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trans-Activation of the Human T-Cell Leukemia Virus Long Terminal Repeat Correlates with Expression of the *x-lor* Protein

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Cell lines established directly from adult T-cell leukemia-lymphoma patients or immortalized by human T-cell leukemia virus type I (HTLV-I) in vitro that do not produce complete HTLV virions were characterized both for the content of viral proteins and for the presence of *trans*-acting factors activating gene expression under the control of the HTLV long terminal repeat. The expression of the 42-kilodalton HTLV *x-lor* product correlated with *trans*-activation of the long terminal repeat. The implications of this study for understanding the role of the HTLV *x-lor* product in the initiation and maintenance of T-lymphocyte transformation are discussed.

Human T-cell leukemia viruses (HTLVs) comprise a retrovirus family associated with lymphoid disorders. HTLV type I (HTLV-I) is associated with adult T-cell leukemia-lymphoma (ATLL) (1, 7, 20, 22, 38). HTLV-II is a rare isolate associated with a benign form of hairy T-cell leukemia (15). Bovine leukemia virus (BLV), the etiological agent of enzootic leukosis in domestic cattle, demonstrates antigenic relatedness to the HTLV viruses in some of the virion proteins (5, 19).

The unique structural and biological properties of HTLV-I, HTLV-II, and BLV have prompted their categorization into a separate family of transforming retroviruses. The terminal repeat regions of these viruses are unusually long (4, 27–29, 33). In addition to the *gag*, *pol*, and *env* genes typical of chronic leukemia viruses, the HTLV-I, HTLV-II, and BLV viruses possess a sequence located between the envelope gene and the 3′ long terminal repeat (LTR) (2, 13, 27, 30). This region of HTLV-I and HTLV-II has been shown to include a gene called *x-lor* that encodes a 42-kilodalton (kd) (HTLV-I) or 38-kd (HTLV-II) protein (6, 17, 31, 35). These proteins partition almost equally between the nucleus and cytoplasm of the infected cell (8; unpublished data). Another distinguishing feature of the HTLV-I, HTLV-II, and BLV family is *trans*-acting regulation of the viral LTR observed in infected cells (23, 32). Cells infected by members of this retroviral family contain factors that greatly stimulate gene expression directed by the viral LTR. These *trans*-acting factors are specific for the LTR of the infecting virus, suggesting that a virus-derived, rather than a host cell-derived, factor might mediate *trans*-activation (23, 32).

We have proposed that the product of the *x-lor* gene *trans*-activates, either directly or indirectly, gene expression under the control of the LTR (32). This hypothesis predicts that cells expressing the *x-lor* protein should display *trans*-activation of the HTLV LTR, whereas cells that do not express the *x-lor* protein should not. The experiments presented here were designed to test this prediction by using HTLV-I-transformed cell lines that do not produce intact virions. Nonproducing cell lines were selected for this study because they offer the opportunity to determine what virus-encoded proteins are necessary for LTR *trans*-activation. For this study we used three cell lines, C81-66-45, C82-15, and E55, established in vitro by either cocultivation or polyethylene glycol-mediated cell fusion with HTLV-I-producing cells (24). Another nonproducer cell line, TL-OmI (34), was established directly from an ATLL patient. All four of these cell lines contain integrated HTLV proviruses (unpublished data). None of the four cell lines releases detectable levels of reverse transcriptase into the culture medium (24). Two HTLV-I virus-producing cell lines, HUT102 and MT2, were also used in these studies.

To determine whether the nonproducing cell lines contained *trans*-acting factors that could specifically stimulate gene expression directed by the HTLV-I LTR, the four nonproducing cell lines were transfected with plasmids containing the LTR sequences of HTLV-I and HTLV-II that were located 5′ to the chloramphenicol acetyltransferase (CAT) gene of *Escherichia coli* (11, 32). At 48 h after transfection, the level of CAT enzymatic activity, which has been shown to correlate with the level of CAT mRNA (10, 14, 36), was determined. To control for differences in the ability of different cell lines to take up foreign DNA, plasmids containing the simian virus 40 (SV40) early promoter located 5′ to the CAT gene (11) were transfected in parallel in these experiments. The CAT activity directed by the HTLV LTR sequences was normalized to that produced after transfection of the same cell lines with the SV40 promoter-containing plasmid.

As reported previously (32), the HTLV-I LTR directs a level of CAT activity ca. three- to fivefold greater than that of the SV40 early promoter in a number of uninfected human T-lymphocyte cell lines (Table 1). The level of CAT activity in these cell lines after transfection of the HTLV-II LTR-containing plasmid was undetectable.

In contrast, in both HTLV-I producer and the C81-66-45, C82-15, and E55 nonproducer cell lines, the level of CAT activity of the HTLV-I-containing plasmid was 30- to 180-fold greater than that of the plasmid containing the SV40 sequences. The relative activity of the HTLV-II LTR in these cell lines was low but slightly higher than that observed

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TABLE 1. Relative activity of the HTLV-CAT plasmids in transfected cell

Human T-lymphocyte Cell line	Description	Relative CAT activity[a]		
		pSV2CAT	pU3R-I	pU3-II
HUT78	Uninfected	1.0	4.5	<0.05
Jurkat	Uninfected	1.0	3.0	<0.05
H9	Uninfected	1.0	2.5	<0.05
MT2	HTLV-I infected	1.0	180	4.8
HUT102	HTLV-I infected	1.0	28	1.4
C81-66-45	HTLV-I immortalized nonproducer	1.0	76	0.1
E55	HTLV-I immortalized nonproducer	1.0	32	2.6
C82-15	HTLV-I immortalized nonproducer	1.0	47	0.6
TL-OmI	ATLL nonproducer	1.0	0.15	<0.05

[a] Relative CAT activity represents the percent conversion of chloramphenicol to acetylated forms per hour after transfection relative to that obtained after transfection of the same cell line with equimolar amounts of pSV2CAT. The pSV2CAT, pU3R-I, and pU3-II plasmids contain sequences derived from the SV40 early region, the HTLV-I LTR, or the HTLV-II LTR, respectively (11, 32).

in uninfected T-lymphocyte cell lines. We conclude that, in contrast to the results in uninfected cell lines, the C81-66-45, C82-15, and E55 cell lines all contain factors that activate HTLV LTR-directed gene expression in *trans*.

The results obtained for the first three nonproducer cell lines contrasted with the results obtained for the TL-OmI cell line as a recipient. In this cell line the CAT activity of plasmids that contained the HTLV-I or HTLV-II LTR sequence was lower than that observed in uninfected cells. We conclude that even though the TL-OmI cell line contains integrated HTLV-I sequences, it does not express *trans*-acting transcriptional factors specific for the HTLV LTR. Whether the low HTLV-I LTR transcriptional ability in this cell line represents a lack of required cellular factors or specific repression is under investigation.

To determine which virus-encoded proteins were present in the nonproducer cell lines, extracts of these cells were immunoprecipitated with an antiserum that had been previously shown to contain antibodies to the *gag*, *env*, and *x-lor* proteins (16, 17). [^{35}S]cysteine-labeled protein extracts were prepared from HUT102 and the four nonproducing cell lines. Virus-specific proteins precipitated from the HUT102 cell line included the gp61 *env* gene precursor and the processed gp45 *env* protein, the *gag* proteins, including the pp55 *gag* gene precursor, the p24 and p19 *gag* proteins, and the p42 *x-lor* gene product (16, 17, 25, 31).

The nonproducing cell line C81-66-45 contained only one of the immunoprecipitable HTLV-I-encoded proteins, the 42-kb *x-lor* gene product (17) (Fig. 1). The amino acid sequence of this protein has been previously shown to correspond to that predicted by the coding sequences of the *x-lor* gene (17). The C82-15 and E55 cell lines also contained 42- to 44-kd (42/44-kd) proteins recognized by the ATLL serum as well as the p55 *gag* gene precursor proteins and some smaller proteins that were probably *gag* gene products. None of the nonproducer cell lines contained proteins of the sizes anticipated for the HTLV-I *env* gene products. To determine whether the 42/44-kb proteins observed in the nonproducer cell lines were derived from the *x-lor* gene, cell extracts were immunoprecipitated with a monospecific rabbit antiserum to the *x-lor* fusion protein (9). This antiserum immunoprecipitates the 42-kd *x-lor* product from the C81-66-45 and HUT102 cell lines and does not react with other viral proteins (Fig. 2). This rabbit antiserum, but not preimmune serum from the same rabbit, recognized the 42/44-kd protein present in the E55 and C82-15 nonproducer cell lines. This observation demonstrates that these proteins are at least partially encoded by the *x-lor* gene. This antiserum did not recognize a corresponding protein in extracts of the TL-OmI cell line (data not shown). No virus-specific proteins were detectable in immunoprecipitates prepared from the TL-OmI cells with the HTLV-I patient antiserum (Fig. 3).

In a separate experiment, extracts prepared from the C81-66-45 cell line (containing the 42-kd *x-lor* product) were used to compete in immunoprecipitation reactions with labeled extracts from the C82-15 and E55 cell lysates. The proteins in the size range of 42 to 44 kd, but not other virus-associated proteins of the C82-15 and E55 cell lysates, competed with the unlabeled C81-66-45 cell lysate. On the basis of these experiments, we conclude that the C81-66-45, C82-15, and E55 cell lines all express the *x-lor* gene product, but the TL-OmI cell line does not. The heterogeneity in the

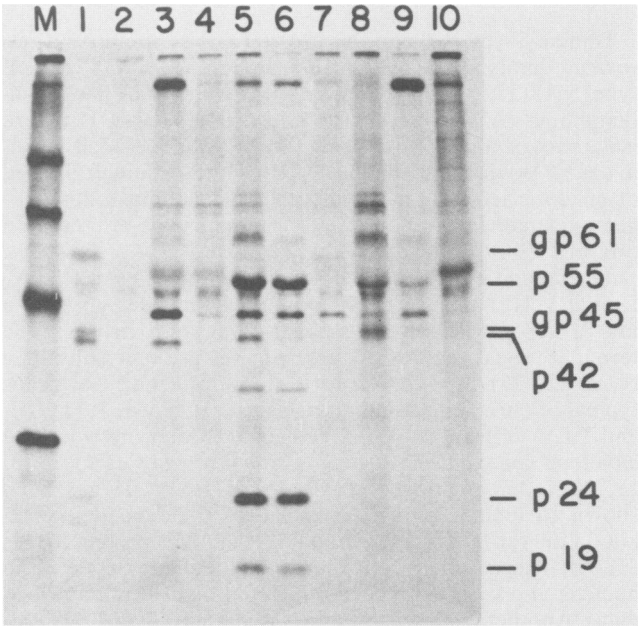

FIG. 1. Immunoprecipitation of proteins from HTLV-transformed cell lines with ATLL patient antiserum. HUT102, an HTLV-I-infected cell line derived from an American ATLL patient, and the three immortalized nonproducer lines C81-66-45, C82-15, and E55 were labeled with [^{35}S]cysteine overnight. Whole-cell lysates were prepared and immunoprecipitated with either ATLL patient antiserum (16, 17) (lanes 1, 3, 5, 6, 8, and 9) or normal human serum (lanes 2, 4, 7, and 10). Cell lysate sources were HUT102 (lanes 1 and 2), C81-66-45 (lanes 3 and 4), E55 (lanes 5, 6, and 7), and C82-15 (lanes 8, 9, and 10). Immunoprecipitation of the E55 and C82-15 cell lines was done without (lanes 5 and 8) or with (lanes 6 and 9) unlabeled cell lysates prepared from C81-66-45. Unlabeled cell lysates were present in a 100-fold excess over the labeled lysates; under similar conditions, unlabeled cell lysates from HTLV-negative T-lymphocyte lines (e.g., HUT78) did not compete for any virus-specific proteins (data not shown). The positions of the *env* (gp61 and gp45), *gag* (p55 and p24), and *x-lor* (p42) gene products are shown. Lane M, Molecular size markers (from the top: myosin, 200 kd; phosphorylase b, 92.5 kd; bovine serum albumin, 69 kd; ovalbumin, 46 kd; and carbonic anhydrase, 30 kd).

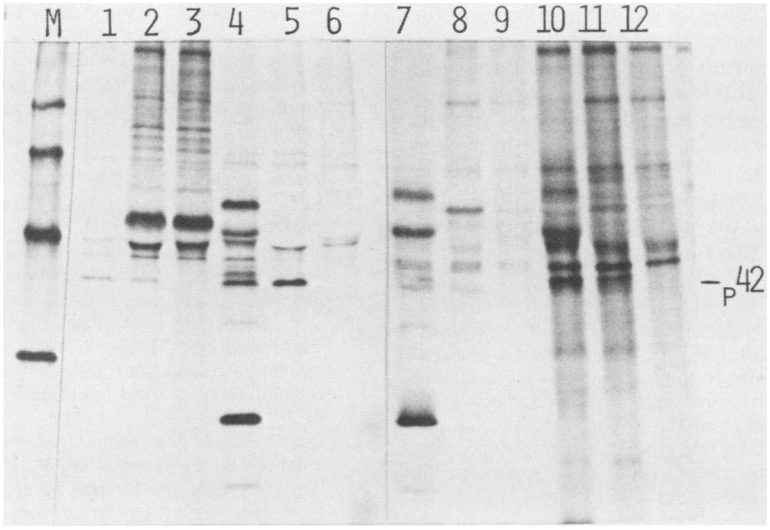

FIG. 2. Immunoprecipitation of nonproducer cell lines with monospecific rabbit antiserum. The C81-66-45 (lanes 1 through 3), HUT102 (lanes 4 through 6), E55 (lanes 7 through 9), and C82-15 (lanes 10 through 12) cell lines were labeled with [^{35}S]cysteine. Cell lysates were immunoprecipitated with either ATLL patient antiserum (16, 17) (lanes 1, 4, 7, and 10), monospecific rabbit antiserum directed against a bacterially synthesized β-galactosidase–*x-lor* fusion protein (lanes 2, 5, 8, and 11) (9), or preimmune serum from the same rabbit (lanes 3, 6, 9, and 12). p42, 42-kd HTLV-I *x-lor* product. Lane M, Molecular size markers (see Fig. 1 legend).

apparent molecular weight of the *x-lor* product in these nonproducing cell lines, varying from 42 to 44 kd, may be due either to variation in the amino terminus of the *x-lor* product or to posttranslational modifications of the *x-lor* product (16, 17).

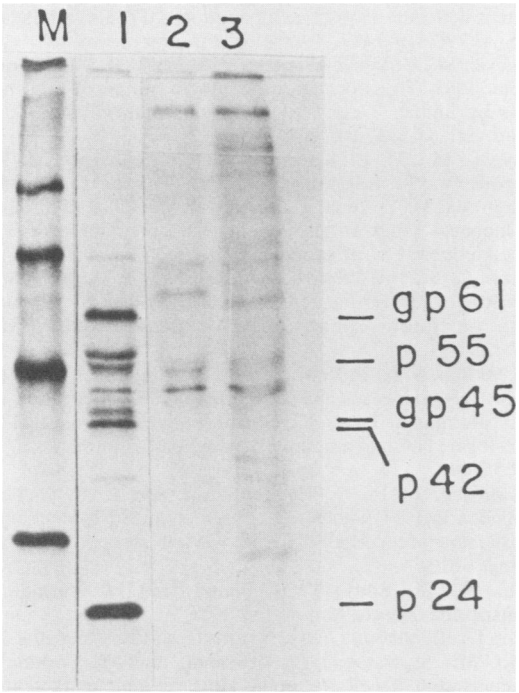

FIG. 3. Immunoprecipitation of the TL-OmI cell line with ATLL patient serum. ATLL patient serum (16, 17) (lanes 1 and 2) or normal human serum (lane 3) was used to immunoprecipitate [^{35}S]cysteine-labeled cell lysates from HUT102 (lane 1) or TL-OmI (lanes 2 and 3) cells. The positions of the gp61 and gp45 *env* gene products, the p55 and p24 *gag* gene products, and the p42 *x-lor* product of the HUT102 cell line are noted. Lane M, Molecular size markers (see Fig. 1 legend).

This report demonstrates that some of the cell lines immortalized by HTLV-I lack a full complement of viral proteins even though they contain factors that act in *trans* to stimulate gene expression directed by the HTLV-I LTR. All three cell lines that contained the 42-kd HTLV-I *x-lor* gene product displayed the phenomenon of LTR *trans*-activation. In one case, the *x-lor* gene product was the only virus-specific protein detectable in the cell. The cell line that contained no detectable HTLV-I-encoded proteins, including no detectable *x-lor* gene product, did not contain *trans*-acting transcriptional factors. These experiments provide indirect evidence for the involvement of the HTLV *x-lor* gene product in *trans*-activation of the HTLV-I LTR. However, the data presented here do not rule out the involvement of HTLV-encoded factors not recognized by ATLL patient sera in *trans*-activation.

We have speculated that the *x-lor* gene product may alter the regulation of cellular as well as viral genes and thereby alter the growth properties of infected cells (32). Specifically, we have proposed that the *x-lor* gene product activates the expression of genes that regulate lymphocyte proliferation (32). This hypothesis can explain the ability of HTLV-I and HTLV-II to immortalize primary human T-lymphocytes in vitro (3, 18, 21, 37) and the lack of specific provirus integration sites in ATLL tumors (12, 26).

The low incidence of ATLL in HTLV-I-infected individuals and the long latent period and monoclonality of HTLV-I-associated leukemias (7) argue that events other than virus infection are necessary for tumorigenesis. We favor a model in which HTLV infection, specifically the expression of the *x-lor* protein, leads to a proliferative response in vivo, a situation mimicked by in vitro immortalization. The presence of the *x-lor* product in HTLV-immortalized cell lines, in some cases to the exclusion of other viral proteins, favors a dual role for the *x-lor* protein in both the initiation and maintenance of this early event in transformation. Such growth deregulation in itself is probably not sufficient to establish the fully malignant phenotype in vivo. Secondary genetic events may allow the monoclonal expansion of a particular cell among a replicating population of virus-

infected T lymphocytes and may obviate the requirement for continued *x-lor* gene expression. This speculation is consistent with the observation that many fresh ATLL tumors do not express HTLV-related mRNAs (6). The hypothesis is also consistent with the observation that cell lines such as TL-Oml that do not express viral proteins can be established from ATLL tumors. This model suggests that the HTLV *x-lor* product would share functional similarities with other nuclear proteins that have the ability to modulate gene expression and to mediate the early immortalizing steps in transformation.

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