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Transactivation of the Human T-Cell Lymphotropic Virus Type 1 Tax_1-Responsive 21-Base-Pair Repeats Requires Holo-TFIID and TFIIA

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The human T-cell lymphotropic virus type 1 (HTLV-1) is the etiological agent for adult T-cell leukemia and tropical spastic paraparesis/HTLV-1-associated myelopathy. The HTLV-1 Tax_1 gene product has been shown to transactivate transcription of viral and cellular promoters. To examine the biochemical mechanism of Tax_1 transactivation, we have developed an in vitro transactivation assay in which wild-type Tax_1 is able to specifically transactivate a polymerase II promoter through upstream Tax_1-responsive elements. The in vitro system utilizes the HTLV-1 21-bp repeats cloned upstream of the ovalbumin promoter and G-free cassette. Purified Tax_1 specifically transactivates this template 5- to 10-fold in a concentration-dependent manner. No transactivation of the ovalbumin promoter (pLovTATA) template control was observed. Tax_1 transactivation was inhibited by low concentrations of α-amanitin and was effectively neutralized by anti-Tax_1 but not control sera. Consistent with in vivo transactivating activity, Tax_1 NF-κB mutant M22, but not cyclic AMP-responsive element-binding protein mutant M47, transactivated the template containing the tandem 21-bp repeat. In a reconstituted in vitro transcription assay, Tax_1 transactivation was dependent upon basal transcription factors TFIIB, TFIIF, Pol II, TFIID, and TFIIA. TATA-binding protein did not functionally substitute for TFIID in the transactivation assay by Tax_1 but was sufficient for basal transcription. Finally, we have used anti-TFIIA antibody (p55) to ask if Tax_1 transactivation required TFIIA activity. Addition of TFIIA antibody to in vitro transcription reactions, as well as depletion of TFIIA by preclearing with antibody, showed that TFIIA was required for Tax_1 transactivation. Only a slight (twofold) drop of basal transcription was observed. Tax_1 transactivation was restored when purified HeLa TFIIA was added back into the reconstituted system. We propose that Tax_1 utilizes a transactivation pathway involving the activator regulated basal transcription factors TFIID and TFIIA.

Infection by the human T-cell lymphotropic virus type 1 (HTLV-1) is associated with an aggressive malignancy of mature $CD4^+$ T cells, adult T-cell leukemia, and the degenerative neuromuscular disease tropical spastic paraparesis/HTLV-1-associated myelopathy (3, 8). HTLV-1 encodes a 40-kDa protein, Tax_1, which is critical for viral replication, transformation, and gene regulation. Tax_1 can transform T cells (26, 27, 29) and fibroblasts in cooperation with an activated *ras* oncogene (54). The Tax_1 transgene can also induce mesenchymal tumors in mice (28, 49). The exact mechanism whereby HTLV-1 influences leukemogenesis is not known but is thought to be due in part to the transcriptional transactivation and transforming activities of the HTLV-1 Tax_1 gene product. In addition to its ability to transactivate the HTLV-1 long terminal repeat (LTR) (9, 17, 33, 34, 44, 45, 51, 58, 62, 64), Tax_1 can influence the expression of cellular genes such as *fos* and c-*myc* and the genes encoding interleukin-2 receptor α, interleukin-2, granulocyte-macrophage colony-stimulating factor, β-polymerase, tumor necrosis factor beta, parathyroid hormone-related protein and mouse αA-crystallin (1, 2, 12, 13, 15, 17, 19, 27, 30, 32, 34, 35, 46, 48, 51, 56, 60, 61, 67, 68, 69). Tax_1 may contribute to the transforming capacity of HTLV-1 in part by the aberrant induction or repression of these genes.

Tax_1 activates HTLV-1 gene expression through Tax_1-responsive *cis* elements TRE-1 and TRE-2 in the viral LTR (9, 44, 45, 51, 55, 58, 60, 64). Tax_1 also activates cellular gene promoters through *cis* elements identified as the targets of sequence-specific DNA-binding proteins such as the cyclic AMP-responsive element-binding protein (CREB), NF-κB, Ets-1, and the serum response factor (1, 2, 4, 5, 16, 24, 40, 52, 56, 68). Rather than bind to DNA elements directly, Tax_1 associates with the transcription factors CREB (65, 71, 72), TIF-1 (44, 45), p67SRF (22), p105, the precursor for the NF-κB protein p50 (30), ATF-1, ATF-2, CCAAT/enhancer-binding protein (67), HEB-1 (6), and p100, the NF-κ B2 gene product (7), to form a ternary complex. It has been postulated that the indirect binding of Tax_1 to the promoter might position the transactivator protein to interact with basal transcription factors. In support of this model, Caron et al. (10) have shown physical interaction between Tax_1 and TATA-binding protein (TBP).

Previous studies of in vitro Tax_1 transactivation have utilized the HTLV-1 LTR as a template (21, 46). We have recently demonstrated that the HTLV-1 template is transcribed by an intermediate polymerase II/polymerase III (Pol II/Pol III) transcription complex (53). In the present paper, we demonstrate that Tax_1-mediated transcription of the HTLV-1 LTR is resistant to low levels of α-amanitin, characteristic of the intermediate transcription complex. In view of these results and in the absence of in vivo experiments to demonstrate the biological significance of the HTLV-1 intermediate promoter, it was important to develop an in vitro transactivation system in which Tax_1 transactivation through Tax_1-responsive elements

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could be studied on a Pol II promoter. Utilizing a template in which the Tax_1-responsive 21-bp repeats were positioned upstream of a basal Pol II promoter, we now demonstrate that Tax_1 specifically transactivates this Pol II template in vitro. Tax_1 transactivation is dependent on the presence of holo-TFIID and TFIIA. When using an extract depleted of TFIIA, we observed no transactivation detectable by Tax_1. The interaction of TFIIA with Tax_1 may provide important insight into the biochemical mechanism by which HTLV-1 Tax activates viral and cellular gene expression.

MATERIALS AND METHODS

Plasmid constructs. The G-free DNAs used in the in vitro transcription assays were pLovTATA, pTRE-1_{Im}, and pTRE-1_{Id}. In the pLovTATA plasmid, the chicken ovalbumin TATA box is inserted upstream of a G-free cassette, containing a stretch of A, C, and T nucleotides (38, 57). Plasmids pTRE-1_{Im} and pTRE-1_{Id} contain one or two copies, respectively, of the promoter-proximal 21-bp repeats from wild-type HTLV-1 LTR inserted upstream of the chicken ovalbumin TATA box. The sequences of 21-bp repeats of pTRE-1_{Im} and pTRE-1_{Id} are 5′-AATTCCGTTGACGACAACCCA-3′ and 5′-AATTCCGTTGACGACAACCCCTCAGGCGTTGACGACAACCC A-3′, respectively. The 21-bp oligonucleotides were cut with *Eco*RI-*Bgl*II and inserted into the pLovTATA construct. Other plasmids, including adenovirus major late promoter (AdML), pU3R, and human immunodeficiency virus (HIV) LTR chloramphenicol acetyltransferase (CAT), are described elsewhere (36, 53). Templates used for in vitro transcription were cut with the appropriate restriction enzymes, phenol-chloroform extracted, ethanol precipitated, and resuspended in H_2O at a concentration of 100 to 500 ng/μl.

Tax_1 purification. Recombinant baculovirus containing the HTLV-1 Tax_1 gene was used to infect SF-9 insect cells. A control baculovirus without any insert was used for infection and preparation of control mock extract. The pblueBac vector expression system (Strategene) was used, and recombinant viruses were selected on the basis of the presence of the *lacZ* gene. Lysates were prepared from the insect cells and secreted material as recommended by the manufacturer. A titration curve was established for in vitro assays in which the same amounts of lysates were run on a 4 to 20% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and Western immunoblotted for the presence of Tax_1 protein. *Escherichia coli* Tax_1 protein was purified by ammonium sulfate precipitation (23, 41). Prior to use in the in vitro transcription assay, Tax_1 was incubated for 30 min at 30°C at a concentration of 200 ng/μl with an equal volume of Tax_1 buffer {2 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 2 mM dithiothreitol, 10 mM β-mercaptoethanol, 1 mM EDTA}. A titration curve of Tax_1 is normally set up for in vitro transcription after each purification, for which protein concentrations of 100 to 150 ng of Tax_1 protein shows an optimal transactivation activity. Tax_1 proteins from both baculovirus and *E. coli* are also checked for in vivo transactivation activity by electroporation into CEM cells (36).

Extracts and in vitro transcription assays. Nuclear extracts of HeLa cells were made by the method of Shapiro et al. (59). Both HeLa and CEM whole-cell extracts (WCE) were made by the procedure of Manley et al. (43).

For the G-free in vitro transcription reactions, incubation was carried out at 30°C for 60 min. Reactions were performed with Shapiro nuclear extract (2 μl of 16-mg/ml extract) or HeLa WCE (15 μl), supercoiled DNA (1.2 μg), and Tax_1 protein (1 or 3 μl, 200 ng). The total volume ranged between 35 and 50 μl. Transcription buffer (32.5 μl per reaction) contained 3 μl of 20% polyethylene glycol 6000, 3 μl of 50 mM $MgCl_2$, 3 μl of 1 mM dithiothreitol, 1 μl 0.2 M Creatinphosphat (Boehringer Mannheim), 1.5 μl of 50 mM ATP/CTP, 1 μl of 20 mM 3′-*O*-methyl-GTP (Pharmacia), 2 μl of [α-^{32}P]UTP (400 Ci/mmol; Amersham), 10 U of RNase T_1 (100 U/μl; Boehringer Mannheim), and 18 μl of buffer D (containing a final concentration of 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.9], 100 mM KCl, 12.5 mM $MgCl_2$, 0.1 mM EDTA, 17% glycerol, and 1 mM dithiothreitol).

Runoff in vitro transcription assays were done in a reaction volume of 16.5 μl, with the addition of template DNA, HeLa WCE, and Tax_1, bringing the volume to a total of 21 μl. All reaction mixtures were incubated for 1 h at 30°C in a buffer containing 2% polyethylene glycol 6000, 5 mM $MgCl_2$, 15 μCi of [α-^{32}P]UTP, 1 mM dithiothreitol, 500 μM nucleoside triphosphates, and 9 μl of buffer D. All transcription reactions were terminated by the addition of 20 mM Tris-HCl (pH 7.8), 150 mM NaCl, and 0.2% SDS. The quenched reaction products were then extracted with an equal volume of phenol-chloroform (50:50) and precipitated with 2.5 volumes of ethanol and 0.1 volume of 3 M sodium acetate. Following centrifugation, RNA pellets were resuspended in 15 μl of formamide denaturation mix containing xylene cyanol and bromophenol blue, heated at 95°C for 3 min, and electrophoresed at 400 V in a 4% polyacrylamide (acrylamide/bisacrylamide ratio, 19:1) gel containing 7 M urea (prerun at 300 V for 30 min) and 1× Tris-borate-EDTA (TBE) running buffer. Gels were exposed to both PhosphorImage and Kodak X-OMAT XAR-5 film at −70°C with intensifying screens for autoradiography.

Purification of HeLa TFIIA, TFIID, and Pol II. For TFIIA and TFIID, HeLa nuclear extracts were fractionated by phosphocellulose column chromatography. TFIIA (0.1 M KCl fraction) was purified further on DEAE-Sephacel, Q-Sepharose, and heparin-agarose by established procedures (20). The TFIIA fraction also contains the elongation transcription factor, TFIIJ. TFIID was prepared from the 1.0 M KCl fraction off of the phosphocellulose fraction and further purified by two sequential rounds of DEAE-52 column chromatography. The semipurified TFIID has its TBP-associated factors (TAFs), which are known to be needed for transactivation with various activators, namely, VP16 and E1A.

The Pol II was purified first on a DEAE-52 column and then by high-pressure liquid chromatography (HPLC) TSK-phenyl and HPLC DEAE-5PW as described previously (42). The RNA Pol II preparation contains both phosphorylated and unphosphorylated forms of polymerase. All three fractions, Pol II, TFIID, and TFIIA, were generous gifts of D. Reinberg.

Transfections and CAT assays. The Tax_1 mutants M22 and M47 were electroporated along with Tax_1, Tat, and reporter plasmid HIV LTR CAT into Jurkat cells as described previously (36). Extracts were prepared 18 h later for CAT assay. Cells were harvested, washed once with phosphate-buffered saline without Ca^{2+} and Mg^{2+}, pelleted, and resuspended in 150 μl of 0.25 M Tris (pH 7.8). The cells were freeze-thawed three times, with vortexing after each thawing. The tubes were incubated for 5 min at 68°C and then centrifuged. The supernatants were transferred to 1.5-ml Eppendorf tubes. After one final spin, the supernatant was again transferred to 1.5-ml Eppendorf tubes and the protein concentration was determined. CAT assays were performed with 10 μg of protein by the method of Gorman et al. (25).

Depletion of extract by Anti-TFIIA antibody. Hydrated and preswollen protein A-Sepharose (200 μl; Pharmacia) was mixed with 200 μl of crude preimmune or immune serum against residues 129 to 376 of hTFIIA/αβ (14) and incubated for 120 min at 4°C. Subsequently, the protein A-Sepharose was washed extensively with buffer D.

HeLa WCE (100 μl) was incubated with 50 μl of immune serum- or preimmune serum-protein A-Sepharose for 4 h at 4°C and then centrifuged. The supernatants (depleted extracts) were used for in vitro transcription assays. Aliquots of the supernatant and protein A-Sepharose-bound proteins were analyzed for TFIIA and TBP by Western blot analysis.

RESULTS

α-Amanitin resistance of basal and Tax_1-transactivated HTLV-1 transcription. We have previously shown that the HTLV-1 basal transcription is a unique promoter in that it contains an overlapping transcription unit which is resistant to α-amanitin in vitro (53). The same promoter has, however, been reported to be sensitive to low levels of α-amanitin when extracts from either HeLa cells or CEM lymphocytes were used in the presence of Tax_1 (46). It was possible, therefore, that the Tax_1-transactivated promoter had the characteristics of a normal Pol II promoter. The purified *E. coli* HTLV-1 Tax_1 used in the experiments presented below are shown in Fig. 1A. The protein is greater than 95% pure when analyzed by SDS-PAGE followed by a Coomassie blue stain. This protein is functional in vivo, as shown by transactivation of the HTLV-1 LTR following electroporation of Tax_1 protein into CEM cells (data not shown). Further, anti-Tax_1 antibodies specifically neutralized the activation and Tax_1 mutants failed to activate transcription (see below). These results suggest that in HeLa WCE, Tax_1 specifically activates HTLV-1 transcription from the α-amanitin-resistant HTLV-1 overlapping transcription unit.

We first compared the α-amanitin sensitivity of basal and Tax_1-activated transcription of the HTLV-1 LTR. As a control for these studies, we included the HIV Pol II promoter (37). In HeLa WCE, transcription from the HIV promoter was inhibited more than 90% in reactions containing 1 μg of α-amanitin per ml (Fig. 1B, lanes 1 and 2). In contrast, but consistent with previous findings from this laboratory on basal transcription of the HTLV-1 promoter (53), Tax_1-activated transcription from the HTLV-1 promoter was resistant to α-amanitin at 1 to 10 μg/ml (lanes 3 to 6). At an α-amanitin concentration of 10 μg/ml, activated transcription from the HTLV-1 promoter was inhibited less than twofold.

Similar results were observed in transcription assays performed in WCE derived from CEM lymphocytes. As observed

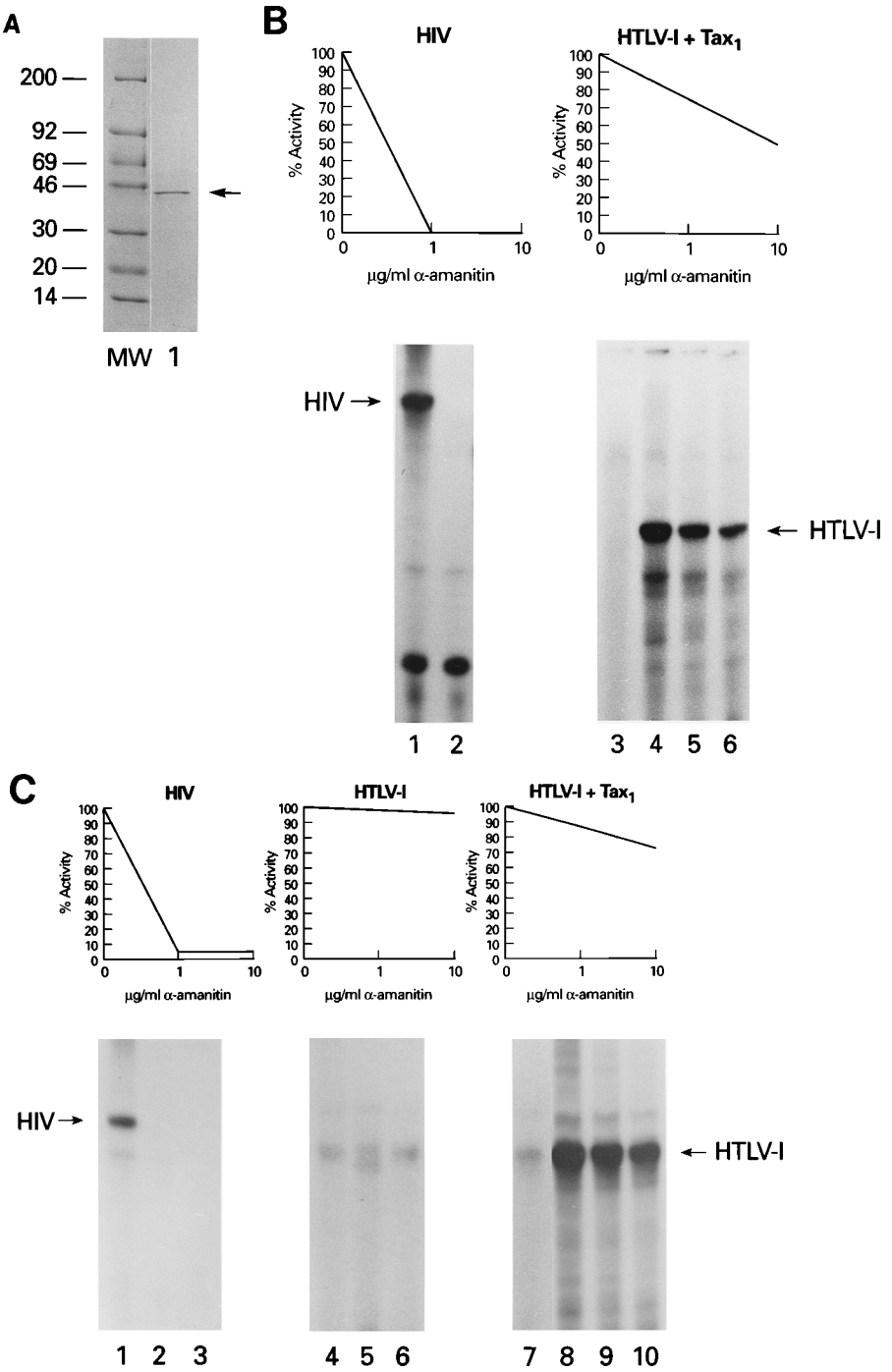

FIG. 1. (A) Coomassie blue stain of 200 ng of purified *E. coli* Tax_1 used for in vitro transcription. Molecular weight (MW) markers are ^{14}C rainbow markers (Bethesda Research Laboratories). (B) In vitro transcription from HeLa WCE (12.5 μg/μl) with the HIV promoter as a control for a classical Pol II promoter and HTLV-1 wild-type promoter. Lanes: 1, HIV template (250 ng), HeLa WCE (7.5 μl); 2, HIV template (250 ng) and HeLa WCE (7.5 μl), α-amanitin (1 μg/ml); 3, HTLV-1 wild-type promoter (250 ng) and HeLa WCE (1 μl); 4, HTLV-1 wild-type promoter (250 ng), HeLa WCE (1 μl), and Tax_1 (100 ng); 5, HTLV-1 wild-type promoter (250 ng), HeLa WCE (1 μl), Tax_1 (100 ng), and α-amanitin (1 μg/ml); 6, HTLV-1 wild-type promoter (250 ng), HeLa WCE (1 μl), Tax_1 (100 ng), and α-amanitin (10 μg/ml). (C) In vitro transcription from CEM (12D7) WCE (10 μg/μl). Lanes: 1, HIV template (250 ng) used as a Pol II control with CEM WCE (50 μl); 2, HIV template (250 ng), CEM WCE (50 μl), and α-amanitin (1 μg/ml); 3, HIV template (250 ng), CEM WCE (50 μl), and α-amanitin (10 μg/ml); 4 and 7, 250 and 500 ng of the HTLV-1 wild-type promoter, respectively; 5, HTLV-1 wild-type promoter (250 ng), CEM WCE (5 μl), and α-amanitin (1 μg/ml); 6, HTLV-1 wild-type promoter (250 ng), CEM WCE (5 μl), and α-amanitin (10 μg/ml); 8 to 10, HTLV-1 wild-type promoter (500 ng), Tax_1 (100 ng), and α-amanitin (0, 1, and 10 μg/ml, respectively).

in the HeLa WCE, transcription from the HIV LTR was sensitive to low concentrations of α-amanitin (Fig. 1C, lanes 1 to 3). Similar to the results presented above, the addition of Tax_1 to transcription reaction mixtures containing the HTLV-1 LTR resulted in a significant increase in HTLV-1 transcription (lanes 7 and 8). The addition of α-amanitin to 10 μg/ml caused less than a twofold decrease in basal or Tax_1-transactivated HTLV-1 transcription (lanes 6 and 10). At higher concentra-

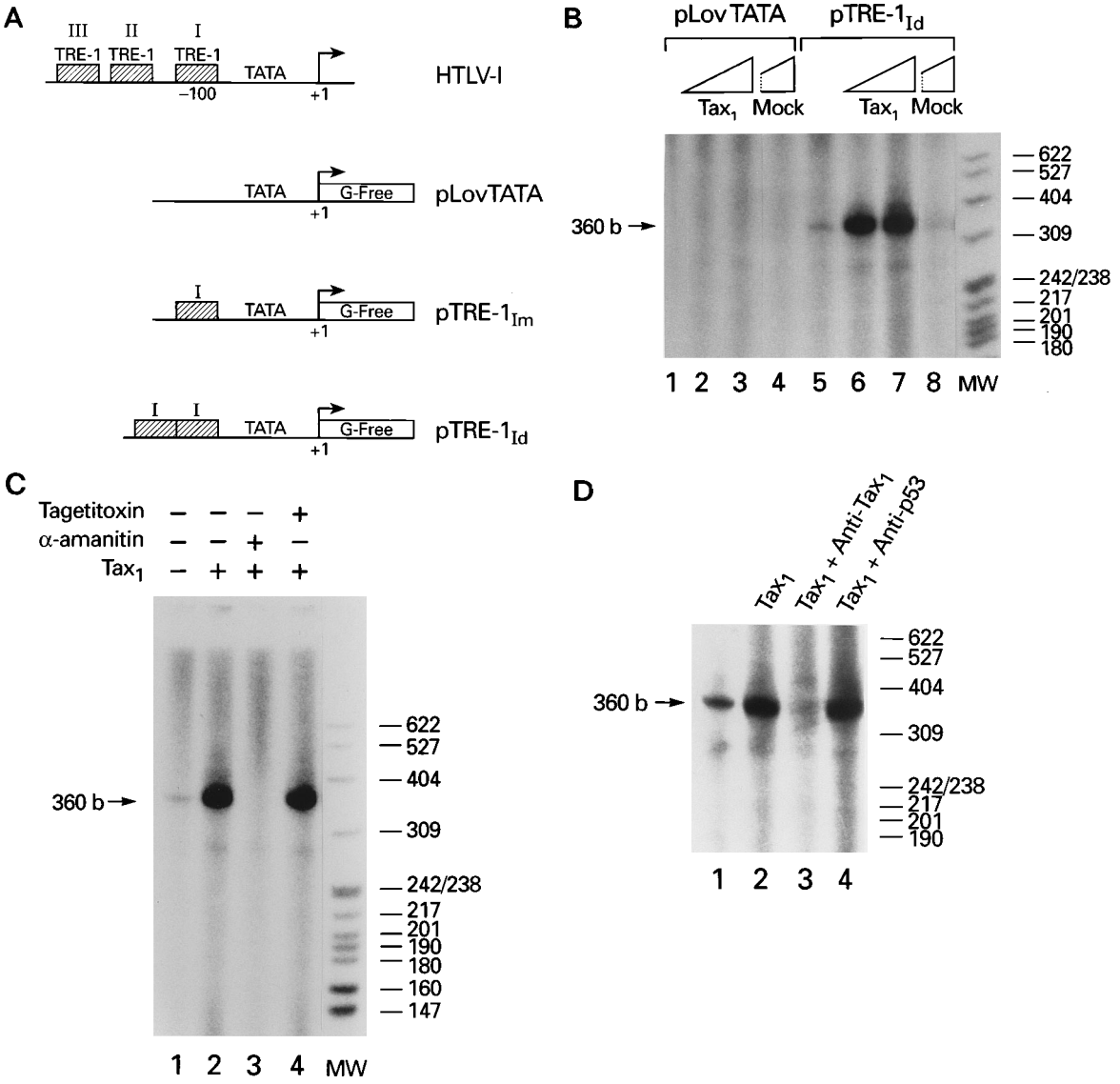

FIG. 2. In vitro transcription with G-free constructs in Shapiro nuclear extract in the presence of baculovirus Tax_1. (A) Plasmid constructs, other than HTLV-1 wild-type promoter, used in in vitro transcription assays were the backbone plasmid, pLovTATA, containing the chicken ovalbumin TATA box inserted upstream of a G-free cassette and plasmids pTRE-1_{Im} and pTRE-1_{Id}, containing one or two copies, respectively, of the 21-bp repeats from wild-type HTLV-1 inserted upstream of the chicken ovalbumin TATA box. (B) In vitro transcription from Shapiro nuclear extract (16 μg/μl) with pLovTATA (1.5 μg) (lanes 1 to 4) in the presence of increasing amounts of baculovirus Tax_1 (amount equivalent to *E. coli* Tax_1, compared on a Western blot) or a control mock WCE. Lanes 2 and 3 contain 1.5 or 3 μl of baculovirus Tax_1; lane 4, contains 3 μl of control mock WCE. Lanes 5 to 8 are identical to lanes 1 to 4 except that template pTRE-1_{Id} (1.5 μg) was added to the reactions. (C) Tax_1-dependent transcription of pTRE-1_{Id}, containing two HTLV-1 21-bp repeats was inhibited by the addition of α-amanitin but not the Pol III inhibitor tagetitoxin. Lanes: 1, pTRE-1_{Id} (1.5 μg); 2, pTRE-1_{Id} (1.5 μg), baculovirus Tax_1 (1.5 μl); 3, pTRE-1_{Id} (1.5 μg), baculovirus Tax_1 (1.5 μl), α-amanitin (5 μg/ml); 4, pTRE-1_{Id} (1.5 μg), baculovirus Tax_1 (1.5 μl), tagetitoxin (10 U). (D) Tax_1 in vitro transactivation was neutralized by preincubation of Tax_1 with TAb172, an anti-Tax_1 antibody, whereas the addition of control p53 (Ab-5) antibody (Oncogene Science) had no effect on Tax_1 transactivation of pTRE-1_{Id}. Lanes: 1, pTRE-1_{Id} (1.5 μg); 2, pTRE-1_{Id} (1.5 μg), Tax_1 (100 ng); 3, pTRE-1_{Id} (1.5 μg), Tax_1 (100 ng), TAb172 (200 ng); 4, pTRE-1_{Id} (1.5 μg), Tax_1 (100 ng), p53 antibody (200 ng). Both antibodies were subtype immunoglobulin G2A. MW, molecular weight.

tions of α-amanitin (50 to 100 μg/ml), inhibition of Tax_1 transactivation was observed (data not shown).

We have further analyzed basal and Tax_1-dependent transcription with a purified LTR fragment for the in vitro transcription template (46). The level of Tax_1 transactivation and the α-amanitin sensitivity curve were identical to the results obtained with the linear plasmid DNA (data not shown). These results demonstrate that while Tax_1 can transactivate the HTLV-1 LTR in HeLa or CEM WCE, the sensitivity to α-amanitin is not that of a typical Pol II promoter. A similar pattern of α-amanitin resistance is also seen when using Tax_1 expressed in baculovirus (data not shown).

In vitro transcription with G-free templates. In view of the above results, plasmids which contained Tax_1-responsive elements upstream of a prototype Pol II promoter were constructed. The 21-bp repeats of the HTLV-1 LTR have been shown to be important for Tax_1 transactivation of the HTLV-1 LTR (10). Furthermore, when these regulatory sequences are positioned upstream of a heterologous promoter, the transcription unit is responsive to Tax_1. Schematic diagrams of the

G-free constructs are shown in Fig. 2A, which shows that the promoter-proximal 21-bp repeat from wild-type HTLV-1 was inserted as a monomer or dimer upstream of the chicken ovalbumin promoter (38). Transcription from the backbone basal promoter, pLovTATA, is low in HeLa nuclear extracts (Fig. 2B, lane 1). The addition of Tax_1 or control Tax_1 extract to in vitro reaction mixtures containing the basal pLovTATA promoter did not increase the level of basal transcription (lanes 2 to 4). Transcription from the pTRE-1_{Id} template, which contains two copies of the 21-bp repeat upstream of the basal promoter, led to a slight increase in basal transcription (lane 5). The addition of Tax_1 to the in vitro transcription reaction led to an eightfold increase in 21-bp-dependent transcription (lanes 5 and 7). In contrast, the addition of a control extract containing no Tax_1 protein stimulated transcription less than twofold (lane 8). Tax_1-dependent transcription of the 21-bp repeat template was inhibited more than 95% by the addition of α-amanitin to 5 μg/ml (Fig. 2C, lanes 2 and 3). In contrast, the Pol III inhibitor tagetitoxin failed to inhibit transcription (lanes 2 and 4). Similar results have been observed with pTRE-1_{Im} (data not shown).

To demonstrate that the transactivation activity was specific to Tax_1, immunoglobulin G2A-purified monoclonal antibody TAb172, an anti-Tax_1 antibody, and an anti-p53 immunoglobulin G2A antibody were tested for their ability to block transactivation (Fig. 2D). Anti-Tax_1 antibody TAb172 efficiently blocked Tax_1 transactivation of pTRE-1_{Id} in vitro (Fig. 2D, lane 3). In contrast, the p53 antibody did not affect Tax_1 transactivation (lane 4).

Effect of wild-type Tax_1 and Tax_1 mutants in vitro and in vivo. To further demonstrate the specificity of Tax_1 proteins in vitro, two Tax_1 mutants, one in the NF-κB activation domain (M22) and the other in CREB activation domain (M47), were used for the in vitro transcription assay. Similar to the results presented above, the addition of wild-type Tax_1 led to a sevenfold increase in the level of transcription from the template containing the HTLV-1 21-bp repeats (Fig. 3A, lanes 1 and 2). The addition of Tax_1 mutant M22 to the in vitro transcription assay led to a fivefold increase in 21-bp-dependent transcription (lane 3). Consistent with in vivo results (63), the transactivation efficiency of the M22 mutant was approximately 50% of that observed with the wild-type Tax_1 protein. In contrast, the addition of the CREB Tax_1 mutant, M47, did not lead to an increase in transcription (lane 4).

It could be argued that the M47 mutant protein had been inactivated during purification. To demonstrate the biological activity of the Tax_1 M47 mutant, we used protein electroporation of the wild-type and mutant proteins on the NF-κB-responsive HIV LTR CAT plasmid. HIV LTR CAT (5 μg) as the reporter plasmid was introduced into Jurkat lymphocytes by electroporation in the presence of wild-type or mutant Tax_1 protein. In this experiment, wild-type Tax_1 protein and Tax_1 mutant M47 transactivated the HIV LTR CAT approximately sevenfold above the level of basal transcription (Fig. 3B, lanes 1, 3, and 5). Tax_1 mutant M22, defective in the NF-κB activation domain, was unable to transactivate the HIV LTR promoter (lane 4). In a reciprocal experiment, we transfected the HTLV-1 pU3R CAT plasmid together with wild-type Tax_1 protein or the Tax_1 mutants into Jurkat cells. As expected, wild-type and mutant M22 Tax_1 were able to transactivate, while M47 Tax_1 could not activate HTLV-1 transcription (data not shown).

Reconstitution of the activated transcription with purified factors. We have used two independent lines of investigation, in vitro reconstitution and antibody depletion, to define basal transcription factors required for Tax_1 transactivation. Tax_1

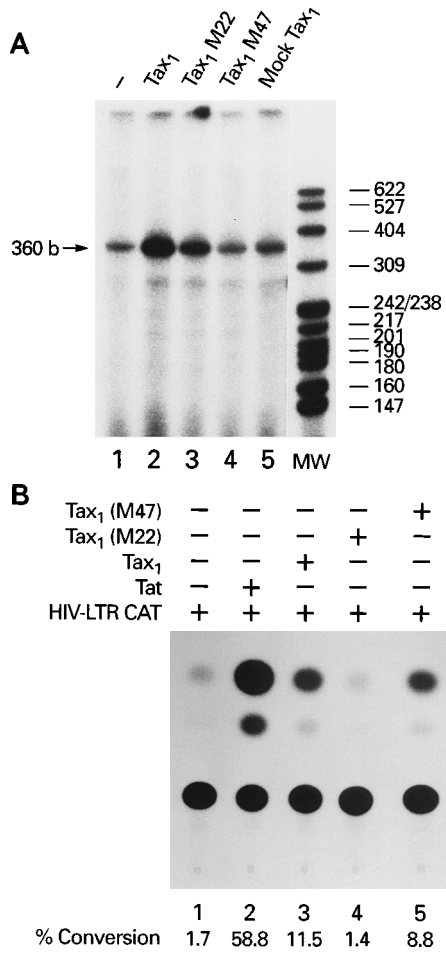

FIG. 3. In vitro and in vivo transactivation experiments with wild-type and mutant Tax_1. (A) In vitro transcription reaction with Shapiro nuclear extract (32 μg) for transcription of pTRE-1_{Id}. Lanes: 1, pTRE-1_{Id} (1.5 μg); 2, pTRE-1_{Id} (1.5 μg) and Tax_1 (100 ng); 3, pTRE-1_{Id} (1.5 μg) and Tax_1 M22 mutant (defective for NF-κB activation pathway) (100 ng); 4, pTRE-1_{Id} (1.5 μg) and Tax_1 mutant M47 (defective for CREB activation pathway) (100 ng); 5, pTRE-1_{Id} (1.5 μg) and control mock extract. (B) Transfection with HIV LTR was performed to check for functional activity of the M47 mutant protein in vivo by electroporation into CEM cells and analysis by the CAT assay (25, 36). Lanes: 1, basal-level transcription by HIV LTR CAT (5 μg); 2, HIV-LTR CAT (5 μg) and Tat protein (0.5 μg); 3, HIV LTR CAT (5 μg) and Tax_1 (2.5 μg); 4, HIV LTR CAT (5 μg) and Tax_1 mutant M22 (2.5 μg); 5, HIV LTR CAT (5 μg) and Tax_1 mutant M47 (2.5 μg). MW, molecular weight.

transactivation can be reconstituted in vitro with transcription factors TFIID, TFIIA, TFIIB, TFIIF, Pol II, and CREB. The Pol II fraction contained the CREB protein. To determine the importance of TFIID and associated TAFs in Tax_1 transactivation, the ability of Tax_1 to transactivate pTRE-1_{Id} in the presence of TFIID or TBP, the 38-kDa basic subunit of TFIID, was compared in the reconstituted transcription assay. In the presence of either TFIID or TBP, transcription from pTRE-1_{Id} basal template was barely detectable (Fig. 4A, lanes 1 and 3). In contrast, when Tax_1 was added to the reconstituted in vitro transcription reaction mixture, a significant increase in transcription was observed in the presence of holo-TFIID but not TBP (lanes 2 and 4). To demonstrate that the TBP protein used in these assays was functional, the same TBP was used to reconstitute in vitro transcription activity for the AdML promoter from phosphocellulose fractions (Fig. 4B) and added back to a HeLa nuclear extract that had TFIID inactivated by

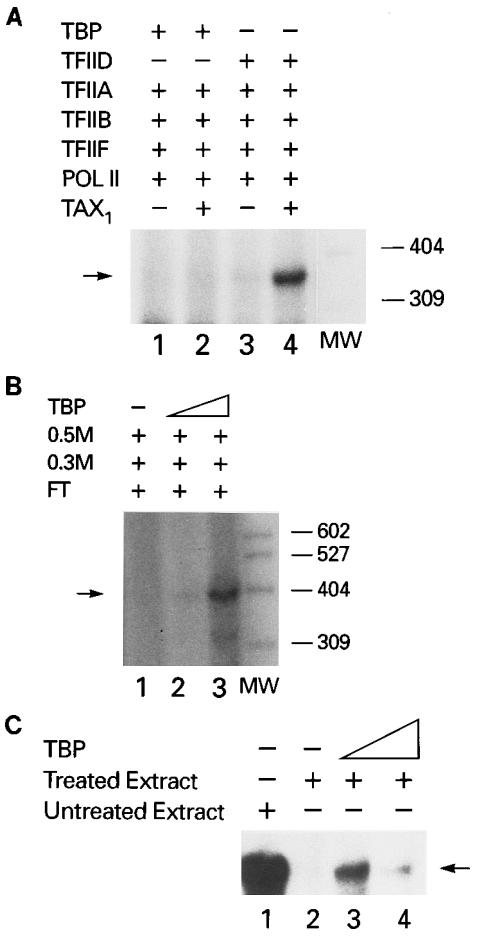

FIG. 4. Reconstitution of pTRE-1_{Id} transcription with purified factors and Tax_1. (A) pTRE-1_{Id} was used as the template to reconstitute Tax_1 activation in vitro. After a series of plasmid and Tax_1 titrations (data not shown), a minimal set of transcription factors, TBP or TFIID, TFIIA, TFIIB, TFIIF, and Pol II, were used to reconstitute transactivation. Lanes: 1, pTRE-1_{Id} (3 μg), TBP (100 ng), TFIIA, TFIIB, TFIIF, and Pol II; 2, pTRE-1_{Id} (3 μg), Tax_1 (100 ng), TBP (100 ng), TFIIA, TFIIB, TFIIF, and Pol II; 3, pTRE-1_{Id} (3 μg), TFIID, TFIIA, TFIIB, TFIIF, and Pol II; 4, pTRE-1_{Id} (3 μg), Tax_1 (100 ng), TFIID, TFIIA, TFIIB, TFIIF, and Pol II. (B) Linear AdML template, phosphocellulose fractions, and limiting amounts of TBP were used as a reconstitution system to determine the functional activity of TBP used in these experiments. Lanes: 1, AdML template (200 ng) and phosphocellulose fractions FT, 0.3 M, and 0.5 M (1 μl each); 2, AdML template (200 ng), phosphocellulose fractions FT, 0.3 M, and 0.5 M (1 μl each), and *E. coli*-purified human TBP (100 ng); 3, AdML template (200 ng), phosphocellulose fractions FT, 0.3 M, and 0.5 M (1 μl each), and *E. coli*-purified hTBP (300 ng). (C) To further determine that TBP is, in fact, a functional transcription factor, a heat-inactivated HeLa nuclear extract (Shapiro), rendering TFIID inactive, was used. Lanes: 1, AdML G-free construct (250 ng) and untreated extract (48 μg); 2, AdML G-free construct (250 ng) and treated extract (48 μg); 3, AdML G-free construct (250 ng), treated extract (48 μg), and *E. coli*-purified hTBP (100 ng); 4, AdML G-free construct (250 ng), treated extract (48 μg), and *E. coli*-purified hTBP (300 ng). MW, molecular weight.

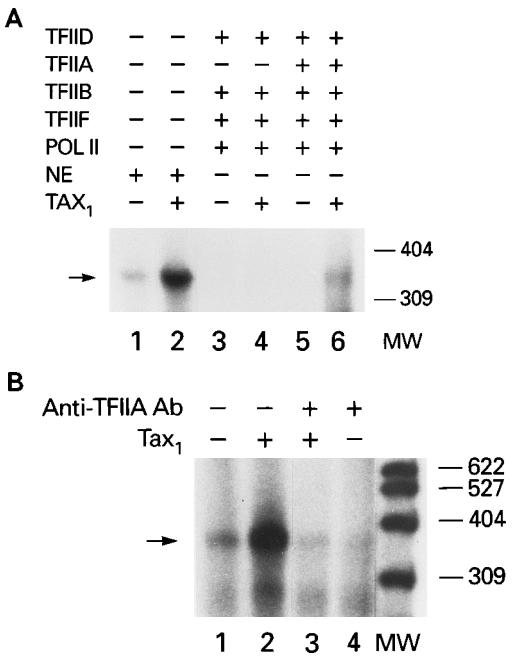

FIG. 5. Reconstitution of Tax_1-activated transcription in vitro. (A) Control lane 1, pTRE-1_{Id} (3 μg) and HeLa nuclear extract (Shapiro) (80 μg); control lane 2, pTRE-1_{Id} (3 μg), HeLa nuclear extract (Shapiro) (80 μg), and Tax_1 (100 ng). Reconstitution with and without TFIIA and Tax_1. Lanes: 3, pTRE-1_{Id} (3 μg), TFIID (2 μl), TFIIB (0.5 μl), TFIIF (1.0 μl), and Pol II (1.0 μl); 4, same as lane 3 with the addition of Tax_1 (100 ng); 5, pTRE-1_{Id} (3 μg), TFIID (2 μl), TFIIA (2 μl), TFIIB (0.5 μl), TFIIF (1.0 μl), and Pol II (1.0 μl); 6, same as lane 5 with Tax_1 (100 ng). (B) Anti-TFIIA antibody (Ab) (anti-p55, polyclonal rabbit) was added at 300 ng per reaction to determine if it could inhibit activated or basal transcription. Lanes: 1, pTRE-1_{Id} (1.5 μg) and HeLa nuclear extract (Shapiro) (80 μg); 2, pTRE-1_{Id} (1.5 μg), HeLa nuclear extract (Shapiro) (80 μg), and Tax_1 (100 ng); 3, pTRE-1_{Id} (1.5 μg), HeLa nuclear extract (Shapiro) (80 μg), Tax_1 (100 ng), and anti-TFIIA antibody (300 ng); 4, pTRE-1_{Id} (1.5 μg), HeLa nuclear extract (Shapiro) (80 μg), and anti-TFIIA antibody (300 ng). MW, molecular weight.

heating (47°C for 15 min) (Fig. 4C). These results indicate that the TBP, which was not able to support Tax_1 transactivation in reconstitution assays, was indeed a functional protein. At higher concentrations of TBP, transcription was inhibited, probably because of squelching of TBP binding proteins (Fig. 4C, lane 4). We conclude from these studies that Tax_1 transactivation requires holo-TFIID.

Similar studies were performed with the basal transcription factor TFIIA. Consistent with the results presented in Fig. 4A, Tax_1 transactivation of pTRE-1_{Id} template was observed in the presence of TFIID, TFIIA, TFIIB, TFIIF, and Pol II (Fig. 5A, lanes 5 and 6). When TFIIA was omitted from the transcription assay, no Tax_1 transactivation was observed (lanes 3 and 4). Tax_1 transactivation was specific to the pTRE-1_{Id} construct, since no Tax_1 transactivation was observed with the pLovTATA construct (data not shown). Therefore, the presence of both a 21-bp repeat and a functional TFIIA is required for Tax_1 activation in vitro.

TFIIA is required for Tax_1 transactivation. The importance of TFIIA in Tax_1 transactivation was further analyzed by antibody neutralization assays in the presence of nuclear extract. The addition of anti-TFIIA antibody to the transcription assay decreased basal transcription by approximately twofold (Fig. 5B, compare lanes 1 and 4). In contrast, the addition of anti-TFIIA antibody to the Tax_1 transactivation assay completely inhibited the function of Tax_1 (compare lanes 2 and 3). A similar experiment, in which a preimmune control antibody was added to the extract, showed no drop in basal or activated transcription (data not shown). The above results suggest that basal transcription is not affected by lack of TFIIA but that activation by Tax_1 is dramatically reduced in the absence of TFIIA.

A similar experiment was performed with a TFIIA-depleted extract with the addition of an antibody raised against the p55 *E. coli* recombinant TFIIA. After depletion, an aliquot of depleted extract and the bound material were run on SDS-PAGE

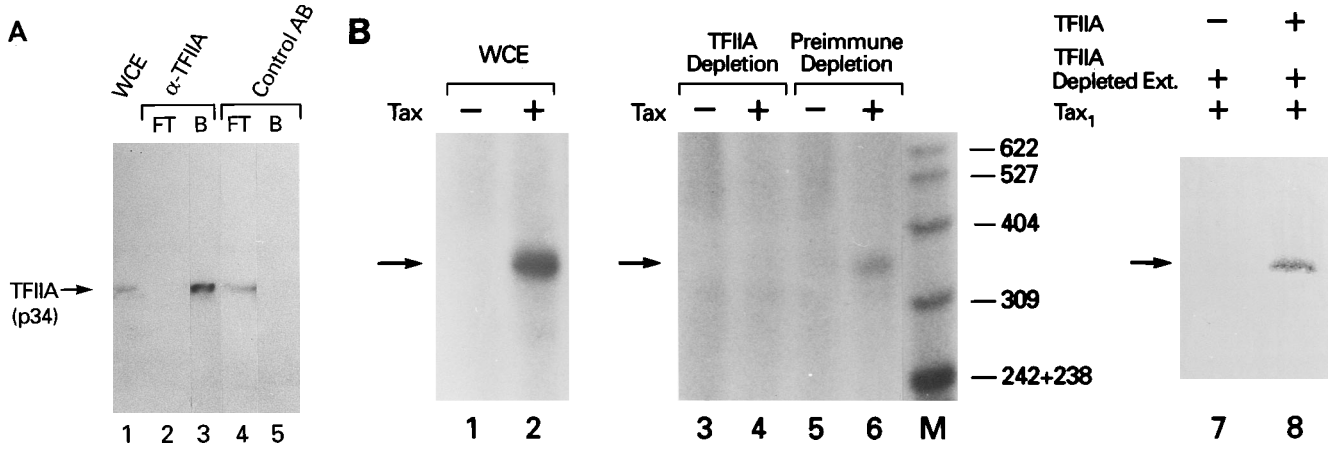

FIG. 6. Reconstitution of activated transcription with TFIIA and TFIIA-depleted extract. (A) Two columns, one with anti-TFIIA antibody (against recombinant p55 TFIIA) and the other with anti-control antibody (rabbit preimmune serum) were mixed with HeLa WCE and rotated to allow binding of TFIIA. The samples were run on 4 to 20% gradient SDS-PAGE, transferred, and Western blotted with anti-TFIIA antibody. The arrow indicates the p34 subunit of TFIIA that is readily and reproducibly detected by anti-TFIIA antibody. Lane 1 contains HeLa WCE (62.5 μg). Lanes 2 and 4 contain 7 μl each of the TFIIA- and control antibody-depleted extracts, respectively). After depletion, the beads were incubated with 50 μl of SDS-BME loading buffer, heated for 5 min at 95°C, and loaded. Lanes 3 and 5 contain one-fifth of the beads that were used to deplete 100 μl of HeLa WCE. (B) In vitro transcription with HeLa WCE and TFIIA-depleted extracts. Lanes: 1, pTRE-1_{Id} (2 μg), HeLa WCE (15 μl); 2, same as lane 1 except for the addition of Tax_1 (100 ng); 3 and 4, same as lanes 1 and 2 except for the addition of TFIIA-depleted extract (15 μl); 5 and 6, same as lanes 1 and 2 except for the addition of preimmune depleted extract (15 μl); 7, pTRE-1_{Id} (2 μg), TFIIA-depleted extract (9 μl), and Tax_1 (100 ng); 8, pTRE-1_{Id} (2 μg), TFIIA-depleted extract (9 μl), Tax_1 (100 ng), and TFIIA (5 μl).

and Western blotted with anti-TFIIA antibody. Figure 6A shows the result of such an experiment in which anti-TFIIA antibody, but not the control antibody, was capable of depleting the endogenous HeLa TFIIA (lanes 3 and 5, respectively). Therefore, we were effectively able to deplete TFIIA from the HeLa extract by the anti-p55 antibody. We next examined the transcriptional activity of the flowthrough fractions and saw that TFIIA-depleted but not preimmune-depleted extract could not support Tax_1 transactivation in vitro (Fig. 6B, lanes 4 and 6, respectively). This was in agreement with our previous result (Fig. 5B), which indicated the importance of TFIIA in Tax_1 transactivation. To unambiguously demonstrate that the depleted transcription was, in fact, TFIIA, we performed experiments in which exogenous purified HeLa TFIIA was added back into the depleted extract. Results of such an experiment are shown in Fig. 6C, where TFIIA restored the Tax_1-activated transcription (Fig. 6B, lanes 7 and 8). It is noteworthy to indicate that TFIIA-depleted extracts may deplete other factors bound to TFIIA, namely, TFIID or other unknown factors. Therefore, a 100% Tax_1 activation from TFIIA-depleted extract may require additional factors other than TFIIA for full reconstitution activity.

DISCUSSION

Previously, we reported that the HTLV-1 LTR contains overlapping promoters. HTLV-1 transcriptional activity was reconstituted in vitro with recombinant TBP, recombinant TFIIB, TFIIA, TFIIE, TFIIF, TFIIH, and Pol II (53). In HeLa WCE, however, HTLV-1 transcription is resistant to low levels of α-amanitin and is not dependent upon RNA Pol II. Both RNA transcripts have the same 5′ end. In this paper, we demonstrate that Tax_1-mediated in vitro transcription from the HTLV-1 LTR is resistant to low levels of α-amanitin in reactions containing either HeLa or CEM lymphocyte extract. Recently, Franklin et al. (21) have reported that CREB and ATF-2 bind to the HTLV-1 21-bp repeat and stimulate HTLV-1 transcription in vitro. Tax_1 was shown to cooperate with CREB and ATF-2 to enhance the level of transcription. In view of our results, the conclusions from these studies regarding Pol II transcription, however, is not straightforward. We would suggest that while CREB and ATF-2 might stimulate HTLV-1 LTR basal and Tax_1-mediated transcription in vitro, this transcription activity is distinct from the normal Pol II transcription unit. To dissect the biochemical mechanism of Pol II-dependent Tax_1 transactivation, we have cloned the Tax_1-responsive 21-bp repeats upstream of a basal Pol II promoter. Using this Pol II template, we demonstrate that transcription factors TFIID and TFIIA are required for Tax_1 transactivation.

TFIID has been shown to be functionally heterogeneous, as a result of the interaction of specific TAFs (66). Following phosphocellulose fractionation of HeLa extracts, TFIID is located in both the 0.3 and 0.85 M elution fractions. The molecular mass of the TFIID complex in the 0.85 M fraction is approximately 750 kDa and is composed of TBP and 8 to 10 TAFs. In contrast, the TFIID complex in the 0.3 M fraction has a molecular mass of approximately 350 kDa and is associated with a distinct set of TAFs important for Pol III transcription. The role of the individual TAFs in basal and activated transcription is apparently dependent on the architectural structure of the promoter and the activator protein. For example, Zhou et al. (73) have demonstrated that while TBP is sufficient for basal transcription, the 0.85 M TFIID fraction is required for response to transactivator proteins such as GAL4-VP16. In contrast, Sharp's laboratory (74) recently reported that the TAFs are disposable for HIV Tat transactivation of the HIV LTR. The results presented in this paper demonstrate that the D-TFIID is required for Tax_1 transactivation of the Tax_1-responsive 21-bp repeats. CREB and ATF-1 are among the activator proteins which interact with the 21-bp repeats and are modulated by Tax_1. Interestingly, the Q2 activation domain of CREB has been shown to interact with $TAF_{II}110$ (18). Since TFIID binding is one of the limiting steps in the assembly of the initiation complex, the induced binding of CREB and interaction of CREB with $TAF_{II}110$ may facilitate incorporation of TFIID into the initiation complex and may represent a critical step in the Tax_1 transactivation pathway.

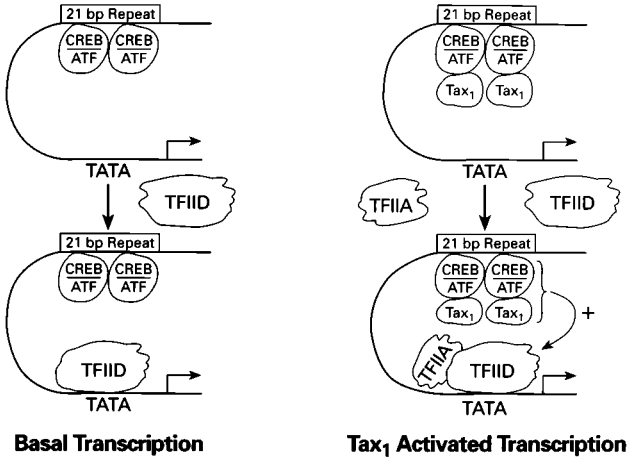

FIG. 7. Diagram of a proposed model for Tax_1 transactivation.

The role of TFIIA in transcription has been the subject of much debate. Until recently, it was proposed that the main function of TFIIA was to counteract the effect of negative cofactors, suggesting that TFIIA may function more as an anti-repressor than an enhancer of basal transcription. Negative cofactors, such as Dr1, Dr2/NC1, and NC2 (31, 47) inhibit interaction of TBP with other basal transcription factors such as TFIIA and TFIIB. Under appropriate conditions, TFIIA cooperates with TBP to counteract these repressors of transcription. The adenovirus $E1A_{12S}$ gene product apparently utilizes the interaction of TBP with Dr1 or TFIIA as a point of regulation in *hsp*70 transcription. Kraus et al. (39) have demonstrated that E1A interacts directly with Dr1 and disrupts the inactive DR1-TBP complex. Whether Tax_1 regulates transcription in a somewhat similar fashion, through physical interaction and activation of TFIIA, is presently under investigation.

It has been demonstrated by kinetic experiments that TFIIA binds to the promoter-TFIID complex and stabilizes the interaction of TBP or TFIID with the template at an early step in transcription, maintaining the committed transcription complex. Therefore, it has also been suggested that TFIIA may function as a true activator of transcription. Consistent with this possibility, Yokomori et al. have recently demonstrated that TFIIA stimulates TFIID-dependent transcription in a reconstituted transcription assay system (70). Moreover, TFIIA significantly enhances transcriptional activation by upstream transcription factors such as Sp1. The authors used these results to suggest that TFIIA works directly as a transcriptional activator, in the absence of negative cofactors. Interestingly, the Epstein-Barr virus EBV Zta activator stimulates the binding of TFIID to the DNA TATA element in a manner that is dependent on TFIIA. Zta-induced TFIID binding was dependent on TAFs and the Zta activation domain, supporting the biological significance of this function (50). These results suggest that Zta may enhance the transcriptional activation function of TFIIA.

At this time, we cannot unambiguously determine whether HTLV-1 Tax_1 functions like the Zta or E1A activators, stabilizing the TFIIA-TFIID complex directly or simply releasing a negative factor from TFIID. The purified TFIIA, TFIID, and Pol II used in the reconstitution transcription assay showed no detectable Dr1 protein by Western blot analysis. Thus, Tax_1 transactivation may resemble the Epstein-Barr virus Zta activation pathway, in which Tax_1 stabilizes the TFIIA-TFIID complex. Interestingly, Tax_1 is not a specific DNA-binding protein like Zta but functions through protein-protein interactions to stimulate transcription factor binding and allow indirect binding of Tax_1 to the DNA. We have recently found that Tax_1 specifically interacts physically with TFIIA. Further, our studies demonstrate that TFIIA enhances Tax_1-dependent transcription in vivo (11).

A model for Tax_1 transactivation is presented in Fig. 7. Tax_1 has been shown by several groups to increase the binding of CREB and ATF proteins to the 21-bp repeats. Interestingly, through protein-protein interaction with CREB or ATF proteins, Tax_1 becomes a part of this DNA-binding complex, interacting with the DNA in an indirect fashion. CREB interaction with TFIID and Tax_1 interaction with TFIIA may facilitate binding of the TFIID-TFIIA preinitiation complex, one of the limiting steps in the assembly of the initiation complex. It is interesting that Tax_1 has also been shown to facilitate the binding and physically interact with other activator proteins such as NF-κB and serum response factor. It will be of interest to analyze the subtle differences in these transcription complexes and determine their requirement for TFIID and TFIIA in Tax_1 transactivation.

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J. F. Duvall and F. Kashanchi contributed equally to this work.

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