

Interaction of the Human T-Cell Lymphotropic Virus Type 1 Tax Transactivator with Transcription Factor TFIIA

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The Tax protein of human T-cell lymphotropic virus type 1 (HTLV-1) is a 40-kDa transcriptional activator which is critical for HTLV-1 gene regulation and virus-induced cellular transformation. Tax is localized to the DNA through its interaction with the site-specific activators cyclic AMP-responsive element-binding protein, NF- κ B, and serum response factor. It has been suggested that the recruitment of Tax to the DNA positions Tax for interaction with the basal transcriptional machinery. On the basis of several independent assays, we now report a physical and functional interaction between Tax and the transcription factor, TFIIA. First, Tax was found to interact with the 35-kDa (α) subunit of TFIIA in the yeast two-hybrid interaction system. Importantly, two previously characterized mutants with point mutations in Tax, M32 (Y196A, K197S) and M41 (H287A, P288S), which were shown to be defective in Tax-activated transcription were unable to interact with TFIIA in this assay. Second, a glutathione-S-transferase (GST) affinity-binding assay showed that the interaction of holo-TFIIA with GST-Tax was 20-fold higher than that observed with either the GST-Tax M32 activation mutant or the GST control. Third, a coimmunoprecipitation assay showed that in HTLV-1-infected human T lymphocytes, Tax and TFIIA were associated. Finally, TFIIA facilitates Tax transactivation *in vitro* and *in vivo*. *In vitro* transcription studies showed reduced levels of Tax-activated transcription in cell extracts depleted of TFIIA. In addition, transfection of human T lymphocytes with TFIIA expression vectors enhanced Tax-activated transcription of an HTLV-1 long terminal repeat-chloramphenicol acetyltransferase reporter construct. Our study suggests that the interaction of Tax with the transcription factor TFIIA may play a role in Tax-mediated transcriptional activation.

Human T-cell lymphotropic virus type 1 (HTLV-1) is associated with adult T-cell leukemia and the degenerative neuromuscular disease tropical spastic paraparesis/HTLV-1-associated myelopathy (28, 66, 72, 108). HTLV-1 encodes a 40-kDa protein, Tax, which transforms rodent fibroblasts (90), immortalizes normal human T cells (34), and can induce a leukemia-like disease in transgenic mice (36). Tax is critical for HTLV-1 gene regulation (44, 68, 75, 85) and has been shown to modulate the expression of a number of cellular genes which include genes encoding cytokines (interleukin-1 [IL-1], IL-2, IL-3, IL-6, IL-8, and granulocyte-macrophage colony-stimulating factor) (35, 37, 60, 61, 65, 83, 100, 103), genes encoding cytokine receptors (IL-2 receptor α) (14, 15, 43, 52, 76), proto-oncogenes (*c-fos*, *c-myc*) (2, 20, 23–25), and genes encoding lymphotoxin (69, 70), parathyroid hormone-related protein (18, 98), and major histocompatibility complex class I proteins (77). The deregulation of these Tax-responsive cellular genes may play an important role in HTLV-1 transformation.

The HTLV-1 Tax protein is able to modulate gene expression by several independent mechanisms. Tax can induce the nuclear localization of the NF- κ B factors c-rel, p65, p50, and p52 (14, 46, 51, 88) by overriding the inhibitory function of I κ B α (38, 46, 51, 56, 62, 88). Recent reports suggest that Tax activation of the NF- κ B pathway involves the induction of phosphorylation and proteasome-mediated degradation of I κ B α (56, 62). In addition, Tax interacts with transcription factors such as the serum response factor (23, 25), several

NF- κ B family members (6, 38, 62, 89), and the bZIP protein, cyclic AMP-responsive element-binding protein (CREB) (1, 33, 104, 105), indirectly forming a complex with the template DNA. Studies have further demonstrated that Tax binds to the DNA-binding basic segment of the bZIP proteins, enhancing their binding affinity and interaction with Tax-responsive, non-consensus CRE sites (1, 4, 5, 22, 71, 96, 111). Interestingly, when fused to a Gal4 DNA-binding domain, Tax was able to activate transcription from a minimal promoter, suggesting that Tax possesses an intrinsic transactivation function (26, 79). On the basis of these studies, it is thought that after localizing to the DNA through interaction with site-specific activators, Tax may affect the dynamics of transcription through contact with the basal transcription machinery.

RNA polymerase II-directed transcription requires the assembly of a large preinitiation transcription complex composed of the basic transcription factors TFIIA, TFIIB, TFIID (TATA-binding protein [TBP] and TBP-associated factors [TAFs]), TFIIF, TFIIH, and TFIIE (reviewed in references 81 and 110). Transcriptionally competent preinitiation complexes formed *in vitro* assemble by an ordered multistep pathway beginning with the binding of TFIID to the TATA box at the promoter region on the DNA template (7, 63, 94). TFIIA, which directly binds TBP, is thought to enter the complex at an early point in the assembly process after or simultaneously with the TFIID-DNA nucleation step (73, 97, 109, 110). The assembly process then continues with the subsequent association of TFIIB, RNA polymerase II complexed to TFIIF, TFIIE, and TFIIH (109).

Transcriptional activators exhibit activation domain-dependent interactions with TBP, TFIIB, TFIIF, TFIIH, and TAFs (9, 11–13, 32, 41, 54, 86, 91, 95, 101, 112) and may function by

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increasing the number of functional preinitiation complexes (12, 39, 54, 109). Recent studies suggest that TFIIA may also be targeted by activators and coactivators (27, 49, 53, 67). TFIIA was initially observed to preclude the association of protein inhibitors with TBP-promoter complexes, whose interaction with TBP can prevent the assembly of functional preinitiation complexes (3, 42, 48, 50, 58, 59, 99). However, additional studies in which highly purified and potentially inhibitor-free transcription factors were used showed a TFIIA enhancement of activated transcription suggesting a direct role for TFIIA in the activation process (87). In these and other systems, TFIIA is also able to enhance basal transcription reconstituted with TBP (16, 48, 55, 87). As pointed out by Goodrich et al. (31a), the prevalence of promoter diversity suggests that interactions between general transcription factors, transcriptional activators, and the DNA template may vary from promoter to promoter.

It is speculated that a critical rate-limiting step in activator-dependent preinitiation complex formation is the formation of the DNA-TFIID-TFIIA complex (10). VP16 and the Epstein-Barr virus transactivator Zta, which both interact with TFIIA in an activation domain-dependent manner (49, 67), increase the stability and rate of formation of DNA-TFIID-TFIIA complexes (49, 53, 67). Another recent study found that a GAL4-VP16 fusion protein stimulated the formation of an activated transcription complex, requiring the presence of TFIIA, the TFIID TAFs, and the nonhistone chromosomal protein HMG-2, a protein that appears to play a role in the architecture of the transcription complex (82). Purified human TFIIA is composed of stoichiometric amounts of a 35-kDa (α) subunit and a 19-kDa (β) subunit, generated from a 55-kDa precursor ($\alpha\beta$), and a 12-kDa subunit (γ), all of which are essential for TFIIA function (16, 17, 55, 67, 87, 106, 107). The cDNAs encoding the highly homologous human, *Drosophila*, and yeast TFIIA subunits have been cloned (16, 17, 45, 55, 67, 87, 106, 107).

To understand the molecular mechanisms by which Tax regulates transcription from RNA polymerase II promoters, we have studied the potential interaction of Tax with the basal transcription factor TFIIA. By using several independent assays, including the yeast two-hybrid system, affinity chromatography, and coimmunoprecipitation analysis, our studies have shown that the HTLV-1 transactivator, Tax, interacts with the 35-kDa (α) subunit of TFIIA. Importantly, two Tax mutants which fail to activate transcription *in vivo* failed to interact with TFIIA. Moreover, we demonstrate that TFIIA is important for Tax transactivation. *In vitro* transcription assays with cell extracts depleted of TFIIA showed a marked reduction in Tax-activated transcription. In addition, Tax-mediated transactivation was enhanced in human T lymphocytes upon coexpression of holo-TFIIA. Our studies suggest that one mechanism of Tax transactivation involves contact with the transcriptional machinery through TFIIA, thereby altering the level of transcription.

MATERIALS AND METHODS

Two-hybrid interaction assay. The LexA DNA-binding domain (DBD) fusion proteins were generated with the 2 μ m *HIS3* yeast shuttle vector, pNL202, which is identical to pEG202 (31) with the addition of a simian virus 40 nuclear localization signal, consisting of amino acids PPKKKRQVA, inserted downstream of the 202-amino-acid LexA moiety. The activation domain (AD) fusion proteins were generated with the 2 μ m *TRP1* yeast shuttle vector, pJG4-5, which directs the synthesis of the simian virus 40 nuclear localization signal (as in pNL202), the B42 acidic activation domain, and the influenza virus HA1 epitope tag (total of 107 amino acids) (31). The LexA DBD fusion proteins are constitutively expressed from the *ADH* promoter, while the AD fusion proteins are expressed from the galactose-induced, glucose-repressed *Gal-1* promoter. The 2 μ m *URA3* reporter plasmid, pJK103, directs the synthesis of the *lacZ* gene from

a minimal *Gall* promoter controlled by two high-affinity *lexA* operator sites (31) which is transcriptionally silent unless bound by a LexA-containing protein or protein complex with activation potential.

Cloning of the fusion protein constructs was conducted by PCR amplification of Tax, TFIIA, and TBP cDNAs with the high-fidelity polymerase V (New England Biolabs) and primers with the appropriate restriction sites designed to omit the initiating methionine. Three independent clones of the Tax-DBD fusion construct (and mutants) were tested with at least two independent clones of the p55 and p35 TFIIA-AD fusion constructs. The identity of the Tax mutants (a kind gift of Warner Greene, University of California, San Francisco) was verified by sequence analysis (Sequenase; U.S. Biochemicals).

Yeast transformations were performed by the standard lithium acetate method as described by Gietz et al. (29) with *Saccharomyces cerevisiae* EGY188 (*MATa trp1 ura3 his3 LEU::pLexop2-LEU2*) previously transformed with pJK103 (31). pEG202, pJG4-5, pJK103, *S. cerevisiae* EGY188, and anti-LexA antibody were generously provided by Roger Brent, Massachusetts General Hospital, Boston.

Levels of β -galactosidase generated from the pJK103 reporter plasmid were used as a measure of the interaction between the AD and DBD fusion proteins. β -Galactosidase activity was determined with exponentially growing yeast cells 12 to 14 h after induction with 2.0% galactose as described by Reynolds and Lundblad (74) and was calculated as follows: $1,000(OD_{420})/(\text{time [in minutes]} \times \text{it takes for substrate to turn yellow})/(\text{volume [in milliliters]} \times \text{cell suspension})/OD_{600}$.

GST-fusion protein-binding assays. Bacterially generated glutathione-S-transferase (GST)-Tax, GST-Tax M32, GST-TBP, and GST fusion proteins were purified by binding to glutathione-Sepharose beads (Pharmacia) and quantitated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis followed by Coomassie blue staining. A 100- μ g sample of the GST-fusion protein-bead complex was incubated with 1.5 mg of HeLa whole-cell extract (WCE) (57) for 2 h at 4°C in buffer A (80 mM KCl, 1% Nonidet P-40, 20 mM Tris [pH 7.9]). After a brief centrifugation, the supernatant (flowthrough) was collected and the protein-complexed beads were washed extensively with buffer B (80 mM KCl, 150 mM NaCl, 0.25% Triton X-100, 20 mM Tris [pH 7.9]) and then washed with 400 μ l of buffer C (300 mM KCl, 1% Triton X-100, 20 mM Tris [pH 7.9]). Cellular proteins which bound to the fusion protein-complexed beads were eluted with buffer D (2.0 M NaCl, 10% glycerol, 20 mM Tris [pH 7.9]). The flowthrough and wash fractions were precipitated with trichloroacetic acid.

A 100- μ g sample of HeLa cell extract, 1/15 of the total flowthrough, and the complete wash and elution fractions were run on an SDS-4 to 20% polyacrylamide gel. Western blot (immunoblot) analysis was conducted with a 1:2,000 dilution of polyclonal antiserum to the 35-kDa subunit of TFIIA (UBI). The blots were stripped in buffer S (67.5 mM Tris [pH 6.8], 2% SDS, 100 mM β -mercaptoethanol) for 30 min at 50°C and then reblotted with a 1:1,000 dilution of antiserum to TFIIB (anti-TFIIB, C18; Santa Cruz Biotechnology, Inc.). TFIIA and TFIIB were visualized by enhanced chemiluminescence (see the section on immunoblot analysis, below).

Immunoprecipitation and immunoblot analysis. HTLV-1-infected C81 human T lymphocytes, which express high levels of Tax, were lysed in TNN buffer (50 mM Tris-HCl [pH 7.5], 120 mM NaCl, 5 mM EDTA, 0.5% P-40, 50 mM NaF, 0.2 mM sodium orthovanadate, 1 mM dithiothreitol, 100 μ g of phenylmethylsulfonyl fluoride, 20 μ g of aprotinin per ml, 10 μ g of leupeptin per ml) for 20 min on ice, and then cell debris was removed by centrifugation at 10,000 \times g for 10 min (30). Immunoprecipitations were conducted by incubating 300 μ g of total-cell protein for 1 h at 4°C with 3 μ l of preimmune rabbit serum, rabbit antibody directed against the 35-kDa (α) subunit of TFIIA (Oncogene Sciences), rabbit antibody directed against the 55-kDa ($\alpha\beta$) TFIIA subunit, a mixture of four anti-Tax monoclonal antibodies with different specificities, or a monoclonal antibody to the influenza virus HA1 epitope (12CA5) as a control. Selected samples were precleared with 40 μ l of nonspecific rabbit antiserum prior to treatment with immune serum. Antibody-bound proteins were collected with 25 μ l of a 0.1-g/ml protein A-Sepharose (Pharmacia) solution in TNN buffer. Bound proteins were washed four times in 500 μ l of TNN buffer, eluted by boiling in Laemmli buffer, and separated on an SDS-10% polyacrylamide gel. The proteins were transferred to Immobilon-P membranes (Millipore) at 100 V for 1 h at 4°C with Western transfer buffer (0.1 M glycine, 12.5 mM Tris [pH 8.3]). Membranes were blocked with 5% milk powder in TNET buffer (10 mM Tris [pH 7.5], 2.5 mM EDTA, 50 mM NaCl, 0.1% Tween 20) for 1 h, washed in TNET without milk, and incubated for 1 h in primary antibody (1:1,000 dilution of Tax monoclonal antibody mix or 1:2,500 dilution of antibody to the 55-kDa [$\alpha\beta$] subunit of TFIIA) at room temperature. After extensive washing, the immunoprecipitated proteins were detected with the appropriate horseradish peroxidase-linked secondary antibody followed by enhanced chemiluminescence (Amersham).

In vitro transcription assay. Assays were conducted with the pTRE-1_{td} plasmid containing two copies of the promoter-proximal 21-bp repeat from the HTLV-1 long terminal repeat upstream of the chicken ovalbumin TATA box and a G-free cassette as described by Duvall et al. (19). Samples were analyzed on a 4% denaturing polyacrylamide gel. HeLa whole-cell extracts were depleted of TFIIA by incubation with protein A-Sepharose-coupled (Pharmacia) preimmune or immune serum specific for the 55-kDa ($\alpha\beta$) subunit of TFIIA (19). The depleted extracts and bound proteins were analyzed for the presence of TFIIA and TBP by Western blot analysis as described by Duvall et al. (19).

Transfection and CAT assays. The cDNA encoding the 55-kDa precursor ($\alpha\beta$) of TFIIA (pCMV55) was cloned as a *HindIII-NotI* fragment and the cDNA encoding 12 kDa (γ) of TFIIA (pCMV12) was cloned as a *HindIII-BamHI* fragment into the pCDNA3 vector (Invitrogen), which directs transcription from the cytomegalovirus promoter. The cDNA for HTLV-1 Tax was expressed from the cytomegalovirus promoter by using the pCTax construct (84). pU3RCAT contains the upstream HTLV-1 long terminal repeat (LTR) driving the chloramphenicol acetyltransferase reporter gene (47). Human Jurkat T lymphocytes (78) were grown in RPMI 1640 medium (Gibco/BRL) supplemented with 10% fetal calf serum. Cells (1.5×10^7) were electroporated as described previously (47) with 0 to 10 ng of pCTax, 0 to 50 ng of pCMVp55 ($\alpha\beta$), 0 to 200 ng of pCMVp12 (γ), and 1 μ g of pU3RCAT. Chloramphenicol acetyltransferase activity was assayed 16 h posttransfection as described previously (40). Results were quantitated with a PhosphorImager and the Image Quant program (Molecular Dynamics).

RESULTS

Tax associates with TFIIA in *S. cerevisiae*. The interaction between Tax and TFIIA was first analyzed in vivo with the yeast two-hybrid interaction system (31). Wild-type Tax and several previously characterized Tax point mutants (84) were generated as fusion proteins with the DBD of LexA by in-frame cloning into the yeast shuttle vector, pNL202. The 55-kDa precursor ($\alpha\beta$), and the 35-, 19-, and 12-kDa (α , β , and γ , respectively) subunits of TFIIA were expressed as fusion proteins with the epitope-tagged (HA1) acidic AD by using the pJG4-5 shuttle vector. Interaction between the protein fused to the LexA DBD and the protein fused to the acidic AD results in the localization of the AD to the LexA operator on the pJK103 reporter plasmid and subsequent activation of the *lacZ* gene.

The Tax DBD, which possessed a low intrinsic level of activation (twofold above that of the DBD vector alone [data not shown]), showed a significant level of interaction with the 35-kDa (α) subunit of TFIIA (p35-AD) (11.4-fold over Tax DBD levels) and with the 55-kDa ($\alpha\beta$) precursor protein (p55-AD) (5.2-fold) (Fig. 1A). Both wild-type and mutant Tax proteins failed to interact with the 19-kDa (β) or 12-kDa (γ) TFIIA subunits (Fig. 1A) (data not shown). Tax was also tested for interaction with the general transcription factors TBP and TFIIIB (Fig. 1A). Although earlier reports suggest an association between Tax and TBP in vitro (8), the interaction between these two proteins in the yeast two-hybrid system was negligible. Similarly, no interaction was observed between Tax and TFIIIB (Fig. 1A). Additionally, when TFIIA was tested for association with the *Drosophila* transactivator Bicoid, no interaction was observed, implying that TFIIA does not bind to transactivators indiscriminately (data not shown). As a further control for these studies, the interaction of Tax with TFIIA was compared with the interaction of TBP and TFIIA, which have been shown previously to interact in the cell. In both vector orientations, TBP and TFIIA interaction levels were approximately twofold higher than the levels of interaction between Tax and TFIIA (data not shown). Expression of the TBP, TFIIIB, Bicoid, and TFIIA fusion proteins were confirmed by Western blot analysis (data not shown).

Several Tax point mutants, previously tested in lymphocytes for transactivation of the HTLV-1 LTR, via the CREB activation pathway, and the human immunodeficiency virus type 1 (HIV-1) LTR, via the NF- κ B activation pathway, were tested for interaction with TFIIA (Fig. 1B) (84). We chose to evaluate a subset of mutants whose previous characterization (84) was not biased as a result of abnormal protein localization or stability (Fig. 1C). The M3 (F13A G14S), M16 (P92A P93S), M19 (Q126A, H127S), and M45 (I315R, P316S) mutants were shown previously to transactivate, often weakly, both the HTLV-1 and HIV-1 LTR promoters. The M47 mutant (L319R, L320S) was able to activate only the HIV-1 LTR

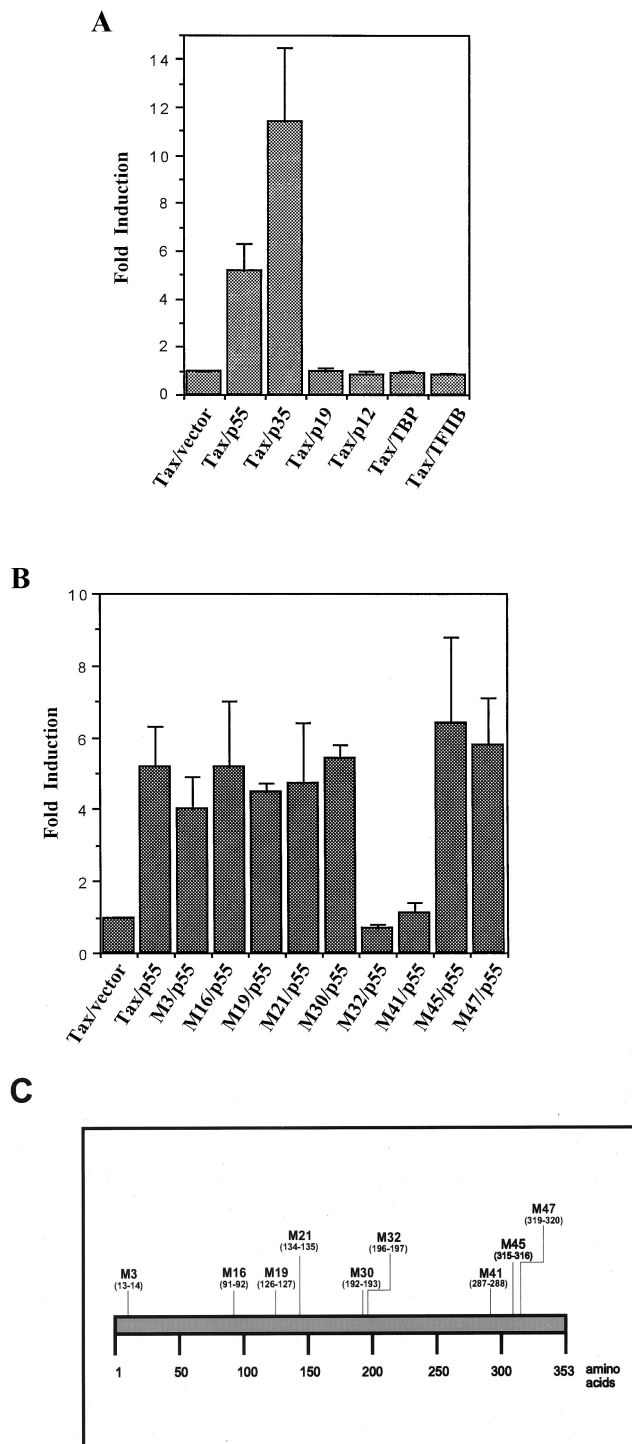


FIG. 1. Tax associates with TFIIA in the yeast two-hybrid interaction system. (A) Levels of interaction between Tax and TFIIA, TBP, and TFIIIB. β -Galactosidase levels, which reflect the extent of interaction between the chimeric DBD and AD fusion proteins, are displayed as the fold increase over levels observed with each chimeric DBD fusion protein and the AD vector alone. (B) β -Galactosidase levels as determined in panel A with cells constitutively expressing the wild-type or M3, M16, M19, M21, M30, M32, M41, M45, or M47 Tax-DBD fusion proteins approximately 12 to 14 h after galactose induction of the TFIIA-AD fusion protein (see Materials and Methods). Results are the mean of three independent experiments. (C) Diagram showing amino acid modifications of the Tax mutants used in this study (84).

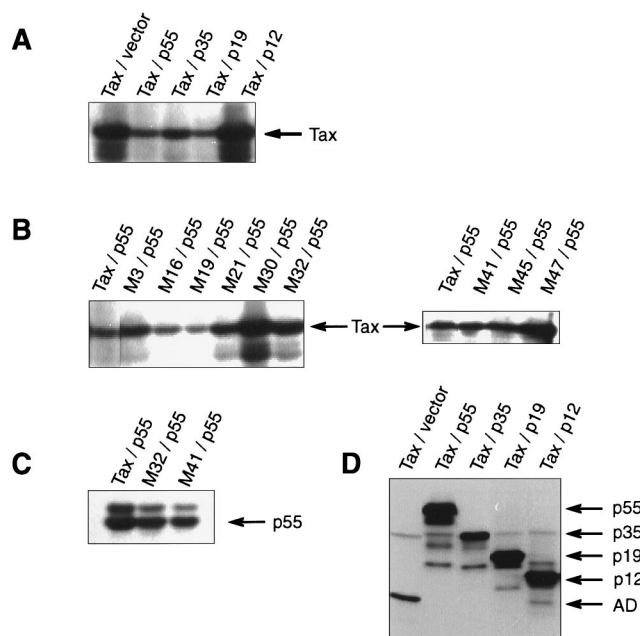


FIG. 2. Western blot analysis of cells coexpressing the Tax-DBD and TFIIA-AD fusion proteins. (A and B) Cells coexpressing both fusion proteins were lysed (see Materials and Methods), and 150 μ g of total-cell protein was analyzed on an SDS-10% polyacrylamide gel. Following electrophoretic separation, the proteins were transferred to Immobilon and immunoblotted with a 1:2,000 dilution of serum directed against the LexA moiety of the DBD-fusion protein (provided by Roger Brent), and proteins were analyzed by enhanced chemiluminescence. Lanes are identified by the DBD/AD fusion proteins that are coexpressed in each sample. (C) Comparison of p55 TFIIA-AD levels in cells coexpressing the wild-type, M32, and M41 Tax-DBD fusion proteins. Total-cell protein (300 μ g) was immunoprecipitated as described in Materials and Methods with 5 μ g of antibody directed against the HA epitope (12CA5) on the AD fusion protein. The precipitated material was run on an SDS-10% polyacrylamide gel, transferred, and immunoblotted with the HA antisera as described for panel B. (D) Same samples as in panel A, except that 50 μ g of total-cell protein was separated on a mini SDS-4 to 20% polyacrylamide gel, transferred to a polyvinylidene difluoride membrane, and immunoblotted with a 1:4,000 dilution of antiserum directed against the HA epitope tag on the AD fusion protein (12CA5; Berkeley).

promoter. The M21 (P134A, D135S), M30 (D192A, G193S), M32 (Y196A, K197S), and M41 (H287A, P288S) mutants were unable to activate either viral LTR (84). Interestingly, the transactivation-defective Tax mutants, M32 (Y196A, K197S) and M41 (H287A, P288S) failed to interact with either the 55-kDa ($\alpha\beta$) precursor or the 35-kDa (α) subunit of TFIIIA. The remaining Tax mutants used in this assay were able to interact with both the p55 and p35 TFIIA proteins.

Western blot analysis demonstrated that all the mutant Tax DBD fusion proteins, including M32 and M41, were expressed at similar levels during coexpression of the TFIIA-AD fusion protein, demonstrating that the lack of interaction is not due to the absence of fusion protein (Fig. 2A and B). In addition, the p55, p35, p19, and p12 TFIIA AD fusion proteins were all expressed in the presence of the wild-type Tax DBD, indicating that the inability of Tax to interact with the p19 and p12 subunits was not due to a change in the stability or expression of the fusion proteins (Fig. 2C and D).

TFIIA from HeLa cells interacts with GST-Tax. We next determined if native TFIIA could interact with the HTLV-1 Tax protein. HeLa WCE was incubated with purified GST-Tax, GST-Tax M32, GST-TBP, or GST protein complexed to glutathione-Sepharose beads. Following a 2-h incubation, the su-

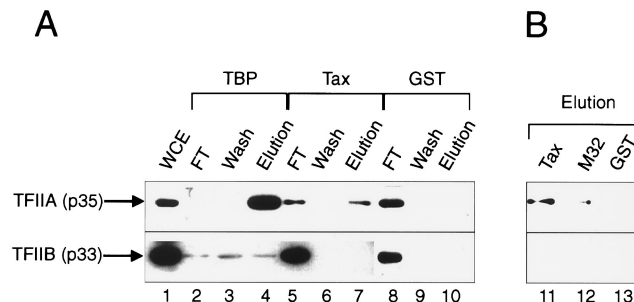


FIG. 3. TFIIA from HeLa cells interacts with GST-Tax. GST-Tax, GST-Tax M32, GST-TBP, or GST fusion proteins (100 μ g) bound to glutathione-Sepharose beads were incubated with 1.5 mg of HeLa WCE. After collection of the supernatant, the beads were washed and bound proteins were eluted with buffer containing 2.0 M NaCl. HeLa WCE (100 μ g), 1/15 of the supernatant (flowthrough [FT]), and the entire wash (Wash) and elution (Elution) fractions were run on an SDS-4 to 20% polyacrylamide gel, and the proteins were transferred to nitrocellulose and immunoblotted with antibody against the p35 subunit of TFIIA. The blot was stripped and reprobed with antibody to TFIIIB (results shown below the TFIIA results).

pernatant was collected (flowthrough fraction) and the beads were extensively washed with a low-ionic-strength buffer (buffer B [containing 80 mM KCl and 150 mM NaCl]) and then washed once with a higher-strength buffer (buffer C [containing 300 mM KCl]). The bound proteins were then eluted from the beads with 2.0 M NaCl (buffer D). Then 1/15 volume of the flowthrough fraction and the entire wash and elution fractions were examined by Western blot analysis for the 35-kDa (α) subunit of TFIIA (Fig. 3A). TFIIA bound to GST-Tax but failed to interact with GST alone (Fig. 3A, lanes 7 and 10). The GST-Tax M32 mutant, which failed to interact with TFIIA in the two-hybrid interaction assay, demonstrated a 20-fold reduction in TFIIA-binding when compared with wild type GST-Tax (Fig. 3B, lanes 11 and 12). As observed in the two-hybrid assay, interaction between TBP and TFIIA was greater than interaction between Tax and TFIIA. However, in the affinity column, the difference in association is more pronounced (TBP bound TFIIA 14-fold more than Tax did) (Fig. 3A, lanes 4 and 7).

As a control for these studies and to confirm results from the two-hybrid assay, the interaction of the GST fusion proteins with TFIIIB was assessed by stripping the original Western blot and reprobing with antibody directed against TFIIIB (33 kDa) (Fig. 3). Consistent with the results of the yeast two-hybrid assay, Tax was unable to interact with TFIIIB (Fig. 3A, bottom, lane 7). The GST-TBP protein did interact with TFIIIB, albeit less efficiently than it interacted with TFIIA.

Tax coprecipitates with TFIIA in HTLV-1 infected cells. We then determined if Tax could associate with native TFIIA by coimmunoprecipitation analysis by using WCE from Tax-expressing C81 T lymphocytes. Proteins were immunoprecipitated with a polyclonal antiserum directed against the 35-kDa (α) subunit of TFIIA (anti-TFIIA) or a nonspecific rabbit serum (PI), and this was followed by Western analysis of the precipitated proteins with an anti-Tax antibody. Tax precipitated with the anti-TFIIA but not with the nonspecific rabbit serum (Fig. 4A). In a reciprocal experiment, we immunoprecipitated proteins from the C81 cell extracts with anti-Tax monoclonal antibodies and subjected them to immunoblotting with TFIIA-specific antisera. The 35-kDa (α) subunit of TFIIA was immunoprecipitated with the monoclonal antibody to Tax (Fig. 4B, lane 3) but not with a control monoclonal antibody against the HA1 epitope (12CA5) or with beads alone (protein

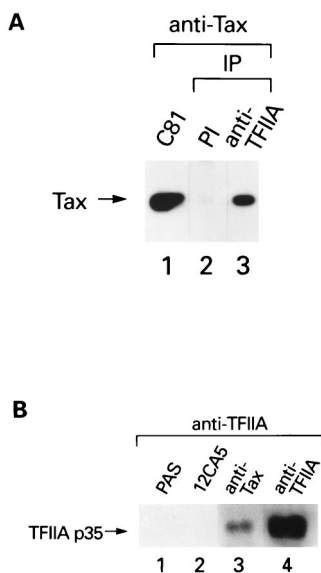


FIG. 4. Coimmunoprecipitation assays demonstrating Tax-TFIIA association in Tax-expressing T lymphocytes (C81 cells). (A) Exponentially growing C81 cells were lysed, and 300 μ g of total-cell protein was immunoprecipitated (IP) with 3 μ l of polyclonal antibody directed against the 35-kDa subunit of TFIIA (anti-TFIIA), or nonspecific polyclonal control antibody (PI). Precipitated proteins along with 50 μ g of untreated C81 cell extract (C81) were separated by SDS-gel electrophoresis followed by immunoblotting with monoclonal anti-Tax antibody. (B) C81 cell extracts were immunoprecipitated with monoclonal anti-Tax antibody (anti-Tax), a control monoclonal antibody against the HA epitope, protein A-Sepharose beads alone (PAS), or anti-TFIIA antibody (anti-p55) as a positive control. Precipitated proteins were analyzed by SDS-gel electrophoresis followed by immunoblotting with anti-TFIIA (p55) antibody.

A-Sepharose), demonstrating the specific coprecipitation of Tax with TFIIA (Fig. 4B, lanes 1 and 2). These results demonstrate that Tax and TFIIA are able to associate in Tax-expressing T lymphocytes.

TFIIA facilitates Tax transactivation in vitro. The functional requirement for TFIIA in Tax transactivation was first addressed by an in vitro transcription assay. Assays were conducted with HeLa whole-cell extracts and pTRE-1_{HTL}, a supercoiled template which contains two 21-bp repeat elements from the HTLV-1 LTR placed upstream of the chicken ovalbumin promoter and a G-free cassette. Upon addition of Tax, transcription was increased approximately sixfold (Fig. 5, lanes 1 and 2). Antibody specific for Tax neutralized this effect,

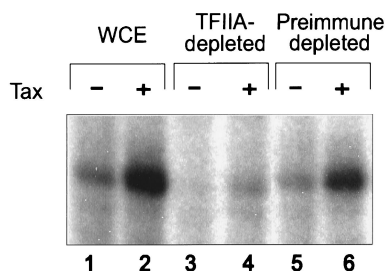


FIG. 5. TFIIA is required for Tax transactivation in vitro. In vitro transcription assays were conducted with the pTRE-1_{HTL} plasmid, which contains two 21-bp repeats from the HTLV-1 LTR, an ovalbumin promoter, and a G-free cassette (see Materials and Methods) (101). HeLa WCE were either untreated (lanes 1 and 2), depleted of TFIIA by preclearing the WCE with anti-TFIIA antibody (lanes 3 and 4), or precleared with control preimmune antibody (lanes 5 and 6). Transactivation by Tax was performed by addition of baculovirus-generated Tax (lanes 2, 4, and 6).

indicating that the enhanced transcription is Tax dependent (reference 19 and data not shown). When the extract was depleted of TFIIA by using a TFIIA-specific antibody (anti-p55), a decrease in both basal and Tax-transactivated transcription was observed (lanes 3 and 4). The decrease in basal transcription to background levels prevented an accurate quantitative comparison of the fold Tax transactivation. We calculate, however, that the level of Tax-transactivated transcription in the TFIIA-depleted extract was reduced 11-fold compared with that of the untreated control (lanes 2 and 4). Treatment of the extract with preimmune serum, which modestly reduced the overall level of basal or Tax-activated transcription, did not significantly reduce the level of Tax transactivation (six- to sevenfold increase) (lanes 5 and 6). As controls for these experiments, Western blot analysis confirmed that TFIIA was absent in the TFIIA antibody-depleted cell extract but present in the preimmune antibody-depleted extract (19). Further, when purified TFIIA was added back to the TFIIA-depleted cell extract Tax transactivation was restored (data not shown) (19). These results suggest that TFIIA is important for Tax-activated transcription, in that Tax transactivation is reduced in the absence of TFIIA.

TFIIA enhances Tax-mediated activation in vivo. We then asked if TFIIA can influence Tax-activated transcription in vivo in a transient-expression assay. Jurkat T lymphocytes were cotransfected with an HTLV-1 LTR-driven CAT reporter (pU3RCAT), a Tax expression vector (pCTax), and plasmids expressing p55 ($\alpha\beta$), equivalent to coexpression of p35 (α) and p19 (β) (17, 55, 106), and the p12 (γ) subunits of TFIIA. We initially established conditions allowing for minimal levels of Tax transactivation such that the effect of TFIIA on Tax transactivation could be evaluated (Fig. 6A). These studies demonstrated that Tax activates transcription in a dose-dependent manner and that suboptimal levels of Tax transactivation could be achieved with 2 to 5 ng of the Tax expression plasmid. Upon coexpression of the p55 ($\alpha\beta$) and p12 (γ) TFIIA subunits, Tax-activated transcription increased to 12-fold above basal levels (Fig. 6B). Importantly, cotransfection of the p55 ($\alpha\beta$) or the p12 (γ) expression plasmid with pU3RCAT and pCTax failed to alter Tax transactivation levels, consistent with the known functional requirement for all three TFIIA subunits (Fig. 6B). In addition, the p55 ($\alpha\beta$) and p12 (γ) subunits, when tested in the absence of Tax, had no effect on the basal activity of the LTR reporter plasmid, suggesting that the effect of TFIIA on LTR transcription was Tax dependent. These in vivo results support our in vitro observations that TFIIA is involved in Tax-activated transcription.

DISCUSSION

Most site-specific activators are modular in structure with distinct and often interchangeable domains required for DNA binding, dimerization, and transactivation (39, 64, 92). Numerous reports describe a physical association between the AD of transactivating proteins and the general transcription factors TBP, TFIIB, TFIIF, TFIID, and the TFIID TAFs (9, 11, 13, 32, 41, 54, 86, 91, 97, 101, 112). The relevance of such interactions has been supported by studies which demonstrate by mutagenic analysis that loss of association between these activators and the general transcription factors corresponds to a reduced capacity of the transactivator to stimulate transcription (41). In this study, we provide evidence for a physical interaction between Tax and the 35-kDa (α) subunit of TFIIA by using the yeast two-hybrid interaction system, by association of GST-Tax with native TFIIA from HeLa cells, and by coimmunoprecipitation of Tax and TFIIA in Tax-expressing T lymphocytes.

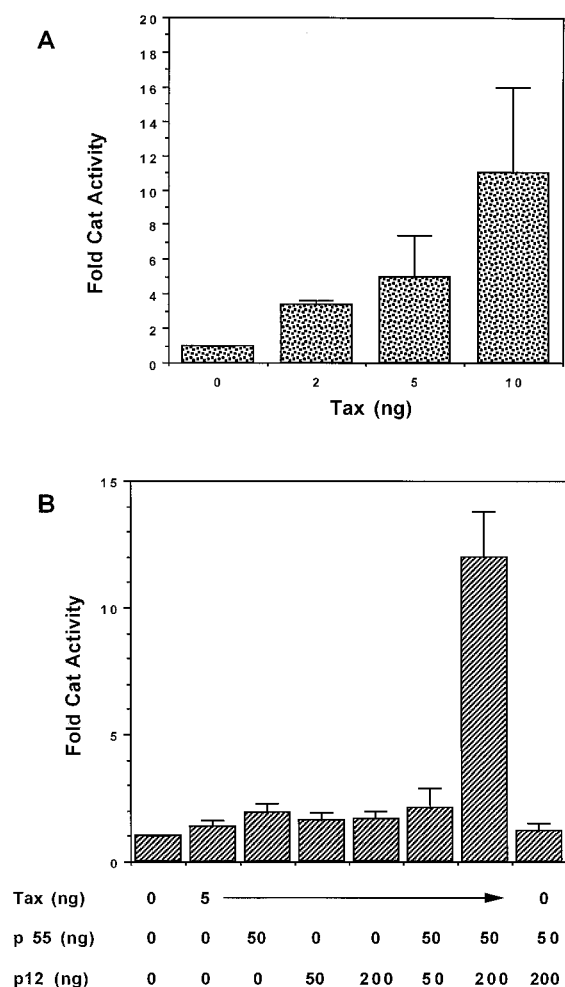


FIG. 6. TFIIA enhances Tax transactivation in vivo. (A) Jurkat T cells were transfected with 1 μ g of pU3RCAT and 0 to 10 μ g of pCTax to determine amounts of pCTax sufficient for minimal Tax transactivation. (B) Jurkat cells were transfected with 5 ng of pCTax, 1 μ g of pU3RCAT, 0 to 50 ng of pCMVp55 ($\alpha\beta$), or 0 to 200 ng of pCMVp12(γ). CAT activities determined are the mean of four independent experiments.

phocytes. We also demonstrate that TFIIA can enhance Tax-activated transcription from a Tax-responsive promoter in vitro and from the HTLV-1 LTR in T lymphocytes. This enhanced activation appears to be specific to the interaction of TFIIA with Tax, since Tax failed to interact with TBP or TFIIB. Interestingly, we identified two previously characterized (84) activation-defective point mutants of Tax, M32 (Y196A, K197S) and M41 (H287A, P288S), which were unable to interact with TFIIA in the yeast two-hybrid assay. When tested in the GST-binding assay, the M32 mutant showed a 20-fold reduction in TFIIA binding compared with wild-type Tax.

Localizing the region(s) of Tax critical to its activation function has been hindered by the pleiotropic nature of Tax. In addition, mutagenesis within multiple regions of Tax often renders the protein transcriptionally inactive, further complicating the structure-function analysis of Tax (80, 84, 93). Nevertheless, several studies involving Tax point and deletion mutants have succeeded in segregating and assigning functions to different domains of Tax (84). Transactivation of the HTLV-1 LTR, mediated by CREB, is affected by mutations in the

amino and carboxy termini, while transactivation of the HIV-1 LTR, involving NF- κ B, is sensitive to mutations in the central region of Tax.

One approach used to define the minimal activation domain of Tax has been to fuse different domains of Tax to a heterologous DBD. One such study in which GAL4-Tax was used to activate transcription from a minimal promoter with upstream GAL4-binding sites found that amino acids 289 to 320 of Tax defined the minimal AD, although flanking regions of Tax enhanced this effect (79). A second study involving GAL4-Tax concluded that two contiguous domains of Tax encompassing amino acids 2 to 255 and 227 to 337 were required for Tax-activated transcription (93). Interestingly, the M32 (Y196A, K197S) and M41 (H287A, P288S) mutants, which failed to interact with the 55-kDa ($\alpha\beta$) or 35-kDa (α) subunits of TFIIA, are defective in transactivating both the HIV-1 and HTLV-1 LTRs. These mutants may interfere with Tax-TFIIA association either by disrupting the structure of a limited region of Tax that is required for this interaction or by exerting a more global effect on the structural integrity of the Tax protein. Significantly, the M32 (Y196A, K197S) mutation resides within a region of Tax rendered transcriptionally inactive by mutagenesis. In contrast, mutants altered in Tax immediately surrounding the M41 (H287A, P288S) point mutant, such as M39, M40, M42, and M43 (84), show nearly wild-type levels of transactivation from both LTRs. It is therefore possible that the M32 mutation affects a region of Tax critical to the structural integrity of the protein. The M41 mutant, within the minimal activation domain, may be important for interaction with TFIIA. It is important to note that the M21 and M30 mutants, defective in transactivation of both the HIV-1 and HTLV-1 LTRs (84), interacted with TFIIA in the two-hybrid assay. It is possible that Tax-activated transcription requires the interaction of Tax with other transcription factors in addition to TFIIA and that the M21 and M30 Tax mutants are deficient in this interaction. For example, Tax may interact with TAFs or other proteins which constitute the holoenzyme.

TFIIA has been shown to enhance the stability of TFIID-promoter complexes as well as to preclude the association of inhibitors that target TBP. The Epstein-Barr virus transactivator Zta and the C-terminal domain of VP16 have been shown to stabilize and increase the rate of formation of DNA-TFIID-TFIIA complexes upon which functional preinitiation complexes are assembled (10, 49). Zta, which, like Tax, lacks a classical transactivation domain, interacts with the p55 ($\alpha\beta$) subunit of TFIIA. In contrast, the C-terminal domain of the acidic activator VP16 interacts with the p12 (γ) TFIIA subunit. A distinct and more N-terminal activation domain of VP16 fails to interact with TFIIA, suggesting that contact with TFIIA represents only one of the pathways through which VP16 operates (49). Since TFIIA is required for many, if not all, activators to stimulate transcription, the requirement may not be manifested as an activator interaction in some cases.

Since Tax operates through contact with DNA-binding transactivators, it is likely that synergy plays an important role in Tax-mediated transactivation. Indeed, interactions between Tax and several site-specific activators, which may interact with the general transcription factors, have been described. For example, CREB, which is involved in Tax-mediated activation, interacts with both TFIIB, perhaps in conjunction with the CREB-binding protein, as well as with TFIID (21, 102). Our results suggest that Tax can interact with TFIIA through the 35-kDa (α) subunit. Hence, it is possible that the multimeric Tax-CREB-CBP complex targets multiple components of the transcription initiation complex including TFIIA, TFIIB, and TFIID. The Tax-CREB interaction, as well as their indepen-

dent interactions with the transcriptional machinery, may confer a greater stability on this multiprotein complex, allowing for the subsequent addition of the remaining transcription factors. Alternatively, the Tax-CREB component may structurally modify the transcription complex such that enhanced transcription from CRE-containing Tax-responsive promoters is favored.

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