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Purification and Characterization of Multiple Nuclear Factors That Bind to the TAX-Inducible Enhancer within the Human T-Cell Leukemia Virus Type 1 Long Terminal Repeat

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Within the human T-cell leukemia virus type I promoter, there are three copies of a 21-base-pair repeat (hereafter called the *tax*-responsive element [TRE]) that both contributes to basal promoter activity and mediates induction by the viral activator TAX. We have identified and separated three nuclear proteins that interact with the TRE. The TRE-binding protein designated TREB-3 bound more avidly to a multimerized TRE than to a single-copy TRE, while the other two TRE-binding proteins, TREB-1 and TREB-2, bound equally well to either TRE. TREB-1 has been purified to near homogeneity, and binding activity was localized to a protein of 35 to 43 kilodaltons. The affinity-purified TREB-1 activated transcription from the human T-cell leukemia virus type I promoter in vitro. The purified TREB-1 fraction contained activating transcription factor binding activity and showed a cooperative interaction with the TATA-binding factor (TFIID) on the adenovirus E4 promoter.

Human T-cell leukemia virus type I (HTLV-I) has been identified as the etiological agent of adult T-cell leukemia or lymphoma (for reviews, see references 60, 62, and 64) and appears to be involved in B-cell chronic lymphocytic leukemia (33) and certain chronic neurological diseases, including the tropical spastic paraparesis (24, 44, 59). In vitro transmission of HTLV-I often results in immortalization of the recipient human and animal T cells (9, 43, 63). The binding of HTLV-I particles to T lymphocytes results in induction of interleukin-2 (IL-2) production and IL-2 receptor (IL-2R) expression, triggering of T-cell proliferation, and colony formation in soft agar (8, 16).

HTLV-I encodes a 40-kilodalton (kDa) nuclear protein (17, 30, 54, 55), now designated TAX_1 (14), that acts in *trans* to increase the rate of transcription from the HTLV-I promoter (10, 50, 56, 57). The ability of HTLV-I TAX, under the control of its own promoter, to induce tumors in transgenic mice establishes TAX as an oncogenic protein and HTLV-I as a transforming virus (39).

The HTLV-I promoter contains three copies of a 21-base-pair (bp) repeat (hereafter called the TAX-responsive element [TRE]) that is involved both in base-level expression and in TAX-mediated induction (4, 13, 41, 42, 45, 51). The 21-bp TRE is also capable of conferring an increased basal expression and a TAX-responsive phenotype on heterologous promoters, independent of position and orientation (13, 42, 45, 51). In addition, the level of activation by TAX is elevated by increasing the TRE copy number (4, 13, 42, 45, 51). This TRE-dependent, TAX-mediated activation has been observed in many types of cells, including HeLa cells (45, 46). TAX has also been shown to activate the promoters of the human IL-2 (T-cell growth factor) receptor (IL-2R) gene (6, 23, 34, 52), the human immunodeficiency virus types 1 and 2 long terminal repeats (LTRs) (2, 53), the simian virus 40 early gene (35, 49), and several lymphokine genes (35), including GM-CSF, IL-2 (34, 52), IL-3, and IL-4. Interestingly, the TAX-responsive regions of the respective promoters (31, 36, 48) do not share any apparent sequence homology with the HTLV-I 21-bp TRE.

Recent results indicate that TAX does not interact directly with the TREs but rather that host cell proteins mediate the TAX response (1, 31, 40, 48). To study the biochemical mechanism(s) of the transcriptional activation mediated by TAX, we have undertaken a systematic study of the nuclear factors that bind to the HTLV-I TRE.

MATERIALS AND METHODS

Plasmids. The plasmids pT_3CAT and pt_3CAT were constructed by inserting the synthetic triple TRE oligonucleotides TTT and ttt, respectively, into the enhancerless SVCAT vector which was obtained by digesting pSV2CAT with the restriction enzymes *Acc*I and *Sph*I.

The HTLV-I plasmids were obtained from E. Gilboa (Sloan-Kettering Cancer Institute, New York, N.Y.) and C. Rosen (Roche Institute of Molecular Biology, Nutley, N.J.). The adenovirus 5 (Ad5) E4 promoter-containing plasmid (pSME4) was obtained from M. R. Green (Harvard University, Cambridge, Mass.). The c-*fos* promoter-containing plasmid (pFC700) was as described previously (11).

Cells. Clone 33 Jurkat cells and C8166 HTLV-I-transformed T cells (obtained from R. Gallo and F. Wong-Staal, National Institutes of Health, Bethesda, Md.) were grown in RPMI medium supplemented with 10% fetal bovine serum plus 4×10^{-5}% β-mercaptoethanol.

Gel retardation assay. The DNA probe was either monomeric (25-bp) or dimeric (48-bp) TRE oligonucleotide. Binding reactions (20 μl) contained 20 mM Tris (pH 7.5), 4% Ficoll, 60 mM KCl, 2 mM EDTA, 0.5 mM dithiothreitol, 1 ng of radiolabeled DNA probe, and 1.0 μg of poly(dI-dC) heteropolymer (or 1.0 μg of salmon sperm DNA). Protein fractions were added last. A mixture of 0.2 μg of poly(dI-dC) and 0.1 mg of bovine serum albumin per ml was used for affinity-purified TRE-binding protein 1 (TREB-1). After 30

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min at 30°C, reactions were loaded directly onto a 4% polyacrylamide gel in 0.25× TBE (25 mM Tris, 25 mM boric acid, 1 mM EDTA) and electrophoresed at 200 V for 1 h at room temperature. The gel was dried and exposed to X-ray film (XAR-5 or BB5; Eastman Kodak Co.).

DNA transfection and CAT assay. DNA transfection and chloramphenicol acetyltransferase (CAT) assay were done as follows. Cells (5×10^6) were cotransfected by the modified DEAE-dextran method (58) with 4 μg of plasmid (either pT_3CAT or pt_3CAT) DNA. At 48 h posttransfection, total cell extracts were prepared by sonication followed by brief centrifugation. Samples containing fixed amounts of protein were used for assay of CAT activity at 37°C followed by thin-layer chromatography (18).

Oligonucleotides. Oligonucleotides were synthesized by the phosphoramidite method on an automated synthesizer (Applied Biosystems, Inc.). The radiolabeled double-stranded oligonucleotide probes were prepared by 3'-end labeling by using Klenow fragment of DNA polymerase I.

5'-GATCCTAAGGCTCTGACGTCTCCCCCCAG-3'
3'-GATTCCGAGACTGCAGAGGGGGGTCCTAG-5'

T^P, a 50-bp promoter fragment containing a monomeric TRE (underlined) that spanned sequences −262 to −213:

5'-GACTGGCCCAGACTAAGGCTCTGACGTCTCCCCCCAGAGGGACAGCTCAGCACC-3'
3'-CCGGGTCTGATTCCGAGACTGCAGAGGGGGGTCTCCCTGTCGAGTCGTGGCTGA-5'

TjT, a 48-bp dimerized TRE oligonucleotide that spanned sequences −252 to −231:

5'-GACTAAGGCTCTGACGTCTCCCCCCAGACTAAGGCTCTGACGTCTCCCCCCA-3'
3'-TTCCGAGACTGCAGAGGGGGGTCTGATTCCGAGACTGCAGAGGGGGGTCTGA-5'

TTT, a 66-bp triple TRE oligonucleotide:

5'-ATACAAGGCCCTGACGTCTCCCCCTAAGGCCCTGACGTCTCCCCCTAAGGCCCTGACGTCTCCCCCTGCATG-3'
3'-TGTTCCGGGACTGCAGAGGGGGATTCCGGGACTGCAGAGGGGGATTCCGGGACTGCAGAGGGGGAC-5'

ttt, a 57-bp mutated triple TRE oligonucleotide:

5'-ATACAAGGCCCTGTCTCCCCCTAAGGCCCTGTCTCCCCCTAAGGCCCTGTCTCCCCCTGCATG-3'
3'-TGTTCCGGGACAGAGGGGGATTCCGGGACAGAGGGGGATTCCGGGACAGAGGGGGAC-5'

The following probes were formed: WT, a 25-bp monomeric TRE oligonucleotide that spanned sequences −254 to −230:

Chromatography. HeLa nuclear extracts were prepared as previously described (7). All chromatography was performed at 4°C. BC buffers contained 20 mM Tris hydrochloride (pH 7.9), 0.2 mM EDTA, 20% glycerol, 0.5 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride, with various KCl concentrations. Protein concentrations were determined by the method of Bradford (3). The salt concentrations were determined by measuring the conductivity relative to known standards. The DNA binding activities were determined by the gel retardation assays.

Typically, 100 ml of HeLa nuclear extract (average 10 mg/ml from 2×10^{10} cells) was adjusted to 100 mM, loaded onto a 50-ml phosphocellulose (P11) column at 1 cv/h (cv, packed column volume), washed with 1 cv of BC100 buffer (BC buffer containing 100 mM KCl), and step eluted at a rate of 2 cv/h with 3 cv each of BC buffers containing 0.25, 0.5, and 1.0 M KCl. Peak protein fractions were pooled from each step elution.

The P11 0.5 M step-eluted fraction was quickly dialyzed against 2 liters of BC0 buffer until the salt concentration was 200 mM and centrifuged at 10,000 × *g* for 10 min to remove precipitated material. This material was loaded onto a 20-ml DEAE-cellulose column, washed with 1 cv of BC200 buffer, and step eluted with 3 cv of BC buffer containing 1.0 M KCl.

The DEAE flowthrough fraction was quickly dialyzed against 2 liters of BC0–Nonidet P-40 (NP-40)–$ZnCl_2$ buffer (BC0 buffer containing 0.1% NP-40 and 0.1 nM $ZnCl_2$) until the salt concentration reached 60 mM and centrifuged as described above. This material was loaded onto a 6-ml mutant oligonucleotide (ttt) column, washed with 1 cv of BC60 buffer, and step eluted with 3 cv each of BC0–NP-40–$ZnCl_2$ buffer containing 0.35 M and 1.0 M KCl.

The 0.35 M step-eluted fraction was diluted into 200 mM KCl by adding an appropriate volume of BC0–NP-40–$ZnCl_2$ buffer and loaded onto an 1-ml specific oligonucleotide (TTT) column (column 1). The column was washed with 8 cv of BC200–NP-40–$ZnCl_2$ buffer and step eluted with 1 cv of a multiple-step gradient (0.2 M to 1.0 M KCl) with a 0.1 M increment of KCl (in BC0–NP-40–$ZnCl_2$ buffer). The B1 (TREB-1) and B2 (TREB-2) active fractions were pooled separately. The pooled B1 active fraction was diluted into 200 mM KCl, loaded onto a 0.2-ml second specific oligonucleotide (TP) column (column 2), and step eluted as described above. The B1 active fractions were pooled. The material was frozen in liquid nitrogen and stored at −70°C.

The P11 flowthrough fraction (B3 active fraction) was loaded onto a DEAE-cellulose column, washed with 1 cv of BC100 buffer, and step eluted with 3 cv of BC300. The DEAE 0.3 M step-eluted fraction was quickly dialyzed against BC0 buffer until the salt concentration was 100 mM and centrifuged as described above. This material was loaded onto a mutant oligonucleotide (ttt) column, washed with 1 cv of BC100 buffer, and step eluted with 1.0 M KCl. The B3 (TREB-3) activity was in the flowthrough fraction.

Preparation of DNA-Sepharose. Specific (wild-type TRE: TTT or T^P) or nonspecific (mutant TRE: ttt) oligonucleotide was coupled to cyanogen bromide-activated Sepharose CL-

4B (Pharmacia), which had been hydrated and washed extensively with (about 200 ml/g of freeze-dried powder) HCl (pH 3.0) at 4°C. Coupling was carried out overnight at room temperature in 10 mM potassium phosphate buffer (pH 8.2) on a rotary shaker. After coupling, the remaining active groups in the resins were inactivated by incubating with 1 M Tris hydrochloride buffer (pH 8.0) at room temperature for 2.5 h. Finally, the coupled resins were washed successively with 0.1 M and 10 mM Tris hydrochloride buffer (pH 8.0) and TE buffer (10 mM Tris hydrochloride [pH 8.0], 1 mM EDTA) containing 1.5 M and 0.1 M NaCl. The oligonucleotide resin storage buffer was the TE buffer containing 0.1 M NaCl and 0.02% sodium azide.

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (28). High-molecular-weight protein markers (Sigma Chemical Co.) were used. Gels were silver stained with Rapid Ag Stain kit (ICN Pharmaceuticals Inc.) by the instructions of the manufacturer.

Renaturation after SDS-PAGE. Protein from each of the SDS-PAGE gel slices was eluted and renatured as described by Hager and Burgess (19) except that 0.1 nM $ZnCl_2$ was included in the dilution buffer and the denaturation step using 6 M guanidium chloride was omitted such that the protein was renatured directly in the dilution buffer. The diluted and renatured samples were concentrated by using a Centricon-30 microconcentrator (Amicon Corp.). The gel retardation assay was performed by using 200 ng of poly(dI-dC) and 100 ng of either a mutant TRE oligonucleotide (MT-4) or a specific TRE oligonucleotide (WT).

In vitro transcription. Transcription reaction mixtures (25 μl each) contained 12 mM Tris (pH 7.9), 12% glycerol, 60 mM KCl, 2 mM $MgCl_2$, 0.6 mM CTP, 0.6 mM UTP, 0.6 mM ATP, 0.025 mM GTP, 0.03 mM [^{32}P]GTP, 0.5 μg of template DNA, and 10 μl of HeLa nuclear extract (80 μg of protein). After incubation for 60 min at 30°C, runoff transcripts were analyzed on a denaturing 4% polyacrylamide gel as described by Dignam et al. (7).

DNase I footprint analysis. The general DNase I footprinting protocol was carried out as previously described (38) except when otherwise noted in the figure legends. The E4 template was prepared as previously described (22). The HTLV-I template was either the *Sma*I-*Taq*I fragment (−322 to +263, upper strand) or the *Xma*I-*Taq*I fragment (−320 to +263, lower strand). The human c-*fos* template was the *Bss*HI-*Eco*RI fragment (−98 to +42).

RESULTS

Identification of three TREBs. To identify nuclear factors that might be involved in TAX-mediated activation, the gel retardation assay (12, 15) was used to screen HeLa nuclear extract-derived chromatographic fractions for proteins that bind specifically to the HTLV-I TRE (Fig. 1). The radiolabeled probe was a dimerized (head-to-tail) 48-bp oligonucleotide (TjT) containing two copies of the TRE. When poly(dI-dC) was used as the nonspecific competitor in the binding assays, two discrete protein-DNA complexes (designated B1 and B2) were observed with the 0.5 M KCl step-eluted phosphocellulose fraction (lane 1). When salmon sperm DNA was used as the nonspecific competitor, a distinct complex (designated B3) was formed with the 0.1 M KCl phosphocellulose flowthrough fraction (Fig. 1, lane 2) but not with the 0.5 M KCl fraction (data not shown). When a monomeric TRE oligonucleotide was used as the probe under the same conditions, exactly the same pattern of

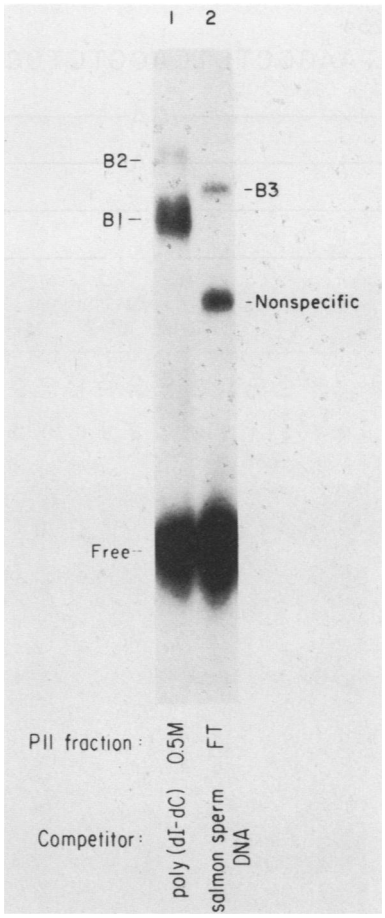


FIG. 1. Gel retardation assay. The probe was a dimerized TRE oligonucleotide (TjT). The nonspecific competitor was either 1 μg of poly(dI-dC) (lane 1) or 1.5 μg of salmon sperm DNA. The assayed protein fractions were either 0.1 M KCl phosphocellulose flowthrough fraction (lane 2) or 0.5 M KCl step-eluted phosphocellulose fraction (lane 1) of HeLa nuclear extracts. B1, B2, and B3, Specific protein-DNA complexes; Free, free probe.

specific complexes (B1 and B2) was observed with poly(dI-dC) as with the nonspecific competitor (Fig. 2B, lane 1). In contrast, complex B3 was barely detectable when a monomeric TRE oligonucleotide was used as the probe and salmon sperm DNA was used as the nonspecific competitor (data not shown), suggesting that complex B3 has a higher affinity for a multimerized TRE than for a single-copy TRE.

Complexes B1 and B2 were detected equally well with either the dimeric or monomeric TRE oligonucleotide probe (Fig. 1, lane 1, and Fig. 2B, lane 1). To demonstrate the sequence specificity of complexes B1 and B2, competition assays were performed (Fig. 2B) in the presence of increasing amounts of an unlabeled wild-type and mutated monomeric TRE oligonucleotide (Fig. 2A). Complexes B1 and B2 showed almost complete competition at a 100-fold molar excess of the specific competitor containing the wild-type sequence (Fig. 2B, lanes 2 to 4). An adjacent fragment derived from the 5′-flanking region (−162 to −121) of the HTLV-I promoter, as well as fragments derived from the Ad2 major late (ML) (−260 to +536) and IL-2R (−280 to −252) promoters, failed to inhibit the formation of complexes B1 and B2 when included as competitors in the binding reactions (data not shown). These results suggested,

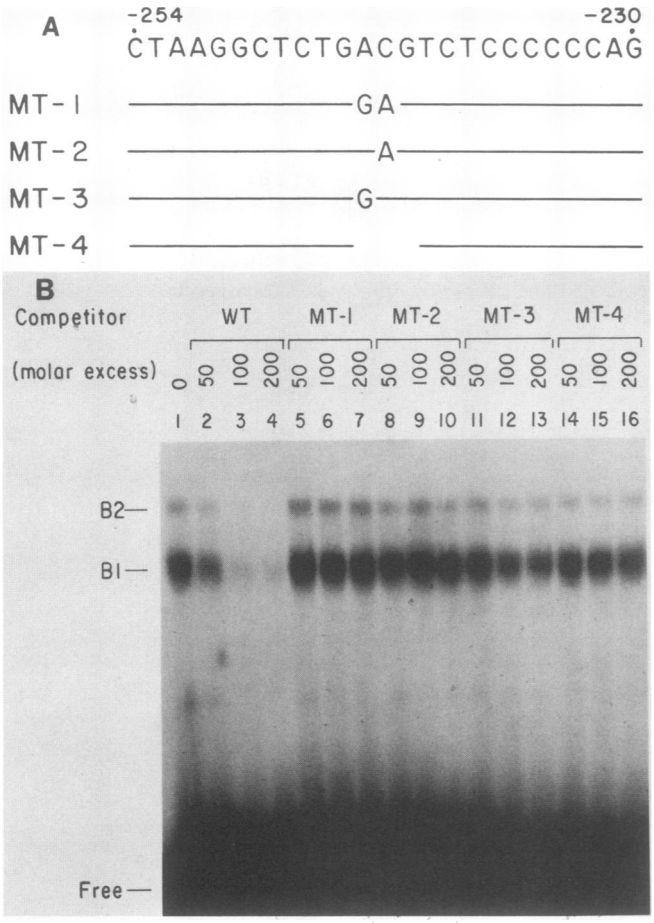


FIG. 2. Competition analysis of the complexes B1 and B2. (A) Substitution and deletion mutations in the center region of TRE. (B) Gel retardation assay using a monomeric TRE oligonucleotide (WT) as the probe and 1 μg of poly(dI-dC) as the nonspecific competitor. The assayed protein fraction was 0.5 M KCl step-eluted phosphocellulose fraction. Fold molar excess of unlabeled competitor in the binding reaction is indicated above each lane. B1 and B2, Specific protein-DNA complexes; Free, free probe.

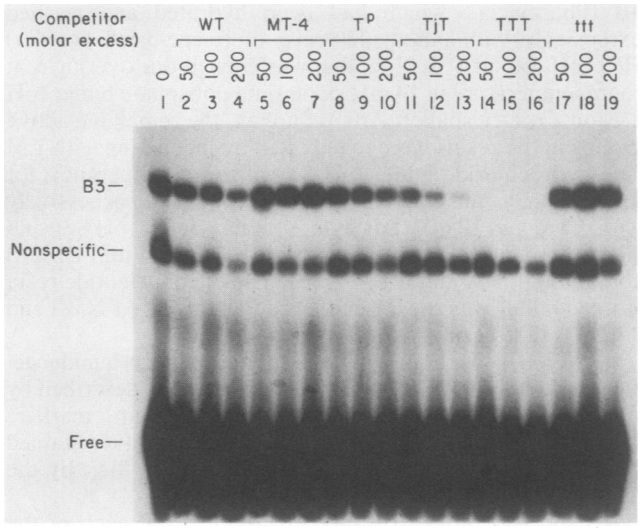


FIG. 3. Competition analysis of the complex B3. The probe was a dimerized TRE oligonucleotide (TjT). Salmon sperm DNA (1.5 μg) was used as the nonspecific competitor in the gel retardation assay. The assayed protein fraction was 0.1 M KCl phosphocellulose flowthrough fraction. Fold molar excess of unlabeled competitor in the binding reaction is indicated above each lane. B3, Specific protein-DNA complex; Free, free probe.

as further substantiated below, that complexes B1 and B2 were specific.

To evaluate the importance of the center of the TRE for formation of complexes B1 and B2, oligonucleotides with substitutions or deletions within the central three base pairs (Fig. 2A) were used in competition assays (Fig. 2B). In contrast to the wild-type oligonucleotide, mutated oligonucleotides MT-1 through MT-4 did not compete effectively with the probe for the B1 and B2 binding proteins in the 0.5 M KCl step-eluted phosphocellulose fraction, even at a 200-fold molar excess of competitor (Fig. 2B, lanes 5 to 16). Thus, the central three residues of the TRE sequences appear to be important for formation of both complexes B1 and B2.

The specific complex (B3) observed with the 0.1 M phosphocellulose flowthrough fraction competed weakly with a 200-fold molar excess of the wild-type TRE oligonucleotide (Fig. 3, lanes 2 to 4) but not with a mutated TRE oligonucleotide MT-4 (Fig. 3, lanes 5 to 7). A longer (50-bp) promoter fragment (T^P) which contained a single TRE element showed a competition profile similar to that of the shorter (25-bp) monomeric TRE oligonucleotide (WT) (lanes 8 to 10). In contrast, an unlabeled oligonucleotide (TjT) identical to the 48-bp probe (containing a dimeric TRE) competed very efficiently with the specific complex B3 (lanes 11 to 13). To eliminate the possibility that complex B3 resulted from spurious interactions of a factor with the junction sequences created by ligation of two monomeric oligonucleotides, a 66-bp synthetic oligonucleotide containing a trimerized TRE and devoid of the junction sequences present in the dimerized probe was used as a specific competitor. This triple TRE was even more effective than the dimerized oligonucleotide in inhibiting the formation of the B3 complex (lanes 14 to 16). However, the introduction of a 3-bp deletion in the center of each of the individual TRE elements abolished the ability of the triple TRE oligonucleotide to act as a specific competitor (lanes 17 to 19). In contrast to the results with complexes B1 and B2, these findings indicate that the factor involved in the formation of complex B3 has a higher affinity for multiple copies of the TRE than for a single copy and that the binding specificity of the factor in complex B3 is related to the factors in complexes B1 and B2. In contrast to the observations with complex B3, both complexes B1 and B2 competed equally well with either monomeric or trimeric TRE oligonucleotide (data not shown).

The nuclear factors responsible for the specific protein-DNA complexes, B1, B2, and B3, were assigned the names of TREB-1, TREB-2, and TREB-3, respectively.

Importance of TGACG motif in TAX inducibility. To examine the importance of the TGACG sequence in the center of the TREs, plasmids pT_3CAT and pt_3CAT were constructed. pT_3CAT was constructed by inserting a synthetic triple TRE oligonucleotide, TTT, into a SVCAT plasmid which lacks an enhancer element but contains an intact simian virus 40 early promoter region. A mutant version of pT_3CAT, pt_3CAT, was constructed by using a synthetic mutant triple TRE oligonucleotide, ttt, with a 3-bp deletion of each of the three TGacg motifs. The inducibility

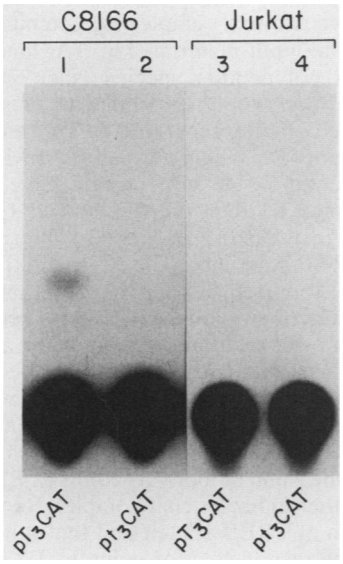


FIG. 4. CAT assays of the inducible expression of T_3CAT by TAX. Jurkat or C8166 cells were transfected with 4 μg of either pT_3CAT or pt_3CAT.

of the wild-type (pT_3CAT) and mutant (pt_3CAT) plasmids was examined by transfecting into either Jurkat (TAX-negative) T cells or C8166 HTLV-I-transformed (TAX-positive) T cells. As shown in Fig. 4, T_3CAT expression was induced in C8166 (TAX-positive) cells (lane 1) but not in Jurkat (TAX-negative) cells (lanes 3), whereas t_3CAT expression was not observed in either case (lanes 2 and 4). Similarly, T_3CAT (but not t_3CAT) expression was induced in Jurkat (and other TAX-negative) cells when cotransfecting with the TAX expression plasmid (data not shown). This demonstrates that the synthetic triple TRE oligonucleotide is sufficient to confer TAX inducibility to a heterologous promoter and that the 3-bp deletion mutation disrupting the TGacgTCT consensus disrupts not only the formation of the three sequence-specific protein-DNA complexes (B1, B2 and B3) in vitro (Fig. 2 and 3) but also the expression of the promoter in vivo.

Purification of TREB-1, TREB-2, and TREB-3. The factors responsible for complexes B1, B2, and B3 were totally or partially purified by using the scheme shown in Fig. 5. Activities of TREB-1, TREB-2, and TREB-3 were monitored by gel retardation assays using radiolabeled monomeric and dimeric TRE oligonucleotides, respectively, as the probes. Because complex B1 was the most abundant of the three complexes, the initial emphasis was on TREB-1. A summary of the entire purification process for this factor is presented in Table 1. The key steps were a mutant oligonucleotide affinity column, on which TREB-1 eluted at low salt concentrations, and two different specific oligonucleotide columns (27, 47), on which the activity eluted at high salt concentrations. The first specific oligonucleotide (column 1) was a triple TRE oligonucleotide (TTT), and the second oligonucleotide (column 2) was the 50-bp promoter fragment (T^P).

Identification of TREB-1. The proteins in the gradient fractions of the second DNA affinity column (column 2) were visualized by silver staining after SDS-PAGE (Fig. 6). The

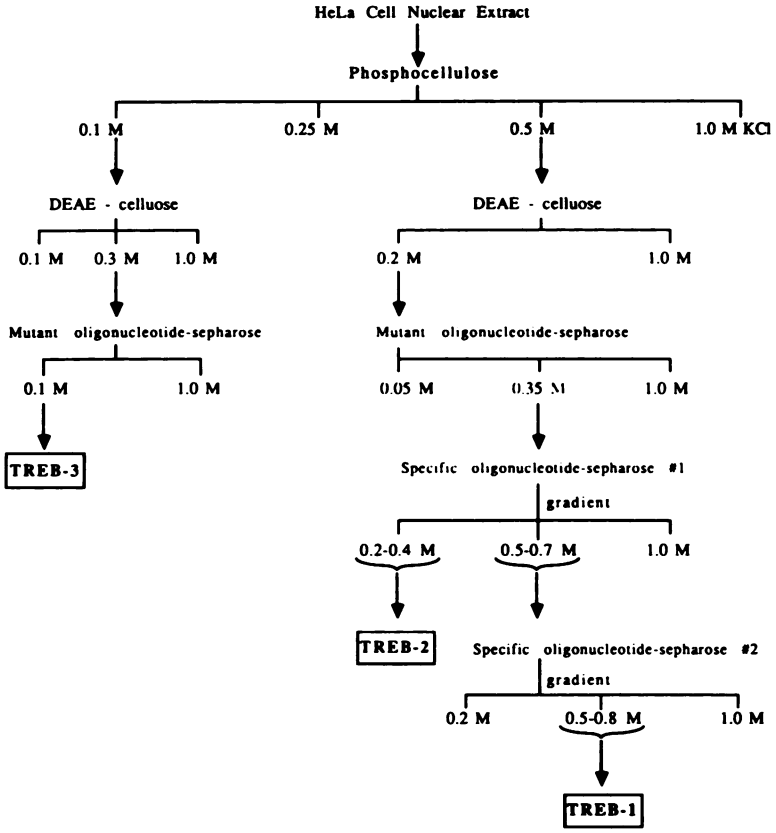


FIG. 5. Purification scheme for the HTLV-I TRE-binding proteins, TREB-1, TREB-2, and TREB-3.

TABLE 1. Purification table of TREB-1

Fraction	Total protein (mg)	Total activity (U)[a]	Sp act (U/mg)	Fold purification	Yield (%)
Nuclear extract	1,000	170,000	170		100
Phosphocellulose	160	100,000	625	3.7	59
DEAE-cellulose	100	70,000	700	1.1	41
Nonspecific DNA affinity	8	40,000	5,000	7.1	24
Specific DNA affinity					
Column 1	0.02	14,000	700,000	140	8
Column 2	0.008	10,000	1,250,000	1.8	6

[a] One unit (binding activity) is the amount of TREB-1 that under standard gel retardation conditions retards 1 fmol of end-labeled probe.

most striking feature was the presence of three prominent polypeptide species, ranging from 35 to 43 kDa, that were consistently present in the fractions containing TREB-1 activity (Fig. 6, lanes 3 to 6). To prove that one or more of the major 35- to 43-kDa polypeptide species purified by the TRE-oligonucleotide affinity column were responsible for TREB-1 activity, we monitored TREB-1 activity following renaturation of the protein eluted from a preparative SDS gel. Thus, after electrophoresis, the gel was cut into five slices, the boundaries of which were marked (Fig. 7A). After isolation and renaturation, the binding activities of these proteins were analyzed by a gel retardation assay (Fig. 7A). The renatured 35- to 43-kDa polypeptides (slice 4) gave rise to a complex (lane 4, nonspecific competitor panel) that had a relative mobility equal to that generated by the input material (data not shown). This complex specifically competed with a monomeric wild-type TRE oligonucleotide (WT; lane 4, specific competitor panel) but not with a mutated monomeric TRE oligonucleotide (MT-4; lane 4, nonspecific competitor panel). This demonstrates that the major 35- to 43-kDa polypeptide species have specific TREB-1 binding activity.

In another experiment, a preparative SDS gel was stained with Coomassie brilliant blue and each of the 43- and 35- to 39-kDa proteins were isolated, renatured, and assayed for TREB-1 activity. The 43-kDa polypeptide alone (slice 6) was

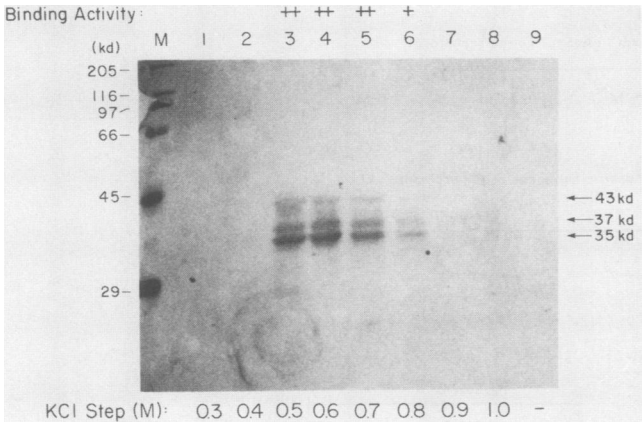


FIG. 6. SDS gel analysis of the chromatographic fractions. Ten microliters of each of the specific oligonucleotide column fractions was loaded on a 10% SDS-polyacrylamide gel; after electrophoresis, the gel was silver stained. The degree of specific TREB-1 binding activity detected by gel retardation assay is indicated (+ or ++) above lanes 3 to 6. kd, Kilodaltons.

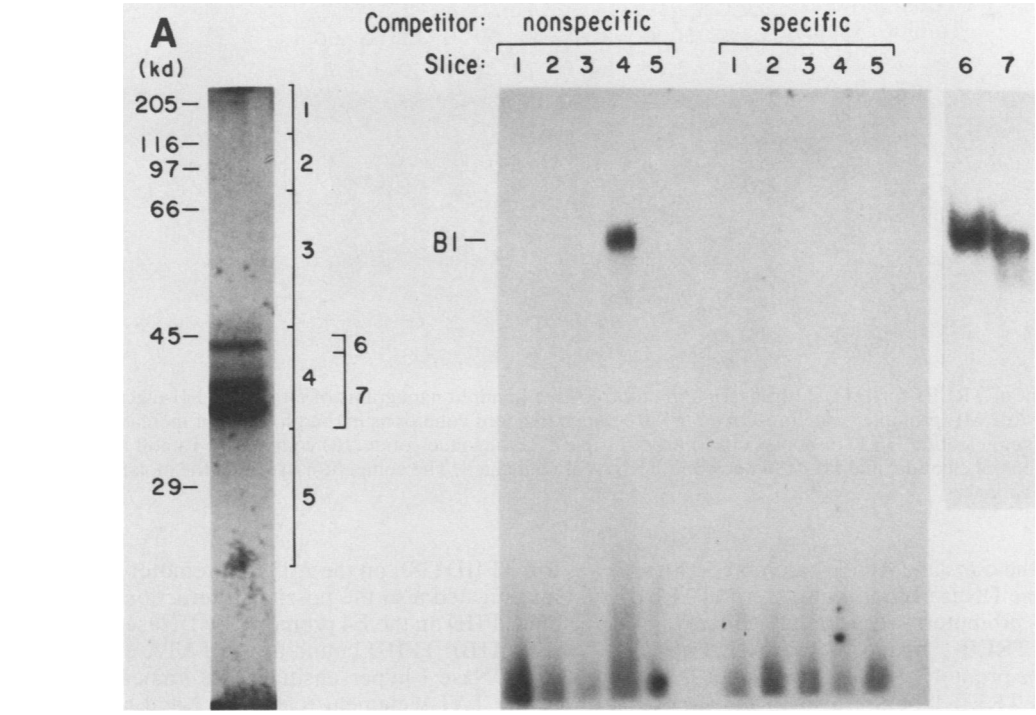
sufficient to give rise to a complex that comigrated with that generated by the input material (Fig. 7A, lane 6), while the 35- to 39-kDa polypeptide species (slice 7) gave rise to complexes that had mobilities similar to or slightly greater than that of the complex generated by the renatured 43-kDa polypeptide (lane 7). When normalized to input levels of protein, the renatured 43-kDa protein gave much greater levels of complex B1 than did the smaller (35- to 39-kDa) proteins. This established that a 43-kDa component had most of the TREB-1 activity. The 35- to 39-kDa polypeptide species could represent either degradation products of TREB-1 or distinct but related TREB-1 polypeptide species.

The result of a gel retardation assay using affinity-purified TREB-1 and a monomeric TRE oligonucleotide probe is shown in Fig. 7B. As expected, TREB-1 formed the specific B1 complex (cf. lanes 4 and 6) but, importantly, increasing amounts of TREB-1 did not give rise to any slower-migrating complex with the same mobility as complex B2 (lanes 1 to 4). This result further indicates that complex B2 was not due to oligomerization of TREB-1 itself and that other polypeptide species must be responsible for complex B2 formation.

Transcriptional activation by purified TREB-1. We have used an HTLV-I promoter-containing template to test transcriptional activation by the affinity-purified TREB-1. The template contained sequences from −350 to +325 and included the entire HTLV-I enhancer-promoter region. When transcribed in HeLa nuclear extracts (7), this template generated a 325-nucleotide runoff transcript corresponding to specific initiation at the in vivo cAP site (Fig. 8, lane 1). The promoter sequence specificity of this transcription was examined by competition with a TRE oligonucleotide (Fig. 8). Increasing amounts of triple TRE oligonucleotide (TTT) virtually abolished transcription from the HTLV-I promoter (cf. lanes 1 to 4) with no effect on transcription from the Ad2 ML promoter (cf. lanes 9 to 12). As expected, equivalent amounts of nonspecific competitor (mutated triple TRE oligonucleotide, ttt) had no effect on transcription from either the HTLV-I (cf. lanes 5 to 8) or ML promoter (data not shown). These data further demonstrate the specificity of the 325-nucleotide runoff transcript and indicate that the binding of TREBs to its recognition site was required for a significant level of transcription from the HTLV-I promoter.

When affinity-purified TREB-1 was added to the TREB-sequestered HeLa extract, transcription from the HTLV-I promoter was activated up to about threefold (Fig. 9, cf. lanes 1 to 3). The endogenous TREBs in HeLa extracts were sequestered by the addition of a 25-fold molar excess of wild-type triple TRE oligonucleotide (determined by the titration experiment shown in Fig. 8). In contrast, purified TREB-1 did not stimulate the transcription from the ML promoter (cf. lanes 4 to 6). This proves that the affinity-purified TREB-1 is transcriptionally active and, along with the above results, indicates that the purified TREB-1 activated transcription from the HTLV-I promoter through the interaction with TREs, rather than in a nonspecific manner.

DNase I footprint analysis of purified TREB-1. The binding of the affinity-purified TREB-1 to the HTLV-I promoter was investigated by DNase I footprint analysis (Fig. 10). The HTLV-I promoter fragment, labeled on either the upper or the lower strand, was assayed in the presence of increasing amounts of the affinity-purified TREB-1 (Fig. 10, lanes 2 to 6). TREB-1 bound to all the three TREs, albeit with a relatively lower affinity for the first TRE (Fig. 10, cf. lanes 1, 4, and 5). Highly purified TREB-1 also partially protected regions downstream and upstream of the second TRE (centered around −170 and −220) and a region downstream of



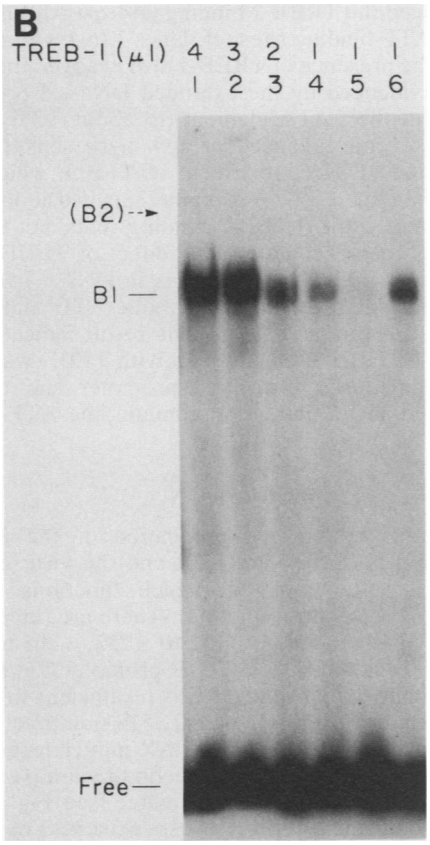


FIG. 7. Renaturation and gel retardation assays of purified TREB-1. (A) Renaturation of TREB-1 binding activity isolated from SDS-polyacrylamide gel. Affinity-purified TREB-1 was run on an SDS-polyacrylamide gel, and slices 1 through 7 isolated from a non-silver-stained lane were run in parallel. The regions of the sample lane that were excised are indicated. After isolation from gel slices and renaturation, the materials were used in a gel retardation assay containing 100 ng of either mutated TRE oligonucleotide (MT-4) (lanes 1 to 5) or wild-type monomeric TRE oligonucleotide (WT) (lanes 6 to 10) as the competitor DNA. kd, Kilodaltons. (B) The binding activity of affinity-purified TREB-1 fraction was tested by the gel retardation assay using radiolabeled monomeric TRE oligonucleotide as a probe. Different amounts of affinity-purified TREB-1 were used as indicated above each lane. The competitors used were monomeric TRE oligonucleotide (WT) (lane 5) and mutated TRE oligonucleotide (MT-4) (lane 6).

the cAP site (around +60 to +80) (Fig. 10, lower, lane 6). Interestingly, a basal promoter element (+6 to +306) also has been mapped to the downstream region of the cAP site (41; see Fig. 12). The significance of TREB-1 binding to this region is, at present, unclear.

Intriguingly, the TGACG motif found in the center of the TRE is also present in activating transcription factor (ATF)-binding sites (29) and cyclic AMP-responsive elements (CREs) (37). Therefore, we have examined potential binding of the affinity-purified TREB-1 to known CREs and ATF-

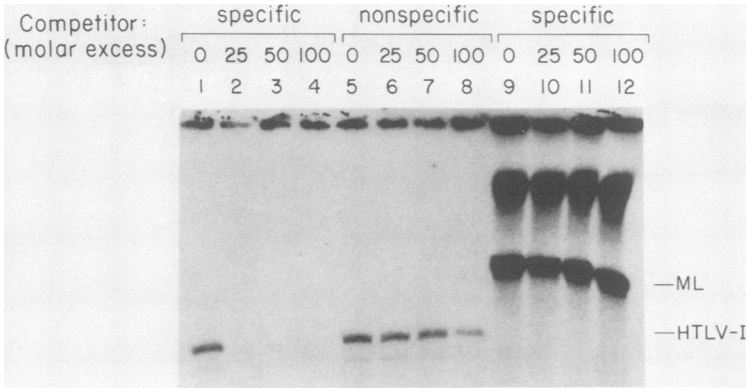


FIG. 8. Requirement of TREBs for HTLV-I transcription in vitro. Five hundred nanograms of either *Hin*dIII-digested HTLV-I (lanes 1 to 8) or *Sma*I-digested Ad2 ML promoter was transcribed in vitro under standard conditions in the presence of increasing amounts of either specific (triple TRE oligonucleotide, TTT) or nonspecific (mutated triple TRE oligonucleotide, ttt) competitor. Runoff transcription products representing HTLV-I (325-nucleotide) and ML (536-nucleotide) RNAs are indicated. The competitor used and the molar excess of competitor are indicated above each lane.

binding sites within the c-*fos* and Ad5 E4 promoters. Figures 11A and 11B show the DNase I footprint pattern of TREB-1 on the c-*fos* and E4 promoters, respectively. Interestingly, the affinity-purified TREB-1 bound to the CRE (−60 element) within the c-*fos* promoter (Fig. 11A, cf. lanes 1 and 5). The affinity-purified TREB-1 bound also to the first (Fig. 11B, ATF-I) and the third (ATF-III) but not the second (ATF-II) ATF-binding sites within the Ad5 E4 promoter (Fig. 11B, cf. lanes 1 and 5).

Since the molecular mass (35 to 43 kDa) of TREB-1 is similar to that of the rat somatostatin gene CRE-binding factor (CREB; 43 kDa) (61) and that of ATF (43 to 47 kDa) (20), and since TREB-1 also binds effectively to both CREs and ATF-binding sites, it is reasonable to speculate that TREB-1, CREB, and ATF may be similar or identical factors. We have shown previously that the affinity-purified 43- to 47-kDa ATF (20) interacted with TATA-binding factor, TFIID (38), on the Ad5 E4 promoter (22). Therefore, we investigated also the possible interactions between TREB-1 and TFIID on the E4 promoter by DNase I footprint analysis (Fig. 11B). TFIID bound to the TATA element and created two DNase I hypersensitive sites immediately downstream of the TATA element (cf. lanes 1 and 6). The presence of TFIID enhanced the TREB-1 binding (at least twofold) to all three of the ATF-binding sites (cf. lanes 1 to 5 with 6 to 10). In addition, the presence of TREB-1 also changed the TFIID binding, as evidenced by the extended DNase I protection downstream of the TATA element (to about +35) and the presence of several enhanced or new hypersensitive sites (cf. lanes 6 and 9). Heat treatment of TFIID, which abolished its binding to the TATA element (38), also abolished its ability to enhance the TREB-1 binding (data not shown). This indicates the specificity of the effect of TFIID on the TREB-1 binding. Identical alterations in DNase I footprint pattern were also observed when both ATF and TFIID bound to the E4 promoter (22). This result indicates that, similar to ATF, TREB-1 can interact with TFIID when both bound simultaneously to the E4 promoter and that the affinity-purified TREB-1 fraction contains the ATF-binding activity.

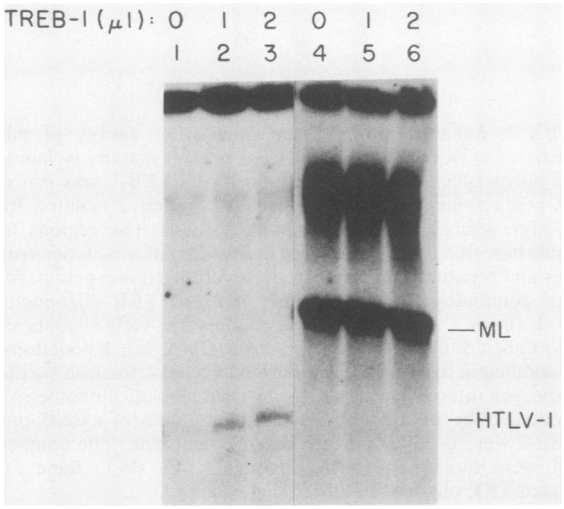


FIG. 9. Transcription activity of purified TREB-1 in vitro. Five hundred nanograms of either HTLV-I (lanes 1 to 3) or ML (lanes 4 to 6) template was transcribed in vitro in the presence of a 25-fold molar excess of triple TRE oligonucleotide, TTT (lanes 1 to 6), and either 1 (lanes 2 and 4) or 2 (lanes 3 and 6) μl of affinity-purified TREB-1. Runoff transcription products representing HTLV-I (325-nucleotide) and ML (536-nucleotide) RNAs are indicated.

DISCUSSION

Efficient transcription and replication of the HTLV-I genome require both the viral LTR and the virus-encoded transcriptional activator TAX, which functions through TREs in the LTR (see Introduction). There are three copies of a 21-bp TRE at sequences −252 to −232, −203 to −183, and −104 to −84 in the HTLV-I LTR promoter (26; Fig. 12). Although a single copy of the TRE is insufficient to effect a significant response to TAX (4, 42, 51), multiple tandem copies of the TRE do act as a TAX-inducible enhancer element (4, 45, 51). In addition, a second region (sequences −160 to −117) of the HTLV-I promoter (see Fig. 12) has been shown to augment the TAX responsiveness of a single 21-bp TRE; however, it alone is not sufficient to confer TAX inducibility (4, 42). To study the biochemical mechanism of the transcriptional activation mediated by TAX, we have conducted a systematic study of the nuclear factors that bind to the HTLV-I TRE.

We have identified and either partially or completely purified three nuclear factors that bind to the TREs within

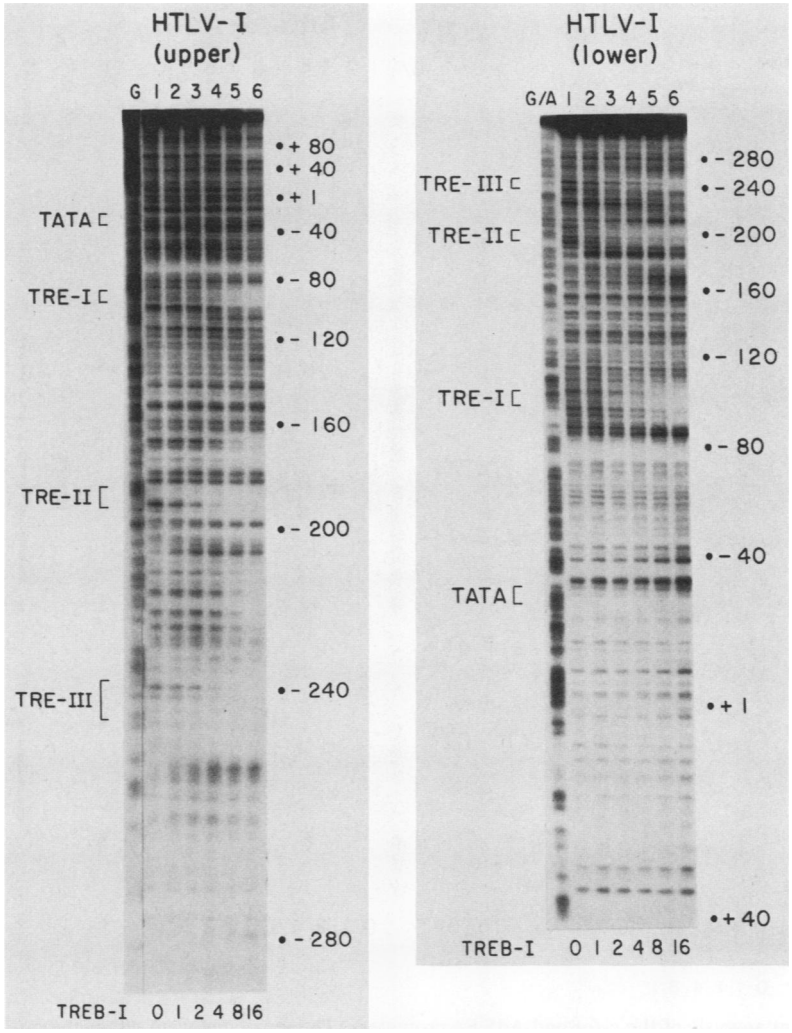


FIG. 10. DNase I footprint analysis of the HTLV-I promoter. The template (−321 to +137) was labeled at the 3′ end of either the transcribed (upper) strand or the nontranscribed (lower) strand. TREs (I to III) are numbered from the site closest to the TATA element. Radiolabeled DNA fragments were incubated with increasing amounts of TREB-1. The presence or absence of TREB-1 and relative amounts of purified TREB-1 in each reaction are indicated below the autoradiogram. G+A or G sequencing reactions were run in adjacent lanes. The locations of the TATA element and TREs are indicated.

the HTLV-I promoter. The factors are designated TREB-1, TREB-2, and TREB-3, and the corresponding specific protein-DNA complexes are designated B1, B2, and B3. Mutations in the center of the TRE disrupted both the formation of the three protein-DNA complexes in vitro (Fig. 2 and 3) and expression from this promoter in vivo (Fig. 4). TREB-1 and TREB-2 appeared to be distinct since they were separable by a specific oligonucleotide affinity column (Fig. 5) and by differential ammonium sulfate precipitation (data not shown); in addition, complex B2 was not formed with an affinity-purified TREB-1 fraction (Fig. 7B). TREB-3 appears to be different from TREB-1 and TREB-2 for the following reasons: (i) different nonspecific competitors were required for the efficient detection of the complex B3 (salmon sperm DNA) and complexes B1 and B2 [poly(dI-dC)] (Fig. 1); (ii) the mobilities of complexes B1, B2, and B3 were clearly different (Fig. 1); (iii) TREB-3 was separable from TREB-1 and TREB-2 by phosphocellulose chromatography (Fig. 5); (iv) TREB-3 activity was heat sensitive, while TREB-1 and TREB-2 were heat stable (data not shown); (v) TREB-3 bound preferentially to a multimerized TRE (Fig. 3), while TREB-1 and TREB-2 failed to show such an apparent preferential binding. After this manuscript was submitted, Jeang et al. (25) reported the binding of a much larger 180-kDa TRE-binding protein. This protein was partially purified, and its molecular weight was identified solely by a UV cross-linking experiment. It is possible that it is identical to either TREB-2 or TREB-3 identified in this report or that the apparent molecular weight determined by the UV cross-linking technique is equivalent to that of the multimeric protein complex (an observation reported previously [61]).

TREB-1 has been purified to near homogeneity by affinity chromatography on a TRE oligonucleotide column, and binding activity was localized to a protein of 35 to 43 kDa. The affinity-purified TREB-1 was shown to activate transcription from the HTLV-I promoter in vitro and is a good candidate for the factor that mediates either basal activation or the *trans*-activation by TAX in vivo. However, this does not preclude the possibility that TREB-2 or TREB-3 is also involved in either basal or TAX-induced transcription. A more interesting possibility is that distinct TREBs are involved in basal activity versus the TAX-induced response.

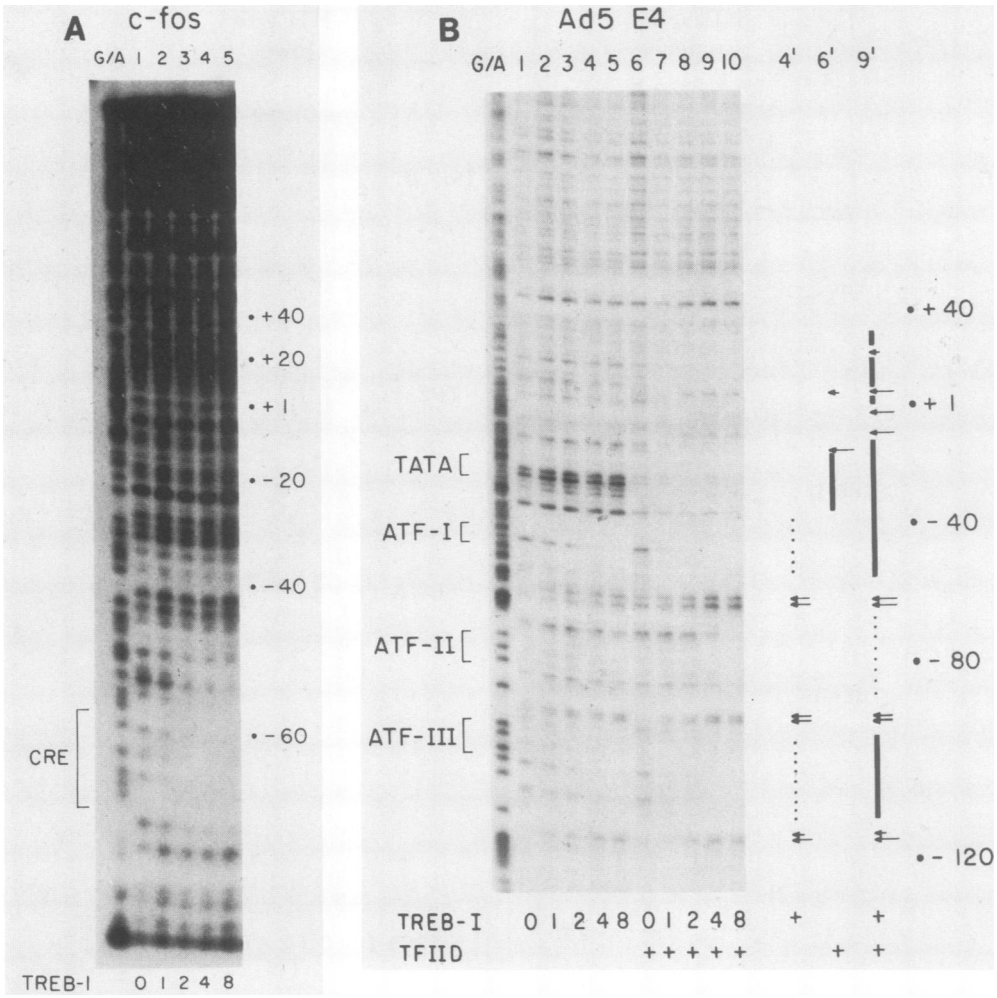


FIG. 11. DNase I footprint analysis of the c-*fos* and Ad5 E4 promoters. The templates were either the c-*fos* promoter fragment (−98 to +42) or the Ad5 E4 promoter fragment (−266 to +250). The locations of the TATA element, CREs, and ATF-binding sites are indicated. The positions of the ATF-binding sites are indicated and numbered (I to III) from the site closest to the TATA element. The lanes on the right summarize the footprint pattern in the representative lanes. The protected regions are marked, and the relative degrees of protection are indicated by the dotted (weak) or solid (strong) lines. The hypersensitive sites are marked by arrows, and the arrow lengths indicate their relative intensities.

The observations that at least two copies of the TRE are necessary to act effectively as the TAX-inducible enhancer (4, 42, 51) and that TREB-3, in contrast to TREB-1 and TREB-2, binds preferentially to multiple copies of the TRE suggest the interesting possibility that TREB-3 may, in fact, play a greater role in mediating the TAX activation.

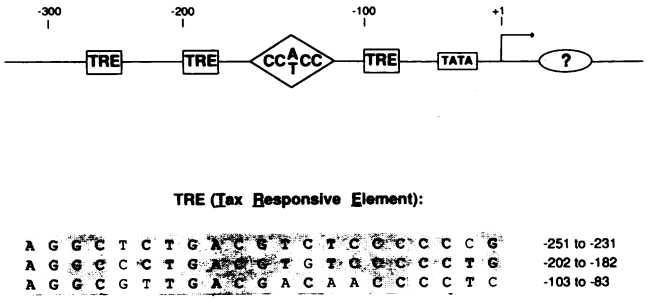


FIG. 12. Schematic representation of the HTLV-I promoter elements.

Interestingly, the TGACG motif found in the center of the TRE is also present in ATF-binding sites (29) and CREs (37). Recently, several laboratories have suggested that ATF and CREB are highly related or identical (21, 32). We have demonstrated TREB-1 binding to the CREs or ATF-binding sites on both the c-*fos* and Ad5 E4 promoters and a cooperative interaction between TREB-1 and TFIID (the TATA-binding factor; 38), as evidenced by both qualitative and quantitative changes in the binding of one factor in the presence of the other on the Ad5 E4 promoters. A similar interaction between TFIID and ATF, purified by virtue of its affinity for the E4 promoter ATF sites, was also observed on the E4 promoter (22). The observations that TREB-1 and ATF (20) have similar molecular masses (35 to 43 kDa and 43 to 47 kDa, respectively) and that affinity-purified TREB-1 binds also to the ATF sites and interacts (similar to ATF) with TFIID on the E4 promoter suggest that TREB-1 and ATF are highly related, if not identical, factors. It may be noted, however, that TREB-1 was purified by using an affinity matrix (HTLV-I promoter sequence) distinct from that (E4 promoter sequence) used for ATF, and that this is

the first report of a purified factor that binds to functional HTLV-I promoter elements. We also show, for the first time, that TREB-1 stimulates transcription from the HTLV-I promoter, indicating the functional relevance of this factor and its promoter interactions.

The mechanism for transcriptional activation of HTLV-I LTR expression by TAX remains unclear. Since similar DNA-protein interactions were observed in the absence or presence of TAX (1, 40), it is unlikely that TAX binds to DNA directly or leads to de novo synthesis of cellular factors that mediate TAX action. More intriguing possibilities are that *tax* expression results in the posttranslational modification of a constitutively expressed transcription factor from an inactive to an active form (e.g., enhanced DNA-binding activity or enhanced functional or transcriptional activity) or that TAX participates directly in the formation of an active transcription initiation machinery. The TGACG motif found in the center of the TRE is also present in the ATF-binding sites (adenovirus E1a responsive) (29) and CREs (37). Interestingly, the CRE within the proenkephalin gene promoter can be induced by either cyclic AMP or phorbol ester, 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (5). This raises the interesting possibilities that the 21-bp TRE could be also induced by adenovirus E1a, cAMP, or TPA and that TAX may enter the HTLV-I regulatory pathway by mimicry of a mitogenic response.

It is an intriguing possibility that the different TREB factors play distinct roles in the HTLV-I gene expression (e.g., basal versus induced expression, activation by different inducing agents, or mediation of T lymphotropism versus neurotropism). Although confirmation of this possibility awaits further investigation, the present study provides a first step in the definition and mechanistic analysis of the transcription factors that mediate the basal or TAX-inducible HTLV-I enhancer function. Further studies of the action of these factors from different sources (e.g., T and neural cells or cells treated with various inducing agents) and in the context of *tax* expression should lead to an understanding of the mechanism of action of TAX and its role in leukemogenesis and pathogenesis of HTLV-I.

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