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A 36-Kilodalton Cellular Transcription Factor Mediates an Indirect Interaction of Human T-Cell Leukemia/Lymphoma Virus Type I TAX_1 with a Responsive Element in the Viral Long Terminal Repeat

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The human T-cell leukemia/lymphoma virus type I (HTLV-I) *trans* activator, TAX_1, interacts indirectly with a TAX_1-responsive element, TRE-2, located at positions −117 to −163 in the viral long terminal repeat. This report describes the characterization of a 36-kilodalton (kDa) protein identified in HeLa nuclear extract which mediates the interaction of TAX_1 with TRE-2. Purification of the protein was achieved by zinc chelate chromatography and preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The renatured 36-kDa protein bound specifically to a TRE-2 oligonucleotide but not to nonfunctional base substitution mutant probes in a gel retardation assay. Renatured proteins of differing molecular weights were unable to form this complex. In addition, the 36-kDa protein specifically activated transcription from the HTLV-I promoter in vitro. Purified TAX_1 protein formed a complex with the TRE-2 oligonucleotide in the presence of the 36-kDa protein, suggesting that indirect interaction of TAX_1 with the viral long terminal repeat may be one of the mechanisms by which HTLV-I transcription is regulated.

Human T-cell leukemia/lymphoma virus type I (HTLV-I) has been associated with adult T-cell leukemia as well as with specific neurological disorders, including tropical spastic paraparesis and HTLV-associated myelopathy (27, 33, 51, 69, 70). In leukemogenic cells, the sites of viral integration are random, suggesting that activation of cellular genes residing near the site of integration is not a mechanism used by HTLV-I to transform cells. In addition, the viral genome does not contain any transduced cellular oncogenes. However, the unique region of the HTLV-I genome encodes two regulatory proteins, TAX_1, a positive regulator of viral transcription, and REX_1, a posttranscriptional regulator involved in splicing and transport of viral mRNAs to the cytoplasm (8, 24, 26, 34, 36, 45, 60, 61, 64–66). The transforming potential of these proteins has been illustrated by their ability to immortalize primary T lymphocytes (21, 53). In addition, transgenic mice carrying the *TAX*$_1$ gene develop neurofibromas (28, 47). In addition to viral genes, TAX_1 (previously known as x-lor, p40x, and tat_1; 18) has also been shown to transactivate certain cellular genes (3, 17, 29, 38, 44, 46, 56). Interestingly, two of these genes, the granulocyte-macrophage colony-stimulating factor and interleukin-2 receptor genes, are transactivated in tissues from transgenic mice carrying the *TAX*$_1$ gene (22). It is possible that the ability of TAX_1 to regulate viral and cellular gene expression is essential for subsequent cellular transformation.

Several distinct mechanisms for TAX_1 transactivation have been proposed and may not be mutually exclusive. The first of these involves direct binding of TAX_1 to control sequences in the long terminal repeat (LTR). Although this possibility cannot be completely ruled out, several laboratories including our own have failed to detect primary complexes of TAX_1 with DNA (unpublished results). Secondly, TAX_1 may induce transcription of cellular proteins which may, in turn, regulate transcription from the viral LTR or cellular promoters. Third, TAX_1 may influence the posttranslational modification of existing cellular proteins, resulting in transcriptional activation. Finally, TAX_1 may form a stable, physical interaction with cellular transcription factors, forming a transcriptionally active complex. It appears that regulation of interleukin-2 receptor gene expression by TAX_1 involves modulation of NF-κB activity via either the second or third mechanism (3, 38, 56). The observation that TAX_1 transactivation of the HTLV-I LTR occurs in the absence of protein synthesis (31) is consistent with all but the second mechanism.

Several examples of complexes comprised of viral transacting polypeptides and cellular regulatory proteins have recently been reported. Adenovirus E1A (68), simian virus 40 T antigen (10), and human papillomavirus type 16 E7 (12) interact independently with the retinoblastoma gene product, which is regarded as a negative regulator of cell cycle progression. Although there has been much speculation, the function of these complexes remains to be established. Additionally, c-FOS (9, 13, 35, 54, 55) has been reported to interact with c-JUN, forming the transcriptionally active complex AP1. These results suggest that the binding and transcriptional activity of AP1 requires the interaction of c-JUN and c-FOS and that the recognition and binding of specific *cis*-acting elements by c-JUN is a prerequisite for c-FOS-mediated stimulation of gene expression (9, 55, 58). Furthermore, these results imply that oncogenic proteins, which exhibit no intrinsic DNA-binding properties, may indirectly influence transcription through interaction with regulatory proteins. Finally, transcription regulation of herpes simplex virus immediate-early genes depends on the interaction of the viral Vmw65 protein with the cellular transcription factor Oct-1 (49).

The HTLV-I LTR contains two previously identified TAX_1-responsive elements. Tandem copies of the 21-base-pair repeats in either orientation, and placed upstream of either a homologous or a heterologous promoter, can be

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transactivated by TAX_1 (5, 30, 50, 63, 65) and are known as TAX_1-responsive element 1 (TRE-1). However, the 21-base-pair repeats are not tandem in the HTLV-I LTR, but rather are separated by either 50 or 100 base pairs. When a random DNA sequence of 40 base pairs was inserted between tandem 21-base-pair repeats, a 97% reduction in TAX_1 responsiveness was observed (40). Thus, TAX_1 responsiveness of the LTR may be mediated not only by the 21-base-pair repeats but also by sequences between these repeats.

We have demonstrated recently that LTR sequences −117 to −163, located between two 21-base-pair repeats, contain a second TAX_1-responsive element (TRE-2). TAX_1 has been shown to associate indirectly with TRE-2 in the presence of HeLa nuclear extract (40). Binding of cellular proteins to HTLV-I LTR sequences, including TRE-1 and TRE-2, has been demonstrated by DNase I footprinting analysis (1, 48). In this study, we have identified a 36-kilodalton (kDa) cellular protein that binds to the HTLV-I TRE-2 site. We demonstrate that this protein stimulates in vitro transcription from the HTLV-I LTR. In addition, the 36-kDa protein forms a complex with the responsive DNA element (TRE-2) and purified TAX_1 protein.

MATERIALS AND METHODS

Oligonucleotide synthesis. DNA sequences were synthesized on an Applied Biosystems DNA synthesizer. Purification of synthetic oligonucleotides has been described previously (40).

Cell culture and extract preparation. HeLa cells were maintained by spinner culture in Eagle minimal essential spinner medium containing 5% horse serum at a density of 5×10^5 cells per ml. Nuclear extracts were prepared from 25 liters of cells as previously described (11). Whole-cell extracts were prepared as previously described by Manley et al. (39).

Southwestern (DNA-protein) blot analysis. Detection of DNA-binding proteins separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed by a modification of the procedure described by Michael et al. (42). Nuclear proteins (100 μg) were separated by SDS-PAGE (10% separating gel) and electrotransferred onto nitrocellulose (4). The nitrocellulose membrane was washed three times for 1 h each time with renaturation buffer (10 mM Tris [pH 7.5], 150 mM NaCl, 10 mM dithiothreitol [DTT], 2.5% Nonidet P-40, 10% glycerol, 5% nonfat dry milk) and rinsed briefly in binding buffer (10 mM Tris hydrochloride [pH 7.5], 40 mM NaCl, 1 mM DTT, 1 mM EDTA, 8% glycerol, 0.125% nonfat dry milk). The nitrocellulose membrane was placed in a heat-sealable pouch in binding buffer plus 60 μg of poly(dI-dC), 5 mM $MgCl_2$, and 5×10^6 cpm of ^{32}P-labeled oligonucleotide per ml. After incubation for 14 h with continuous agitation, the nitrocellulose was washed four times (30 min each time) in 10 mM Tris hydrochloride (pH 7.5) and 50 mM NaCl.

Zinc chelate chromatography. The zinc chelate affinity column was produced by using an iminodiacetic acid epoxy-activated Sepharose 6B matrix (Sigma Chemical Co., St. Louis, Mo.). The 4-ml column was loaded with zinc by passing 20 column volumes of $ZnCl_2$ (1 mg/ml) over the matrix. The column matrix has the capacity to bind 30 μmol of zinc per ml of bed volume. The column was washed with 20 column volumes of wash buffer (20 mM sodium phosphate buffer [pH 7.5], 0.5 M NaCl, 0.5% Tween 20) to remove free zinc ions. Crude HeLa nuclear extract (100 mg) was passed over the column three times. The column was washed with 5 column volumes of wash buffer to remove cellular proteins lacking affinity for zinc. Bound proteins were removed from the column with elution buffer (20 mM sodium phosphate buffer [pH 7.5], 0.5% Tween 20, 0.5 M NaCl, 250 mM EDTA) and collected as 500-μl fractions. Elution fractions were dialyzed overnight at 4°C against a 1,000-fold excess of buffer D (20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.9], 100 mM KCl, 20% glycerol, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride) and stored at −70°C for subsequent analyses.

Protein renaturation. The fraction from zinc affinity chromatography containing peak amounts of the 36-kDa protein was separated by preparative SDS-PAGE (10% separating gel), and the proteins were visualized by using 0.3 M $CuCl_2$ (37). Proteins of various molecular weights were excised from the gel and destained with three 10-min changes of a buffer containing 0.25 M Tris hydrochloride (pH 9) and 0.25 M EDTA. Proteins were passively eluted from the gel slices in 2 ml of buffer containing 50 mM Tris hydrochloride (pH 7.9), 0.1 mM EDTA, 0.1% SDS, 5 mM DTT, and 150 mM NaCl by incubation overnight at room temperature with gentle rotation (59). The eluate was precipitated with 4 volumes of acetone (−20°C) and centrifuged for 30 min at 10,000 rpm in an HB-4 rotor. The pelleted precipitate was washed with 80% acetone (−20°C) and centrifuged again to collect the precipitate. The pellet was suspended in 200 μl of X50 buffer (20 mM HEPES [pH 7.9], 20% glycerol, 50 mM KCl, 1 mM EDTA, 1 mM DTT, 0.5 mM phenylmethyl sulfonyl fluoride) containing 6 M guanidine. The samples were dialyzed over a 48-h period at 4°C against a 2,000-fold excess of X50 buffer containing 0.5 mg of bovine serum albumin per ml. Renatured proteins were stored at −70°C for subsequent analyses.

Gel retardation analysis. Gel retardation analyses were modified from the procedure of Fried and Crothers (16) and Garner and Revzin (19). Oligonucleotide probes were labeled with [^{32}P]dCTP by using Klenow enzyme. Labeled oligonucleotides (20 ng) were incubated with either 10 μg of HeLa nuclear extract or 0.5 μg of an elution fraction from the zinc affinity column and 3 μg of poly(dI-dC) in 10 mM Tris (pH 7.5)–40 mM NaCl–1 mM DTT–1 mM EDTA (gel shift binding buffer) for 20 min at 24°C. For gel retardation analysis of renatured proteins, approximately 100 ng of protein was substituted for HeLa extract in the reaction mixture described above, and the amount of poly(dI-dC) was reduced to 0.5 μg. For competition studies, a 25-fold excess of unlabeled oligonucleotide was added to the incubation mixture. After incubation, DNA-protein complexes were analyzed on a 4% native acrylamide gel at a constant current of 25 mA in buffer containing 6.7 mM Tris (pH 7.5), 3.3 mM sodium acetate, and 1 mM EDTA.

Indirect DNA-binding analysis. A synthetic oligonucleotide containing TRE-2 sequences was end labeled with biotinylated dATP. The biotinylated oligonucleotide (60 ng) was incubated with either 40 μg of HeLa nuclear extract, 25 μg of an elution fraction from the zinc column, or approximately 1 μg of renatured protein, 1.5 μg of purified TAX_1 protein generated in *Escherichia coli* (20), and 3 μg of poly(dI-dC) in gel shift binding buffer containing 0.5% Triton X-100. After a 20-min incubation period at 24°C, streptavidin-agarose was added and incubated for 10 min with gentle agitation. The incubation mix was diluted 50-fold with gel shift binding buffer containing 0.5% Triton X-100 and centrifuged for 2 min. The supernatant was removed, and the pelleted proteins were separated by SDS-PAGE (10% separating gel) and

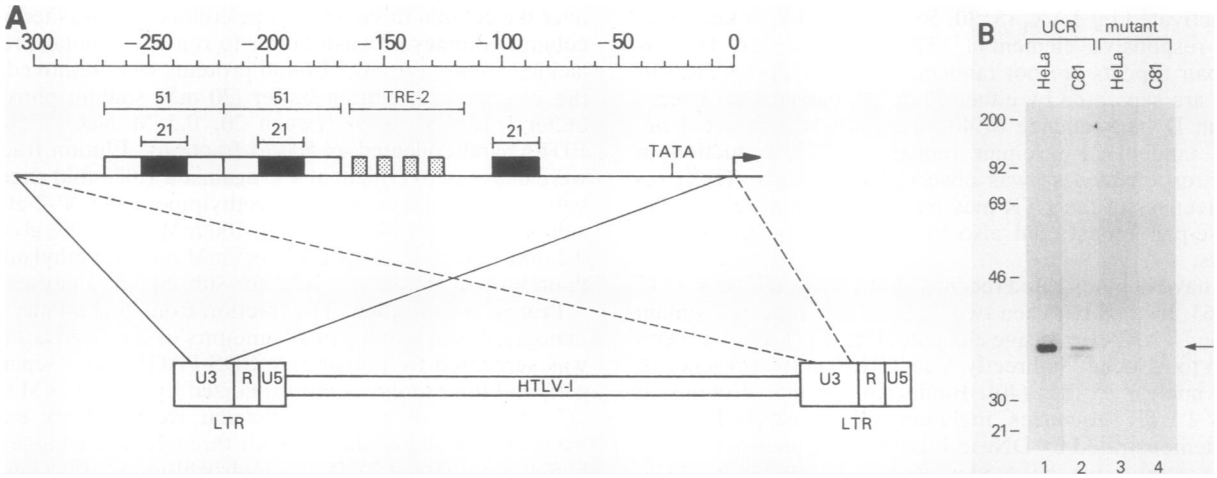

FIG. 1. Identification of a cellular protein that interacts with the TRE-2. (A) Schematic diagram of the HTLV-I upstream regulatory region. The HTLV-I enhancer is located within the U3 region of the viral genome. The enhancer consists of tandem 51-base-pair repeats (□) containing two of the three 21-base-pair repeats (■) and an upstream control region containing TRE-2 with four pentanucleotide repeats (▩). (B) Southwestern blot analysis of nuclear extracts. Nuclear extracts were prepared from HeLa (lanes 1 and 3) and C81-66-45 (lanes 2 and 4) cells. A 100-μg sample of each extract was separated by SDS-PAGE, transferred to a nitrocellulose membrane, and probed as described in Materials and Methods. The probes were either a ^{32}P-labeled TRE-2 probe (lanes 1 and 2) or a ^{32}P-labeled base substitution mutant of the TRE-2 in which the second and third pentanucleotide repeats were altered (40). Positions of the molecular weight markers are indicated on the left in kilodaltons. The position of TIF-1 is indicated by an arrow on the right. UCR, Upstream control region which contains TRE-2.

transferred to an Immobilon-P (Millipore Corp., Bedford, Mass.) membrane. The membrane was probed with polyclonal anti-TAX_1 serum followed by ^{125}I-protein A.

In vitro transcription assay. HeLa cell extracts were prepared by the method of Manley et al. (39), using cells that were harvested at a cell concentration of 5×10^5 to 6×10^5 cells per ml. Each standard transcription reaction mixture (15 μl) contained 10 mM HEPES (pH 7.9), 50 mM KCl, 6.25 mM $MgCl_2$, 0.05 mM EDTA, 1.5 mM DTT, 8.5% glycerol, 0.5 mM each ATP, GTP, and CTP, 15 μCi of [^{32}P]UTP (500 Ci/mmol), and 0.3 μg of template DNA. TIF-1 was dialyzed against 20 mM HEPES (pH 7.9)–50 mM KCl–1 mM EDTA–1 mM DTT–20% glycerol with 250 μg of bovine serum albumin per ml. Where indicated, 5 or 10 ng of renatured TIF-1 was added to the reaction mixture. Transcription assays were incubated for 60 min at 30°C. RNA was purified by phenol-chloroform-isoamyl (50:50:1) extraction, followed by chloroform extraction and ethanol precipitation. Radiolabeled RNA samples were then analyzed by electrophoresis in 4% acrylamide-urea gels.

RESULTS

TIF-1 binds to TRE-2. Previous work has demonstrated that TAX_1 is capable of forming an indirect, stable complex with TRE-2 in the presence of HeLa nuclear extract (40). TRE-2 (Fig. 1A) contains four pentanucleotide repeats, the center two of which lie within an imperfect 10-base-pair repeat. Previous studies have demonstrated that sequences within the second and third pentanucleotide repeats are necessary to maintain TAX_1 responsiveness of TRE-2 in transient expression chloramphenicol acetyltransferase assays as well as to compete for specific protein binding to TRE-2 in gel retardation assays (5, 40). The first and fourth pentanucleotide repeats are not required for specific protein binding by gel retardation analysis. To identify the transcription factor required for complex formation with TRE-2 and TAX_1, we first analyzed nuclear extracts from HeLa and

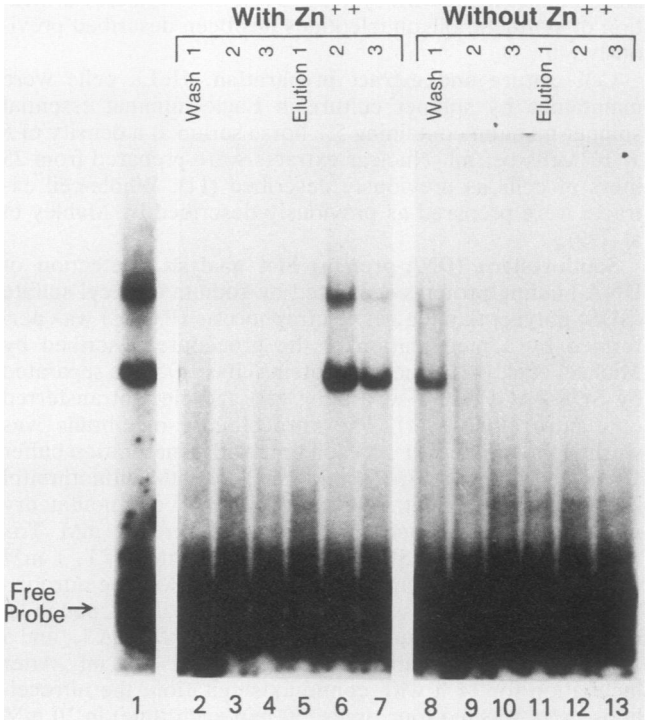

FIG. 2. Gel retardation analysis of zinc column fractions. HeLa nuclear extract was applied to a column preloaded with zinc (lanes 2 to 7) or lacking zinc (lanes 8 to 13). Preparation of the zinc affinity column and details of the fractionation protocol are given in Materials and Methods. The columns were washed to remove unbound proteins, and the proteins that remained bound to the column were eluted with 250 mM EDTA. A ^{32}P-labeled TRE-2 oligonucleotide probe (20 ng) was incubated with either crude HeLa nuclear extract (lane 1), wash fractions from the zinc column (lanes 2 to 4 and 8 to 10), or elution fractions (lanes 5 to 7 and 11 to 13) in the presence of 3 μg of poly(dI-dC) in gel shift binding buffer. After incubation, the complexes were resolved on a 4% native acrylamide gel.

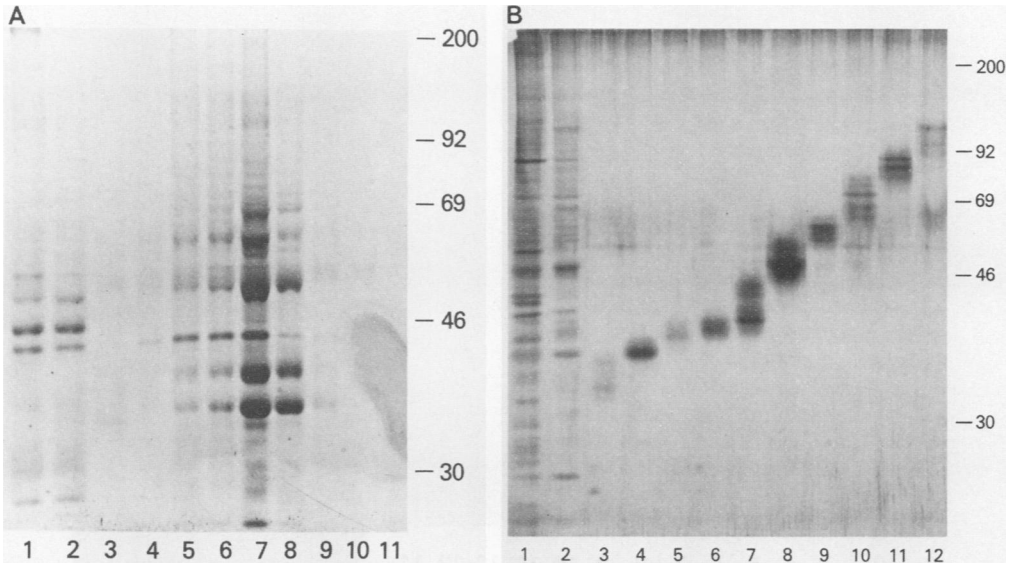

FIG. 3. Purification of proteins from a zinc column elution fraction. (A) SDS-PAGE analysis of fractions from zinc chelate chromatography. Equal volumes of zinc column wash fractions (lanes 1 to 3) or elution fractions (lanes 4 to 11) from the zinc chelate column were separated by SDS-PAGE and silver stained to analyze the protein content of each fraction. Positions of molecular weight markers are shown on the right in kilodaltons. (B) SDS-PAGE analysis of purified, renatured proteins. Proteins from a peak zinc column elution fraction (such as in panel A, lane 7) were separated by preparative SDS-PAGE. Individual proteins or regions of the gel were excised, eluted, and renatured as described in Materials and Methods. Protein recovery and purity was determined by analytical SDS-PAGE of 100 ng of crude HeLa nuclear extract (lane 1), 50 ng of a peak zinc column elution fraction (lane 2), or equal volumes of renatured proteins with molecular sizes of 30 to 35 kDa (lane 3), 36 kDa (lane 4), 37 to 41 kDa (lane 5), 42 kDa (lane 6), 43 to 46 kDa (lane 7), 47 to 53 kDa (lane 8), 54 to 63 kDa (lane 9), 64 to 75 kDa (lane 10), 76 to 90 kDa (lane 11), and 91 to 110 kDa (lane 12). Proteins were visualized by silver staining. Positions of molecular weight markers are shown on the right in kilodaltons.

C81-66-45, an HTLV-I immortalized, non-virus-producing cell line (23, 57), by Southwestern blot analysis. Nuclear extracts (100 μg) from either HeLa or C81-66-45 cells were electrophoresed through a 10% SDS-polyacrylamide gel and transferred to an Immobilon-P filter. Proteins were renatured and probed with ^{32}P-labeled synthetic oligonucleotides representing either the TRE-2 region of the LTR or a base substitution mutant in which the second and third pentanucleotide repeats were altered (40). The TRE-2 probe bound specifically to a 36-kDa cellular protein, which we have designated TIF-1, in both HeLa (Fig. 1B, lane 1) and C81-66-45 (lane 2) nuclear extracts. The mutant TRE-2 oligonucleotide, in which the center two pentanucleotide repeats were altered, failed to bind to cellular proteins in either the HeLa (lane 3) or C81-66-45 (lane 4) nuclear extracts. Thus, the DNA-binding properties of the 36-kDa protein are consistent with the sequence requirements for in vivo activity of TRE-2. From densitometry scans of the Southwestern autoradiograms, HeLa nuclear extracts were found to contain approximately 17-fold more 36-kDa protein than did C81-66-45 nuclear extracts. HeLa nuclear extract was used for subsequent procedures because of the abundant expression of TIF-1.

Purification of TIF-1. Zinc chelate chromatography (25, 52) was used to fractionate a crude HeLa nuclear extract. A $ZnCl_2$ solution was used to load an iminodiacetic acid epoxy-activated Sepharose 6B matrix with zinc ions. The fully loaded column had the capacity for 120 μmol of zinc. HeLa nuclear extract (100 mg) was passed over the zinc-loaded column three times, and proteins lacking affinity for zinc were collected as flowthrough. After the wash, proteins with affinity for the zinc column were eluted with 250 mM EDTA. Eluted protein fractions were dialyzed to remove residual zinc and EDTA before analysis. Gel retardation analysis with a TRE-2 probe was used to monitor the DNA-binding activity. The TRE-2 oligonucleotide (20 ng), labeled with [^{32}P]dCTP, was incubated with either crude nuclear extract (10 μg) or individual zinc column fractions in the presence of nonspecific poly(dI-dC) DNA (3 μg). Incubation of the TRE-2 probe with HeLa nuclear extracts resulted in formation of two distinct gel retardation complexes (Fig. 2, lane 1). TIF-1 gel retardation activity bound the zinc affinity column (lanes 2 to 7) and was eluted with 250 mM EDTA (lanes 5 to 7). Both the upper and lower gel retardation complexes were observed in fractions eluted from the column with EDTA. When the extract was passed over a control column in which no zinc was present (lanes 8 to 12), TIF-1 activity was found in the wash fractions, indicating that TIF-1 interaction with the column required the presence of metal ions.

The wash and elution fractions from the zinc affinity column were analyzed further by SDS-PAGE. The column afforded partial purification of several proteins (Fig. 3A). Proteins with molecular sizes of 36, 42, 50, 65, and 69 kDa were enriched in the fractions eluted from the column (Fig. 3A, lanes 7 and 8) and were absent in wash fractions (lanes 1 to 3). Preparative SDS-PAGE of the peak TRE-2 fraction (lane 7) was used to isolate larger quantities of enriched proteins. Proteins were visualized by $CuCl_2$ staining (37), and proteins of 36 and 42 kDa were excised individually from the gel, while other regions of the gel, including proteins of 30 to 35, 37 to 41, 43 to 46, 47 to 53, 54 to 63, 64 to 75, 76 to 90, and 91 to 110 kDa, were excised as groups. Each of these gel slices was destained, and proteins were eluted and renatured by the procedure of Schreiber et al. (59). The renatured proteins were analyzed subsequently by SDS-PAGE to determine their purity. Distinct groups of proteins or individual proteins were recovered by this procedure (Fig.

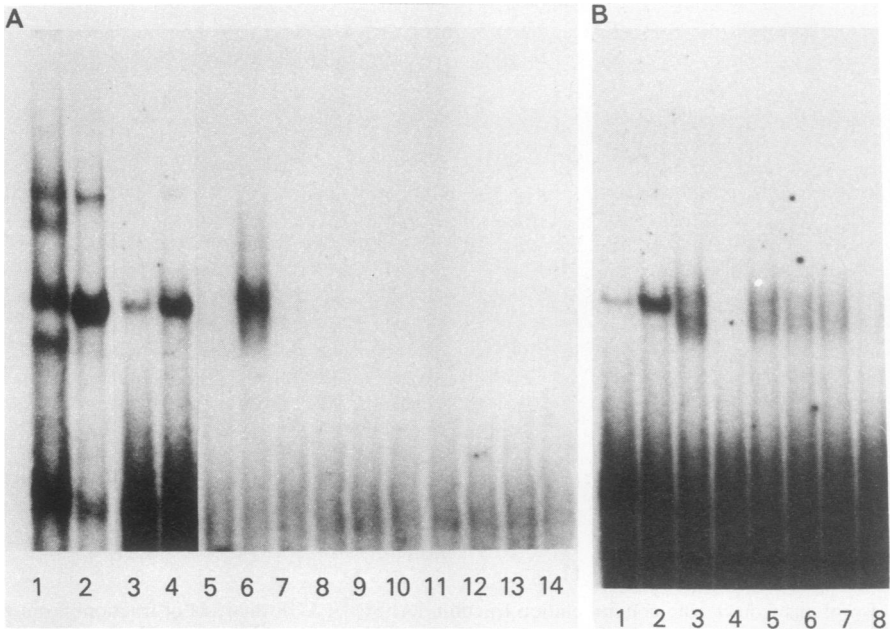

FIG. 4. Binding of a 36-kDa protein to the TRE-2. (A) Gel retardation analysis of purified, renatured proteins. The ability of crude HeLa nuclear extract (lane 1), a peak zinc column fraction (lane 2), a 100-fold dilution of crude HeLa nuclear extract (lane 3), a 100-fold dilution of a peak zinc column fraction (lane 4), or individual renatured proteins (30 to 35 kDa [lane 5], 36 kDa [lane 6], 37 to 41 kDa [lane 7], 42 kDa [lane 8], 43 to 46 kDa [lane 9], 47 to 53 kDa [lane 10], 54 to 63 kDa [lane 11], 64 to 75 kDa [lane 12], 76 to 90 kDa [lane 13], and 91 to 110 kDa [lane 14]) to bind a ^{32}P-labeled TRE-2 probe was analyzed by gel retardation as described in Materials and Methods. The exposure times were either 1 h (lanes 1 and 2) or 24 h (lanes 3 to 14). (B) Binding of the 36-kDa protein specifically to TRE-2. A ^{32}P-labeled TRE-2 probe was used to analyze the specificity of DNA protein complexes formed by a 100-fold dilution of crude HeLa nuclear extract (lane 1), a 100-fold dilution of a peak zinc column elution fraction (lane 2), or the purified 36-kDa protein (lanes 3 to 8). A 25-fold excess of unlabeled competitor oligonucleotide representing TRE-2 (lane 4), TRE-1 (lane 5), a mutant TRE-2 containing base substitutions in the second and third pentanucleotide repeats (lane 6), a TRE-2 probe containing base substitutions in the third pentanucleotide repeat (lane 7), and a mutant TRE-2 containing only the second and third pentanucleotide repeats (lane 8) were added to the reaction mix as indicated to demonstrate specificity of the 36-kDa binding to the TRE-2.

3B). The faint band across the silver-stained gel at 65 to 69 kDa represents an artifact of the silver-staining procedure that has been reported previously (41). Variation in recovery of individual proteins was noted and may have resulted from minor differences in the technical handling of samples during the procedure.

Functional activity of renatured TIF-1. To determine whether the purified 36-kDa protein bound to the TRE-2 probe, gel retardation analysis was performed. Approximately 10 ng of each renatured protein was incubated with 20 ng of the TRE-2 probe and 0.5 μg of poly (dI-dC). For the 36-kDa protein, these conditions represent a two-fold molar excess of oligonucleotide probe to protein. After incubation, DNA-protein complexes were analyzed on a 4% polyacrylamide gel. A gel retardation complex was formed with the renatured 36-kDa protein (Fig. 4A, lane 6) but not with the other renatured proteins (lanes 5 and 7 to 14). The position of the DNA-protein complex formed by TIF-1 and TRE-2 migrated similarly to the position of the lower gel retardation complex seen with HeLa nuclear extract (lanes 1 and 3) and a peak fraction from the zinc affinity column (lanes 2 and 4). At dilute concentrations, both crude HeLa nuclear extract and the peak zinc column fraction formed primarily the lower gel retardation complex (Fig. 4A, lanes 3 and 4, respectively).

Specificity of the gel retardation complex formed by the 36-kDa protein was demonstrated by competition analysis using a 25-fold excess of specific or nonspecific competitor. The gel retardation complex formed with purified TIF-1 (Fig. 4B, lane 3) was competed for by the specific TRE-2 competitor (lane 4) but not by the TRE-1 competitor (lane 5) or by TRE-2 base substitution mutants that lacked in vivo activity (lanes 3, 6, and 7). Consistent with our previous results, specific competition was observed with the complete TRE-2 sequence (lane 4) as well as with a DNA fragment containing only the second and third pentanucleotide repeats (lane 8). These results demonstrated that purified TIF-1 was capable of binding specifically to a TAX_1-responsive element in the HTLV-I LTR.

Purified TIF-1 stimulates in vitro transcription from the HTLV-I LTR. To determine whether the purified TIF-1 protein would stimulate specific transcription from the HTLV-I LTR, templates for in vitro transcription assays were prepared by *Bam*HI restriction digestion of the control adenovirus major late plasmid (Pϕ4) and *Hin*dIII restriction digestion of the HTLV-I LTR plasmid (pU3RCAT). Accurate initiation at the adenovirus major late promoter and the HTLV-I LTR templates was expected to produce transcripts of 375 and 278 nucleotides, respectively. The authenticity of these runoff transcription products were confirmed by S1 nuclease analysis using a 5′-end-labeled probe (data not shown). After restriction enzyme digestion, template DNAs were purified, ethanol precipitated, and suspended at a concentration of 0.1 μg/μl. HeLa transcription extracts were prepared by the method of Manley et al. (39). The amount of HeLa extract added to the transcription assay was first titrated to determine the minimum quantity of extract required to detect transcription from the HTLV-I LTR and the adenovirus major late promoter. Each assay received 0.3 μg of template DNA and either 7.5, 15, 37.5, or 55 μg of HeLa

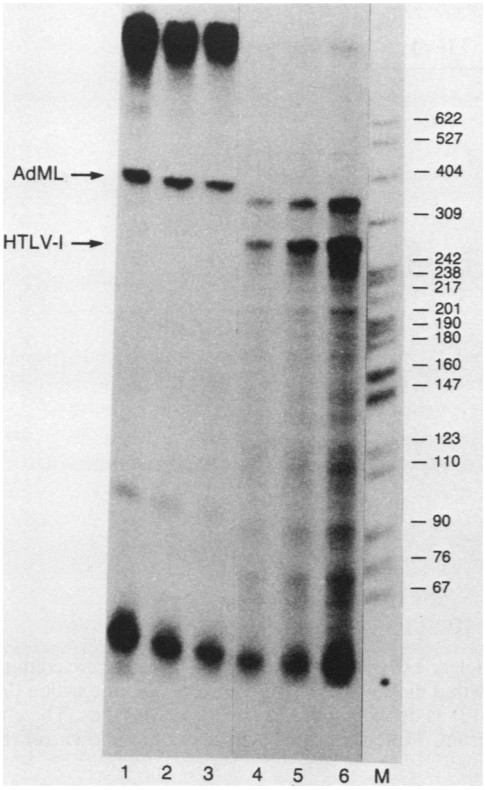

FIG. 5. Transcriptional activation analysis of the HTLV-I LTR by TIF-1. An adenovirus major late promoter template (pϕ4) (lanes 1 to 3) or an HTLV-I LTR template (pU3RCAT) (lanes 4 to 6) was incubated with 0 μg (lanes 1 and 4), 0.125 μg (lanes 2 and 5), or 0.25 μg (lanes 3 and 6) of purified TIF-1 in the presence of [^{32}P]UTP and HeLa whole-cell extract as described in Materials and Methods. Positions of molecular weight markers are shown in lane M and indicated on the right as nucleotide lengths. The positions of the adenovirus major late transcript (AdML) and the HTLV-I transcript (HTLV-I) are indicated on the left.

extract. From these assays, it was determined that the minimal quantity of HeLa extract required to obtain basal transcription was 15 μg for the HTLV-I template and 37.5 μg for the adenovirus major late promoter (data not shown). This amount of HeLa nuclear extract was used in subsequent transcription assays. The addition of approximately 5 or 10 ng of renatured TIF-1 to the in vitro transcription assays containing the adenovirus major late template did not stimulate transcription (Fig. 5, lanes 1 to 3). In contrast, addition of the same quantity of TIF-1 stimulated transcription from the HTLV-I LTR, as demonstrated by the increase in the level of the [^{32}P]UTP-labeled 285-nucleotide transcript (lanes 4 to 6). On the basis of densitometry of several autoradiogram exposures, we estimated that transcription was elevated approximately 8- to 10-fold in the reactions containing 10 ng of purified TIF-1. These experiments provide direct evidence that TIF-1 is a transcription factor which positively regulates transcription from the HTLV-I LTR.

Purified TIF-1 mediates the binding of TAX_1 to TRE-2. We have previously demonstrated that formation of a complex containing TAX_1 and TRE-2 requires the presence of HeLa nuclear extract and that the complex was unable to form on mutant DNA which lacked TAX_1 responsiveness in vivo (40). Since TIF-1 possessed specific TRE-2-binding characteristics and the ability to stimulate in vitro transcription, the ability of renatured TIF-1 to replace crude nuclear extract in this complex was investigated. To evaluate this possibility, the TRE-2 oligonucleotide probe was end labeled with biotinylated dATP. The biotinylated probe (60 ng) was mixed with either 40 μg of nuclear extract, 25 μg of zinc affinity-enriched nuclear extract, or 100 ng of purified TIF-1. Purified TAX_1 (1.5 μg) from an *E. coli* expression system was added as indicated. The conditions for incubation were identical to those described above for the gel retardation assays except that 0.5% Triton X-100 was included to reduce nonspecific protein aggregation. After incubation for 20 min at 24°C, streptavidin-agarose was added, and incubation continued for an additional 10 min with gentle, intermittent agitation. The incubation mix was centrifuged briefly, washed with 50 volumes of binding buffer, and analyzed by Western immunoblot for the presence of TAX_1 in the pelleted fraction. The relative levels of TAX_1 were determined by densitometry.

TAX_1 protein was present in the pelleted fraction in the complete reaction mixture (Fig. 6, lane 1). TAX_1 was not detected when either the HeLa extract, TAX_1 protein, or biotinylated TRE-2 DNA was absent from the reaction mixture (lanes 2 to 4, respectively). In a parallel set of assays, the peak fraction from the zinc affinity column (Fig. 2, lane 8) containing the highest binding activity for TRE-2 was substituted for the HeLa extract (Fig. 6, lanes 5 to 8). Specific precipitation of TAX_1 was observed in the complete reaction mixture (lane 5) but not when the column fraction, TAX_1, or DNA was omitted (lanes 6 to 8, respectively). In the final set of assays, the purified TIF-1 protein was added to the binding reactions. As seen with the whole HeLa extract and the zinc affinity column fraction, specific precipitation of the TAX_1 protein was observed in the complete reaction mixture (lane 9) but not when either TIF-1, TAX_1 or biotinylated DNA (lanes 10 to 12, respectively) was omitted from the reaction. The other renatured proteins were unable to form a complex with TAX_1 and TRE-2 in this assay (data not shown). These results demonstrated that the indirect TAX_1-binding complex can be reconstituted by using the purified complex components TAX_1, TIF-1, and biotinylated TRE-2 DNA probe.

DISCUSSION

We have demonstrated that a 36-kDa HeLa nuclear protein (TIF-1) bound specifically to a TAX_1-responsive element in the HTLV-I LTR and stimulated transcription from the viral LTR in vitro. We have also demonstrated that TIF-1 mediated the indirect interaction of TAX_1 with a responsive element in the LTR. These results suggest that TAX_1, similar to c-FOS (9, 13, 35) and herpes simplex virus Vmw65 (49), may regulate gene expression by specific interaction with a sequence-specific transcription factor. c-FOS is apparently required to form a functional complex (AP1) with the DNA-binding protein c-JUN. In this complex, c-FOS directly interacts with DNA as part of the AP1 complex (54, 55, 58). A different relationship apparently exists between TAX_1 and TIF-1 because TIF-1 is capable of binding TRE-2 and stimulating transcription in the absence of TAX_1. This observation is important because cellular factors, such as TIF-1, may allow early rounds of viral transcription generating messages for viral regulatory proteins, including TAX_1. The TAX_1–TIF-1 complex with TRE-2 could form after TAX_1 synthesis and could affect either the affinity or the stability of the complex on DNA. We are

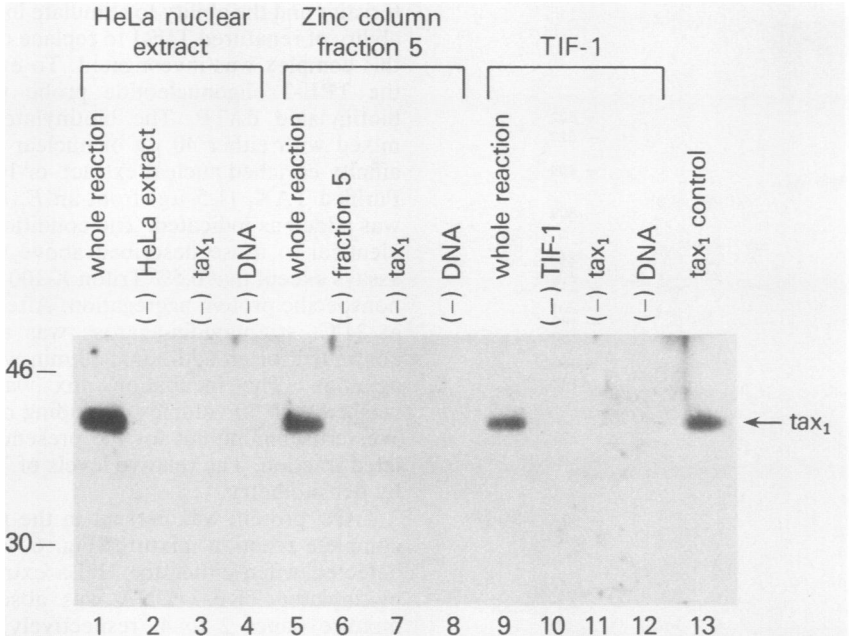

FIG. 6. Indirect interaction of TAX_1 with TIF-1 and the TRE-2. Crude HeLa nuclear extract (lanes 1, 3, and 4), a peak zinc column elution fraction (lanes 5, 7, and 8) or purified TIF-1 (lanes 9, 11, and 12) was incubated with a biotinylated TRE-2 oligonucleotide probe (lanes 1 to 3, 5 to 7, and 9 to 11) and purified TAX_1 protein (lanes 1, 2, 4 to 6, 8 to 10, 12, and 13) as described in Materials and Methods. The complexes were analyzed by SDS-PAGE and then by Western blot using anti-TAX_1 antiserum. Molecular weight markers are shown on the left in kilodaltons. The position of TAX_1 is indicated on the right.

currently investigating the affinity and stability of this complex as well as its activity in in vitro transcription assays. It is important to note that this mode of transactivation may not be applicable to all TAX_1 activated genes.

The ability of TIF-1 to bind to a zinc affinity column suggests that it contains a metal-binding domain (7, 43). Three observations suggest that the affinity of this protein for the zinc column does not result simply from charge interactions between the cationic column and the protein which may contain negatively charged amino acid domains. First, the wash buffer used in zinc chromatography contained 0.5 M NaCl, a relatively high salt concentration that reduces potential charge interactions. Second, on a column matrix that lacks zinc, TIF-1 activity did not bind to the column and was found in the flowthrough and wash fractions. Third, a DNA-binding protein (Oct-1) that lacks affinity for heavy metals (67) does not bind the zinc column (data not shown).

Metal binding by proteins may facilitate either DNA-protein interactions as illustrated by TFIIIA (7, 43) or protein-protein interactions as illustrated by human immunodeficiency virus type 1 TAT protein (14, 15). Since the HTLV-I TAX_1 protein has recently been shown to interact with zinc (P. Lindholm and S. Gitlin, unpublished results), it is interesting to speculate that the zinc-binding capabilities of TIF-1 and TAX_1 may facilitate the interaction of these two factors. We are currently investigating this model in further detail.

HeLa cells provide an easily obtainable and abundant source of material for transcription factor isolation. In the case of TIF-1, we observed that HeLa nuclear extracts contain 17 times more TIF-1 protein per microgram of total protein than do nuclear extracts from C81-66-45 cells. From the experiments presented in Fig. 6, we can determine the following. The binding activity of 100 ng of purified TIF-1 contains approximately 25% of the activity present in 40 μg of HeLa nuclear extract. On the basis of control experiments, we calculate that 10% of the renatured TIF-1 is functional. Thus, we can estimate that 10 ng of TIF-1 is present in approximately 10 μg of nuclear extract, representing approximately 0.1% of the total protein. For comparison, other transcription factors such as C/EBP and Sp1 are approximately 0.002% of the total protein (6, 32). Therefore, in our HeLa cell line, TIF-1 is approximately 50 times more abundant than these other transcription factors. However, the level of TIF-1 in the C81 cell line is within a factor of 3 of the cellular concentration of Sp1 or C/EBP. It is also possible that the 36-kDa protein is not homogeneous and the binding and transcriptional activities are attributed to a minor 36-kDa species. Clearly, the analysis of additional cell lines and tissues is needed to determine the natural abundance of the TIF-1 transcription factor.

It is interesting to note that renatured TIF-1 produced a gel retardation complex which migrated to a position similar to that of the lower complex produced by whole nuclear extract. At this point, we have not been able to obtain the upper gel retardation complex with purified TIF-1. One explanation for these results is that the upper complex may result, in more concentrated extracts, from dimerization of TIF-1. It is possible that the metal-binding potential of TIF-1 may mediate its ability to form homodimers as well as heterodimers with TAX_1. Alternatively, it is possible that the upper complex results from the interaction of a unique protein with the TRE-2 or from the additive effect of TIF-1 and another nuclear protein.

TIF-1 is one of two cellular proteins known to interact with TRE-2 in the viral LTR. Purified Sp1 (95 to 105 kDa) has been shown by DNase footprint analysis to protect the third pentanucleotide repeat (W. Dynan, personal communication). Until amino acid sequence data have been obtained for the purified protein, the possibility that the 36-kDa

TIF-1 is a proteolytic breakdown product of the 95- to 105-kDa Sp1 factor can not be dismissed. However, three experimental results discount this possibility. First, cells that were scraped from a tissue culture plate immediately into SDS-PAGE sample buffer containing protease inhibitors for Southwestern blot analysis exhibited a 36-kDa reactive band but lacked any reactivity in the 95- to 105-kDa range (K. Brown, unpublished data). Second, when proteins migrating in the 95- to 105-kDa range of an SDS-PAGE were excised, eluted, and renatured, no gel retardation activity was observed with a TRE-2 probe. Finally, in lectin-binding experiments, Sp1 but not TIF-1 specifically bound wheat germ agglutinin, whereas TIF-1 bound specifically to concanavalin A, indicating that TIF-1 may be glycosylated differently than Sp1 (data not shown). It appears likely that TIF-1 and Sp1 are two distinct proteins that bind a similar nucleotide motif. The binding of two different proteins to identical nucleotide sequences is not without precedent. NF-κB and H2TF1 each bind a single site within the simian virus 40 enhancer as well as a single site upstream of the $H\text{-}2K^b$ major histocompatibility complex gene (2, 62). In addition to TIF-1 and Sp1, our laboratory has preliminary evidence that another cellular protein binds specifically to TRE-2. Further investigation of these proteins, including their relative abundance and binding affinity, will be required to fully understand the contribution of TRE-2 to transcriptional regulation of HTLV-I gene expression.

The relative contributions and possible interactions of proteins that bind to TRE-1 and TRE-2 in TAX_1-mediated transactivation of the LTR in vivo remains to be established. We can envision a model in which TRE-2 serves as a binding site for proteins whose function is to stabilize the interaction of proteins with TRE-1. The fact that tandem, but not separated, copies of the TRE-1 are required for transactivation in oligonucleotide constructs suggest that protein binding requires stabilization. In the native LTR structure, TRE-2 bridges two TRE-1 elements. It is intriguing to speculate that TAX_1 stabilizes the interaction of proteins bound to TRE-2 and TRE-1 and that this complex activates transcription in vivo. Experiments to analyze this model are currently in progress.

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