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Transcriptional Activation of RNA Polymerase III-Dependent Genes by the Human T-Cell Leukemia Virus Type 1 Tax Protein

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The human T-cell leukemia virus-encoded Tax protein is a potent activator of many viral and cellular genes transcribed by RNA polymerase II. We find that both chromatin and cell extracts derived from human T-cell leukemia virus type 1-infected human T lymphocytes support higher levels of 5S rRNA and tRNA gene transcription than chromatin or extracts from uninfected T lymphocytes. The viral protein Tax was likely responsible for this higher level of class III gene transcription, as purified Tax was found to stimulate both genes when added to the uninfected cell extract or in reconstituted systems. Both limiting-component transcription assays and DNA binding assays identified the class III gene transcription factor TFIIIB as the principal target of Tax activity. Surprisingly, we find that Tax increases the effective concentration of active TFIIIB molecules. These data suggest that Tax stimulates RNA polymerase III-dependent gene expression by accelerating the rate and/or extent of transcription initiation complex assembly.

Human T-cell leukemia virus type 1 (HTLV-1) is the causative agent of an aggressive and fatal malignancy called adult T-cell leukemia (39; for a review, see reference 21). The virus is also associated with a variety of additional clinical disorders in humans, including a neurodegenerative disease called tropical spastic paraparesis/HTLV-1-associated myelopathy (31). The pathogenesis of HTLV-1 appears directly linked to the virally encoded transcriptional activator protein Tax. Tax is essential to the life cycle of the virus, as it strongly stimulates RNA polymerase II (pol II)-dependent transcription of the HTLV-1 genome, thus leading to efficient viral replication (14, 15, 19, 23, 44, 48). Tax has also been shown to deregulate the expression of a wide variety of class II cellular genes, leading to the hypothesis that HTLV-1-associated pathogenesis may result from the highly pleiotropic deregulation of cellular gene expression by Tax.

Tax does not bind DNA directly but instead utilizes a variety of structurally unrelated cellular DNA-binding proteins to mediate transcriptional deregulation. For example, Tax strongly up-regulates HTLV-1 gene expression apparently mediated through the cellular protein CREB, a member of the basic leucine zipper (bZIP) family of transcription factors (7, 12, 20, 59). Tax also stimulates the expression of a wide variety of class II viral and cellular genes mediated through additional cellular transcription factors. These processes include the transcriptional up-regulation of human immunodeficiency virus, interleukin 2, and interleukin 2 receptor α genes mediated through members of the NF-κB family of proteins (5, 10, 41, 45) and transcriptional up-regulation of the c-*fos*, *Krox-20* and *Krox-24* genes mediated through serum response factor (1, 2, 22). Interestingly, the basic helix-loop-helix proteins, which play critical roles in cellular proliferation and differentiation, appear to be involved in Tax-mediated repression of gene expression (51).

The precise molecular steps leading to deregulated gene expression by Tax are not fully understood; however, the details of one possible mechanism are beginning to emerge. Several reports suggest that Tax may activate transcription through enhancement of the DNA binding activities of certain target cellular transcription factors, thus increasing the concentration of the promoter-bound activator proteins at Tax-responsive genes (3, 4, 12, 20, 52, 58, 62). In studies examining the effect of Tax on the bZIP family of transcription factors, enhancement in DNA binding appears to be mediated through an interaction between Tax and the basic DNA binding domains of these proteins (6, 20, 52, 58). This interaction appears to result in the stabilization of a structure that promotes both DNA binding and dimerization (3, 6). These studies have been unable to identify specific bZIP amino acids involved in the interaction with Tax, suggesting that Tax may recognize a protein structure rather than a specific amino acid sequence. This interpretation is consistent with the observation that Tax is highly pleiotropic, enhancing the activity of a wide variety of unrelated cellular transcription factors involved in class II gene expression.

In this report, we extend these studies to examine whether Tax might additionally affect genes transcribed by RNA pol III (class III genes). Because Tax is highly pleiotropic, strongly deregulating many viral and cellular genes through interaction with RNA pol II-dependent transcription factors, we reasoned that Tax may also deregulate class III gene expression in a similar way. Furthermore, previous studies have demonstrated that viral infection by adenovirus and pseudorabies virus results in enhancement in the expression of RNA pol III-dependent genes (8, 25, 30, 60). RNA pol III is responsible for the transcription of small RNAs that are involved in protein synthesis and RNA processing. These include the tRNAs, 5S rRNA, and U6 small nuclear RNA. Transcription of the tRNA genes requires, in addition to RNA pol III, the general factors TFIIIC and TFIIIB. Transcription of the 5S gene requires the additional 5S gene-specific factor TFIIIA.

We demonstrate in this study that the levels of transcription of both 5S and tRNA genes are higher in chromatin derived from an HTLV-1-infected human T-cell line than in chromatin derived from an uninfected human T-cell line. We further

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show that highly purified Tax protein strongly stimulates transcription by RNA pol III in transcription reactions using both crude extracts derived from an uninfected T-cell line and fractionated components derived from both human and *Xenopus* cells. This stimulation appears to result from a Tax-dependent increase in the activity of TFIIIB, one of the general class III gene transcription initiation factors. Together, these data provide strong evidence in support of a role for the HTLV-1 Tax protein in transcriptional activation of RNA pol III-dependent genes.

MATERIALS AND METHODS

Recombinant proteins. Tax was purified to near homogeneity from *Escherichia coli* HB101, using the pTaxH_6 expression vector (61), by two rounds of Ni^{2+} chelate chromatography (Ni^{2+}-nitrilotriacetic acid agarose; Qiagen) followed by gel filtration on a Superdex 75 fast protein liquid chromatography column (Pharmacia) in a buffer containing 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.9), 12.5 mM $MgCl_2$, 10 μM $ZnSO_4$, 150 mM KCl, 4 mM β-mercaptoethanol, 20% (vol/vol) glycerol, and 0.1% (vol/vol) Nonidet P-40. Peak fractions containing Tax were aliquoted and stored at −70°C. The purity of Tax was determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and Coomassie blue staining. Tax protein concentration was determined to be approximately 80 ng/μl. Purified recombinant His_6-tagged 9-*cis* retinoic acid receptor protein was the generous gift of Peter Ghazal and James LeBlanc (The Scripps Research Institute).

DNA templates and oligonucleotides. Templates for transcription reactions were the somatic 5S RNA gene contained in plasmid pXIs 11 (38) and the somatic $tRNA^{TyrD}$ gene (49), both of *Xenopus laevis*, and the $tRNA^{Arg}$ gene of *Drosophila melanogaster* (18). Plasmid pBluescript SK+ (Stratagene) was used as nonspecific carrier DNA. A 26-bp double-stranded TATA box oligonucleotide (derived from the sequence of the adenovirus major late promoter) has the top-strand sequence 5′-GATCGGGGCTATAAAAGGGGGTG-3′. The mutant TATA-box oligonucleotide had the top-strand sequence 5′-TCCTGAA GGGGGGCTAGAGAAGGGGGTGGGG-3′ (the mutated positions within the TATA box are underlined). A 24-bp double-stranded oligonucleotide corresponding to the consensus B-block TFIIIC binding site within tRNA-like pol III genes (26) has the top-strand sequence 5′-GATCGATGGTTCGAATCCAT CCTC-3′.

Cell extracts and nuclei. Cytoplasmic extracts and nuclei were prepared from CEM cells, an uninfected human T-lymphocyte cell line, and MT2 cells, an HTLV-1-infected human T-lymphocyte cell line. Cells were harvested by centrifugation, washed twice in phosphate-buffered saline, and lysed in hypotonic lysis buffer (10 mM Tris-HCl [pH 7.9], 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM dithiothreitol), using a Dounce homogenizer. The cytoplasmic fraction was removed following centrifugation, and the pelleted nuclei were resuspended in hypotonic lysis buffer containing 20% glycerol. The cytoplasmic extracts were clarified by centrifugation at 14,000 × *g* for 15 min at 4°C. One-ninth volume of a 10× transcription buffer was added to each extract to give final concentrations of 10 mM Tris-HCl (pH 7.6), 65 mM KCl, and 6 mM $MgCl_2$. For transcription experiments with nuclei, the amounts of nuclear DNA were quantitated by measuring the A_{260} of appropriate dilutions in 1% SDS. Aliquots of nuclei were diluted in transcription buffer (see above) and used as templates with exogenous RNA pol III and nucleoside triphosphates (see below). HeLa cells were grown, and a cytoplasmic S-100 extract was prepared as described previously (34).

Transcription factors and transcription reaction conditions. Partial purification of *Xenopus* and HeLa transcription factors by chromatography on phosphocellulose P-11 was done as described previously (43). RNA pol III was purified from the *Xenopus* oocyte S-150 PC-B fraction (phosphocellulose fraction containing TFIIIB) by chromatography on DEAE-Sepharose as described previously (46). TFIIIA was purified from immature *Xenopus* oocytes as described previously (47), with the modification that heparin-agarose was used in place of BioRex 70. TFIIIA was included in the 5S RNA transcription reactions at a 5- to 10-fold molar excess over genes. TFIIIA-5S DNA complexes were allowed to form for 15 min prior to the addition of the other transcription components. Transcription reaction mixtures contained the components indicated in the figure legends in a final reaction volume of 20 μl (or 30 μl for the reactions with cytoplasmic extracts), and nucleoside triphosphates were included at 0.6 mM for ATP, UTP, and CTP; GTP was included at a final concentration of 0.02 mM along with 10 μCi of [α-^{32}P]GTP (DuPont NEN). Reaction mixtures also contained 20 U of recombinant RNasin (Promega) and were maintained at ambient temperature (22 ± 1°C) for 2 h. In some experiments, after termination of the reactions with a stop buffer, consisting of 20 mM Tris-HCl (pH 7.5), 1% SDS, 0.3 M sodium acetate, and 10 mM EDTA, equal amounts of a radiolabeled 5S rRNA or tRNA transcript were added to each reaction to act as a recovery standard. RNAs were purified by extraction with RNAzol (Tel-Test Inc., Friendswood, Tex.) and precipitation with isopropanol and were analyzed by electrophoresis on denaturing 6 to 10% polyacrylamide gels (29:1 acrylamide to bisacrylamide) containing 8.3 M urea in 88 mM Tris-borate (pH 8.3)–2 mM EDTA. After electrophoresis, the gels were dried and subjected to autoradiography with Kodak Biomax film. For quantitation, dried gels were subjected to phosphorimage analysis with a Molecular Dynamics instrument, and the relative amounts of radioactivity in the RNA transcripts were determined using the Molecular Dynamics Imagequant software.

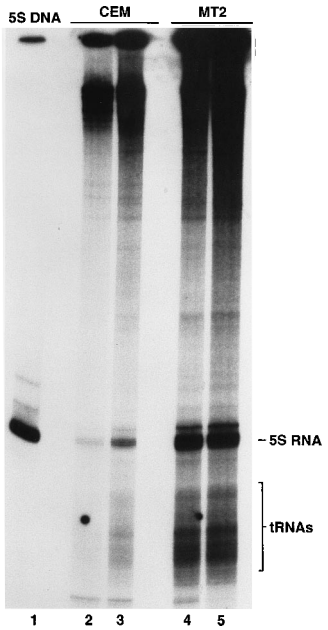

FIG. 1. Higher levels of RNA pol III transcription from nuclei isolated from HTLV-infected MT2 cells than from control CEM cells. Nuclei (10 μg of DNA equivalents [lanes 2 and 4] or 25 μg of DNA equivalents [lanes 3 and 5]) were prepared and used as templates for transcription with exogenous *Xenopus* RNA pol III (2 μl per reaction) and labeled and unlabeled nucleoside triphosphates as described in Materials and Methods. The products of the transcription reaction were purified and analyzed on a 10% sequencing gel. Lane 1 represents radiolabeled *Xenopus* somatic 5S RNA (120 nucleotides in length) transcribed in a reaction mixture containing 10 ng of plasmid pXIs11 DNA, 2 ng of TFIIIA, 290 ng of pBluescript DNA, and *Xenopus* PC-B and PC-C fractions. The number of 5S genes in this reaction (3×10^9) was similar to the number of genes in 10 μg of chromatin (6×10^9). The positions of 5S RNA and the tRNAs are shown.

Gel mobility shift assays. DNA binding reaction mixtures contained the amounts of radiolabeled double-stranded oligonucleotides indicated in the figure legends and 200 to 250 ng of nonspecific carrier DNA (pBluescript DNA for the TATA box oligonucleotide and poly(dI-dC) for the B-block oligonucleotide) in a final reaction volume of 20 μl. Oligonucleotides were labeled with polynucleotide kinase and [γ-^{32}P]ATP by standard methods. The binding buffer consisted of 20 mM HEPES-OH (pH 7.6), 50 mM KCl, 1 mM $MgCl_2$, 10 mM dithiothreitol, 50 μM $ZnSO_4$, and 12% (vol/vol) glycerol. Binding reactions were allowed to proceed at ambient temperature for 45 min to 1 h (except where noted otherwise) prior to electrophoresis on 6% nondenaturing polyacrylamide gels (29:1 acrylamide to bisacrylamide) containing the following buffers. For the B-block oligonucleotide, the electrophoresis buffer was 88 mM Tris-borate (pH 8.3). For the TATA box oligonucleotide, the gel electrophoresis buffer consisted of 25 mM Tris, 190 mM glycine (pH 8.5), and 1 mM EDTA (13). After electrophoresis, gels were transferred to Whatman paper and subjected to autoradiography. For quantitation, autoradiograms (taken within the linear range of the X-ray film) were scanned with an LKB laser densitometer or dried gels were subjected to phosphorimage analysis.

RESULTS

Chromatin templates from HTLV-infected cells support higher levels of transcription by RNA pol III than chromatin from uninfected cells. The template activities of nuclei from two unrelated human T-lymphocyte cell lines, CEM (uninfected) and MT2 (HTLV-1 infected), were monitored by using exogenous RNA pol III isolated from *Xenopus* oocyte extracts (Fig. 1). This experiment measures the occupancy of class III genes by transcription factors in cellular chromatin (42). In the absence of the exogenous polymerase, only low levels of 5S

RNA and tRNA transcripts were observed (data not shown). However, when these nuclei were incubated with exogenous polymerase, high levels of these RNAs were transcribed from the chromatin templates. We calculate that comparable levels of 5S RNA were synthesized from the chromosomal 5S RNA genes in the MT2 chromatin and from similar numbers of cloned 5S RNA genes, using a fractionated transcription system (Fig. 1; compare lane 1 with lanes 4 and 5). At 10 μg of nuclear DNA template, the level of 5S RNA transcription observed for the MT2 cell nuclei was 10- to 15-fold higher than that observed for the CEM cell nuclei. At 25 μg of template, this stimulation was reduced to four- to fivefold, suggesting that polymerase may be limiting under these conditions. Similar to results for 5S RNA transcription, significantly higher levels of tRNA transcription were observed with the MT2 nuclei than with the CEM nuclei. On the basis of previous chromatin transcription studies (42), these data suggest that a larger number of 5S RNA and tRNA genes are assembled in active transcription complexes in the MT2 cell nuclei than in CEM cell nuclei. Consistent with these observations, Northern (RNA) blot analysis revealed higher levels of 5S RNA in the MT2 cells than in the CEM cells (per microgram of total RNA), indicating that the steady-state levels of 5S RNA are elevated in the HTLV-1-infected cell line (data not shown).

Cytoplasmic extracts prepared from HTLV-infected cells are more active in RNA pol III transcription than extracts from uninfected cells. Cytoplasmic extracts prepared from both the MT2 and CEM cells were tested for the ability to support transcription of cloned class III genes (Fig. 2A). When similar amounts of cytoplasmic proteins were used in transcription reactions with the *Xenopus* 5S RNA gene, the HTLV-1-infected MT2 cell extract gave approximately 10-fold-higher levels of transcription than did the uninfected CEM cell extract (Fig. 2A; compare lanes 1 and 2). Comparable levels of 5S RNA transcription were observed when 8- to 13-fold-higher levels of CEM extract protein were used (compare lane 1 with lanes 5 and 6). Similar results were obtained with a *Drosophila* $tRNA^{Arg}$ gene (data not shown). These data suggest that, on a per-unit-of-total-protein basis, the MT2 cell extract contains a higher level of pol III transcription factor activity or polymerase than does the CEM cell extract. Assays for nonspecific RNA pol III activity in the two cytoplasmic extracts indicated similar levels of polymerase activity in the two extracts (on a per-unit-of-protein basis; data not shown).

One difference between the HTLV-1-infected and uninfected cells is the presence of the virus-encoded transcriptional activator protein Tax in the infected cells and in the extracts derived from these cells. To determine whether Tax is responsible for the observed difference in the transcriptional activities of the two extracts, we tested the effect of recombinant His_6-tagged Tax protein on the transcriptional activity of the MT2 and CEM cell extracts (Fig. 2B). Addition of 160 ng of Tax to the infected cell extract results in a 2.4-fold stimulation of tRNA transcription, while addition of a similar amount of Tax to the uninfected CEM extract resulted in a 8.9-fold stimulation of transcription. Similar levels of tRNA transcription were observed with both extracts in the presence of Tax (Fig. 2B; compare lanes 5 and 10). Addition of a similar amount of another recombinant protein, His_6-tagged 9-*cis* retinoic acid receptor, had no significant effect on transcription (lanes 6 and 11). These results suggest that the viral protein Tax is responsible for the increased pol III transcriptional activity observed with extracts prepared from HTLV-1-infected cells.

Extracts from HTLV-1-infected cells contain higher levels of TFIIIB/TBP TATA box binding activity than uninfected cell extracts. It is well documented that extracts from certain other

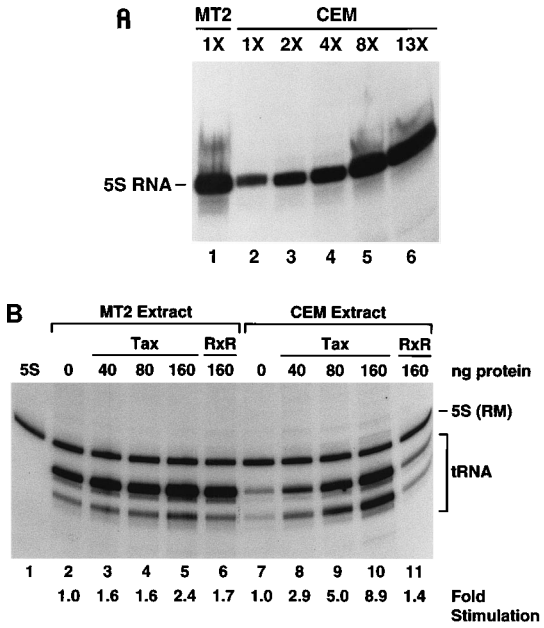

FIG. 2. (A) Cytoplasmic extracts from HTLV-infected MT2 cells support higher levels of 5S RNA transcription than similar extracts from control CEM cells. Each reaction mixture contained 10 ng of plasmid pXIs11 DNA (containing the *Xenopus* somatic 5S RNA gene), 2 ng of TFIIIA (providing a 10-fold molar excess), and 290 ng of pBluescript DNA in a final volume of 30 μl. The reaction mixture of lane 1 contained 22.5 μl of MT2 cytoplasmic extract, while the reaction mixtures of lanes 2 to 6 contained 1.7, 3.4, 6.8, 13.6, and 22.5 μl of CEM cytoplasmic extract, respectively, and transcription buffer to make up 22.5 μl. The protein concentration of the CEM extract was 13-fold higher than that of the MT2 extract, resulting in the relative protein concentrations shown at the top. The products of transcription were purified and analyzed on a 6% sequencing gel. (B) Effect of recombinant Tax on tRNA transcription in the MT2 and CEM cytoplasmic extracts. Each reaction mixture contained 20 ng of a $tRNA^{TyrD}$ gene plasmid DNA, 280 ng of vector DNA, and either 11 μl of MT2 cell extract or 0.9 μl of CEM extract in a final volume of 20 μl. The reaction mixtures were supplemented with the indicated amounts of His_6-tagged Tax or 9-*cis* retinoic acid receptor (RxR) protein or buffer and incubated for 30 min prior to the addition of nucleotides. After an additional 2 h of incubation, the reactions were stopped and identical amounts of a radiolabeled 5S RNA transcript were added to each reaction to serve as a recovery marker (RM; lane 1). The fold stimulation of tRNA transcription, indicated at the bottom, was determined by phosphorimage analysis with appropriate correction for 5S RNA recovery. The positions of 5S RNA and tRNA are shown at the right.

virus (e.g., adenovirus and pseudorabies)-infected cells have increased levels of activity of the general pol III transcription factor TFIIIC (8, 25, 29, 30). To assay for the levels of TFIIIC DNA binding activity in the HTLV-1-infected and uninfected T-cell extracts, we performed gel mobility shift assays with a B-block double-stranded oligonucleotide, corresponding in sequence to the major binding site for TFIIIC within tRNA-like class III genes (26). The PC-C fraction (phosphocellulose fraction containing TFIIIC) from the HeLa cell extract (Fig. 3, lanes 2 to 4) shows two major gel shift complexes which likely correspond to the two forms of TFIIIC described by Hoeffler et al. (29), in which the more slowly migrating band corresponds to the transcriptionally active form of TFIIIC. The CEM extract shows a preponderance of the active form, whereas the MT2 cell extract shows only the active form. Quantitation of these binding assays indicated that the MT2 cell extract contains 1.7-fold-higher levels of B-block DNA-binding activity than the comparable amount of protein from the CEM extract (Fig. 3A). This small difference in B-block DNA binding activity was unlikely to account for the large difference in pol III transcriptional activity of the MT2 cell

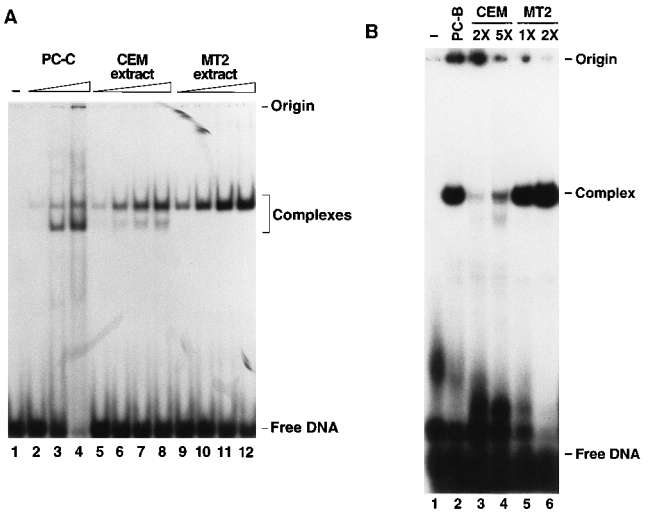

FIG. 3. (A) B-block DNA binding activity in HTLV-infected MT2 and control CEM cytoplasmic extracts. The autoradiogram represents a gel mobility shift assay in which increasing amounts of cytoplasmic protein from MT2 and CEM cells were incubated along with 60 fmol of a radiolabeled double-stranded B-block oligonucleotide and 200 ng of poly(dI-dC) in a total volume of 20 μl. Lane 1 shows DNA in the absence of protein; lanes 2 to 4 show reaction mixtures with 1, 2, and 4 μl, respectively, of HeLa 0.6 M PC-C fraction. The reaction mixtures of lanes 5 to 8 contained 1, 2, 3, and 4 μl, respectively, of a 1:12 dilution of CEM cytoplasmic extract to give the same amounts of protein as undiluted MT2 extract, shown in the reactions of lanes 9 to 12. (B) TATA box DNA binding activity in HTLV-infected MT2 and control CEM cytoplasmic extracts. The indicated relative amounts of cytoplasmic protein from MT2 and CEM cells were incubated along with 14.5 fmol of a radiolabeled double-stranded TATA box oligonucleotide and 250 ng of pBluescript DNA in a total volume of 20 μl. Lane 1 shows DNA in the absence of protein, and lane 2 shows a reaction with 2 μl of HeLa 0.35 M PC-B fraction.

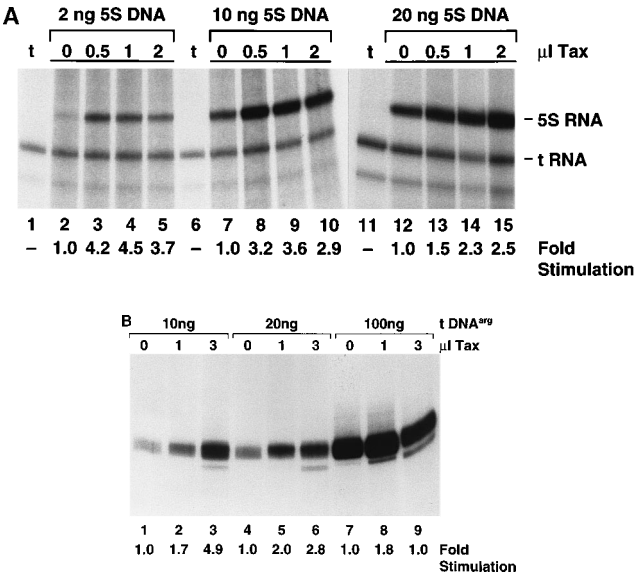

FIG. 4. HTLV Tax stimulates tRNA and 5S RNA gene transcription in reconstituted systems. Transcription reactions were carried out with *Xenopus* transcription components in panel A (with the somatic 5S RNA gene as the template) and with HeLa-derived transcription components in panel B (with the $tRNA^{Arg}$ gene as the template) as described in Materials and Methods. (A) Each reaction mixture contained 300 ng of total DNA with 2 ng (lanes 2 to 5), 10 ng (lanes 7 to 10), or 20 ng (lanes 12 to 15) of 5S gene plasmid DNA (pXIs11) per reaction. The remaining DNA was pBluescript vector DNA. The reaction mixtures were not supplemented (lanes 2, 7, and 12) or supplemented with 0.5 μl (lanes 3, 8, and 13), 1 μl (lanes 4, 9, and 14), or 2 μl (lanes 5, 10 and 15) of recombinant Tax protein (at 80 ng/μl). (B) Each reaction mixture contained 300 ng of total DNA with 10 ng (lanes 1 to 3), 20 ng (lanes 4 to 6), or 100 ng (lanes 7 to 9) of $tRNA^{Arg}$ gene plasmid DNA per reaction. The reactions were unsupplemented (lanes 1, 4, and 7) or supplemented with 1 μl (lanes 2, 5, and 8) or 3 μl (lanes 3, 6, and 9) of recombinant Tax protein (at 80 ng/μl). After termination of the reactions, equal amounts of a radiolabeled tRNA transcript were added to the reactions shown in panel A to serve as a recovery standard. Lanes 1, 6, and 11 of panel A show this RNA (t). The fold stimulation of transcription was quantitated by phosphorimage analysis and is shown at the bottom. In three separate experiments, 2 ng of 5S template and 1 μl of Tax per reaction produced a 4.1 ± 0.3-fold stimulation of transcription. At higher template concentrations (10 ng to 100 ng per reaction), twofold or lower stimulation of transcription was consistently observed.

nuclei and cytoplasmic extracts. We next assayed for the TATA-box-binding protein (TBP) DNA-binding activity of TFIIIB in these extracts with a TATA box double-stranded oligonucleotide. TFIIIB contains TBP and pol III-specific TBP-associated factors (TAFs) (for a review, see reference 28), and TATA box oligonucleotides can effectively compete TFIIIB transcriptional activity (40, 55); hence, the TBP component of TFIIIB can be monitored by gel mobility shift assays with a TATA box DNA probe (27, 35). Figure 3B shows this assay with TFIIIB from a phosphocellulose fraction (PC-B; lane 2) and the cytoplasmic proteins from both the CEM and MT2 cells (lanes 3 to 6). The cytoplasmic extracts each produced a single gel shift complex with a mobility coincident with the TFIIIB complex from the HeLa cell PC-B fraction. Quantitation of these and other binding experiments indicated that the MT2 cell extract contained ~10-fold higher levels of TFIIIB TATA box binding activity than the corresponding amount of cytoplasmic protein from the uninfected CEM cell extract. Purified TBP does not form a stable complex with DNA in the buffer system used in this gel shift experiment (13); thus, the protein-DNA complex observed with the TATA box oligonucleotide probe and proteins in the cytoplasmic extract and with the PC-B fraction represented TBP assembled with TAFs in the TFIIIB complex (35). We do not know whether this complex represents the complete assembly of TBP and pol III TAFs that comprise TFIIIB or whether this gel shift complex represents only a subset of the components of TFIIIB. These results suggest that the increased transcriptional activity of the MT2 cell extract may be due to higher levels of TFIIIB in these extracts than in the uninfected CEM cell extract.

HTLV-1 Tax protein stimulates class III gene transcription in reconstituted systems. To further test whether Tax might be responsible for the stimulation of class III gene transcription in chromatin and extracts, we performed in vitro transcription experiments with cloned class III genes in reconstituted transcription systems derived from *Xenopus* (Fig. 4A) and HeLa (Fig. 4B) cell extracts. We observed increased levels of both 5S RNA gene transcription (Fig. 4A) and $tRNA^{Arg}$ gene transcription (Fig. 4B) upon the addition of recombinant Tax protein to these systems. The maximal effect of Tax on class III transcription was observed at low input levels of gene-containing plasmid. With both the 5S RNA and tRNA genes, we found a ~5-fold-higher level of transcription in the presence of Tax at low template concentrations, in contrast to a ~2-fold enhancement at higher template concentrations. At high levels of template (greater than 100 ng/20-μl reaction) at which maximal levels of transcription were observed, Tax was without effect. Single-round transcription assays were also performed to monitor the rate of assembly of transcription complexes in the presence and absence of Tax. In this experiment, transcription factors were preincubated with Tax or Tax buffer for 20 min, and then transcription complexes were allowed to form on a 5S RNA gene for various times prior to the initiation of transcription by the addition of nucleotides. Transcription was

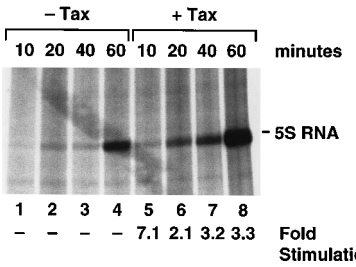

FIG. 5. Single-round transcription assays. Each reaction mixture contained 20 ng of 5S gene plasmid DNA, 280 ng of pBluescript DNA, and *Xenopus* transcription components. Reaction mixtures were supplemented with either 1 μl of Tax buffer (lanes 1 to 4) or 1 μl of Tax (lanes 5 to 8), incubated for 20 min prior to the addition of the DNA, and then incubated for the indicated times prior to the addition of labeled and unlabeled nucleoside triphosphates. After 2 min, 1 μl of heparin (2 mg/ml) was added to each reaction mixture, and the reactions were terminated after an additional 5-min incubation. The fold stimulation of transcription was determined by phosphorimage analysis and is defined as the ratio of counts in 5S RNA in the presence versus the absence of Tax (for each time point).

limited to a single round with the addition of heparin (11). Quantitation of the levels of RNA synthesized after various times of complex assembly indicated that Tax has maximal effects at the earliest incubation times (Fig. 5). These results indicate that Tax promotes the assembly of active transcription complexes. The full effect of Tax is on the assembly process rather than on any subsequent transcription event such as elongation or termination.

Since Tax stimulates both 5S RNA and tRNA gene transcription to similar extents (Fig. 4), it is unlikely that Tax acts on the 5S gene-specific factor TFIIIA. To distinguish which component of the class III gene transcription apparatus is affected by Tax in the reconstituted system, we performed transcription assays with a tRNA gene under limiting transcription factor conditions. In this assay, the amounts of each of the phosphocellulose fractions PC-B and PC-C were varied in separate reactions. Figure 6 shows that much higher levels of stimulation by Tax were observed when the PC-B fraction was limiting (>50-fold; Fig. 6B) than when the PC-C fraction was limiting (6-fold; Fig. 6A). As both fractions contained RNA polymerase III, and the catalytic activity of pol III was unaffected by Tax, this result suggests that TFIIIB is the primary target of Tax in the class III gene system.

HTLV-1 Tax stimulates the TATA box DNA binding activity of TFIIIB. Since the HTLV-infected T-cell extracts contained higher levels of the TATA box DNA binding activity of TFIIIB than the uninfected T-cell extracts (Fig. 3B), we were interested in determining whether Tax directly affected this DNA binding activity of TFIIIB. A TFIIIB titration (using the HeLa PC-B fraction) was performed in the presence and absence of recombinant Tax (Fig. 7A). This experiment shows that higher levels of TATA box DNA binding activity were observed in the presence of Tax than in the absence of Tax (compare lanes 2 to 7 with lanes 8 to 13). Similar experiments with TFIIIC and TFIIIA failed to show any significant effect of Tax on the DNA binding activities of these class III transcription factors for their specific DNA recognition elements (data not shown). As was observed with the transcription experiments shown above, the fold stimulation of TATA box DNA binding activity by Tax was greatest at the lowest input amounts of TFIIIB, and the fold stimulation of binding was reduced as near-saturating levels of TFIIIB were added. Interestingly, Tax did not appear to be part of the gel shift complex, as the mobility of the TATA box DNA-protein complex was unaffected by Tax. Similar results have been reported for class II gene transcriptional activators (4, 20, 52, 58). These data are consistent with TFIIIB being the primary target of Tax activity, thus accounting for the enhanced levels of class III gene transcription.

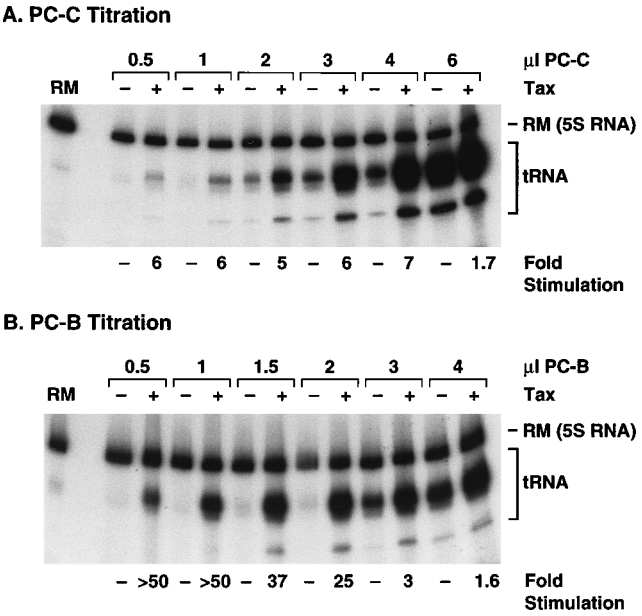

FIG. 6. Effect of Tax on tRNA gene transcription with limiting amounts of *Xenopus* transcription factors. Each reaction mixture contained 100 ng of $tRNA^{TyrD}$ gene plasmid DNA, 200 ng of pBluescript DNA, the indicated volumes of phosphocellulose fractions PC-B and PC-C, and 1 μl of Tax in a total volume of 20 μl. In panel A, each reaction mixture contained 4 μl of the PC-B fraction and the volume of PC-C was varied as indicated. In panel B, each reaction mixture contained 6 μl of the PC-C fraction and the volume of PC-B was varied as indicated. DNA, PC fractions, and Tax were incubated for 20 min prior to the addition of nucleotides. After an additional 90-min incubation, the reactions were terminated and equal amounts of radiolabeled 5S RNA were added to each reaction to serve as a recovery marker. This 5S RNA was run alone in the lanes marked RM. The positions of the recovery marker (RM) and tRNA transcripts are indicated at the right. The fold stimulation of transcription, as determined by phosphorimage analysis, is shown for each set of reaction conditions. Fold stimulation was corrected for 5S RNA recovery in each reaction, and the phosphorimage units for each tRNA reaction were also corrected for the background intensity at the tRNA position in the recovery marker lane.

To determine whether the stimulation of the TATA box binding activity of TFIIIB by Tax could be due to a direct increase in the intrinsic affinity of the TBP component of TFIIIB for DNA, we examined the effect of Tax on the TATA box binding activity of recombinant human TBP. Unlike the case for TFIIIB, however, no significant effect of Tax was observed on DNA binding activity of TBP (data not shown). These findings suggest that Tax affects the TATA box binding activity of the TBP subunit of TFIIIB only when TBP is assembled with pol III-specific TAFs. Three interpretations of these data are (i) that Tax facilitates the binding of TFIIIB to DNA by changing either the on rate and/or the off rate for this binding reaction, (ii) that Tax promotes the assembly of TFIIIB from preexisting subunits present in the TFIIIB fraction or renatures inactive TFIIIB molecules, and (iii) that Tax neutralizes an inhibitor of TFIIIB binding activity. In any of these situations, a change in rate constant would be reflected by a change in the apparent dissociation constant for the binding reaction. To distinguish among these interpretations, we performed a TATA box DNA titration experiment at a constant input concentration of TFIIIB both in the absence (Fig. 7B) and in the presence (Fig. 7C) of Tax. As before, more

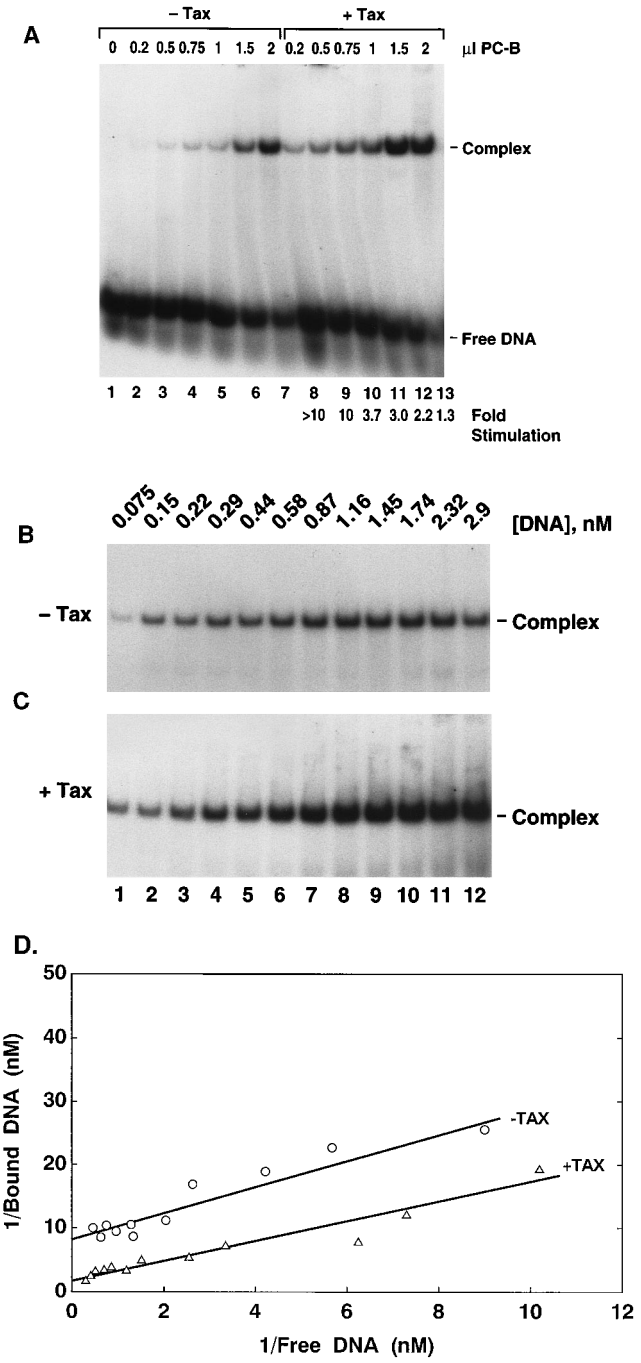

FIG. 7. (A) Tax stimulates the TATA box DNA binding activity of TFIIIB. The indicated volumes of the HeLa PC-B fraction were incubated along with 14.5 fmol of a radiolabeled double-stranded TATA box oligonucleotide and 250 ng of pBluescript DNA in a total volume of 20 μl. The reaction mixture also contained 2 μl of Tax buffer (lanes 1 to 7) or 2 μl of Tax (lanes 8 to 13). The fold stimulation of DNA binding activity is the ratio of radiolabeled DNA in the complex in the presence versus the absence of Tax for each volume of the PC-B fraction (as determined by densitometry of the autoradiogram). (B and C) Effects of Tax on the TATA-box DNA binding activity of TFIIIB at various DNA concentrations. Shown at the top are the concentrations of the radiolabeled double-stranded TATA box oligonucleotide in a total volume of 20 μl per reaction. Each reaction mixture contained 1 μl of HeLa PC-B fraction, 250 ng of pBluescript DNA, and either 2 μl of Tax buffer (B) or 2 μl of Tax (C). Panels B and C show only the region of the gel containing the protein-DNA complex. (D) Double-reciprocal plot of the data shown in panels B and C. The amounts of bound and free DNA at each DNA concentration were determined by densitometry of the autoradiogram. Data points at the lowest concentration of input DNA (0.075 nM) were omitted from this analysis since the amount of bound DNA in the absence of Tax was too low to be determined with confidence.

DNA molecules were shifted into the TFIIIB-DNA complex in the presence of Tax than in the absence of Tax. In titration experiments of this type, both the apparent dissociation constant and concentration of active DNA-binding protein molecules can be calculated from a double-reciprocal plot of the binding data (Fig. 7D). The *y* intercept of this curve is the reciprocal of the concentration of active molecules, and the slope of the line is the apparent dissociation constant divided by the concentration of active molecules. This analysis revealed that the apparent K_d for the reaction of TFIIIB binding to the TATA box oligonucleotide was not changed significantly by Tax (0.6 nM in the presence of Tax; 0.8 nM in the absence of Tax). However, the concentration of active DNA-binding molecules was increased ~3-fold by Tax. Thus, Tax stimulates the DNA binding activity of TFIIIB by increasing the apparent concentration of active molecules.

Tax stimulates the DNA binding activity of TFIIIB in a slow process that is independent of DNA. To address the mechanism whereby Tax increases the apparent concentration of active TFIIIB molecules, we performed order-of-addition experiments to determine whether Tax acts directly on TFIIIB or whether the DNA binding site must be present in the reaction for Tax to elicit its effect (Fig. 8A). The results of this experiment indicated that Tax acts directly on TFIIIB in the absence of DNA. After Tax and TFIIIB were incubated for 1 h, no difference in DNA binding activity was observed whether these mixtures were then incubated with the DNA for 1 min or 1 h (Fig. 8A; compare lanes 4 and 5 with lanes 7 and 8). Thus, the association of active TFIIIB molecules with DNA is very rapid, but the apparent generation of active molecules by Tax is a slow process. We next monitored the rate at which Tax affects the DNA binding activity of TFIIIB by varying the incubation time prior to gel electrophoresis (Fig. 8B). In this experiment, Tax and TFIIIB (or TFIIIB alone) and the DNA probe were incubated for various times prior to electrophoresis. When the incubation period was limited to 1 min, very little difference in binding activity was observed between the reactions containing and lacking Tax. Therefore, Tax appeared to elicit its effect on the TATA box DNA binding activity of TFIIIB in a slow process that proceeded over the course of the experiment.

DISCUSSION

It is well established that the HTLV-1-encoded Tax protein is a potent transcriptional activator of many RNA pol II-dependent cellular genes. In this report, we examined the effect of Tax on cellular genes transcribed by RNA pol III. We demonstrate that the expression of both 5S rRNA and tRNA genes is significantly increased in nuclei derived from a Tax-expressing HTLV-1-infected T-cell line, relative to an uninfected T-cell line, suggesting that higher numbers of these class III genes are programmed in active transcription complexes in the HTLV-1-infected cells than in uninfected cells. When transcription extracts from the two cell lines were tested with purified 5S rRNA and tRNA gene templates, the infected cell extracts supported significantly higher levels of transcription than did the extracts from the uninfected cells. This increased level of class III transcription did not correlate with increased RNA pol III activity or with the DNA binding activity of the class III transcription factor TFIIIC. However, we observed significantly higher TFIIIB/TBP DNA binding activity in extracts from the HTLV-1-infected cells than in extracts from a control uninfected T-cell line.

We provide evidence that the transcriptional stimulation observed in HTLV-1-infected T cells is likely due to the virally encoded Tax protein. The addition of purified recombinant

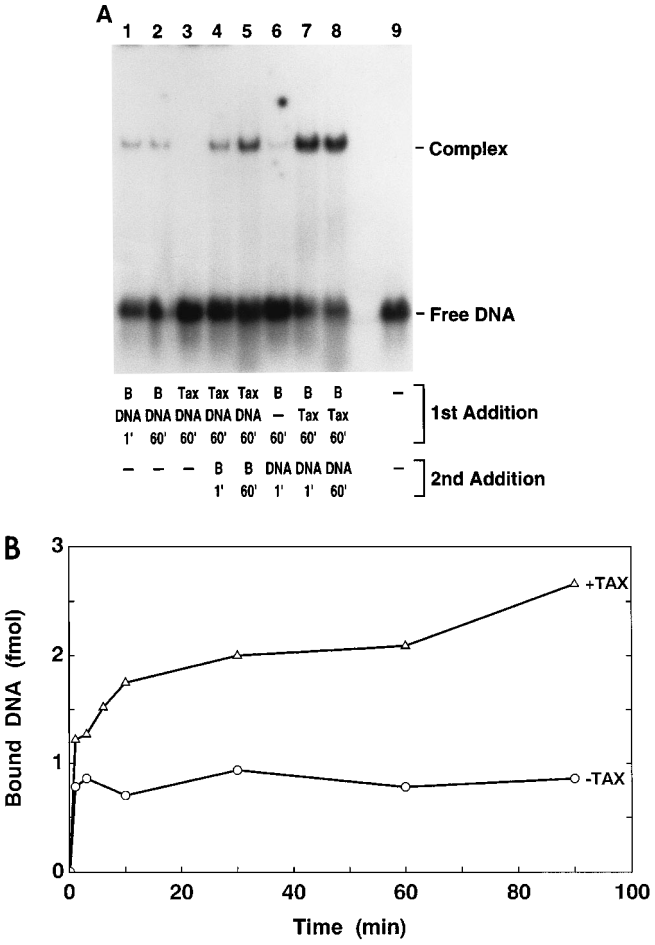

FIG. 8. (A) Order of addition of reaction components determines the effect of Tax on the TATA box DNA binding activity of TFIIIB. Reaction mixtures contained 29 fmol of TATA box oligonucleotide, 250 ng of pBluescript DNA, and the components listed at the bottom. B indicates 1 μl of HeLa PC-B; Tax indicates 2 μl of Tax protein. Reaction mixtures lacking any of the components contained the corresponding volume of the appropriate buffer. The order of addition of components and times of incubations are shown at the bottom. (B) Effect of Tax on the rate of formation of TATA box DNA-TFIIIB complexes, determined by a graphical analysis of a gel mobility shift experiment in which 20-μl aliquots of reaction mixtures containing 29 fmol of TATA box oligonucleotide, 250 ng of pBluescript DNA, 0.5 μl of the HeLa PC-B fraction, and either 2 μl of Tax buffer or 2 μl of Tax were incubated for the times indicated prior to gel electrophoresis. The amounts of DNA bound at each time point were determined by densitometry of an autoradiogram.

Tax protein to the uninfected cell extracts stimulated tRNA transcription to the level observed with the HTLV-1-infected cell extracts. Tax markedly stimulated both 5S rRNA and tRNA gene transcription in reconstituted systems, with maximal effects observed under conditions of limiting TFIIIB or under conditions of limiting template. When saturating amounts of TFIIIB or DNA template were used, only small effects of Tax protein were observed. Similarly, under conditions of limiting TFIIIC, only a modest stimulation of transcription by Tax was observed. These data suggest that the effect of Tax is on the assembly of active transcription complexes and that the primary target of Tax is the general pol III transcription factor TFIIIB. Consistent with this hypothesis, single-round transcription experiments showed that Tax increased the assembly of active transcription complexes.

We have used a gel mobility shift assay with a TATA box oligonucleotide, derived from the adenovirus major late promoter, to monitor the DNA binding activity of the TBP component of TFIIIB. Although 5S RNA and tRNA genes do not contain TATA boxes, and TFIIIB is positioned on these genes through interactions with promoter-bound TFIIIC, oligonucleotides corresponding to this sequence have been shown to inhibit transcription of class III genes, presumably by binding to the TBP subunit of TFIIIB (40, 55). In the case of TATA-containing class III genes, such as the U6 small nuclear RNA gene, the TBP subunit of TFIIIB most likely binds directly to the TATA element (reviewed in reference 28). Moreover, the same TFIIIB species is thought to participate in transcription of TATA-containing and TATA-less class III genes. We have also shown that a TATA box DNA-Sepharose column will retain TFIIIB (27). Competition experiments with specific, nonspecific, and mutant TATA box oligonucleotides showed that this gel shift complex was specific for the wild-type TATA box sequence. The single gel shift complex represents TFIIIB, since TFIIIB transcriptional activity and TATA box DNA binding activity cofractionate under conditions that separate TFIIIB from the other TBP-containing complexes (B-TFIID, TFIID, SL1, and $SNAP_c$ [35]). Therefore, the gel shift assay that we have used in this work reflects the abundance of TFIIIB in our extracts and in chromatographic fractions (35). Using this assay, we found that Tax stimulated the TATA box binding activity of the TBP component of TFIIIB. Tax protein did not stimulate the DNA binding activity of TBP alone when monitored under gel conditions in which stable TBP-DNA complexes can be observed (data not shown). Tax stimulated the TATA box DNA binding activity of TFIIIB under conditions of limiting protein or limiting DNA probe (Fig. 7). Surprisingly, the Tax-dependent increase in TFIIIB/TBP DNA binding activity resulted from an increase in active TFIIIB protein concentration rather than in a change in the apparent dissociation constant for this binding reaction. The full effect of Tax on TFIIIB activity was observed in the absence of DNA (Fig. 8), suggesting that Tax either facilitated the assembly of TFIIIB from its polypeptide constituents present in the purified fraction, neutralized an inhibitor of TFIIIB, or promoted the renaturation of inactive TFIIIB molecules. The lack of detectable alterations in the mobility of the TFIIIB-DNA gel shift complex in the presence of Tax is consistent with the idea that Tax promotes an increase in the number of active TFIIIB molecules in the absence of DNA. Future studies will be aimed at elucidation of the mechanism of activation of TFIIIB by Tax.

The precise nature of the interaction between Tax and the class II target cellular transcription factors remains controversial and may depend on the protein. For example, in studies examining enhancement in the DNA binding activities of a variety of bZIP proteins, Tax appeared to improve both DNA binding and protein dimerization, thus increasing the rate of bZIP protein association with the DNA (3, 6, 52). Other studies examining the interaction between Tax and the bZIP protein CREB suggest that an additional mechanism may be involved (12). While Tax has been demonstrated to enhance CREB binding to a variety of cAMP response element-containing sequences (20), a specific interaction between Tax and CREB was observed only in the context of specific DNA sequences immediately adjacent to the CREB recognition element (12, 36). This interaction with Tax resulted in a decrease in the dissociation rate of CREB from the DNA (12). In the work presented here, we provide evidence showing that Tax increased the TATA box DNA binding activity of TFIIIB in the absence of DNA. While this result appears initially inconsistent with the studies described above, it is conceivable that through recognition and stabilization of specific protein struc-

tures, Tax functions as an assembly factor facilitating protein-DNA as well as protein-protein interactions.

Previous studies on the effects of viral infection on RNA pol III transcription have documented increases in the activity of the general pol III transcription factor TFIIIC. Notably, the adenovirus E1A protein has been shown to stimulate both the transcriptional activity and DNA binding activity of TFIIIC (8, 17, 25, 29, 30, 37, 60). Similarly, both the herpes simplex virus immediate-early protein ICP27 and the human immunodeficiency virus Tat protein have been reported to increase TFIIIC transcriptional activity (32, 33). On the other hand, poliovirus infection leads to the down-regulation of pol III transcription through inactivation of TFIIIC (16). Several studies have documented negative effects of growth conditions, cell cycle control, and general repressor proteins on pol III transcription mediated through TFIIIB activity (27, 50, 54, 56, 57). Previous studies on the mode of action of phorbol esters and the hepatitis B virus X protein have demonstrated stimulation of pol III transcription mediated through TFIIIB (24, 53); however, this is the first report showing direct stimulation of pol III transcription by the action of a viral transactivator protein on TFIIIB. In studies of the pathway of assembly of pol III transcription complexes in higher eukaryotic cells (9), TFIIIB recruitment to the template has been shown to be the rate-limiting step in formation of the active complex. Increases in TFIIIB activity, then, would correlate with increased complex assembly. Under conditions of limiting transcription factors or under low-template conditions, we observed the most pronounced effects of Tax, as would be expected from our current understanding of the pathway of transcription complex assembly. Under conditions of excess transcription factors or high-template concentrations, the assembly of transcription complexes is accelerated and Tax is without significant effect on transcription.

Since many of the genes transcribed by RNA pol III are involved in protein synthesis, the effect of Tax on class III genes may be relevant to the life cycle of HTLV-1. During the early phase of viral activation, Tax stimulates the expression of viral mRNAs encoding virion polypeptides. We speculate that the concurrent Tax stimulation of tRNA and 5S cellular gene expression would result in greater translational capacity in the infected cell, leading to increased synthesis of viral polypeptides and enhanced virion production.

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