Activation of E2F-mediated Transcription by Human T-cell Leukemia Virus Type I Tax Protein in a p16<sup>INK4A</sup>-negative T-cell Line*

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The human T-cell leukemia virus type I (HTLV-I) is a causative agent of adult T-cell leukemia. Although the exact mechanism by which HTLV-I contributes to leukemogenesis is still unclear, the Tax protein is thought to play a major role in this process. This 40-kDa polypeptide is able to interact with the tumor suppressor p16<sup>INK4A</sup>. Consequently, Tax can activate the signaling pathway that lead to the release of E2F that in turn induces expression of factors required for cell cycle progression. In this paper, we demonstrate that Tax can also activate E2F-mediated transcription independently of p16<sup>INK4A</sup>. Indeed, when Tax is coexpressed with the E2F-1 transcription factor in CEM T-cells, which lack expression of p16<sup>INK4A</sup>, it strongly potentiates the E2F-dependent activation of a reporter construct driven by a promoter containing E2F binding sites. This stimulation is abrogated by mutations affecting the E2F-binding sites. In addition, Tax also stimulates the transcription of the E2F-1 gene itself. Using Tax mutants that fail to activate either ATF- or NF-κB-dependent promoters and different 5′ truncation mutants of the E2F-1 promoter, we show that the Tax-dependent transcriptional control of the E2F1 gene involves, at least in part, the ATF binding site located in the E2F-1 promoter.

Human T-cell leukemia virus type I (HTLV-I) is the etiologic agent of adult T-cell leukemia (ATL). The viral genome codes for regulatory proteins including the 40-kDa Tax protein, which transactivates its own promoter. Tax transactivation involves three 21-base pair regulatory elements containing imperfect cyclic AMP response element (CRE), localized in the U3 region of the long terminal repeat (1–4). Tax interacts directly with proteins of the activating transcription factor/CRE-binding protein (ATF/CRE) family (5–7) and increases their activ-

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The abbreviations used are: HTLV-I, human T-cell leukemia virus type I; mAb, monoclonal antibody; ATL, adult T-cell leukemia; P13K, phosphatidylinositol 3-kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CRE, cAMP response element; CREB, CRE-binding protein; IL, interleukin; RT, reverse transcriptase; PCR, polymerase chain reaction; DHFR, dihydrofolate reductase.
myc, N-myc, erb-B and B-myb.

A detailed study of E2F protein complexes in T-cells demonstrated that the prevailing E2F complexes contain E2F-1 and E2F-4 associated with DP-1 (30). The cellular oligonucleotide primers used in this study are as follows: E2F-1 I (5’-CAGATCTCCCTTAAGAGC-3’), nucleotides 1041–1058, E2F-1 II (5’-CAGTCGAAGAGCTTCTG-3’), nucleotides 1582–1599, antisense mRNA, GAPDH I (5’-TTGAGAATGTGACAACAGC-3’), nucleotides 3806–3824, and GAPDH II (5’-TCCACCAGTACAGTTG-3’), nucleotides 4394–4411, antisense mRNA. The oligonucleotide primer pair Tax2/TRU2 (17) was used to detect retrotranscribed Tax mRNA.

Transfections and Luciferase Assays—CEM cells were transiently cotransfected according to the previously published procedure (49). 5 μg of a β-galactosidase-containing plasmid (pACβ1) was included in each transfection for controlling of the transfection efficiency. The total amount of DNA in each series of transfection was equal, the balance being made up with empty pSG-5 vector without Tax. Cell extracts equalized for protein content were used for luciferase and β-galactosidase assays. Western Blot Assay—Nuclear extracts were prepared as described previously (49). 20 μg of protein from nuclear extracts were electrophoresed onto 10% sodium dodecyl sulfate-polyacrylamide gel and blotted to nitrocellulose filter membranes (Millipore). The blot was then incubated for 1 h at room temperature with a blocking solution (phosphate-buffered saline (PBS) containing 10% milk and 0.05% Tween 20) prior to addition of antiserum. After 1 h at 20 °C, the blot was washed three times with PBS + 0.05% Tween 20 and incubated for 30 min with goat anti-mouse immunoglobulin-peroxidase conjugate (Immunochem, Marseille, France). After three washes, the membrane was incubated with enhanced chemiluminescence (ECL) reagent (Amersham Pharmacia Biotech). The membrane was then exposed for 0.5 to 5 min to Hyperfilms-ECL (Amersham Pharmacia Biotech). Anti-E2F-1 mAb and anti-actin mAb C4 were purchased, respectively, from Santa Cruz Biotechnology Inc. (Santa Cruz, CA), and ICN Biomedicals Inc. (Costa Mesa, CA); anti-Tax was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIADDK, NIH.

Tax Stimulates E2F1-dependent Transcription in the p16INK4A-negative CEM T-cell Line—To test whether Tax might activate E2F-mediated transcription independently of p16INK4A pathway, we performed transient cotransfection assays in a T-cell line defective in expression of p16INK4A. It has been described that some tumor cell lines are unable to express the p16INK4A and among them various transformed T-cell lines having homozygous deletions of the p16INK4A gene (33). In agreement with these observations, no p16INK4A could be detected in the CEM cell line used in our study (data not shown).

At first, we tested the effects of Tax on the promoters of the DHFR and cyclin E genes, which are known to be controlled by the E2F factors (29, 45). Transient cotransfection assays were carried out using luciferase expression plasmids. The transfection assays were performed in CEM cells in the presence or absence of the Tax expression vector pSG-Tax. Tax synthesis was controlled by inclusion of the DHFR-galactosidase-containing plasmid (pAC β1) was included in each transfection for controlling the transfection efficiency. The total amount of DNA in each series of transfection was equal, the balance being made up with empty pSG-5 vector without Tax. Cell extracts equalized for protein content were used for luciferase and β-galactosidase assays. Western Blot Assay—Nuclear extracts were prepared as described previously (49). 20 μg of protein from nuclear extracts were electrophoresed onto 10% sodium dodecyl sulfate-polyacrylamide gel and blotted to nitrocellulose filter membranes (Millipore). The blot was then incubated for 1 h at room temperature with a blocking solution (phosphate-buffered saline (PBS) containing 10% milk and 0.05% Tween 20) prior to addition of antiserum. After 1 h at 20 °C, the blot was washed three times with PBS + 0.05% Tween 20 and incubated for 30 min with goat anti-mouse immunoglobulin-peroxidase conjugate (Immunochem, Marseille, France). After three washes, the membrane was incubated with enhanced chemiluminescence (ECL) reagent (Amersham Pharmacia Biotech). The membrane was then exposed for 0.5 to 5 min to Hyperfilms-ECL (Amersham Pharmacia Biotech). Anti-E2F-1 mAb and anti-actin mAb C4 were purchased, respectively, from Santa Cruz Biotechnology Inc. (Santa Cruz, CA), and ICN Biomedicals Inc. (Costa Mesa, CA); anti-Tax was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIADDK, NIH.

To study possible involvement of E2F in Tax transactivation of these cellular promoters, we first tested whether Tax might cooperate with E2F in transactivation. E2F1 and Tax were coexpressed in presence of a reporter construct driven by a minimal promoter with upstream E2F-binding sites (p3xE2F-WT-luc). This construct contains three E2F binding sites cloned immediately upstream of a TATA box controlling the transcription of the luciferase gene. Fig. 2 shows that this reporter was stimulated 8.5-fold in the presence of the expression vector pCMV-E2F-1 alone or pCMV-E2F-1 plus pSG-Tax alone. No stimulation was detected with the mutants Tax M9 (Fig. 2) and Tax M21 (data not shown). This Tax stimulation was likely mediated by endogenous E2F factor. Cotransfection of pSG-Tax and pCMV-E2F-1 induced a 44-fold increase in luciferase activity, indicating that Tax was able to stimulate E2F-1 activity in the CEM cell line. Cotransfections with the transcriptionally defective Tax mutants produced no stimulation of E2F-1 activity. To assess whether transactivation by Tax may be ascribed to
Tax Transactivation of E2F-dependent Transcription

**Fig. 1.** Tax stimulation of the luciferase reporter gene driven by the DHFR or cyclin E promoters. CEM cells (5 × 10⁶) were transfected with 2 μg of luciferase gene driven by cyclin E promoter (pCycE-luc) or by DHFR promoter (pDHFR-luc) + 5 μg of the Tax expression vectors (producing either the wild type Tax or the transcriptionally defective mutant Tax M9) or empty pSG-5 vector. 5 μg of a β-galactosidase containing plasmid (pACβ1) was included in each transfection for controlling of the transfection efficiency. After 48 h, cell extracts prepared and equalized for protein content as described previously (49) were used for luciferase and β-galactosidase assays. Luciferase values were normalized for product, we tested whether Tax would also stimulate the E2F1 gene expression. Although expression of E2F-4 in cells transfected with the expression vectors (E2F-Box1) gave rise to a 1.5-fold enhancement by Tax, which corroborates previous published results (see, e.g., Refs. 51–53), and represents low level of Tax induction on a minimal TATA box.

**Fig. 2.** Tax stimulation of gene expression mediated by E2F-1. Assays were performed in CEM cells using for each transient cotransfection 10 μg of p3xE2F-WT-luc bearing three copies of E2F binding site (mentioned E2F-Box in the figure) and 5 μg of pACβ1. The effects of Tax and E2F-1 were analyzed by using either 0.1 μg of pCMV-E2F-1 + 0.5 μg of pDP1 (E2F-Box + E2F-1), or 5 μg Tax expression vectors (E2F-Box +Tax or E2F-Box + Tax M9), or 0.1 μg of pCMV-E2F-1 + 0.5 μg of pDP1 + 5 μg of Tax expression vectors (E2F-Box + E2F-1 + Tax or E2F-Box + E2F-1 + Tax M9), the balance of total amount of transfected DNA being made up with pSG-5. Luciferase values were normalized for β-galactosidase activity. The activity of luciferase gene driven by E2F-Box in the absence of E2F-1 or/and Tax was arbitrarily given a value of 1, and the activities of the other transfections were adjusted relative to this activity. Values represent the mean ± S.D. (n = 3).

a direct effect of the binding of E2F-1 to the tested minimal promoter, Tax stimulation of the luciferase gene driven by three mutated E2F binding sites were analyzed with the plasmid p3xE2F-MUT-luc. The design of this mutant, which converts the E2F binding site 5'-TATTCCCGC-3' to 5'-TTgCtCGa-3', was based on mutations that are known to abolish binding and activity of E2F (44). As shown in Fig. 3, the mutant promoter was very poorly induced by Tax and E2F-1 compared with the wild type construct. The efficiency of Tax-mediated induction of luciferase activity was reduced by 5.5-fold with Tax alone and 39-fold with Tax and E2F-1 compared with control promoter. The mutant promoter still showed a slight 1.5-fold enhancement by Tax, which corroborates previous published results (see, e.g., Refs. 51–53), and represents low level of Tax induction on a minimal TATA box.

**Fig. 3.** Comparative sensitivity of wild-type or mutant E2F binding sites to activation by E2F-1 or by E2F-1 + Tax. Transient cotransfection assays were carried out and luciferase values normalized as described in the legend of Fig. 2, with 10 μg of p3xE2F-WT-luc or p3xE2F-MUT-luc bearing, respectively, wild-type (E2F-Box) or mutated copies (MUT-Box). Values represent the mean ± S.D. (n = 2).
To address this question, we first checked the level of endogenous E2F-1 protein in two human T-cell lines infected with HTLV-I, MT4, and C8166 cells. As shown in Fig. 4, immunoblotting analysis using an anti-E2F-1 mAb indicated that expression of E2F-1 was increased in nuclear extracts of Tax-positive cells compared with the level seen in uninfected Jurkat and HSB-2 cells. This result suggests that Tax expression could stimulate E2F-1 gene expression.

In order to test whether this effect was due to a direct transcriptional effect of Tax on E2F-1 gene expression, we then compared E2F-1 mRNA levels in presence or absence of Tax protein. This experiment was carried out with the JPX-9 clone of Jurkat cells containing the Tax gene under a promoter whose expression is stimulated by heavy metal ions (42). As shown in Fig. 5A, the level of E2F-1 mRNA was clearly increased in JPX-9 cells treated with either ZnCl₂ or CdCl₂, whereas the expression of control GAPDH mRNA was unchanged. JPX/M cells that expressed nonfunctional Tax were treated in the same way but did not show any increase in the level of E2F-1 mRNA (Fig. 5B). These results indicate that stimulation of E2F-1 mRNA synthesis correlates with the presence of Tax.

We finally tested whether Tax can stimulate E2F-1 promoter activity. Transfection assays were performed with the plasmid pGL2-AN that contains the E2F-1 promoter cloned upstream of the luciferase gene. Cotransfection of pSG-Tax and pGL2-AN (48) were cotransfected with pSG-Tax and pGL2-AN vector containing the E2F binding sites was still stimulated by Tax, confirming the results already described in this paper with the plasmid p3xE2F-WT-luc that contained three copies of the E2F binding site.

Taken together, these results confirm the involvement of an ATF-dependent pathway, at least in part, in the transcriptional control of E2F-1 gene by Tax.
ATF Pathway Is Necessary but Not Sufficient to Explain the Transactivation by Tax—In this paper, we show that Tax is able to activate E2F-1-dependent transcription in CEM cells (Figs. 2 and 3) and to stimulate E2F-1 gene transcription through the ATF-responsive element localized in the promoter (Figs. 6 and 7). Stimulation of the E2F-1 gene by Tax could explain how Tax transactivates E2F-1-dependent transcription. To determine effects of this stimulation in our tests, transfection assays already described in Fig. 2 were performed again, but in these assays cells were cotransfected with the plasmid p3xE2F-WT-luc containing E2F-binding sites, the E2F-1 expression vector pCMV-E2F-1, together with the mutant Tax M47. As shown in Fig. 8, the wild type Tax and the mutant Tax M22 induced about a 40-fold increase in luciferase activity, whereas Tax M47 stimulated luciferase activity by 14-fold. Since Tax M47 fails to activate ATF-dependent promoters, this stimulation cannot be explained by the effect of Tax on the stimulation of the E2F-1 gene through the ATF pathway.

This result confirms the importance of the ATF pathway but also indicates that other activation mechanisms could contribute to E2F-1 stimulation by Tax.

DISCUSSION

The regulation of E2F is a key target for oncoviruses. Binding of pRB and the other pocket proteins to ligands such as, adenovirus E1A, simian virus large T antigen, and papillomavirus E7, leads to a stimulation of E2F-dependent transcription and cellular transformation (54–56). The human cytomegalovirus IE72 protein is able to phosphorylate E2F-1–3 and the pocket proteins p107 and p130, and this phosphorylation step would play an essential role in the mechanism of cell proliferation (57). Induction of E2F DNA binding activity in HTLV-I-infected T-cell lines and leukemic cells obtained from ATL patients (31) suggests that the activation of E2F-dependent transcription by HTLV-I could also be involved in the proliferative response during HTLV-I infection. Suzuki et al. (33) demonstrated that Tax interacted with p16INK4A and suggested that the inactivation of p16INK4A by Tax would contribute to cellular immortalization and transformation induced by HTLV-I infection. The observations that Tax released T-lymphocytes from cell cycle arrest induced by p16INK4A but also that p16INK4A overexpression blocked Tax-dependent stimulation of DNA synthesis (34) are effectively consistent with a deregulation of cell cycle progression by Tax in a p16INK4A-dependent manner. However, these results did not exclude the possibility that Tax could also activate E2F-dependent transcription in a p16INK4A-independent manner.

In this report, we demonstrate that Tax stimulates the activity of E2F-1 and E2F-4 in CEM T-cells, which lack expression of p16INK4A. This stimulation is abrogated by mutations affecting the E2F binding sites, thereby confirming that stimulation by Tax is due to direct effect of the binding of E2F-1 and E2F-4 to E2F boxes. In addition, we demonstrate that (i) Tax stimulates E2F-1 mRNA synthesis in Jurkat cells, (ii) Tax transactivates the E2F-1 promoter, and (iii) this transactivation is dependent, at least in part, on the ATF pathway. Tax is well known to interact directly with proteins of the ATF/CREB family and to stimulate their activity (5–7). Tax interaction with the ATF pathway is important in the development of the neoplastic phenotype in the case of adult rat fibroblasts (19), and mutants of Tax, which failed to activate the endogenous

![Fig. 6. Tax transactivation of the E2F-1 promoter is dependent on the ATF pathway.](image)

![Fig. 7. 5′ deletion analysis of the activation of the human E2F-1 promoter by Tax.](image)
ATF pathway, had a reduced ability to induce tumors in transgenic mice (58). Stimulation of E2F-1 mRNA at the transcriptional level may be a critical step in cell cycle control. Overproduction of E2F-1 can induce cell proliferation, presumably by titrating pRB from the relevant promoter and thus leading rest in entry into S phase (59, 60), but can also induce p53-dependent apoptosis (61, 62). However, it has been shown that p53 is fully inactivated by Tax in the HTLV-I transformed T-cells (63, 64), confirming that stimulation of E2F-1 by gene can be Tax and induce phosphorylation of cyclin D3 in T-cells null for p16INK4A. They suggested that this Tax-associated phosphorylation of cyclin D3 might stabilize the cyclin-cdk complexes, and thus enhance the pRB phosphorylation and increase the E2F transcriptional activity.

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Addendum—During the submission of this paper, Neuveut et al. (77) published that a construct containing p16INK4A inhibits phosphorylation of pRB, induces cyclin D3, and degradation of p27kip1 (73). Altogether, these results establish a crucial PI3K/protein kinase B-mediated link between the IL-2 receptor and the cell cycle machinery. Thus, the direct activation of this pathway by Tax could contribute to explain the p16INK4A-independent activation of E2F-mediated transcription by Tax and the switch to IL-2 independence in HTLV-I immortalized cells. Yet other mechanisms could explain the effects of Tax including, for example, the direct interaction of Tax with E2F-1, as described for several cellular transcriptional factors. Experiments are under way to further elucidate the role of Tax in E2F-mediated transcription independently on p16INK4A. The identification of this novel mechanism will provide a useful tool to understand how HTLV-I could induce T-cell transformation.

FIG. 8. Effect of Tax M47 on the transactivation of E2F-1. CEM cells were cotransfected with 0.1 μg of pCMV-E2F-1, 0.5 μg of pDP1, and 10 μg of pcxE2F-WT-luc (E2F-Box) together with 5 μg of either pSG-Tax (E2F-Box + E2F-1), or pSG-Tax (E2F-Box + E2F-1 + Tax), or pSG-Tax M22 (E2F-Box + E2F-1 + Tax M22), or pSG-Tax M47 (E2F-Box + E2F-1 + Tax-M47). Luciferase values were normalized for β-galactosidase activity. The activity of luciferase gene driven by E2F-Box in the absence of E2F-1 was arbitrarily given a value of 1, and the activities of the other transfections were adjusted relative to this activity. Values represent the mean ± S.D. (n = 3).
