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c-*fos* promoter trans-activation by the tax_1 protein of human T-cell leukemia virus type I

(transcription/regulatory elements/oncogenesis)

MASAHIRO FUJII, PAOLO SASSONE-CORSI, AND INDER M. VERMA

Molecular Biology and Virology Laboratory, The Salk Institute, P.O. Box 85800, San Diego, CA 92138

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ABSTRACT **To understand the mechanisms of oncogenesis by human T-cell leukemia virus type I, we have investigated the ability of the tax_1 protein to modulate transcription of protooncogenes. By using a transient cotransfection assay, we report that the protooncogene *fos* promoter is trans-activated by tax_1 in a variety of cell types. Two regions containing upstream sequences between positions −362/−324 and −323/−276 of the c-*fos* promoter responded to this activation and also conferred tax_1 responsiveness to the heterologous herpesvirus thymidine kinase promoter. These two sequences include elements mediating the induction by v-sis-conditioned medium and serum, phorbol ester, or epidermal growth factor, respectively. Furthermore, expression of the endogenous c-*fos* gene was activated by tax_1 in human T-cell leukemia virus type I-infected cell lines. In contrast, no trans-activation of the c-*myc* or c-Ha-*ras* promoter was observed.**

Human T-cell leukemia virus type I (HTLV-I) is the etiological agent of an aggressive form of human malignancy, adult T-cell leukemia, and transforms normal human T lymphocytes *in vitro* (1–3). Unlike many other acutely transforming retroviruses, the HTLV-I genome does not contain an oncogene homologous to cellular sequence (4). In addition to the structural genes (*gag*, *pol*, and *env*) characteristic of replication-competent retroviruses, the HTLV-I genome encodes a 40-kDa protein designated as tax_1 (p40, pX, TA-I, or tat-I) that is not only critical for transformation but has been shown to trans-activate the viral promoter in the long terminal repeat (LTR) (5–8). Furthermore, tax_1 may regulate transcription of some cellular genes, such as interleukin 2 (IL-2) and the IL-2 receptor (9, 10).

Expression of several protooncogenes, including the nuclear oncogenes c-*fos*, c-*myc*, and c-*myb*,* is transiently induced in peripheral blood lymphocytes after stimulation with lectins, suggesting that their products might be involved in the molecular events leading to T-cell proliferation and activation (11, 12). Since tax_1 trans-activates its cognate promoter in the LTR and other cellular genes, we tested whether it would also stimulate the expression of protooncogenes implicated in T-cell proliferation. Here, we show that the HTLV-I tax_1 activates expression of the c-*fos* protooncogene in a transient cotransfection assay. The endogenous *fos* gene expression is also increased upon transfection with a tax_1 expression plasmid or infection with HTLV-I. c-*fos* encodes a nuclear protein that is thought to be involved in cell growth and differentiation (13). An accumulating body of evidence suggests that the c-*fos* gene product regulates the transcription of several genes, including itself (14, 15). Therefore, our results suggest that trans-activation by tax_1 may initiate a network of complex transcriptional regulation leading to transformation.

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MATERIALS AND METHODS

Plasmids. The plasmids pMAXneo, pMAXneo/M, pCHL4, c-Ha-ras–CAT, c-myc–CAT, and FC1–FC10 have been described (8, 16–19). Briefly, pMAXneo contains tax_1 from HTLV-I under the control of the mouse metallothionein I promoter (8–16). pMAXneo/M was constructed by filling in the unique *Mlu* I site [182 nucleotides (nt) from the initiation codon AUG], resulting in premature termination in the p40 coding region (16). The pCHL4 (referred to in Fig. 1 as HTLV-I LTR) contains the bacterial chloramphenicol acetyltransferase (CAT) gene under the control of HTLV-I LTR (from position −325 to position +306) (16). The c-fos–CAT (FC) constructs (FC1–FC10) contain various *fos* promoter sequences from position −2250 (FC1) through position −124 (FC10) to position +42 (17); the c-Ha-ras–CAT construct contains the c-Ha-*ras* sequence between positions −420 and +130 (18); the c-myc–CAT construct contains c-*myc* sequence between positions −2325 and +36 (19).

The plasmids p1tk, p2tk, p3tk, pBtk, and p5tk were constructed by insertion of five sequential fragments from position −705 to position −100 of the c-*fos* promoter into pTEN plasmid. pTEN contains the herpesvirus thymidine kinase promoter (positions −109 to +51) linked to the CAT gene (20). The five restriction fragments were cloned directly upstream of the thymidine kinase promoter in the sense orientation (see Fig. 2*B*). Similarly, four fragments from pBtk sequence were inserted into the pTEN vector and designated pCtk, pDtk, pFtk, and pGtk (see Fig. 2*B*).

Cells and Transfections. Human HeLa cells and HepG2 cells, a hepatocellular carcinoma cell line, were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) fetal calf serum (FCS). HuT 102 is a HTLV-I-transformed human T-cell line (1). K562 is a HTLV-I-uninfected human erythroleukemia cell line and CEM and Jurkat are T-cell lines. HOS cells are a human osteosarcoma cell line (21). HOS/PL was obtained by infecting parent HOS cells with HTLV-I, and thus HOS/PL cells express a functional tax_1 protein (21, 22). They were cultured in RPMI 1640 medium supplemented with 10% FCS.

For transfection, HeLa and HepG2 cells were seeded at 5–10 × 10^5 cells per 100-mm plate in Dulbecco's modified Eagle's medium supplemented with 10% FCS. Five micrograms of the indicated CAT construct DNA was transfected along with 1–5 μg of pMAXneo, pMAXneo/M, or pGEM-4 by using the calcium phosphate coprecipitation technique as described (23). K562 cells were transfected by using the DEAE-dextran technique as described (16). CAT activity in cell extracts was determined as described (24) and quantified by densitometer scanning of autoradiograms. Fold activation shows the ratio of CAT activity in the cells cotransfected with

Abbreviations: HTLV-I, human T-cell leukemia virus type I; IL-2, interleukin 2; CAT, chloramphenicol acetyltransferase; LTR, long terminal repeat; nt, nucleotide(s); FCS, fetal calf serum.
*Human gene symbols are not used in this report.

pMAXneo with respect to cells cotransfected with pGEM-4 and is an average of a series of experiments.

RNA Analysis. Cytoplasmic RNA was extracted from growing cells or from cells transfected with pMAXneo, pMAXneo/M, or pGEM-4 and ≈30 μg was hybridized to a specific complementary RNA probe. RNase protection analysis was performed as described (25, 26). A 1073-nt complementary RNA from the human c-*fos* gene (positions −100 to +973) and a 340-nt complementary RNA from FC4 were used as probes for endogenous c-fos and c-fos–CAT mRNA, respectively. The expected fragments that were protected after RNase digestion were 297 nt and 310 nt, respectively.

RESULTS

Trans-Activation of the c-*fos* Promoter by the tax_1 Protein. To examine the effect of tax_1 on protooncogene promoters, we cotransfected HeLa cells with a tax_1 expression plasmid (pMAXneo, refs. 8 and 16) and with constructs containing the CAT gene under the control of promoters from the oncogenes c-*fos*, c-Ha-*ras*, or c-*myc* (17–19). In addition to the 40-kDa tax_1 protein, the pMAXneo plasmid can potentially encode p27 and p21 products (27). Only p40 has been shown to manifest trans-activation properties to date, although p27 has been reported to act as a posttranscriptional regulator of viral gene expression (28). Fig. 1*A* shows that CAT activity from the HTLV-I promoter was greatly increased by cotransfection with pMAXneo as reported (compare lanes 1 and 2, ref. 7). Similarly, expression from the c-*fos* promoter was also increased by tax_1 (lanes 3–6). Since c-*fos* promoter activity is serum dependent (ref. 29; and compare also lanes 4 and 6), we tested whether stimulation by tax_1 would occur at low (0.1% FCS) as well as at high (10% FCS) serum concentrations. As shown in lanes 3–6, the c-*fos* promoter transactivation is serum independent, although the extent of stimulation is higher in low serum presumably because of the lower basal level of transcription of the c-*fos* promoter. In contrast, tax_1 had little effect on either c-Ha-*ras* or c-*myc* promoter activity (lanes 7–10).

To confirm that the activation of the c-*fos* promoter by pMAXneo is mediated by tax_1, a frame-shift mutant (pMAXneo/M) within the tax_1 coding region (16) was cotransfected with a c-fos–CAT construct into HeLa cells. No effect on c-*fos* expression was observed, indicating the requirement for a functional tax_1 protein (Fig. 1*B*). The c-*fos* promoter was also trans-activated by tax_1 in HepG2, a human hepatoma cell line, and in K562, a human erythroleukemia cell line (Fig. 1*C*). Therefore, the effect of tax_1 on the c-*fos* promoter, like that for the HTLV-I promoter, is not tissue-specific.

RNase protection analysis with an SP6 complementary RNA probe from the c-*fos*–CAT gene exhibited an increased level of correctly initiated RNA in cotransfection assays with pMAXneo but not with pGEM-4 or with mutant pMAXneo/M (Fig. 1*D*). It thus appears that trans-activation of the c-*fos* promoter by tax_1 protein is at the transcriptional level.

Identification of c-*fos* Promoter Regions Required for tax_1 Trans-Activation. To delineate the sequences required for tax_1 activation, we used a series of 5′ deletion mutants of the c-*fos* promoter linked to the CAT gene. Fig. 2*A* shows both

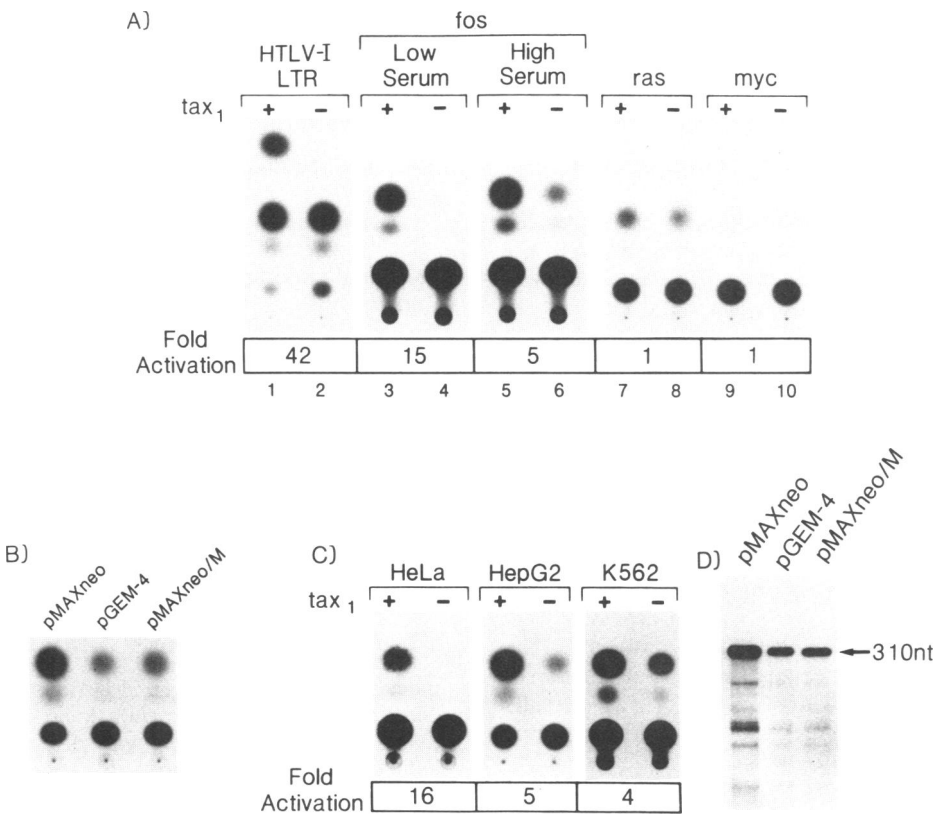

FIG. 1. Trans-activation of the c-*fos* promoter by tax_1 protein. (*A*) Cotransfection experiments of promoter–CAT recombinants with the tax_1 expression vector pMAXneo in HeLa cells. Representative experiment of the effect of tax_1 on the cognate HTLV-I LTR promoter (lanes 1 and 2), the c-*fos* promoter (FC4, lanes 3–6), the c-Ha-*ras* activity (lanes 7 and 8), and the c-*myc* (lanes 9 and 10) activity. Low serum is 0.1% FCS, and high serum is 10% FCS. The extent of the promoter activation by tax_1 is indicated and is the average value of four experiments. (*B*) Frame-shift mutation in the tax_1 coding sequence impairs trans-activation. pMAXneo (intact tax_1), pGEM-4 (control plasmid), and pMAXneo/M (frame-shift mutant) were cotransfected with FC4. (*C*) c-*fos* promoter is trans-activated by tax_1 in a variety of cell types. A representative experiment in which FC4 was cotransfected with pMAXneo in HeLa, HepG2 (human hepatoma), and K562 (human erythroleukemia) cells is shown. The extent of trans-activation is the average value of three experiments. (*D*) RNase protection analysis of promoter trans-activation by tax_1. The 310-nt long fragment represents c-fos–CAT mRNA.

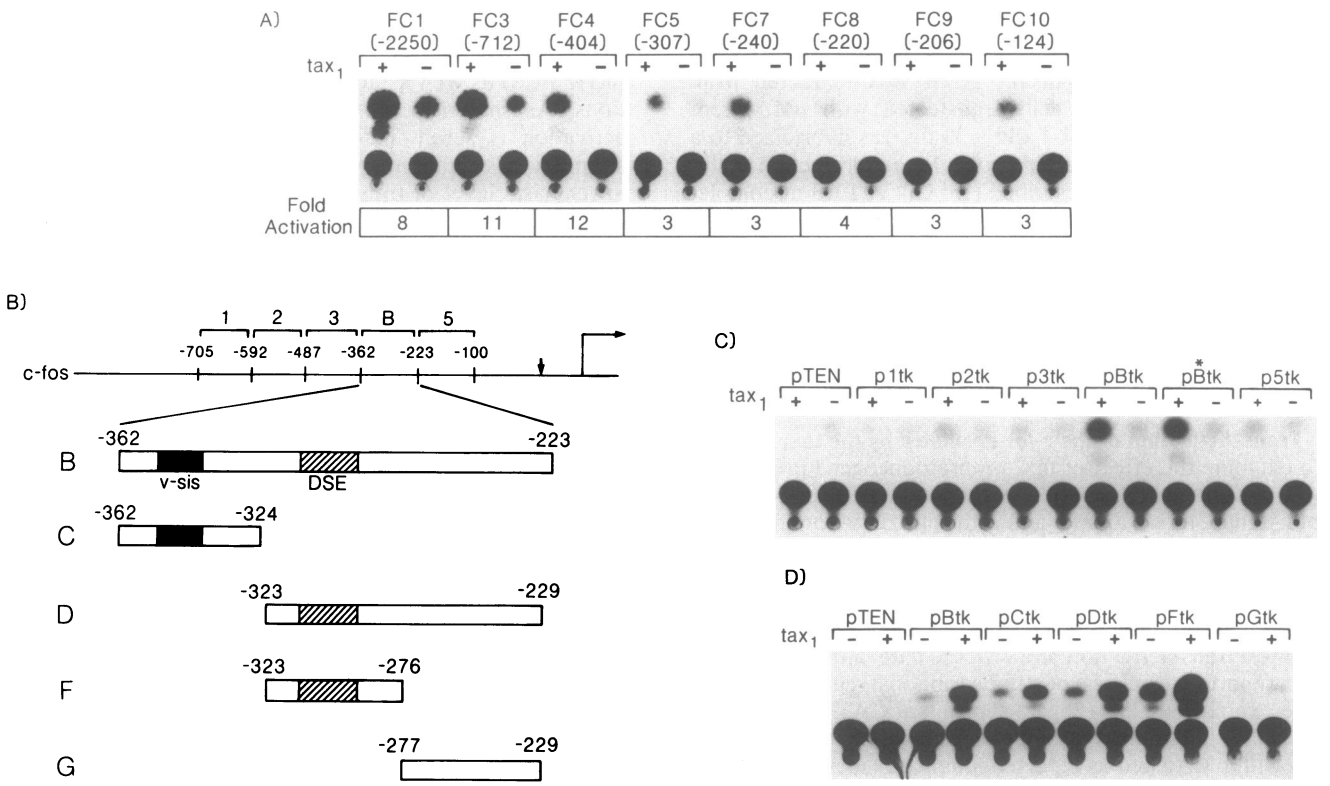

FIG. 2. Identification of the c-*fos* promoter region required for tax_1 trans-activation. (*A*) HeLa cells were transfected with the indicated construct with pMAXneo (+) or with pGEM-4 (−). The fold activation is an average of four experiments. The 5′ endpoints of the deletions are indicated. (*B*) c-*fos* promoter structure. The solid box and hatched region represent the v-sis-conditioned medium responsive element and the dyad symmetry element (DSE), respectively. (*C*) The recombinants pTEN, p1tk, p2tk, p3tk, pBtk, and p5tk were cotransfected in HeLa cells with pMAXneo (+) or with pGEM-4 (−). In pB*tk, the B fragment is cloned in the nonsense orientation. (*D*) The plasmids pTEN, pBtk, pCtk, pDtk, pFtk, and pGtk were cotransfected in HeLa cell with pMAXneo (+) or with pGEM-4 (−). The endpoints of each construct are indicated in *B*. The large arrow indicates the start site of transcription, and the small arrow indicates the TATA box.

the endpoint of the deletions and CAT activities in HeLa cells after cotransfection with pMAXneo plasmid, compared to the basal level. The expression from FC1, FC3, and FC4 plasmids was augmented by tax_1, whereas constructs FC5, FC7, FC8, FC9, and FC10 showed both lower basal levels and weak activation. These data suggest that the majority of activation by tax_1 is mediated by c-*fos* upstream sequences between positions −404 and −307. Similar results were obtained in K562 cells (data not shown).

To further delineate the c-*fos* sequences required for tax_1 activation, we inserted five sequential fragments of the c-*fos* promoter (between positions −705 and −100) into pTEN, a plasmid that has the CAT gene under the control of the herpesvirus thymidine kinase promoter (20) and does not respond to tax_1 trans-regulation (Fig. 2*C*). Upon cotransfection of HeLa cells with pMAXneo plus one of the five plasmids containing various c-*fos* upstream promoter fragments, only pBtk-CAT showed increased CAT activity. The increased transcription was independent of the orientation of the fragment (compare pBtk with pB*tk-CAT). These results indicate that the 140-base-pair fragment of the c-*fos* upstream region between positions −362 and −223 contains cis-acting regulatory elements that are responsive to trans-activation by tax_1. Since this 140-base-pair fragment contains multiple promoter elements, including the dyad symmetry element that encompasses the serum responsive element (29), an AP-1-like element (30), the v-sis-conditioned-medium responsive element (31), and an E1A-inducible element (32), plasmids containing subfragments of the *B* element were constructed (Fig. 2*B*, constructs pCtk, pDtk, pFtk, and pGtk). The activation by tax_1 was detected for all plasmids except pGtk. As indicated in Fig. 2*B*, pCtk and pFtk contain elements required for the induction by v-sis-conditioned medium and serum, respectively.

Transcriptional Activation of the Endogenous c-*fos* Gene. To address whether c-*fos* trans-activation could be due to the episomal conformation of the c-*fos*–CAT fusion gene plasmid, we investigated whether an increase of c-*fos* transcription could be detected in HTLV-I-infected cells. Fig. 3*A* shows that, in two HTLV-I-infected cell lines (HuT 102, lane 1, and HOS/PL, lane 4) that produce functional tax_1, c-*fos* gene transcription is induced, as determined by RNase protection analysis (see the 297-nt protected fragment in Fig. 3*A*). In uninfected cell lines, no c-*fos* transcripts could be detected (lanes 2, 3, and 5). Furthermore, the transfection of the tax_1 expression plasmid into K562 cells increased the number of c-*fos* transcripts compared with those with pGEM-4 or pMAXneo/M, a frame-shift mutant (Fig. 3*B*). The low level of induction of the c-*fos* gene in pMAXneo-transfected cells as compared to HTLV-I-producing HuT 102 cells likely represents the lower efficiency of DNA transfection. These results indicate that tax_1 activates endogenous c-*fos* gene expression constitutively, although the data do not rule out the possibility that other viral components may also contribute toward the induction of the c-*fos* gene.

DISCUSSION

In the present study, we have examined the effect of the HTLV-I trans-activating protein tax_1 on c-*fos* gene expression. By using cotransfection in culture cells, we showed that c-*fos* promoter trans-activation by tax_1 requires sequences located between positions −362 and −276. By using deletion mutants and isolated fragments encompassing various promoter regions, we have demonstrated that at least two

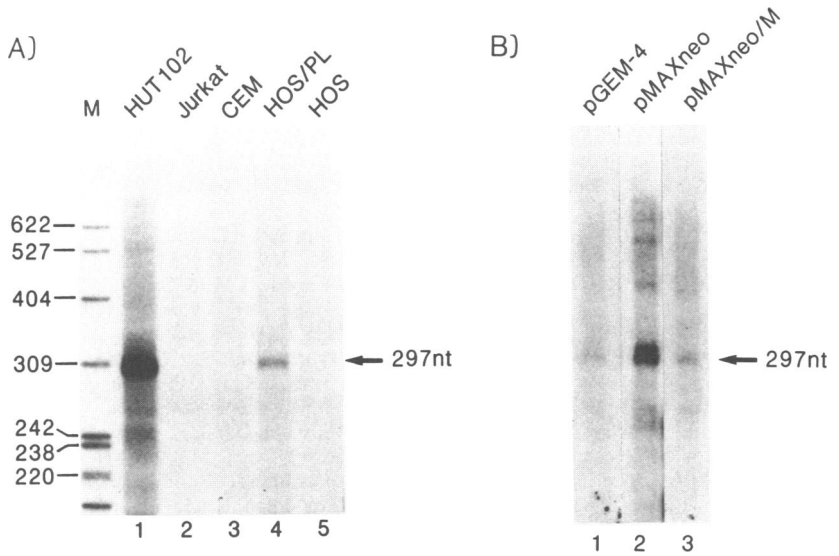

FIG. 3. Activation of the endogenous c-*fos* gene in HTLV-I-infected cells or by tax_1. (*A*) Cytoplasmic RNA (30 μg) was extracted from HuT 102 (lane 1), Jurkat (lane 2), CEM (lane 3), HOS/PL (lane 4), or HOS (lane 5) cells in the logarithmic growth phase and was hybridized with a ^{32}P-labeled human *FOS* cytoplasmic RNA probe as template. The 297-nt fragment represents the endogenous c-*fos* expression. (*B*) K562 cells were transfected with pGEM-4, pMAXneo, or pMAXneo/M by using the DEAE-dextran procedure. Thirty-six hours after transfection, cells were harvested, and cytoplasmic RNA was extracted and used for RNase protection analysis.

cis-acting elements mediate trans-activation of the c-*fos* promoter by tax_1. Fragment C (Fig. 2*B*) contains a putative cAMP-responsive element (33, 34) that is similar to a sequence in the HTLV-I 21-base-pair element required for trans-activation by tax_1 (22). However, one copy of the cAMP-responsive element sequence (−60 elements, ref. 29) linked to herpesvirus thymidine kinase promoter did not confer tax_1 responsiveness in HeLa cells (data not shown), suggesting that at least one copy of the cAMP-responsive element is not sufficient for tax_1 activation. Additionally, Hayes *et al.* (31) have identified a nuclear factor whose binding to a sequence in fragment C is increased by v-sis-conditioned medium. Therefore, tax_1 activation might involve interactions with this factor.

It has been pointed out (35) that a consensus sequence "C-C-(A+T-rich)-G-G" (CCArGG) is a protein-binding site common to transcription-regulatory regions of the cardiac actin, c-*fos*, and IL-2 receptor genes. This sequence forms part of the dyad symmetry element and is required for induction of the c-*fos* gene with serum and other growth factors (29). Cross *et al.* (10) have identified a region in human IL-2 receptor gene containing the CCArGG sequence that is essential for trans-activation by tax_1. Furthermore, this sequence competes for binding with the serum-responsive factor (35), implying the possibility that the dyad symmetry element may mediate the trans-activation of two genes by tax_1 protein. In addition, as reported (36), trans-activation of the IL-2 receptor promoter by tax_1 may also occur through a NF-κB-like transcriptional factor, although this result does not rule out a possible participation of the nuclear factor serum-responsive factor in tax_1 activation.

The mechanism by which HTLV-I induces T-cell transformation remains obscure since its genome does not contain an oncogene homologous with a cellular gene and its random integration in the cellular genome is unlikely to activate protooncogenes by promoter/enhancer insertion (4, 37). tax_1, in addition to trans-activating the HTLV-I LTR, may also activate cellular genes required for T-cell growth. In fact, tax_1 can activate promoters of IL-2 and IL-2 receptor genes (9, 10). Although HTLV-I-transformed cells constitutively express IL-2 receptor, simultaneous IL-2 production is rarely observed. IL-2 receptor activation may be critical for the initial step (IL-2-dependent state) of T-cell transformation. On the other hand, a fibrosarcoma was induced in transgenic mice carrying a tax_1 expression plasmid (5). Since the activation of IL-2 and IL-2 receptor is unlikely to induce a fibrosarcoma, perhaps tax_1 trans-activates other genes leading to abnormal proliferation. Another mechanism of HTLV-I-induced transformation may entail the trans-activation of protooncogenes. It has been shown that c-fos protein and nuclear transcription factor AP-1 are associated (38, 39). We have observed that the c-fos protein is required for increased AP-1 function (39). One scenario would be that tax_1 activates c-*fos* which activates AP-1 or the NF-κB protein to regulate transcription of promoters, including IL-2 and IL-2 receptor. The results presented here would argue that the trans-activation of the c-*fos* gene can influence T-cell proliferation because its product can act as a transregulator of other cellular genes involved in T-cell proliferation.

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