responding primarily to long-term O_3 pollution. The situation is complex, however, since all forest ecosystems are composed of unique combinations of biotic, geologic, edaphic, and climatic factors.

The results of this study do not conclusively answer the question of how present levels of atmospheric pollution are affecting vegetation. In general, improved monitoring of ambient O_3 concentrations in agricultural and forested regions throughout the country must occur before good estimates of the extent of O_3-induced reductions in P_n and growth can be made. In addition, O_3 concentrations may reach peak values in ambient air at potentially critical times, such as during periods of leaf expansion, flowering, or seed maturation. Furthermore, many questions about the long-term effects of acidic rain on soils, plants, or soil microorganisms remain unanswered. The results of this and other studies do, however, indicate that O_3 is an important pollutant affecting vegetation. Researchers working toward identifying whether North American forests or agro-ecosystems are suffering a general decline and understanding the causes of such a decline must assess the impact of O_3 pollution as well as that of acidic deposition.

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30. Ozone concentration and dose were correlated for a given species in these studies; not enough information is available to permit one to use dose to predict P_n in all cases. To our knowledge, no one has yet tested whether the same dose applied in the form of several low concentrations for differing exposure periods (for example, 0.08 ppm for 3 hours per day versus 0.03 ppm for 8 hours per day) would have the same effects or not. However, our data comparing different species suggest that, at relatively low concentrations, dose should be an effective measure of O_3 exposure.
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Functional Relation Between HTLV-II *x* and Adenovirus E1A Proteins in Transcriptional Activation

Abstract. *The mechanism of cellular transformation by the human T-cell leukemia viruses (HTLV) is thought to involve a novel gene known as the* x *gene. This gene is essential for HTLV replication and acts by enhancing transcription from the HTLV long terminal repeat. The HTLV* x *gene product may also cause aberrant transcription of normal cellular genes, resulting in transformation of the infected cells. Although there is no evidence as yet for such a mechanism, it was shown that the HTLV-II* x *gene product can activate transcription from adenovirus E1A-dependent early promoters and therefore has the potential to activate cellular genes. It was also shown that the adenovirus and herpes pseudorabies immediate early proteins activate expression from the HTLV-I and HTLV-II long terminal repeats, though at lower levels than with the* x *gene product. These findings indicate possible common mechanisms of action for transcription-regulatory genes of distinct viruses.*

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The human T-cell leukemia viruses type I (HTLV-I) and type II (HTLV-II) are associated with specific human T-cell malignancies (*1*). HTLV-I is the apparent etiologic agent of adult T-cell leukemia, a highly malignant leukemia/lymphoma endemic to parts of Japan, the Caribbean, and Africa (*1*). HTLV-II has been associated with a single case of T-cell variant hairy-cell leukemia (*1–4*). Both types of HTLV transform normal human cord or peripheral blood T lymphocytes in vitro, as defined by the continued proliferation of the T cells in the absence of exogenous interleukin-2 (*5*). The mechanism of cellular transformation by HTLV is unknown. HTLV is unlike acutely transforming retroviruses in that no viral oncogene is present in the HTLV sequence (*3, 4*), and it is unlike the nonacute leukemogenic retroviruses in that it does not appear to induce malignancies by association with cellular oncogenes (*6*). Therefore, HTLV appears to transform cells by a mechanism different from that of other retroviruses.

Both HTLV and bovine leukemia virus (BLV), an oncogenic virus of cattle sharing many biological and molecular features with HTLV (*7*), have a gene termed *x* or *lor* (*3, 4*) that is not present in the genomes of other animal retroviruses. This gene, which is hypothesized to be the transforming gene of HTLV and BLV, encodes a protein of 40 kD and 37 kD in HTLV-I– and HTLV-II–infected cells, respectively (*8*). The *x* gene is essential for HTLV replication and acts to enhance transcription by the HTLV long terminal repeat (LTR) (*9*). Mutants of HTLV that are defective for *x* gene functions transcribe only very low levels of HTLV RNA. The *x* gene product has a nuclear subcellular localization consistent with its proposed function in transcriptional activation (*10*).

Certain DNA viruses have gene products that act to enhance transcription by some viral promoters (*11*). For example, a gene product expressed by the E1A gene of adenovirus early after viral infection facilitates transcription by six unique adenovirus promoters (*11*), several endogenous cellular genes (*12*), and newly transfected genes (*13*). In addition to those genes transcribed by RNA polymerase II, the E1A proteins can also activate genes transcribed by RNA polymerase III (*14*). Adenovirus mutants lacking the E1A gene can transcribe

E1A-dependent promoters; however, the expression is delayed, compared to expression in the presence of the E1A gene (*15*). The E1A gene is also required for the transformation of rodent cells (*16*).

Aberrant transcriptional activation has been proposed as a possible mechanism for cellular transformation [see (*17*) for reviews], with viral and cellular transforming genes such as E1A (*16*), papovavirus T-antigens (*18*), and *myc* (*19*) having a role in carcinogenesis. The analogies between the function of adenovirus E1A genes and the HTLV *x* gene suggest that transformation by HTLV may also involve transcriptional activation of cellular genes, although there is no direct evidence for this hypothesis.

We examined the possibility that the *x* gene of HTLV acts to regulate the transcription of genes other than the HTLV LTR by testing the ability of the HTLV-II *x* gene to activate transcription by promoters normally dependent on the E1A gene product of adenovirus. Our results demonstrate that transcription by the promoters of the two early region adenovirus genes, early region II (E2) and early region III (E3), can be activated by the *x* gene product of HTLV-II.

The *x* gene of HTLV-I and HTLV-II has *trans*-acting transcription-regulatory (*trans*-regulatory) functions (*9, 20, 21*). Mutations within the *x* gene abolish the ability of otherwise normal HTLV-II proviruses to transcribe messenger RNA efficiently (*9*). A useful assay for *trans*-regulatory functions of the HTLV genome makes use of recombinant constructs between the LTR and the chloramphenicol acetyltransferase gene (CAT), for which expression of CAT is dependent on the efficient initiation of transcription by the LTR. The use of these constructs revealed that efficient LTR function occurred only in cells infected with HTLV-I or HTLV-II and did not occur in uninfected cells (*9, 20*).

Using assays similar to those in the LTR studies, we examined the *trans*-regulatory functions of HTLV with respect to the activation of transcription by other promoters by testing for activation of the E1A-dependent E3 promoter of adenovirus fused to the CAT gene. We used DNA transfection to introduce an E3 CAT construct (*22*) into a T-cell line transformed by HTLV-II (Mo-T) (Fig. 1A). E3 promoter activity was detected in the HTLV-II–infected cell lines, although it was not as efficient as LTR-II CAT expression in HTLV-II–infected cells. The SV40 promoter expressed CAT efficiently in both infected and uninfected cells. Other T-cell lines not infected with HTLV-II, HUT78 (Fig. 1), MOLT 4, and CEM, were not competent for E3 CAT expression although the SV-CAT constructs containing the SV40 promoter functioned efficiently in these cells. These results indicate that functions associated with HTLV-II infection regulate the transcriptional activity of the E3 promoter.

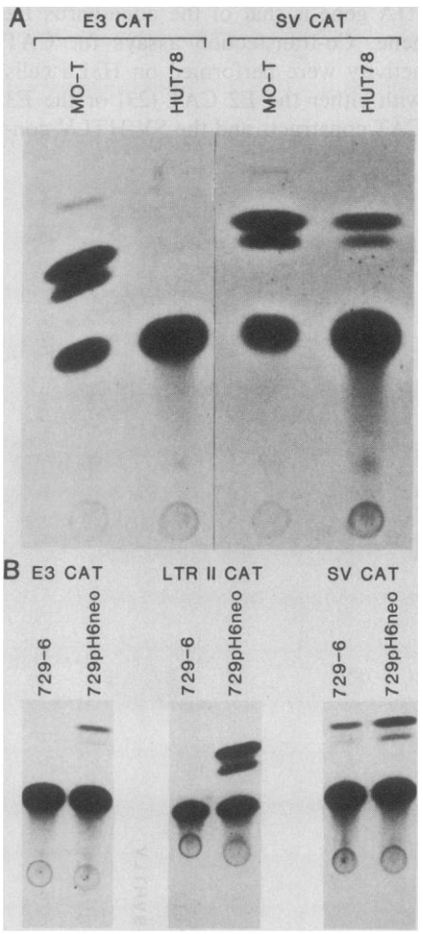

Fig. 1. Induction of E3 CAT expression in HTLV-II–infected and transformed cell lines. (A) The HTLV-II–transformed T-cell line, Mo-T, has been described (*5*). HUT78 is a mature T-cell line that is not infected with HTLV (*26*). The recombinant constructs E3 CAT, SV CAT and LTR-II CAT have been described (*9, 22*). E3 CAT contains sequences which include both the E3 promoter and adjacent E2 promoter sequences in the opposite orientation from adenovirus 5 (*22*). LTR-II CAT contains the entire HTLV-II LTR (*9*). SV CAT is a modification of pSV2 CAT (*27*) in pUC 8. (B) The B-cell lines, 729-6 and 729pH6neo, are uninfected or infected with HTLV-II, respectively. 729pH6neo, which produces infectious HTLV-II, has previously been described (*4, 9*). Of the indicated cells, 10^7 were transfected with 10 μg of the indicated plasmids by the DEAE-dextran transfection procedure. These cells were collected 42 hours after transfection and assayed for CAT activity as described (*9*). The migration of [^{14}C]chloramphenicol is from bottom to top, with the acetylated forms of chloramphenicol having greater mobility. In three experiments, the average conversion relative to SV CAT was (A) 0.92 for E3 CAT/Mo-T and <0.01 for E3 CAT/HUT78; (B) <0.05 for E3 CAT/729-6, 0.32 for E3 CAT/729pH6neo, <0.05 for LTR-II CAT/729-6, and 1.6 for LTR-II CAT/729pH6neo.

Activation of the E3 promoter was tested further by infecting Epstein-Barr virus (EBV)–transformed B-cell lines with HTLV-II. The use of these cell lines allowed us to compare E3 promoter function directly in an infected and uninfected pair derived from the same parent cells. The B cells are not transformed by HTLV-II but express HTLV-II products, including the *x* gene products of HTLV-II (*8, 9*). E3 promoter function was evident in the HTLV-II–infected B-cell lines, but not in the uninfected parental cell line (Fig. 1B). CAT activity from the plasmid SV CAT was detected in both infected and uninfected cell lines. These results demonstrate that the activation of expression by the E3 promoter occurs in HTLV-II–infected B cells or HTLV-II–transformed T-cell lines.

We demonstrated earlier that the *x* gene of HTLV-II is directly responsible for transcriptional activation of the HTLV-II LTR (*9*). Therefore, the *x* gene of HTLV-II is probably responsible for the activation of the E3 promoter in the HTLV-II–infected cells. This was directly tested by using a transient transfection assay for HTLV-II *trans*-regulatory functions in which CAT expression constructs and HTLV-II genes expressed by an SV40 promoter (SV-HTLV) were co-transfected (*21*). Since the HTLV-II LTR is relatively weak in most cell lines (*4*), substitution of the SV40 promoter for the LTR allows more efficient expression of HTLV-II genes in the construct (*21*). This construct activates the transcription of HTLV-I and HTLV-II LTR CAT constructs when introduced into COS or HeLa cells by co-transfection (*21*). Mutations in SV-HTLV that abolish *x* gene function result in a loss of transcriptional activation functions (*21*).

We used the SV-HTLV construction to determine whether the *x* gene was responsible for the enhanced transcription by the E3 adenovirus promoter. Co-transfection of E3 CAT and SV-HTLV into COS cells produced a marked increase in CAT activity, indicating that functions of the HTLV-II genome were capable of enhancing transcription of the E3 promoter (Fig. 2A). That this activity was due to the *x* gene of HTLV was shown by using a mutant of SV-HTLV (SV-HTLV Cla 5) defective for *x* gene functions. This mutant contains a 2–base pair insertion in the *x* gene that completely abolishes its ability to enhance expression by the LTR (*21*). This mutant was also incapable of enhancing expres-

sion by the E3 promoter. We showed that the activation of E3 expression was due to increased transcription by S1 nuclease analysis of E3-specific RNA in the co-transfected cells. Using an S1 nuclease probe that spans the cap site of the E3 promoter, we detected increased levels of correctly initiated RNA in co-transfection with E1A and with SV-HTLV, but not with SV-HTLV Cla 5 (Fig. 2C). These results demonstrate that the *x* gene of HTLV-II is capable of enhancing transcription by promoters other than the LTR.

We tested whether other adenovirus genes activated by the adenovirus E1A gene product could also be activated by the HTLV-II *x* gene functions. A second promoter dependent on the adenovirus E1A gene is that of the adenovirus E2 gene. Co-transfection assays for CAT activity were performed on HeLa cells with either the E2 CAT (*23*) or the E3 CAT constructs and the SV-HTLV construct (Fig. 2B). These experiments were performed on HeLa cells to exclude potential complicating effects of the SV40 T-antigen present in COS cells. The results were similar to those obtained in COS cells. E2 CAT expression was activated when E2 CAT was co-transfected with SV-HTLV in HeLa cells (Fig. 2B), although in general, the level of activation was lower than that for E3 CAT. Co-transfection with the SV-HTLV Cla 5 mutant did not enhance E2 CAT or E3 CAT expression. These results demonstrate that other E1A-dependent promoters can also be activated by the HTLV-II *x* gene product.

The ability of the HTLV-II *x* gene product to activate expression by E1A-inducible adenovirus promoters suggested that the HTLV LTR may be responsive to the *trans*-activating proteins of DNA viruses (*11, 13, 24*). We used the co-transfection assay in HeLa cells to test the response of transcription by the HTLV LTR to these other gene products. Both the HTLV-I LTR and the HTLV-II LTR were reproducibly responsive to the adenovirus E1A gene product and the pseudorabies immediate early (IE) gene product, although the degree of transcriptional induction was weak. The level of LTR-I and LTR-II CAT induction was usually only 10 to 20 percent of that observed with the HTLV-II *x* gene construct SV-HTLV (Fig. 3). The induction was also weaker than the response of the E3 promoter to E1A and IE. Similar results were obtained when the response of the LTR's to the E1A protein was compared in HeLa cells infected with either wild-type adenovirus or dl 312, an adenovirus mutant in the E1A gene, in the presence of arabinosylcytosine to induce the E1A proteins to high levels (*25*).

These results provide direct evidence that the transcription-regulatory functions of the HTLV-II *x* gene can act to enhance expression from promoters other than the HTLV LTR, in this case, adenovirus E1A-dependent early promoters. We also show that the HTLV-I and HTLV-II LTR's are weakly responsive to the E1A and herpes pseudorabies IE gene product, indicating that functional similarities exist between the *x* protein of HTLV and other transcription-regulatory proteins. The similar response of these inducible promoters to heterologous transcription-regulatory genes may indicate similar mechanisms of transcription induction. A gross comparison of nucleic acid and amino acid sequences of the adenovirus E1A gene product and the HTLV-I or HTLV-II *x* gene product reveals no homology.

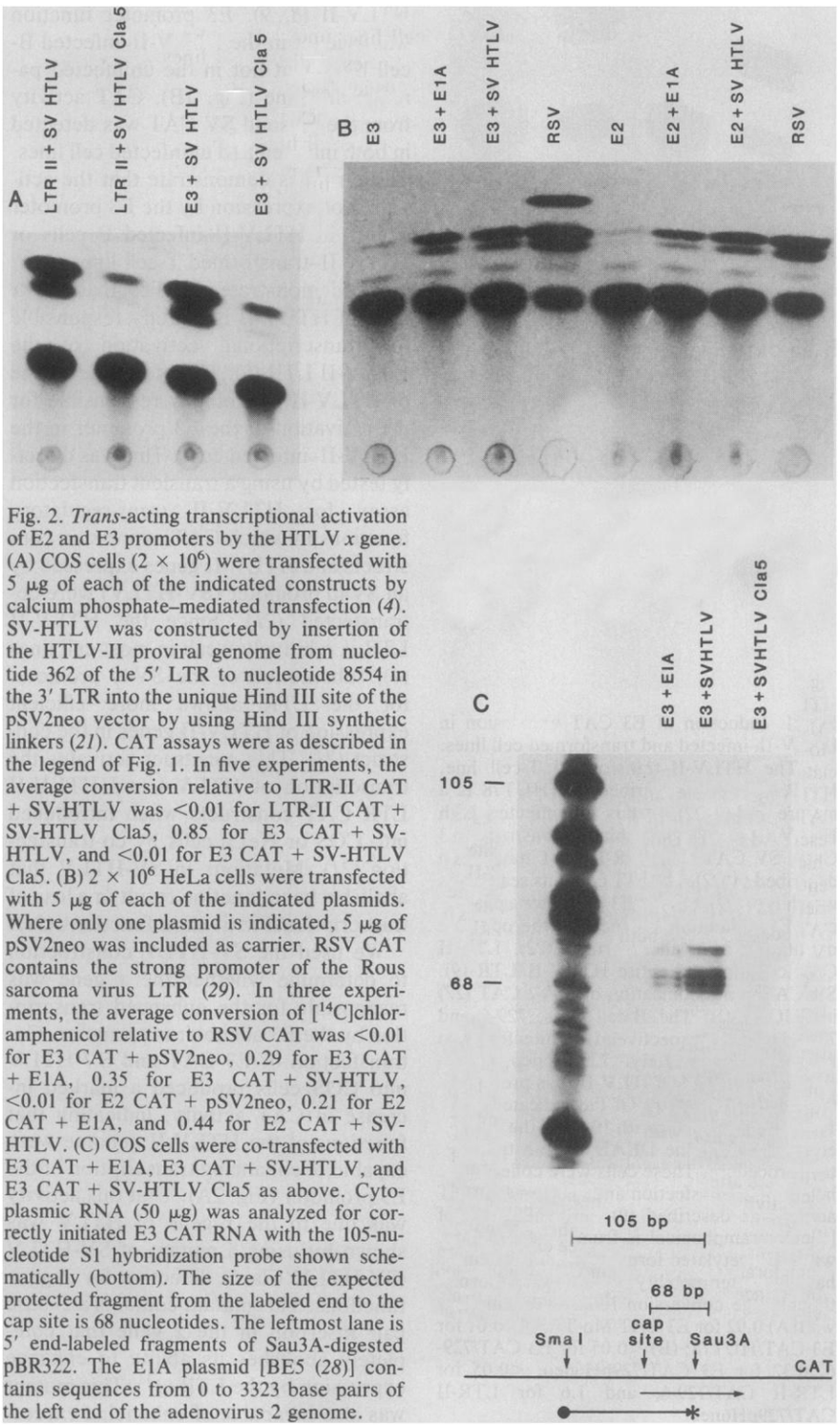

Fig. 2. *Trans*-acting transcriptional activation of E2 and E3 promoters by the HTLV *x* gene. (A) COS cells (2×10^6) were transfected with 5 μg of each of the indicated constructs by calcium phosphate–mediated transfection (*4*). SV-HTLV was constructed by insertion of the HTLV-II proviral genome from nucleotide 362 of the 5′ LTR to nucleotide 8554 in the 3′ LTR into the unique Hind III site of the pSV2neo vector by using Hind III synthetic linkers (*21*). CAT assays were as described in the legend of Fig. 1. In five experiments, the average conversion relative to LTR-II CAT + SV-HTLV was <0.01 for LTR-II CAT + SV-HTLV Cla5, 0.85 for E3 CAT + SV-HTLV, and <0.01 for E3 CAT + SV-HTLV Cla5. (B) 2×10^6 HeLa cells were transfected with 5 μg of each of the indicated plasmids. Where only one plasmid is indicated, 5 μg of pSV2neo was included as carrier. RSV CAT contains the strong promoter of the Rous sarcoma virus LTR (*29*). In three experiments, the average conversion of [^{14}C]chloramphenicol relative to RSV CAT was <0.01 for E3 CAT + pSV2neo, 0.29 for E3 CAT + E1A, 0.35 for E3 CAT + SV-HTLV, <0.01 for E2 CAT + pSV2neo, 0.21 for E2 CAT + E1A, and 0.44 for E2 CAT + SV-HTLV. (C) COS cells were co-transfected with E3 CAT + E1A, E3 CAT + SV-HTLV, and E3 CAT + SV-HTLV Cla5 as above. Cytoplasmic RNA (50 μg) was analyzed for correctly initiated E3 CAT RNA with the 105-nucleotide S1 hybridization probe shown schematically (bottom). The size of the expected protected fragment from the labeled end to the cap site is 68 nucleotides. The leftmost lane is 5′ end-labeled fragments of Sau3A-digested pBR322. The E1A plasmid [BE5 (*28*)] contains sequences from 0 to 3323 base pairs of the left end of the adenovirus 2 genome.

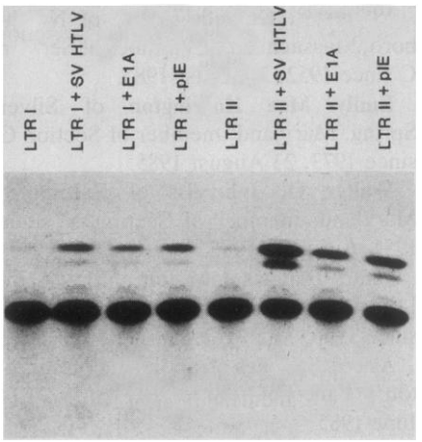

Fig. 3. Activation of HTLV-I LTR and HTLV-II LTR expression by adenovirus E1A and pseudorabies virus IE genes. HeLa cells (2×10^6 were co-transfected with 5 μg of each of the indicated constructs by calcium phosphate–mediated transfection. Where only one construct is indicated, pSV2neo was included as carrier. The E1A (BE5) and IE (pIE) genes used in the transfections have been described (*13, 24, 28*). The IE plasmid contains a 15-kbp Sal I fragment containing the pseudorabies virus IE gene in pBR325 (*13*). The LTR-I CAT construct was derived from the 3′ LTR of an HTLV-I provirus and includes approximately 1.0 kbp additional of 3′ viral sequences. This construct expresses CAT activity in cells infected with HTLV-I and HTLV-II and in co-transfection assays with SV-HTLV (*21*). In three experiments, the average conversion of [^{14}C]chloramphenicol relative to LTR-I CAT + SV-HTLV was <0.01 for LTR-I CAT + pSVneo, 0.15 for LTR-I CAT + E1A, 0.20 for LTR-I CAT + pIE, and relative to LTR-II CAT + SV-HTLV was <0.01 for LTR-II CAT + pSVneo, 0.12 for LTR-II CAT + E1A, and 0.16 for LTR-II CAT + pIE.

However, limited sequence homology was detected in short regions of the adenovirus E2 promoter and the HTLV-I and HTLV-II LTR's. Nine of 12 nucleotides within an imperfectly repeated region upstream from the adenovirus E2 cap site (at −121 to −110 (ATGGCGCTGACG) and repeated at −108 and −83) are homologous to the 21-nucleotide repeats of the HTLV-I and HTLV-II LTR's. A similar sequence is not present immediately before the E3 promoter; however, the E3 CAT construct includes the upstream sequences of the E2 promoter (*22*), since the E3 and E2 transcriptional units are adjacent and oriented in opposite directions in the adenovirus genome. The significance of this short sequence is unclear since it does not appear to be a unique sequence responsible for E1A induction (*22, 23*).

The similar functions of structurally distinct gene products, the E1A gene product, the HTLV *x* gene product, and pseudorabies IE gene product (*11, 13*) present in distinct families of viruses, indicate that the adenoviruses, retroviruses, and herpesviruses have independently evolved genes critical for their own replication. The adenovirus E1A gene has been shown to facilitate transcription from early adenovirus genes (*11*), although adenovirus transcription will occur in the absence of the E1A gene products with delayed kinetics (*15*). The *x* gene is required for efficient transcription of HTLV-II proviral templates; however, low levels of transcription (less than 1/100 the level of wild-type HTLV-II) are present in the absence of a functional *x* gene product (*9*). The precise function of such genes in these viruses is unknown; they may relate to mechanisms that evolved to allow regulation of viral transcription in response to differing host cell environments.

The adenovirus E1A proteins induce transcription of a few endogenous cellular genes (*12*), indicating that the induction of aberrant transcriptional regulation may be important in cellular transformation. The demonstration that the *x* gene of HTLV-II can act in *trans* to regulate expression of other promoters provides support for the hypothesis that anomalous transcriptional regulation of cellular genes may be the mechanism of HTLV leukemogenesis. The human T-cell leukemia viruses transform normal human T lymphocytes in vitro (*5*), resulting in the continued proliferation of the infected T cells. These cells have many of the characteristics of T cells stimulated by lectin or antigen. The ability of the HTLV-II *x* gene to enhance expression by the E1A-dependent promoters of E2 and E3 suggest that the *x* gene product may act on promoters in the T cells that normally respond to cellular transcription factors induced by antigenic stimulation of the cells. A transcriptional regulation model of carcinogenesis may be applicable to oncogenesis by HTLV and oncogenic DNA viruses such as herpesviruses, adenoviruses, and papovaviruses, as well as to some nonviral cancers.

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