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U3 Sequences from HTLV-I and -II LTRs Confer *px* Protein Response to a Murine Leukemia Virus LTR

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Human T-cell leukemia virus (HTLV) types I and II are unusual among replication-competent retroviruses in that they contain a fourth gene (*x*) necessary for replication. The *x* gene product, p*x*, transcriptionally transactivates the viral long terminal repeat (LTR), and is thus a positive regulator. To investigate p*x* transactivation, sequences from the U3 regions of the LTRs of HTLV-I and -II were inserted into the Moloney murine leukemia virus (M-MuLV) LTR by recombinant DNA techniques. Transient expression assays of the chimeric LTRs indicated that the HTLV sequences conferred to the M-MuLV LTR responsiveness to HTLV p*x* protein. M-MuLV enhancers were not required for function of the chimeric LTRs. Infectious recombinant M-MuLVs containing chimeric LTRs were also generated. These viruses showed higher infectivity when assayed in mouse cells expressing HTLV-II p*x* protein compared to normal mouse cells. Thus the HTLV sequences were able to confer p*x* responsiveness to infectious M-MuLV. The generation of a virus dependent on a transactivating protein for its replication has implications for the evolution of the human T-cell leukemia viruses.

HUMAN T-LYMPHOTROPIC VIRUSES types I and II (HTLV-I and -II) are replication-competent retroviruses that induce two forms of T-lymphoma in humans (*1*). In addition to the three standard retroviral genes *gag, pol*, and *env*, these viruses contain an additional gene called *x, lor*, or *tat* (*2*). This gene encodes proteins px^{I} and px^{II} of 40 kD and 37 kD, respectively (*3*); these proteins transcriptionally transactivate the HTLV-I or -II long terminal repeats (LTRs) (*4–7*). As a result, transcription from the HTLV LTRs is much more efficient in HTLV-infected cells that express p*x* compared with matching uninfected cells (*8*). Also, deletions in the *x* gene of HTLV-II destroy infectivity of the virus (*9*). Thus p*x* protein is a positive regulator of HTLV-I and -II expression, and is required for viral replication. The sequences in the HTLV LTRs that respond to the p*x* protein have also been investigated. The presence of three copies of conserved 21-bp repeats in the U3 regions of both of these viruses suggested that these repeats might have important enhancer or regulatory function (*2, 10*). The HTLV LTRs also contain regions with transcriptional enhancer activity, as well as sequences that respond to p*x* transactivation (*11–13*). In the experiments reported here, U3 sequences from the HTLV-I and -II LTRs were inserted into the U3 region of the LTRs of Moloney murine leukemia virus (M-MuLV). This resulted in the formation of chimeric LTRs that responded to p*x*. In addition, replication-competent M-MuLVs containing HTLV-II U3 sequences were responsive to p*x* and required it for efficient replication.

The regions of the HTLV-I and -II LTRs used for insertion into the M-MuLV LTR are indicated in Fig. 1A by the cross-hatching. These sequences contained the three copies of the 21-bp repeats but lacked the HTLV TATA sequences at −30. Thus insertion of these sequences into an M-MuLV LTR would not be expected to introduce an additional start site for transcription. In the wild-type M-MuLV LTR, the enhancer sequences are indicated by the stippled box; in addition, another LTR, ΔMo, which lacks the M-MuLV enhancer sequences (*14*), is shown. The HTLV-I and -II U3 sequences were inserted into the wild-type M-MuLV and ΔMo LTRs by means of Xba I linkers, resulting in the recombinant constructs indicated (Fig. 1A). After the chimeric LTRs were generated they were introduced into a plasmid for expressing the chloramphenicol acetyltransferase (CAT) gene and used in transient expression assays (*15*). The LTRs were also used to generate plasmids containing a complete M-MuLV proviral organization with the chimeric LTRs at both ends (Fig. 1A).

The effect of placing the HTLV sequences into the wild-type and ΔMo M-MuLV LTRs was first investigated by transient expression assays in mouse NIH 3T3 cells. Insertion of either HTLV-I or -II sequences into the wild-type M-MuLV LTR did not significantly affect the LTR transcriptional activity (Fig. 1B). In addition, when the HTLV sequences were inserted in either orientation into the ΔMo LTR (which shows no transcriptional activity because it lacks the enhancer sequences), weak but detectable activity was observed. This indicated that the inserted HTLV-I and -II sequences have weak enhancer activity in NIH 3T3 cells, even in the absence of p*x* protein.

To test the effect of HTLV-I p*x* on the chimeric LTRs, we transfected the CAT expression plasmids into human osteosarcoma (HOS) and HTLV-I–infected HOS (HOS/PL) cells (*16*). In these experiments,

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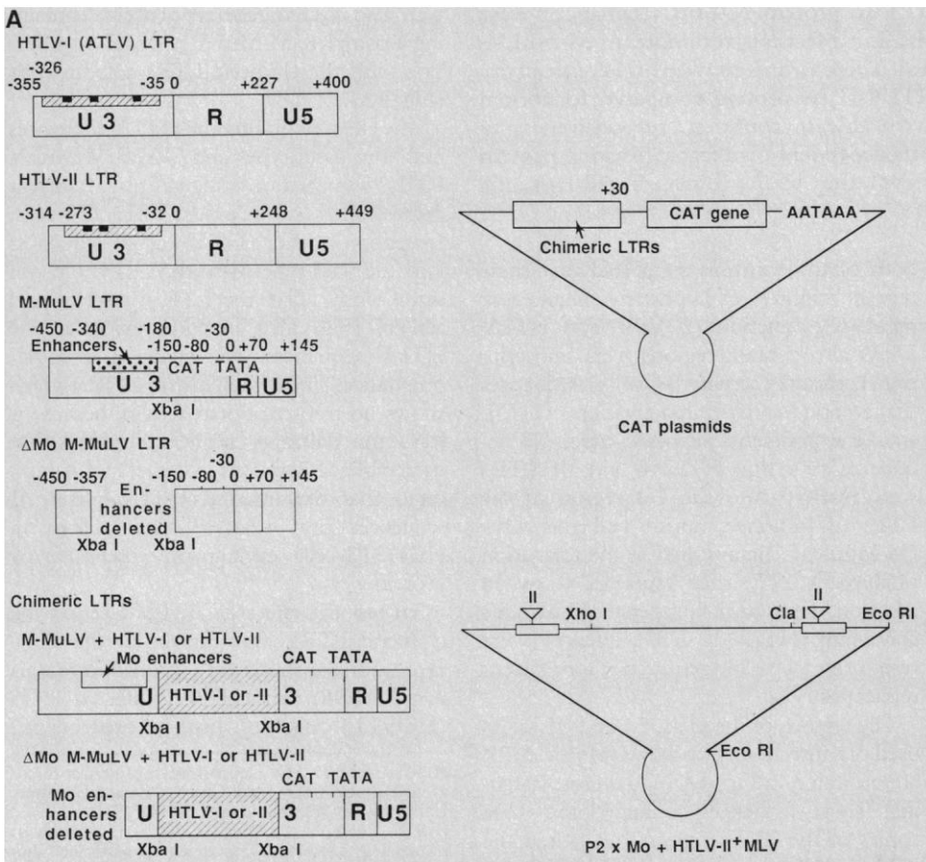
the reference plasmid pSVCAT, which contains the CAT gene driven by the SV40 viral promoter (*17*), was used. HOS/PL cells appeared to be more transcriptionally active than HOS cells, as indicated by the higher activity of the reference pSVCAT plasmid. The wild-type M-MuLV LTR also showed a slight (two- to threefold) induction in comparison to pSVCAT. However, as shown in Fig. 2, the Mo+HTLV-I$^+$ and Mo+HTLV-II$^+$ CAT plasmids showed significantly higher CAT activity than the wild-type M-MuLV LTR CAT plasmid in the HOS/PL cells when compared to HOS cells. Thus insertion of the HTLV sequences into the M-MuLV LTR conferred responsiveness to px^I. More dramatically, while the ΔMo LTR was inactive in either uninfected or infected HOS cells, the ΔMo+HTLV-II$^+$ and ΔMo+HTLV-I$^+$ LTRs were inactive in the HOS cells but strongly active in the HOS/PL cells. Transactivation of the ΔMo+HTLV-II$^+$ LTR was somewhat less than the ΔMo+HTLV-I$^+$ LTR in the HTLV-I-infected HOS/PL cells, consistent with the behavior of the HTLV-I and -II LTRs in these cell lines (*4*). The results with the ΔMo plasmids indicate that the inserted HTLV sequences could function as px^I-dependent enhancers.

The chimeric CAT expression plasmids were also tested in HTLV-II–infected human B-lymphoid cells (J-WIL) (*17*), as well as the uninfected parent line WIL-2 (Fig. 3). Neither the wild-type nor ΔMo M-MuLV LTRs had activity in either infected or uninfected WIL-2 cells. Insertion of either HTLV sequence into either the wild-type or deleted M-MuLV LTRs (in either orientation) again resulted in very high activity, but only in the infected J-WIL cells. Thus, the chimeric LTRs were responsive to HTLV-II infection as well. The lack of activity of the wild-type M-MuLV LTR in the human B-lymphoid cells was particularly noticeable in the J-WIL cells. Raji cells, another human B-lymphoid cell line, also showed no activity with the wild-type M-MuLV LTR, although activity of pSVCAT was also low. The inactivity of the wild-type M-MuLV LTR may be characteristic of human B-lymphoid cells.

While the experiments with the matched infected cell lines strongly suggested that the chimeric LTRs were directly responding to virus-coded gene products, it was theoretically possible that other differences between the matched cell lines were involved in the transactivation. Therefore, chimeric LTR-CAT plasmids were cotransfected into NIH 3T3 cells together with plasmids that express either HTLV-I or -II p*x*s (SV3.9 and SV HTLV, respectively) (*4*). As shown in Fig. 4, cotransfection with the p*x* expression plasmids resulted in transactivation of the chimeric LTRs. The results also indicated that the chimeric LTRs are responsive to p*x* in mouse NIH 3T3 cells, consistent with previous results (*18*). However, it is interesting that the entire HTLV-II LTR was not activated by px^I (*18*), whereas ΔMo+HTLV-II$^+$ was activated to a minor extent.

To test the responsiveness of the chimeric LTRs in a situation more closely resembling that of an HTLV infection, we used infectious M-MuLVs containing the chimeric LTRs. We first prepared the proviral plasmids indicated in Fig. 1A, and transfected these plasmids into NIH 3T3 cells. The cells were passaged until confluently infected with the virus. Two such viruses, Mo+HTLV-II$^+$ and ΔMo+HTLV-II$^+$ M-MuLVs, were generated. In the case of Mo+HTLV-II$^+$ M-MuLV, NIH 3T3 cells were transfected, since the data in Fig. 1B indicated that the LTR was functional in these cells. In the case of ΔMo+HTLV-II$^+$ M-MuLV, the transfection was performed on a line of NIH 3T3 cells expressing HTLV-II p*x* protein (introduced by a retro-



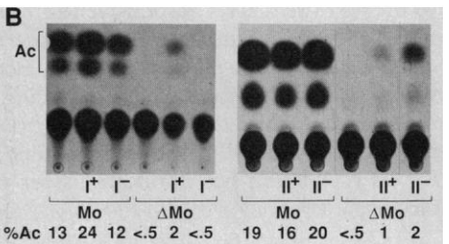


Fig. 1. CAT assays on NIH 3T3 cells. (**A**) Diagrams of the HTLV-I and -II (*2*) and M-MuLV LTRs (*23, 24*). All numbering is in base pairs from the RNA cap site (the 5′ end of R). Hatched boxes in the HTLV-I and -II LTRs, sequences used to construct the chimeric LTRs; closed boxes, 21-bp repeats. The HTLV-I sequences were a Sma I–Hinf I fragment from −326 to −35 bp; the HTLV-II sequences were a Hinf I fragment from −273 to −32 bp. M-MuLV enhancers, the stippled box in U3; the M-MuLV Xba I site is at −150 bp. In the ΔMo M-MuLV LTR, the enhancers as well as some surrounding sequences have been deleted (*14*). For the chimeric LTRs, the HTLV-I or -II sequences were inserted into the M-MuLV or ΔMo LTRs at the Xba I sites via Xba I linkers, in either orientation; + indicates HTLV sequences inserted in the same transcriptional direction as the M-MuLV LTR. The structure of the CAT expression plasmids, and of the proviral DNA form is also shown. In the CAT expression plasmids, the chimeric LTRs were fused to the CAT gene at their nucleotide position +30 [for detailed constructions of equivalent plasmids, see (*24*)]. (**B**) Transient CAT assays in NIH 3T3 cells [see (*25*) for details]. An autoradiogram of a chromatogram is shown, and the position of the acetylated chloramphenicol (Ac) is indicated. Repetition of the CAT assays indicated no significant difference between the activity of the ΔMo+HTLV-II$^+$ and ΔMo+HTLV-II$^-$ LTRs. The lack of activity for the ΔMo+I$^-$ LTR is not significant—in other experiments this plasmid gave the same CAT activity as the ΔMo+I$^+$ LTR.

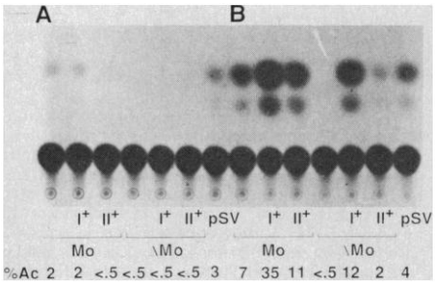


Fig. 2. CAT assays on (**A**) HOS and (**B**) HOS/PL cells. For this experiment, transfection was performed by using the DEAE dextran method (*26*). Each transfection contained 10^6 cells, and 10 μg of plasmid DNA was introduced in 1 ml. After incubation for 1.5 hours, cells were washed and refed with medium containing 0.1 m*M* chloroquine for 2.5 hours. Cells were then washed and incubated in medium for an additional 2 days. Cell extracts were then assayed for CAT enzyme activity as described in Fig. 1. All CAT activities should be compared to the expression of pSVCAT in the two cell lines.

viral expression vector), 15S-1 (*19*). The two chimeric viruses were then titered for infectivity [by the XC plaque assay (*20*)] on normal NIH 3T3 as well as two different px^{II} positive cell lines, 15S-1 and 15S-5a. For comparison, wild-type M-MuLV was also infected and titered on the same three cell lines. Wild-type M-MuLV showed essentially the same relative titers on the three cell lines, indicating that it was not px-responsive (Table 1). In contrast, both Mo+HTLV-II$^+$ and ΔMo+HTLV-II$^+$ M-MuLVs were more infectious on the px^{II}-positive cells. In the case of Mo+HTLV-II$^+$ M-MuLV, there was a 20- to 50-fold increase in infectivity on the 15S-5a cells. 15S-1 cells, which apparently had a lower level of px^{II} protein, showed an intermediate increase. The result was even more dramatic for ΔMo+HTLV-II$^+$ M-MuLV. This virus showed extremely low infectivity on the standard NIH 3T3 cells, whereas it showed 3 to 4 logs higher infectivity on the 15S-5a cells. Again, an intermediate increase was seen on the 15S-1 cells. Thus, insertion of HTLV sequences resulted in px-responsive M-MuLVs.

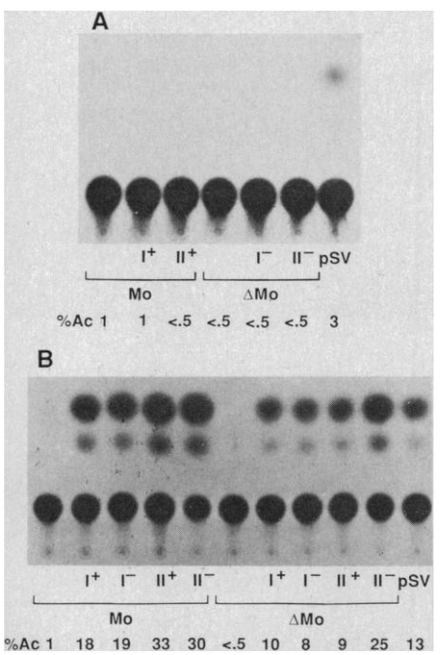


Fig. 3. CAT assays on (**A**) WIL-2 and (**B**) J-WIL cells. For these assays, DEAE-dextran transfection was used with modifications for nonadherent cells (*26*), namely, 10^7 cells were used per transfection, and incubations were done in 50-ml tubes.

The results reported here indicate that U3 sequences from HTLV-I and -II can confer responsiveness to viral px protein when introduced into the heterologous M-MuLV LTR. The transactivation was demonstrated by transient assays as well as by generating infectious M-MuLV containing the chimeric LTRs. The insertions into the ΔMo LTR were particularly informative. They indicated that the U3 region of these HTLVs upstream of the TATAA sequences can provide px-responsive enhancer activity to an enhancerless promoter. This result is consistent with results of Fujisawa *et al.* (*13*) for the HTLV-I LTR. In the case of HTLV-I, there is some controversy as to whether the enhancer and px-responsive sequences within the U3 region of the LTR are one and the same, or distinct (*11, 13*). Since the constructs described here contained relatively large portions of the HTLV U3 regions, no conclusions could be drawn as to whether enhancer function and px responsiveness reside in the same or different HTLV DNA sequences.

The great differences in infectivity for the chimeric viruses in the presence and absence of px^{II} did not necessarily imply that viral transcription in the infected cells showed

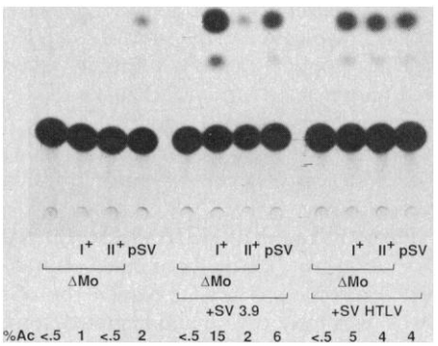


Fig. 4. Cotransfection with px expression plasmids. For these assays, NIH 3T3 cells were cotransfected with 5 μg each of CAT plasmid with or without an equal amount of pSV 3.9 or pSV HTLV plasmid DNAs (*4*). The ability of px^{II} to activate the HTLV-I and -II LTRs with equal efficiency has been previously reported (*4*).

exactly the same magnitude of response. An XC plaque assay reflects both cell-to-cell spread of infectious virus and also multiplication of infected cells. Viruses with moderate alterations in LTR activity can show more pronounced effects in infectivity assays, and the absolute magnitude of the effects can be influenced by the particular infection protocol (*21*). Indeed, repetition of the infectivity assays shown in Table 1 consistently showed px responsiveness of Mo+HTLV-II$^+$ and ΔMo+HTLV-II$^+$ M-MuLVs, but the magnitude of the effect varied with the conditions of the infection (115-fold px induction of ΔMo+HTLV-II$^+$ M-MuLV in the experiment showing the least response).

The infectivity of Mo+HTLV-II$^+$ M-MuLV on normal NIH 3T3 cells appeared to be somewhat lower than wild-type M-MuLV (20- to 50-fold, when corrected for amount of virus particles in the respective stocks). However, the CAT assays of Fig.

Table 1. Infectivity of chimeric viruses on px-positive cells. 15S-5a and 15S-1 cells are NIH 3T3 cells that express HTLV-II px protein introduced by a retroviral vector (*18*). 15S-5a cells were also infected with amphotropic MuLV 4070A. Virus stocks were obtained by clarifying tissue culture supernatants from subconfluent cultures of cells producing the different viruses (*27*). Wild-type M-MuLV was obtained from the A9 cell line (*27*). Mo+HTLV-II$^+$ M-MuLV was obtained from NIH 3T3 cells transfected with proviral DNA containing the chimeric LTRs, and ΔMo+HTLV-II$^+$ M-MuLV was obtained from 15S-1 cells transfected with the corresponding proviral DNA.

Virus	Cell line		
	NIH 3T3	15S-5a	15S-1
Wild-type M-MuLV	8.2×10^5*	5.5×10^5 (0.67×)†	2.9×10^5 (0.35×)
Mo+HTLV-II$^+$ M-MuLV	2.1×10^3	8.1×10^4 (38×)	1.6×10^4 (7.6×)
ΔMo+HTLV-II$^+$ M-MuLV	5×10^1	1.3×10^5 (2.6×10^3×)	5.5×10^3 (110×)

*Titers are given as XC plaque-forming units per milliliter (*20*). Serial dilutions of the virus stocks (10^{-1} to 10^{-6}) were prepared; each dilution was divided into three equal parts and infected onto tissue culture dishes of each of the indicated cell lines. Four days after infection, the infected cells had reached confluency; they were then irradiated with ultraviolet light and overlayed with rat XC cells. Two days later, the cultures were fixed and stained, and syncytial plaques were counted. Titers of virus were then calculated and are shown in the table. Each of the titers represents the average of the plaque counts from the cultures infected at the two or three highest dilutions that showed plaques. †The numbers in parentheses indicate the relative virus titer measured in the 15S-5a or 15S-1 cells, in comparison to the same virus stock titered on NIH 3T3 cells. The increases in infectivity for Mo+HTLV-II$^+$ and ΔMo+HTLV-II$^+$ M-MuLVs have not been corrected for the lower infectivity of wild-type M-MuLV on these cells.

1B indicated similar LTR activities for wild-type and Mo+HTLV-II$^+$ M-MuLV LTRs. One possibility is that the infectivity assays were more sensitive to small changes in LTR promoter activity. Alternatively, transient assays for LTR activity might not predict the biological behavior of retroviruses in every detail.

Previous studies of HTLV-I and -II transactivation have usually been based on transient expression assays, in which the test DNA is introduced in unintegrated form and at high copy number. When used alone, these assays might over- or underestimate effects, for instance by titration of limiting cellular factors with high amounts of transfecting DNA (*22*). The use of p*x*-responsive M-MuLV containing the HTLV-II U3 sequences should facilitate the transactivation studies. Infection of the chimeric M-MuLVs results in integration of low numbers of proviral DNA copies in standard chromatin configuration, similar to the form of the integrated HTLV DNAs in infected human cells. These chimeric LTRs may also provide a means to express other genes in a conditional fashion when introduced into cells by retroviral vectors.

The distinguishing feature of human T-cell leukemia viruses and also bovine leukemia virus is that they contain a fourth gene (*x*) essential for replication, in addition to the usual complement of retroviral genes. The results reported here show that the U3 sequences of the LTR, when inserted in an unrelated retrovirus, confer dependence for replication on p*x* protein. Thus, the evolution of an HTLV-like retrovirus from other oncoviruses may have occurred by acquisition of promoter elements responsive to cellular transactivating factors. Mouse mammary tumor virus may represent such a retrovirus, where efficient expression is dependent on interaction of the cellular glucocorticoid receptor with response elements in the LTR. Subsequent acquisition of a transactivating function within the viral genome would free the virus from constraints placed by cellular transactivating genes. This process could result in the generation of an HTLV-like retrovirus.

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19. The 15S-1 cells were produced by transfection of a retrovirus vector (inxcess) containing the complete coding sequences of the HTLV-II *x* gene (*18*) into NIH 3T3 cells. G418-resistant cell clones were selected for *x* gene expression by transfection with LTR-I CAT. Virus containing the *x* gene was rescued by superinfection of the cells with amphotropic MuLV; this virus was used to infect fresh NIH 3T3 cells, which were selected for G418 resistance. Resulting clones were selected again for expression of *x* gene by transfection with LTR-I CAT. One of these clones was designated 15S-5a.
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25. Details of the CAT assays have been described previously (*15, 24*). Briefly, each transfection plate (10^6 cells) received 20 μg of plasmid DNA as a calcium phosphate precipitate in the presence of chloroquine. After 4 hours of incubation, the precipitate was removed and the cells were incubated in growth medium for an additional 2 days. Cell extracts were then assayed for CAT enzyme activity by incubation with ^{14}C-labeled chloramphenicol. Acetylated and unacetylated forms of chloramphenicol were then separated by ascending thin-layer chromatography on silica gel plates. Percentage acetylation was determined by scintillation counting of the unacetylated and acetylated spots.
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