRNA in the cells (*12, 21*). However, in contrast to the variable phenotype of *trans*-activation exhibited by *x*II Leu^5, *x*II Leu^5/Cla is not able to induce expression from any of the promoter/CAT recombinants tested (Table 1). The *x*II Leu^5/Cla mutant was also assayed for inhibition of HTLV-II LTR *trans*-activation by wild-type $p37^{xII}$. As shown in Table 2, *x*II Leu^5/Cla had little effect on the *trans*-activation of the HTLV-II LTR by $p37^{xII}$. Thus, the protein product of the *x*II Leu^5 mutant directly mediates the inhibition of HTLV-II LTR induction.

The same constructs were tested for inhibition of HTLV-I LTR induction by $p40^{xI}$ and $p37^{xII}$ (Table 2). When *x*II Leu^5 was cotransfected with the SV-HTLV and HTLV-I LTR/CAT recombinants, there was only a small reduction in LTR *trans*-activation compared with control cotransfections with either *x*II, *x*II Leu^5/Cla, or *x*IIΔ(2–17). Similar studies performed with a recombinant (SV 3.9) that expresses the HTLV-I *x* protein $p40^{xI}$ (*12*) showed less than a twofold inhibition of HTLV-I LTR *trans*-activation by the Leu^5 mutant of either *x*I or *x*II. These results show that under comparable experimental conditions, the most dramatic inhibition of *trans*-activation is by *x*II Leu^5 upon *x*II. Furthermore, inhibition of the HTLV-II LTR is more pronounced than of the HTLV-I LTR.

Trans-activation by the HTLV *x* protein is due to an increase in the level of mRNA initiated from a specific promoter (*7, 12–15*). To ascertain whether the inhibition of wild-type *trans*-activation by the Leu^5 mutant protein occurs at the level of transcription, we performed S_1 nuclease analysis for mRNA transcribed from the HTLV-II LTR. The oligonucleotide probe spans the HTLV-II LTR cap site located at base pair 314 in the provirus, resolving a protected fragment of 70 nucleotides (see control Mo-T lane in Fig. 4). In the absence of the HTLV-II *x* construct SV-HTLV, no detectable RNA was expressed from the HTLV-II LTR (Fig. 4). When SV-HTLV and HTLV-II LTR/CAT were cotransfected, RNA was expressed from the LTR cap site. In contrast, the presence of *x*II Leu^5 in the cotransfection with SV-HTLV and HTLV-II LTR/CAT did not result in the presence

of detectable RNA expressed from the HTLV-II LTR cap site. These results are consistent with a mechanism in which the action of $p37^{xII}$ Leu^5 inhibits the induction of HTLV-II LTR by the wild-type protein at the level of transcription.

We and others have shown that $p40^{xI}$ from HTLV-I and $p37^{xII}$ from HTLV-II specifically enhance transcription. The data presented here demonstrate that the amino terminus of the *x* protein is part of a functional domain critical for *trans*-activation. Within this domain, a single amino acid change at position 5 can have dramatic effects on the function of the *x* protein. Others have constructed recombinants expressing the *x* gene with leader sequences that either ablate or add nonspecific coding sequences to the amino terminus of the *x* molecule (*13, 14*). Given the importance of this region of the *x* protein, care must be taken in interpreting results obtained with these recombinants, because they may not accurately reflect the native behavior of $p40^{xI}$ or $p37^{xII}$.

One protein of the Leu^5 mutation on the HTLV *x* protein is the variable effect of the mutation on *trans*-activation of different promoters. One explanation for these differences is that the *x* protein may influence a common step in transcription for these promoters, but this step may contribute differentially to overall *trans*-activation of each one. Alternatively, the variable *trans*-activating capacity of the mutant protein suggests that the *x* protein may affect a different rate-limiting step in the transcription process for each of these promoters.

Table 2. Inhibition of HTLV LTR induction by *x*II Leu^5. CAT activity was measured in COS cells as described in Table 1. Cells were transfected with 3.3 μg of each of the indicated constructs. Each result represents the average of at least three assays. The conversion of [^{14}C]chloramphenicol is normalized to SV-HTLV + p91023-B + HTLV-II LTR/CAT or HTLV-I LTR/CAT for the SV-HTLV–based cotransfection series or to SV 3.9 + p91023-B + HTLV-I LTR/CAT for the SV 3.9–based series. A less than threefold difference in CAT activity was not considered meaningful.

Construct	Acetylation (%)	
	HTLV-II LTR/CAT	HTLV-I LTR/CAT
pSVneo + p91023-B	1.1	2.3
SV-HTLV + p91023-B	100	100
SV-HTLV + *x*II	98.9	129.1
SV-HTLV + *x*II Leu^5	5.3	33.3
SV-HTLV + *x*II Leu^5/Cla	45.6	49.7
SV-HTLV + *x*IIΔ(2–17)	37.8	41.6
SV-HTLV + *x*I	49.8	63.9
SV-HTLV + *x*I Leu^5	54.4	67.0
pSV + p91023-B		4.8
SV 3.9 + p91023-B		100
SV 3.9 + *x*II		223.2
SV 3.9 + *x*II Leu^5		59.6
SV 3.9 + *x*I		107.9
SV 3.9 + *x*I Leu^5		71.0

The Leu^5 mutant HTLV-II *x* protein is able to inhibit *trans*-activation by the wild-type protein. One hypothesis for its mechanism of action is that the mutant protein acts by competing with the wild-type protein for a substrate involved in a rate-limiting step of *trans*-activation. Several mechanisms could account for the phenotype of the $p37^{xII}$ Leu^5 mutant. (i) If the *x* protein functions in *trans*-activation as a dimer or multimer, the Leu^5 mutant protein could form a complex with native *x* proteins that is unable to *trans*-activate the promoter. Such a mechanism is observed with dominant mutants of the lac repressor (*23*). (ii) Mutant Leu^5 and wild-type *x* proteins may compete for binding to the promoter. Mutant forms of phage λ and Lex A repressor proteins have been characterized that interfere with the normal function of the wild-type protein by irreversibly binding to the operator (*24*). (iii) The mutant *x* protein could compete for cellular proteins that normally function together with the wild-type *x* protein as a transcription complex. A similar competition mechanism has been proposed for the interaction between core RNA polymerase and different σ factors in *Escherichia coli* to regulate the heat-shock response (*25*).

An alternative hypothesis is that the observed inhibition may represent an autoregulatory function normally present in the wild-type protein. Thus, the Leu^5 mutation may unmask a distinct autoregulatory function of the *x* protein, which is not distinguished in routine assays for *trans*-activation with the wild-type gene. Such a function would be critical to the HTLV life cycle to autoregulate *x* protein expression, thereby limiting the positive self-control circuit involved in HTLV proviral transcription. If uncontrolled, high levels of transcriptional activating proteins, like *x*, might be cytotoxic to the infected host cell by depletion of cellular cofactors needed for maintenance of transcription. A negative regulatory role has been demonstrated for the *trans*-acting regulatory proteins of cytomegalovirus (*26*), herpes simplex virus (*27*), and adenovirus (*28*). In addition, a negative regulatory domain in the *x* protein could serve a role in the process of viral latency.

Note added in proof: Subsequent to acceptance of this report, Inoue *et al.* (*29*) reported that $p27^{xIII}$, which is produced from an alternative overlapping reading frame, is also involved in the regulation of proviral expression. The *x* constructs described in this report do not code for $p27^{xIII}$, and the mutant contains a stop codon for that reading frame. However, we cannot rule out expression of other alternative reading frames.

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