In Vitro Activation of Transcription by the Human T-Cell Leukemia Virus Type I Tax Protein

MAURA-ANN H. MATTHEWS, RHEA-BETH MARKOWITZ, AND WILLIAM S. DYNAN*

Department of Chemistry and Biochemistry, University of Colorado, Boulder, Colorado 80309-0215

Received 16 October 1991/Accepted 7 February 1992

The human T-cell leukemia virus type I (HTLV-I) regulatory protein Tax activates transcription of the proviral long terminal repeats and a number of cellular promoters. We have developed an in vitro system to characterize the mechanism by which Tax interacts with the host cell transcription machinery. Tax was purified from cells infected with a baculovirus expression vector. Addition of these Tax preparations to nuclear extracts from uninfected human T lymphocytes activated transcription of the HTLV-I long terminal repeat approximately 10-fold. Transcription-stimulatory activity copurified with the immunoreactive 40-kDa Tax polypeptide on gel filtration chromatography, and, as expected, the effect of recombinant Tax was diminished in HTLV-I-infected T-lymphocyte extracts containing endogenous Tax. Tax-mediated transactivation in vivo has been previously shown to require 21-bp-repeat Tax-responsive elements (TxREs) in the promoter DNA. Stimulation of transcription in vitro was also strongly dependent on these sequences. To investigate the mechanism of Tax transactivation, cellular proteins that bind the 21-bp-repeat TxREs were prepared by DNA affinity chromatography. Recombinant Tax markedly increased the formation of a specific host protein-DNA complex detected in an electrophoretic mobility shift assay. These data suggest that Tax activates transcription through a direct interaction with cellular proteins that bind to the 21-bp-repeat TxREs.

Human T-cell leukemia virus type I (HTLV-I) is the etiologic agent of adult T-cell leukemia (22, 28, 57) and is also associated with several neurologic and muscular degenerative diseases, including HTLV-associated myelopathy and tropical spastic paresis and polymyositis (reviewed in reference 48). As with all retroviruses, proviral DNA is transcribed into viral mRNA and new genomic RNA by host cell RNA polymerase II. Transcription originates from the long terminal repeat (LTR) of the proviral genome. Numerous cotransfection experiments have shown that the *tax* gene product (Tax), a 40-kDa protein encoded at the 3' end of the HTLV-I viral genome, increases transcription from the proviral LTR (5, 6, 9, 10, 65, 66, 71) and a number of other promoters (7, 18, 32, 44, 47, 52, 61, 64, 67, 68). Tax elicits a variety of pathological changes in transgenic mice (18, 19, 21, 49–51, 80) and contributes to the transformation of cultured cells (17, 60, 77, 80). Because *tax* functions as a viral oncogene, the mechanism by which it interacts with the host cell transcriptional machinery is of considerable interest.

The Tax-responsive elements (TxREs) in the HTLV-I LTR include three imperfect 21-bp sequence repeats (Fig. 1B, sites 1, 2, and 3) (5, 11, 14, 27, 42, 43, 45, 66). In addition, a region between the second and third repeats increases response to Tax when in the context of at least one 21-bp repeat (42, 43). The mechanism of interaction between these elements and Tax remains incompletely understood. Tax-mediated transactivation does not require the synthesis of new host cell proteins (14, 27). A comparison of nuclear extracts from HTLV-I-transformed T lymphocytes that do and do not express Tax shows that none of the observed features of the DNase I footprint pattern correlate directly with the presence of Tax in the extract (1, 54). These results suggest that the primary recognition of promoter elements in the LTR involves specific interactions with preexisting host

cell proteins and that Tax influences the activity of one or more of these proteins. For example, Tax might modify a host cell protein in a manner that alters its ability to bind DNA or to be recognized by the general transcriptional machinery. Alternatively, Tax might bind indirectly to the promoter via host cell proteins, perhaps serving as an adaptor or mediator of the interactions between transcription factors bound at different sites.

Several experiments suggest that localization of Tax at the promoter may be important for its activity. A GAL4-Tax fusion protein, but not Tax alone, transactivated a construct containing GAL4 binding sites (12). Tax has been shown to bind indirectly to the HTLV-I promoter via a host cell factor that binds between the second and third 21-bp-repeat TxREs (42). Interpretation of this finding is complicated, however, since the recognition site for the host cell factor is not essential in all contexts and since, by using this same technique, indirect binding to the 21-bp TxRE could not be demonstrated (42).

All three HTLV-I 21-bp-repeat TxREs contain a TGACG core sequence that also occurs in the E1a-activated early genes of human adenoviruses (30) and in the cyclic AMP (cAMP) response element (CRE) (46). The 21-bp repeats of HTLV-I direct cAMP-dependent transcription (14, 24, 59, 76); however, an element containing the 8-bp dyad-symmetric consensus CRE, TGACGTCA, is not Tax responsive (11). Moreover, HTLV-I expression is superinduced by cAMP in the presence of Tax (59). Together, these data suggest that although a similar sequence is recognized by transcription factors concerned with the cAMP response and Tax transactivation, these two pathways of induction are independent.

A number of cellular proteins that bind the HTLV-I 21-bp-repeat TxRE have been identified. Four proteins, TREB5, TREB7, TREB36, and TAXREB67 (with molecular sizes of 29, 55, 29, and 52 kDa, respectively) were identified by screening cDNA libraries for expression of proteins that bind a 21-bp-repeat TxRE oligonucleotide (78, 81). TREB7 is

* Corresponding author.

FIG. 1. In vitro transcription with uninfected or HTLV-I-infected cell extracts. (A) Transcription reaction mixtures contained SPTK3 reference template (lanes 1 and 2) or HTLV-I templates (lanes 3 to 18) coupled to agarose beads as described in Materials and Methods. Transcription reactions were performed with nuclear extract from the uninfected T-lymphocyte cell line CEM (odd-numbered lanes) or the HTLV-I-infected T-lymphocyte cell line SLB-I (even-numbered lanes) as described in Materials and Methods. Radiolabeled runoff transcripts were analyzed by urea-PAGE and visualized by autoradiography. Position of the correctly initiated runoff transcript for the reference template is indicated by the arrow at the left; position of the correctly initiated HTLV-I transcript is indicated by the arrow at the right. A slightly longer HTLV-I transcript, which initiates approximately 30 bp upstream of the in vivo RNA cap site, is a reproducible characteristic of SLB-I extracts and is sometimes observed with CEM extracts. Radiolabeled single-stranded DNA fragments were used to estimate the length of runoff transcription products. Marker length is shown in nucleotides at the left. (B) Sequences present in HTLV-I templates. Construct names indicate the number of nucleotides of HTLV-I sequence present upstream of the cap site and whether additional sequences (21-bp-repeat TxREs or site 2a) have been inserted. Stippled boxes represent the three imperfect 21-bp-repeat TxREs, which are located as follows in the wild-type HTLV-I, relative to the in vivo RNA cap site indicated by the arrow (nucleotide positions are indicated): site 1, -253 to -233; site 2, -203 to -183; and site 3, -104 to -84. Open boxes represent site 2a sequence, nucleotides -160 to -117 (see text).

identical to CRE-BP1 and differs by two amino acids from ATF-2 (20, 38, 81). TREB36 is identical to ATF-1 and related to rat CREB (16, 20, 81); TAXREB67 is identical to ATF-4 (20, 78). Other 21-bp-repeat TxRE-binding proteins have been identified by using DNA affinity chromatography. TREB-1, a group of polypeptides with molecular sizes in the range of 35 to 43 kDa, has been purified from HeLa cell extracts (75). HEF-IT, a group of polypeptides with molecular sizes of 41 to 43 and 59 kDa, has been purified from lymphocyte extracts (53). Lymphocyte polypeptides with molecular sizes 32, 36 to 42, 50, and 110 kDa were identified as binding the 21-bp-repeat TxRE by DNA affinity chromatography, glycerol gradient sedimentation, and UV cross-linking experiments (4). Although the preparations in these reports are not identical, they may have several polypeptides in common. Less well characterized host cell factors, some of which may be identical to those identified by screening cDNA libraries or by DNA affinity chromatography, including HEF-IB, TREB-2, TREB-3, HEB1, HEB2, PKAF, NF21, and unnamed 110-, 120-, and 180-kDa polypeptides (24, 45, 53, 56, 58), have been identified by electrophoretic mobility shift assays (EMSA) and UV cross-linking. Other proteins, including AP-2, Sp1, NF-1, HEF-4C, TIF-1, and Ets1 (15, 43, 55), bind to the HTLV-I promoter at sequences outside the 21-bp-repeat TxRE. Identifying which of these proteins, if any, mediate the Tax response is difficult since such a large number of proteins can interact with the HTLV-I promoter. For example, Jun/AP-1 appears to bind the 21-bp-repeat TxRE but evidently is not essential for Tax-mediated transactivation (25). TREB7/CRE-BP1 was recently shown to mediate transactivation by the adenovirus transactivator E1a but not by Tax (37).

Tax transactivates several viral and cellular promoters in addition to the HTLV-I LTR. Most of these promoters do not appear to have sequences homologous to the 21-bp-repeat TxRE found in the HTLV-I LTR (7, 32, 47, 61, 62, 67, 68). Tax transactivation of the interleukin-2 receptor α -chain and human immunodeficiency virus promoters appears to be mediated by the transcription factor NF- κ B (3, 31, 35, 61). Mutants of the Tax protein that are selectively defective for transactivation of the HTLV-I LTR or NF- κ B-responsive promoters have been identified, suggesting that the mechanisms by which Tax transactivates the HTLV-I LTR and NF- κ B-responsive promoters are different (62, 70). Experiments presented here consider only Tax-mediated transactivation of the HTLV-I LTR and not NF- κ B-dependent promoters.

To begin to elucidate the mechanism of Tax-mediated transactivation of the HTLV-I LTR, we have developed a Tax-dependent *in vitro* transcription system. Purified recombinant Tax typically activated transcription of the HTLV-I LTR 10-fold, and Tax-mediated transactivation showed a strong dependence on the 21-bp-repeat TxRE. Tax increased the binding of at least one affinity-purified host cell protein to the 21-bp-repeat TxRE in an EMSA, suggesting that activation of transcription involves a direct interaction with host cell TxRE-binding proteins.

MATERIALS AND METHODS

Lymphocyte cell culture and extract preparation. CEM T lymphocytes (69) were grown in spinner culture at 37°C, using Iscove's medium supplemented with 5% fetal bovine serum, 5% calf serum, and glutamine. SLB-I cells (29) were grown in T-150 flasks at 37°C, using the same medium. Nuclear extracts were prepared as described previously (8),

with the following modifications. All buffers contained the protease inhibitors phenylmethylsulfonyl fluoride, leupeptin, aprotinin, and soybean trypsin inhibitor (1 μ g/ml each). To prepare extracts for chromatography, isolated nuclei were resuspended in 2 packed-cell volumes (PCV) of nuclear extraction buffer (0.05 M Tris-HCl [pH 7.9], 0.42 M KCl, 0.005 M MgCl₂, 0.001 M EDTA, 0.002 M dithiothreitol [DTT], 20% [vol/vol] glycerol, 10% [vol/vol] sucrose). Nuclei were stirred on ice for 60 min and centrifuged for 30 min at 17,000 rpm in a Dupont Sorvall SS-34 fixed-angle rotor. High-speed supernatant was diluted to 0.15 M KCl and applied to a heparin-agarose column. To prepare extracts for *in vitro* transcription experiments, isolated nuclei were resuspended in 4 PCV of nuclear extraction buffer and centrifuged as described above, and proteins were precipitated with 0.33 g of ammonium sulfate per ml for 60 min on ice. Precipitated proteins were pelleted by centrifugation for 10 min at 15,000 rpm in the SS-34 rotor, and the pellet was resuspended in 0.05 high-speed supernatant volumes of TM buffer (0.050 M Tris-HCl [pH 7.9], 0.0125 M MgCl₂, 0.001 M EDTA, 0.001 M DTT, 20% [vol/vol] glycerol) containing 0.1 M KCl, dialyzed against the same buffer overnight, and frozen at -70°C.

Expression and purification of recombinant Tax. *Spodoptera frugiperda* (Sf9) cells were maintained in suspension culture at 28°C in Grace's Antheraea medium (GIBCO) containing 3.33 g of lactalbumin hydrolysate (Difco) per liter, 3.33 g of Yeastolate (Difco) per liter, 60 μ g of penicillin G per liter, 60 μ g of streptomycin sulfate per liter, 0.1% Pluronic F-68 (GIBCO), and 10% heat-inactivated fetal bovine serum. Infections with the Tax-expressing baculovirus vAcPx (26) were carried out in monolayer cultures in medium as described above but without antibiotics or Pluronic F-68, following the methods of Summers and Smith (74). For large-scale preparation of the HTLV-I Tax protein, 10 to 30 150-mm cell culture flasks were used. Infection was monitored visually, and cells were harvested 48 to 72 h after infection.

Cells were centrifuged for 5 min at 1,000 rpm in a Beckman GH 3.7 rotor, washed with serum-free Grace's medium, resuspended in 4 PCV of Nonidet P-40 (NP-40) lysis buffer (0.1% [vol/vol] NP-40, 0.15 M NaCl, 0.01 M Tris-HCl [pH 7.9], 0.0015 M MgCl₂), and incubated on ice for 3 min. The lysed cell suspension was centrifuged at 3,000 rpm in a Beckman GH 3.7 rotor for 5 min, the cytoplasmic fraction was removed, and the pelleted nuclei were washed with 2 PCV of NP-40 lysis buffer. Washed nuclei were resuspended in 4 PCV of nuclear extraction buffer, stirred on ice for 30 min, and centrifuged at 17,000 rpm in a Sorvall SS-34 rotor for 30 min. The high-speed supernatant (HSS1) was reserved. The pellet was resuspended in a high-salt extraction buffer (0.05 M Tris-HCl [pH 7.9], 1 M NaCl, 1% [vol/vol] NP-40, 10% [vol/vol] glycerol, 0.004 M β -mercaptoethanol) and subjected to 15-s bursts of sonication three times, using a microtip (model W-225; Heat Systems-Ultrasonics Inc.). Sonicated nuclei were stirred on ice for 30 to 60 min and centrifuged at 17,000 rpm in a Sorvall SS-34 rotor. The high-speed supernatant (HSS2) was diluted 1:3 with high-salt extraction buffer, and proteins were precipitated with 0.33 g of ammonium sulfate per ml for 60 min at 4°C. Precipitated proteins were pelleted by centrifugation at 15,000 rpm in a Sorvall SS-34 rotor for 10 min, resuspended in chromatography buffer (10 mM Tris-HCl [pH 7.4], 1 mM EDTA, 500 mM NaCl, 10% [vol/vol] glycerol, 1% [vol/vol] NP-40, 1 mM DTT), and dialyzed against the same buffer overnight at 4°C. Proteins were chromatographed over a

Sephacryl 300 column (30 by 1.5 cm) or a Superdex-200 H16/160 column (Pharmacia), using the same buffer. Fractions were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), and Tax protein was detected by Western immunoblotting. In some cases, Tax-containing fractions were dialyzed overnight against TM buffer containing 0.1 M KCl and concentrated by ultrafiltration (Amicon ultrafiltration cell model 8010, YM30 ultrafiltration membrane). Tax-containing fractions were stored at -70°C .

In some experiments, Tax from HSS1 was used. In these cases, HSS1 was adjusted to 1 M $(\text{NH}_4)_2\text{SO}_4$ and stirred for 90 min at 4°C (14). The mixture was centrifuged at $100,000 \times g$ for 60 min at 4°C . Precipitated proteins were resuspended, dialyzed overnight against 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–0.1% (vol/vol) NP-40, and subjected to chromatography, using a Superdex-200 H16/160 column (Pharmacia) equilibrated in the same buffer. Tax-containing fractions were stored at -70°C .

In vitro transcription assay. To prepare HTLV-I templates, plasmid pU3RCAT and various derivatives (gift of J. Brady [5]) were digested with *Hind*III and *Pvu*II, and the promoter fragment was isolated by preparative PAGE. TK0 and SPTK3 templates contained zero and three Sp1 binding sites, respectively, inserted upstream of the herpes simplex virus thymidine kinase TATA box. Promoter fragments were excised from the TK0 and SPTK3 vectors with *Eco*RI and *Eco*RV and isolated by preparative PAGE. Immobilized templates were prepared by coupling streptavidin-agarose beads to a biotinylated nucleotide incorporated at the upstream end of the promoter as described previously (2). Preinitiation transcription complexes were formed in the presence of 300 to 500 ng of immobilized template, 50 to 200 μg of nuclear extract, and TX buffer (25 mM Tris-HCl [pH 7.9], 6.25 mM MgCl_2 , 0.5 mM EDTA, 0.5 mM DTT, 50 mM KCl, 10% [vol/vol] glycerol) in a 50- μl reaction at 30°C for 60 min. Immobilized templates were washed three times with 1 ml of TX buffer and resuspended in 50 μl of TX buffer. RNA synthesis was initiated by the addition of 250 μM each ATP, GTP, and UTP and 12.75 μM $[\alpha\text{-}^{32}\text{P}]\text{CTP}$ (15.7 Ci/mmol). Reaction mixtures were incubated at 30°C for 5 min, Sarkosyl (*N*-lauroyl sarcosine; Sigma) was added at a final concentration of 0.4% (vol/vol), and incubation was continued for 10 min. Reactions were terminated, and RNA transcripts were isolated and analyzed by urea-PAGE as described previously (2).

In vitro transcription reactions were also performed by using templates in solution. Promoter fragments were isolated as described above but were not biotinylated. Preinitiation complexes were formed in the presence of 50 to 75 ng of DNA template, 50 to 100 μg of nuclear extract, and TX buffer in a 50- μl reaction at 30°C for 60 min. Following this incubation, α -amanitin was added to a final concentration of 1 $\mu\text{g}/\text{ml}$ where indicated, and RNA synthesis was initiated by the addition of 250 μM each ATP, GTP, and UTP and 6.32 μM $[\alpha\text{-}^{32}\text{P}]\text{CTP}$ (31 Ci/mmol). Reaction mixtures were incubated for 15 min at 30°C , and RNA was isolated and analyzed by urea-PAGE as described above. Relative amounts of RNA were quantitated by using a Molecular Dynamics Phosphor Imager.

Western blot analysis. Analysis of samples for Tax was carried out by boiling in SDS sample buffer (80 mM Tris-HCl [pH 6.8], 2% [wt/vol] SDS, 100 mM DTT, 0.004% bromophenol blue, 12% [vol/vol] glycerol) and subjecting samples to

SDS-PAGE, using a 10% resolving gel. Proteins were transferred to nitrocellulose (0.45- μm pore diameter; Bio-Rad) with a Genie electrophoretic blotter (Idea Scientific Co.) at 500 mA for 30 to 90 min and visualized by Ponceau S (Sigma) staining. The filter was blocked with 1% (wt/vol) bovine serum albumin (Sigma grade VIII) and 3% (wt/vol) Carnation low-fat powdered milk in TTBS buffer (0.9% NaCl, 10 mM Tris-HCl [pH 7.2], 0.02% [wt/vol] sodium azide, 0.05% [vol/vol] Tween 20). The filter was probed with a polyclonal rabbit serum containing antibodies against a 13-amino-acid peptide from the carboxyl terminus of Tax and washed with TTBS buffer. Antibody binding was detected with ^{125}I -protein A (8.9 $\mu\text{Ci}/\mu\text{g}$; ICN) and visualized by autoradiography or was detected with an alkaline phosphatase-conjugated goat anti-rabbit antibody (ICN) and visualized by incubation with 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt and nitroblue tetrazolium chloride (GIBCO/BRL) chromogenic substrates. Relative amounts of bound ^{125}I -protein A were quantitated by using a Molecular Dynamics Phosphor Imager.

DNA affinity chromatography and EMSA. Twenty-one-base-pair-repeat-TxRE-binding proteins were purified from CEM T lymphocytes. Nuclear extracts were subjected to heparin-agarose chromatography followed by two rounds of DNA affinity chromatography, using a matrix representing the second 21-bp repeat of HTLV-I and flanking sequences (–204 to –174) as described previously (53). AP-1 was affinity purified from HeLa cells as described elsewhere (41).

DNA binding activity of affinity-purified proteins was determined by EMSA. Binding reaction mixtures contained 0.17 to 0.33 nM ^{32}P -end-labeled double-stranded oligonucleotides. A wild-type oligonucleotide contained core TxRE sequences from the second 21-bp repeat of HTLV-I (–199 to –185), 5'-GATCTCCCTGACGTGTCCCCA-3' and 5'-GATCTGGGGACACGTCAGGGA-3'. A mutant TxRE oligonucleotide contained four substitutions in the TxRE core sequence (mutations underlined), 5'-GATCTCCCACTGGTGTCCCCA-3' and 5'-GATCTGGGGACACTGACGGGA-3'. An AP-1 consensus recognition oligonucleotide contained sequences 5'-GATCATGGTGTGCTGACTAATTGA GA-3' and 5'-GATCTCCTCAATTAGTCAGCAACCAT-3'. In addition to oligonucleotides, binding reaction mixtures contained 0.5 to 1.0 μg of poly(dI-dC), 25 mM Tris-HCl (pH 7.9), 6.25 mM MgCl_2 , 0.5 mM EDTA, 0.5 mM DTT, 60 to 120 mM KCl, and 10% (vol/vol) glycerol in a 30- μl reaction volume. Some reaction mixtures also contained 5 μl of affinity-purified host cell proteins, 50 to 400 ng of Tax protein, and 100 μM ATP as indicated in the figure legends. Reaction mixtures were incubated on ice for 10 min and analyzed on 5% nondenaturing polyacrylamide gels (49:1, acrylamide/*N,N*-methylenebisacrylamide). The electrophoresis buffer contained 0.04 M Tris, 0.306 M glycine (pH 8.5), and 0.1% NP-40. Gels were dried and autoradiographed.

RESULTS

Nuclear extracts from HTLV-I-infected cells direct more transcription from the HTLV-I LTR than do extracts from uninfected cells. Nuclear extracts were prepared from the HTLV-I-infected, Tax-producing T-lymphocyte cell line SLB-I and from the transformed, uninfected T-lymphocyte cell line CEM. CEM and SLB-I cell lines appear to be functionally well matched, despite their independent genetic origins. We have shown previously that nuclear extracts from both of these T-lymphocyte cell lines give similar

DNase I footprinting patterns on the HTLV-I 21-bp-repeat TxRE (53).

In vitro transcription activity of each extract was assayed on immobilized DNA templates as described in Materials and Methods. Extract from the Tax-producing SLB-I cells was much more active than extract from CEM cells on a wild-type HTLV-I template, containing 306 bp of HTLV-I sequence upstream from the in vivo RNA cap site (Fig. 1A; lanes 3 and 4). The correctly initiated HTLV-I transcript is indicated by the arrow at the right in Fig. 1A. A second transcript, which initiates approximately 30 bp upstream of the HTLV-I in vivo RNA cap site, is a reproducible characteristic of SLB-I nuclear extracts and is sometimes observed with CEM extracts. The two extracts were equally active on a reference template, SPTK3, which contained no HTLV-I sequence.

To determine which HTLV-I sequences were responsible for the increased transcriptional activity of the Tax-containing SLB-I extract, deletion analysis was performed by using the mutants diagrammed in Fig. 1B, which have been previously characterized in vivo (5). With both CEM and SLB-I extracts, there was a progressive decline in transcription as upstream sequences were deleted (Fig. 1A, lanes 5 to 10). The SLB-I extract was still more active than the CEM extract on an HTLV-I mutant that retained 101 bp upstream of the in vivo RNA cap site and a single 21-bp-repeat TxRE (lanes 7 and 8), whereas no clear difference between extracts was observed on a mutant that retained only 52 bp of upstream sequence and no TxREs (lanes 9 and 10). The difference in transcriptional activity between CEM and SLB-I extracts was enhanced or restored by adding a site 1 21-bp-repeat TxRE oligonucleotide upstream of the 101 and 52 deletion mutants (lanes 13, 14, 17, and 18). Activity was also increased by adding an oligonucleotide containing sequences between the second and third 21-bp-repeat TxREs that enhances the Tax response in vivo (5, 42) (lanes 11, 12, 15, and 16).

It is likely that the greater transcriptional activity of the SLB-I extract is attributable at least in part to the presence of Tax, since the level of transcriptional activity in both extracts was the same on a reference promoter, and the difference in activity between CEM and SLB-I extracts was seen only in HTLV-I constructs containing 21-bp-repeat TxREs or other sequences known to affect the Tax response. This conclusion is supported by later experiments in which the effects of adding purified Tax to SLB-I and CEM extracts were compared (see Fig. 4).

The use of immobilized template in these reactions allowed us to analyze, in parallel, the amount of Tax protein that remained associated with the template after unbound proteins were removed by washing. Bound Tax was detected by SDS-PAGE and immunoblotting. The amount of bound Tax increased with increasing numbers of TxREs (data not shown), consistent with the idea that localization of Tax at the promoter is important for its activity (12, 42). However, there was appreciable Tax binding even with the mutant that retained only 52 bp upstream of the start site and with the reference promoter, suggesting that binding was not strictly TxRE specific under the conditions tested. The interaction of Tax with cellular TxRE-binding proteins was investigated more directly in experiments presented later.

Purified Tax protein stimulates transcription in vitro. To further investigate the mechanism of Tax-mediated transactivation, Tax was purified from a baculovirus expression system. We chose this system because the baculovirus-

FIG. 2. Expression and purification of Tax. Sf9 cells were infected with recombinant baculovirus vAcPx (gift of K. T. Jeang) and extracted as described in Materials and Methods. All lanes represent protein from 2.3×10^5 cells. (A) Analysis of subcellular fractions by SDS-PAGE with Coomassie blue staining. The position of Tax is indicated by the arrow at the right. Positions (kilodaltons) of protein molecular weight standards (lane M) are shown at the left. Whole cell lysates of uninfected Sf9 cells (lane 1) and Sf9 cells infected with a recombinant Tax-expressing baculovirus, vAcPx (lane 2), are shown along with cytoplasmic fraction (lane 3), low-salt nuclear extract (lane 4), and high-salt nuclear extract (lane 5) from infected cells. (B) Western blot. The procedure was carried out as described in Materials and Methods. Immune complexes were visualized with alkaline phosphatase and chromogenic substrates. Lanes are as in panel A.

expressed Tax was previously reported to be correctly posttranslationally modified and active in vivo (26).

Nuclear extracts were prepared from Sf9 cells infected with vAcPx (gift of K. T. Jeang) as described in Materials and Methods. Tax was quantitatively localized in the nuclear fractions (Fig. 2B; compare cytoplasmic fraction [lane 3] with nuclear fractions [lanes 4 and 5]). Approximately 30% of the Tax protein was extracted from the nuclei under conditions typically used to prepare extracts for in vitro transcription experiments (Fig. 2A and B, lanes 4). Much of the remaining Tax was extracted when nuclei were sonicated in the presence of high salt and detergent (Fig. 2A and B, lanes 5). Although Tax is the major component in this second extract, this material was not active in functional assays until subjected to gel filtration chromatography to separate aggregated and monomeric Tax as described in Materials and Methods. Only the monomeric fraction was active.

The transcriptional activity of purified Tax is shown in Fig. 3. Increasing amounts of a monomeric Tax fraction were added to transcription reaction mixtures containing CEM nuclear extract and either wild-type HTLV-I template or control SPTK3 template. Runoff transcripts were electrophoresed on a denaturing urea gel, and the relative amounts of RNA synthesized were quantitated by Phosphor Imager analysis. In the presence of 1 μ g of Tax, transcription of the wild-type HTLV-I template was activated approximately 10-fold, while the reference template was activated less than 2-fold (Fig. 3). The degree of HTLV-I activation varied in different experiments and was sometimes as great as 40-fold. Activation of HTLV-I transcription was typically fivefold greater than activation of reference template transcription. These experiments were performed under conditions in which template was limiting. The amount of transcription with the reference promoter was approximately 10-fold less than the maximal amount seen with saturating levels of template (data not shown). Thus, the lack of an effect of Tax on the reference promoter could not be attributed to limiting amounts of general transcription factors in the extract.

FIG. 3. Activation of transcription by purified Tax protein. (A) Increasing amounts of Tax protein were added to in vitro transcription reaction mixtures containing CEM nuclear extract and either an HTLV-I or SPTK3 reference template. Reactions were performed, and radiolabeled runoff transcripts were analyzed by urea-PAGE and visualized by autoradiography as described in Materials and Methods. Single-stranded DNA markers are shown at the left, with length given in nucleotides. (B) Quantitation of RNA synthesis. Relative amounts of radiolabeled runoff transcripts from the gel shown in panel A were determined by using a Molecular Dynamics Phosphor Imager. Fold activation was determined by dividing the amount of RNA synthesized in the presence of Tax by that synthesized in the absence of Tax.

To demonstrate that the higher levels of transcription previously observed with infected cell extracts were attributable at least in part to Tax, the effect of recombinant Tax was measured in extracts from HTLV-I-infected and uninfected cells (Fig. 4). In extract from uninfected CEM cells, the baculovirus-expressed Tax preferentially activated transcription of the HTLV-I template. In extract from Tax-producing SLB-I cells, the recombinant Tax had less effect and did not preferentially activate transcription of the HTLV-I template relative to the reference template. Presumably, transcription in the SLB-I extract was nearly saturated with endogenous Tax and could not respond to additional recombinant Tax.

To demonstrate that Tax, rather than a contaminating protein, was responsible for the activation of transcription, fractions from a gel filtration column profile were analyzed.

FIG. 4. Effect of exogenous recombinant Tax on transcription in nuclear extracts from uninfected and Tax-producing HTLV-I-infected T lymphocytes. Runoff transcription was performed and quantitated as described in Materials and Methods in the absence and presence of 1 µg of Tax. Fold activation is the ratio of RNA synthesized in the presence of Tax to that synthesized in its absence.

FIG. 5. Coelution of Tax protein and transcriptional activation activity during gel filtration chromatography. (A) Quantitation of Tax present in column fractions. Western blotting was performed as described in Materials and Methods. Immune complexes were visualized with ^{125}I -protein A and quantitated by using a Molecular Dynamics Phosphor Imager. (B) Quantitation of transcriptional activation activity in column fractions. In vitro transcription reaction mixtures contained 15 µl of each column fraction, CEM nuclear extract, and wild-type HTLV-I template. Radiolabeled RNA transcripts were analyzed by urea-PAGE, and relative amounts of RNA synthesis were quantitated as in panel A. The transcriptional activity of each column fraction was normalized to the level of transcription observed when column buffer alone was added (represented by the dashed line). (C) SDS-PAGE analysis of column fractions. A 15-µl sample of each column fraction was subjected to SDS-PAGE and visualized with silver staining. Molecular weight standards are in lane M, with sizes in kilodaltons indicated at the right. In lane OP, 5 µl of onput to the column was analyzed. Column fractions are indicated. Arrow at left indicates position of Tax.

The amount of transcriptional activation closely correlated with the amount of Tax in each fraction, as determined by immunoblotting (Fig. 5A and B). In addition, Tax was the only polypeptide visible in the peak fraction when proteins were analyzed by SDS-PAGE and silver staining (Fig. 5C, fraction 40). No transcription was detected when α -amanitin was included in a reaction mixture containing this peak Tax fraction (data not shown), indicating that the observed Tax-mediated activation of transcription is specific for RNA polymerase II.

The 21-bp-repeat TxRE mediates Tax transactivation in vitro. One copy, and in some genetic backgrounds, two

FIG. 6. Determination of sequences required for stimulation of in vitro transcription by Tax. (A) Transcription of wild-type and deletion mutants of the HTLV-I LTR (diagrammed in Fig. 1B) in the presence and absence of 1 μ g of purified Tax. Reactions were performed with extract from CEM T lymphocytes. Radiolabeled runoff transcription products were analyzed by urea-PAGE and quantitated by using a Molecular Dynamics Phosphor Imager as described in Materials and Methods. (B) Effect of number of 21-bp-repeat sequences on transcription in the presence and absence of Tax. Reactions were performed as in panel A, using HTLV-I deletion mutant templates containing TxRE oligonucleotide inserts as diagrammed in Fig. 1B.

copies, of the 21-bp-repeat TxRE are necessary for Tax-mediated transactivation in vivo (5, 45, 55, 66). To determine the sequences required for Tax-mediated transactivation in vitro, wild-type and deletion mutants of the HTLV-I LTR were transcribed in CEM extract in the absence and presence of Tax (Fig. 6). Transcription was strongly activated by Tax on the 306, 242, and 101 deletion mutants, which contain three, two, and one copies, respectively, of the 21-bp-repeat TxRE. Tax had no effect on the transcription of the 52 deletion mutant, which does not contain a 21-bp-repeat TxRE.

To confirm the requirement for the 21-bp-repeat TxRE, we examined the effect of Tax on constructs in which a 21-bp-repeat TxRE oligonucleotide was inserted into mutants containing 101 or 52 bp of HTLV-I upstream promoter sequence. Transcription of all templates containing the 21-bp-repeat TxRE was activated when purified Tax was added to reactions containing CEM extract (Fig. 6B). Most notable was the difference between the 52 mutant, which contained no 21-bp-repeat TxRE and showed no response to Tax, and the 52+21 mutant, which contained a single 21-bp-repeat TxRE and was activated more than sevenfold. These results are generally similar to those obtained with transfection experiments (5), although there are some quantitative differences. In particular, constructs containing a single 21-bp-repeat TxRE were virtually inactive in vivo in the absence and presence of Tax. By contrast, these constructs showed measurable basal activity in vitro and were activated by Tax.

Tax increases binding of a factor to the 21-bp-repeat TxRE.

As discussed in the introduction, it is likely that Tax affects transcription by interacting with cellular proteins that bind the 21-bp-repeat TxRE. To further investigate the nature of these interactions, factors that bind the second 21-bp-repeat TxRE were purified from a CEM nuclear extract by chromatography on heparin-agarose and a 21-bp-repeat TxRE DNA affinity matrix. Results of the purification were similar to those described previously (53). DNA binding activity of the preparation was determined by EMSA. Two complexes were formed (Fig. 7A, lanes 2 to 9). The upper complex was competed for by a wild-type oligonucleotide probe (lanes 3 to 5) but not by a mutant oligonucleotide in which the TxRE core sequence TGACG was altered to ACTGG (lanes 7 to 9). This mutant TxRE does not support transactivation in vivo (55). By contrast, the prominent lower complex was only slightly affected by either competitor DNA. On the basis of these and subsequent results (see below), we suggest that the upper complex is TxRE specific and that the lower complex either is nonspecific or reflects binding to other sequences in the oligonucleotide.

To determine whether Tax affected the binding of host cell proteins to the TxRE, EMSA was carried out in the absence and presence of Tax. Addition of Tax to the reaction mixtures markedly increased the amount of the upper complex but did not affect the amount of the lower complex (Fig. 7B, lanes 4 to 7). Complex formation was dependent on affinity-purified host cell proteins. In the absence of host cell proteins, neither the upper nor the lower complex was observed (lane 8), and a faint complex of intermediate mobility was observed only with the highest amount of Tax protein added. No stimulation of binding activity was detected when similar amounts of transcriptionally inactive, aggregated Tax were added to the reaction mixture, suggesting that an activity of the native Tax protein is required for the observed effect on binding (data not shown).

The effect of Tax on host protein DNA binding was TxRE dependent. This is shown in Fig. 7C, in which the wild-type TxRE probe was compared with the mutant containing the altered TxRE core sequence. The upper complex was not formed with the mutant probe in either the absence or presence of Tax (compare lanes 8, 9 with lanes 2 and 6). By contrast, the lower complex appeared to be unaffected or slightly increased by the mutation.

As a further control for the TxRE specificity of the Tax effect, Tax was added to EMSA reaction mixtures containing the transcription factor AP-1 and an oligonucleotide representing the consensus AP-1 recognition sequence. This sequence is related to the 21-bp-repeat TxRE, but AP-1 is evidently not involved in the Tax response in vivo (25). Tax had no effect on the DNA binding of partially purified AP-1 (Fig. 7D; compare lanes 2 and 3 with lanes 4 to 6).

The data presented here suggest that Tax modifies the behavior of an existing host cell 21-bp-repeat-TxRE-binding protein. Phosphorylation has been implicated in the in vivo Tax response, since a protein kinase inhibitor blocks Tax-mediated transactivation (76). In addition, a different viral transactivator protein, hepatitis virus X, has an intrinsic protein kinase activity (39). To determine whether ATP had any direct effect on the interaction of Tax with TxRE-binding proteins, EMSA was performed in the absence and presence of ATP. In the presence of Tax, ATP had no effect on complex formation (Fig. 8; compare lanes 4 and 5). In the absence of Tax, slightly more upper complex formed in the presence of ATP (lanes 2 and 3). This may not be significant, since the amount of upper complex formed in the absence of

FIG. 7. Binding of affinity-purified host cell factors to 21-bp-repeat sequence in the absence and presence of Tax. (A) Effect of competitor DNA on 21-bp-repeat-TxRE-binding activity. Reaction mixtures contained 5 fmol of ³²P-end-labeled wild-type 21-bp-repeat TxRE oligonucleotide probe alone (lane 1) or with 5 μl of affinity-purified host cell TxRE-binding proteins (TxRE BP) (lanes 2 to 9). Reaction mixtures contained unlabeled wild-type (lanes 3 to 5) or mutant (lanes 7 to 9) TxRE oligonucleotide in the amounts indicated. Protein-DNA complexes were resolved by nondenaturing PAGE and visualized by autoradiography as described in Materials and Methods. Arrow marked U indicates the TxRE-specific upper complex; arrow marked L indicates the lower complex (see text). (B) Effect of increasing amounts of purified Tax. Reaction mixtures contained 10 fmol of ³²P-end-labeled wild-type 21-bp-repeat TxRE oligonucleotide probe alone (lane 1) or with 5 μl of affinity-purified host cell TxRE-binding proteins in the absence of Tax (lanes 2 and 3) or in the presence of increasing amounts of Tax, as indicated (lane 4 to 7). Tax was also incubated with the oligonucleotide probe in the absence of host cell proteins in the absence of Tax (lanes 2 and 8) or in the presence of increasing amounts of Tax, as indicated (lanes 3 to 6 and 9). Tax was also incubated with oligonucleotide probe in the absence of host cell proteins (lanes 7 and 10). Upper and lower complexes are indicated as in panel A. (C) Binding of host cell factors to wild-type and mutant TxREs. Reactions were performed as in panel B with the wild-type 21-bp-repeat TxRE oligonucleotide (lanes 1 to 7) or a mutant 21-bp-repeat TxRE oligonucleotide (lanes 8 to 10) (see text). Reaction mixtures contained 10 fmol of ³²P-end-labeled probe alone (lane 1) or with 5 μl of affinity-purified host cell proteins in the absence of Tax (lanes 2 and 8) or in the presence of increasing amounts of Tax, as indicated (lanes 3 to 6 and 9). Tax was also incubated with oligonucleotide probe in the absence of host cell proteins (lanes 7 and 10). Upper and lower complexes are indicated as in panel A. (D) Tax does not affect AP-1-DNA interactions. Reaction mixtures contained 10 fmol of ³²P-end-labeled AP-1 oligonucleotide probe alone (lane 1) or with 2.5 μl of affinity-purified AP-1 in the absence of Tax (lanes 2 and 3) or in the presence of increasing amounts of Tax, as indicated (lane 4 to 6). Tax was also incubated with the oligonucleotide probe in the absence of AP-1 (lane 7).

Tax was variable in other experiments (for example, Fig. 7B, lanes 2 and 3).

In a separate experiment, no labeling of proteins in either complex was seen when [γ -³²P]ATP was included in the binding reaction mixtures, suggesting that neither Tax nor the host cell TxRE-binding proteins have an intrinsic kinase activity under the conditions tested (data not shown).

DISCUSSION

We have developed an *in vitro* system to characterize interactions between the HTLV-I transactivating protein Tax and the host cell transcriptional machinery. *In vitro* transcription of the HTLV-I LTR was typically stimulated 10-fold when purified recombinant Tax was added to nuclear extract from uninfected T lymphocytes. Tax protein co-eluted with increased transcriptional activity in gel filtration chromatography, and, as expected, the amount of activation by exogenous recombinant Tax was diminished when nuclear extracts containing endogenous Tax were used. Tax-mediated activation of transcription *in vitro* was dependent

FIG. 8. Effect of ATP on complex formation. Protein-DNA complexes were allowed to form and were analyzed as in Fig. 7 in the absence (lanes 1, 2, 5, and 6) or presence (lanes 3, 4, and 7) of 100 μM ATP. Reaction mixtures contained 10 fmol of 21-bp-repeat TxRE oligonucleotide probe alone (lane 1) or with 10 μl of second affinity-purified host cell protein in the absence (lanes 2 and 3) or presence (lanes 4 and 5) of 0.1 μg of Tax. Tax was also incubated with the oligonucleotide probe in the absence of host cell proteins (lanes 6 and 7). BP, binding protein.

on the 21-bp-repeat TxRE, which also mediates Tax transactivation *in vivo*. These findings suggest that the *in vitro* system presented here is a good model for characterization of Tax-mediated transactivation.

To begin to investigate the mechanism by which Tax activates transcription *in vitro*, we used DNA affinity chromatography to isolate cellular factors that bind the 21-bp-repeat TxRE. Tax increased the DNA-binding activity of at least one of the proteins in the preparation, as evidenced by an increase in the amount of a protein-DNA complex specific for the conserved core sequence of the 21-bp-repeat TxRE. It is likely, although not yet proven, that this increased binding contributes to the Tax-dependent increase in RNA synthesis observed in the *in vitro* transcription reaction.

It appears that Tax interacted only transiently with host cell TxRE-binding proteins under the conditions used for our binding experiments. Tax increased the amount of a specific protein-DNA complex but did not further retard its electrophoretic mobility, as would have been expected if Tax were a stable component of the complex. These results are in agreement with the data of Marriott and coworkers, who also failed to detect a stable complex between Tax and a 21-bp TxRE oligonucleotide (42, 43). Our results should be interpreted with some caution, however, since it is possible that stable association occurs when multiple TxREs are present or when TxREs are present in the context of the whole promoter. Our results do not rule out the possibility that stable association might occur under other conditions. While this report was in preparation, a different laboratory reported an association of Tax with a probe containing two 21-bp-repeat TxREs in the presence of proteins from a T-cell nuclear extract (82). The discrepancy between these results and our own is unexplained but could relate to differences in the way in which the experiments were performed.

As discussed in the introduction, a number of cellular proteins bind to the 21-bp-repeat TxRE. It is likely that many of these are not constitutive activators of transcription, since the integrated provirus is known to remain latent for long periods. However, some of the 21-bp-repeat TxRE-binding proteins are known to participate in cAMP and protein kinase C-mediated pathways of signal transduction and could thus promote an initial burst of viral gene expression in response to a variety of stimuli. The physiological role of Tax is evidently to maintain expression after the initial induction. The present results suggest that Tax does so by inducing the binding of a particular cellular factor, which may be different from the factors that were bound during the latent phase of infection. By doing so, Tax would promote the function of the LTR as a bistable molecular switch.

Whether this is the only mechanism of Tax transactivation remains to be determined. For example, Tax might also interact with general transcription factors bound near the start site for transcription. This would be consistent with the activation of transcription by a GAL4-Tax fusion protein in a promoter lacking known TxREs (12). It would also be consistent with the approximate twofold activation of transcription that we have seen *in vitro* with use of simple promoters lacking TxREs, including the synthetic SPTK3 promoter (Fig. 3 and 4) and the adenovirus type 2 major late promoter (data not shown).

Tax is one of several viral regulatory proteins that activate transcription without binding directly to DNA in a sequence-specific manner. Some of the other viral proteins in this group have already been shown to interact with both promoter-specific and general transcription factors. For example, the adenovirus transactivating protein, Ela, interacts

with the promoter-specific activators E2F, ATF-2, and AP-1/Jun and with the general transcription factor TFIID (23, 33, 36, 37, 40). The herpes simplex virus transactivating protein, VP16, interacts with the promoter-specific activator OTF1 and with the general transcription factors TFIID and TFIIB (13, 34, 72, 73). The hepatitis B virus transactivating protein, X, interacts with the promoter-specific factors ATF-2 and CREB (39). It is not known whether hepatitis virus X protein interacts with general transcription factors, but, as with other viral transactivating proteins, localization of the hepatitis virus X protein at the promoter is important for its activity (12, 33, 63, 79). These observations suggest that viral transactivating proteins often function as adaptors or mediators between promoter-selective transcription factors and the general transcriptional machinery. The *in vitro* system described here will be useful for investigating directly whether Tax also functions by such a mechanism.

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REFERENCES

- Altman, R., D. Harrich, J. A. Garcia, and R. B. Gaynor. 1988. Human T-cell leukemia virus types I and II exhibit different DNase I protection patterns. *J. Virol.* **62**:1339-1346.
- Arias, J. A., and W. S. Dynan. 1989. Promoter-dependent transcription by RNA polymerase II using immobilized enzyme complexes. *J. Biol. Chem.* **264**:3223-3229.
- Ballard, D. W., D. W. Bohnlein, J. W. Lowenthal, Y. Wano, R. B. Franza, and W. C. Green. 1988. HTLV-I Tax induces cellular proteins that activate the κ B element in the IL-2 receptor- α gene. *Science* **241**:1652-1655.
- Beimling, P., and K. Moelling. 1990. Tax-independent binding of multiple cellular factors to Tax-response element DNA of HTLV-I. *Oncogene* **5**:361-368.
- Brady, J., K.-T. Jeang, J. Duvall, and G. Khoury. 1987. Identification of p40^x-responsive regulatory sequences within the human T-cell leukemia virus type I long terminal repeat. *J. Virol.* **61**:2175-2181.
- Cann, A. J., J. D. Rosenblatt, W. Wachsmann, N. P. Shah, and I. S. Y. Chen. 1985. Identification of the gene responsible for human T-cell leukemia virus transcriptional regulation. *Nature (London)* **318**:571-574.
- Cross, S. L., M. B. Feinberg, J. B. Wolf, N. J. Holbrook, F. Wong-Staal, and W. J. Leonard. 1987. Regulation of the human interleukin-2 receptor α chain promoter: activation of a non-functional promoter by the transactivator gene of HTLV-I. *Cell* **49**:47-56.
- Dynan, W. S. 1985. DNase I footprinting as an assay for mammalian gene regulatory proteins, p. 75-87. *In* J. K. Setlow and A. Hollaender (ed.), *Genetic engineering: principles and methods*. Plenum Publishing Corp., New York.
- Felber, B. K., H. Paskalis, C. Klienman-Ewing, F. Wong-Staal, and G. N. Pavlakis. 1985. The pX protein of HTLV-I is a transcriptional activator of its long terminal repeats. *Science* **229**:675-679.
- Fujisawa, J.-I., M. Seiki, M. Sato, and M. Yoshida. 1986. A transcriptional enhancer sequence of HTLV-I is responsible for trans-activation mediated by p40^x of HTLV-I. *EMBO J.* **5**:713-718.
- Fujisawa, J.-I., M. Toita, and M. Yoshida. 1989. A unique enhancer element for the trans activator (p40^{tax}) of human T-cell leukemia virus type I that is distinct from cyclic AMP- and 12-O-tetradecanoylphorbol-13-acetate-responsive elements. *J. Virol.* **63**:3234-3239.

12. Fujisawa, J.-I., M. Toita, T. Yoshimura, and M. Yoshida. 1991. The indirect association of human T-cell leukemia virus *tax* protein with DNA results in transcriptional activation. *J. Virol.* **65**:4525-4528.
13. Gerster, T., and R. G. Roeder. 1988. A herpes virus transactivating protein interacts with the transcription factor OTF-1 and other cellular proteins. *Proc. Natl. Acad. Sci. USA* **83**:6347-6351.
14. Giam, Z.-C., and Y.-L. Xu. 1989. HTLV-I *Tax* gene product activates transcription via pre-existing cellular factors and cAMP responsive element. *J. Biol. Chem.* **264**:15236-15241.
15. Gitlin, S. D., R. Bosselut, A. Gegonne, J. Ghysdael, and J. H. Brady. 1991. Sequence-specific interaction of the Ets1 protein with the long terminal repeat of the human T-lymphotropic virus type I. *J. Virol.* **65**:5513-5523.
16. Gonzalez, G. A., K. Yamamoto, W. H. Fischer, D. Karr, P. Menzel, W. Biggs, W. Vale, and M. Montminy. 1989. A cluster of phosphorylation sites on the cyclic-AMP regulated nuclear factor CREB predicted by its sequence. *Nature (London)* **337**:749-752.
17. Grassmann, R., C. Dengler, I. Muller-Fleckenstein, B. Fleckenstein, K. McGuire, M.-C. Dokhelar, J. G. Sodroski, and W. A. Haseltine. 1989. Transformation to continuous growth of primary human T lymphocytes by human T-cell leukemia virus type I X-region genes transduced by a *Herpesvirus saimiri* vector. *Proc. Natl. Acad. Sci. USA* **86**:3351-3355.
18. Green, J. E., C. G. Begley, D. K. Wagner, T. A. Waldmann, and G. Jay. 1989. *trans* activation of a granulocyte-macrophage colony-stimulating factor and the interleukin-2 receptor in transgenic mice carrying the human T-lymphotropic virus type I *tax* gene. *Mol. Cell. Biol.* **9**:4731-4737.
19. Green, J. E., S. H. Hinrichs, J. Vogel, and G. Jay. 1989. Exocrinopathy resembling Sjogren's syndrome in HTLV-1 *tax* transgenic mice. *Nature (London)* **341**:72-74.
20. Hai, T., F. Liu, W. J. Coukos, and M. R. Green. 1989. Transcription factor ATF cDNA clones: an extensive family of leucine zipper proteins able to selectively form DNA-binding heterodimers. *Genes Dev.* **3**:2083-2090.
21. Hinrichs, S., M. Nerenberg, R. K. Reynolds, G. Khoury, and G. Jay. 1987. A transgenic mouse model for human neurofibromatosis. *Science* **237**:1340-1343.
22. Hinuma, Y., K. Nagata, M. Misoka, M. Nakai, T. Matsumoto, K. Kiroshita, S. Shirakawa, and I. Miyoshi. 1981. Adult T-cell leukemia: antigen in ATL cell line and detection of antibodies to the antigen in human sera. *Proc. Natl. Acad. Sci. USA* **78**:6476-6480.
23. Horikoshi, N., K. Maguire, A. Kralli, E. Maldonado, D. Reinberg, and R. Weinmann. 1991. Direct interaction between adenovirus E1A protein and the TATA box binding transcription factor IID. *Proc. Natl. Acad. Sci. USA* **88**:5124-5128.
24. Jeang, K. T., I. Boros, J. Brady, M. Radonovich, and G. Khoury. 1988. Characterization of cellular factors that interact with the human T-cell leukemia virus type I p40x-responsive 21-base-pair sequence. *J. Virol.* **62**:4499-4509.
25. Jeang, K. T., R. Chiu, E. Santos, and S. J. Kim. 1991. Induction of the HTLV-I LTR by Jun occurs through the Tax-responsive 21-bp elements. *Virology* **181**:218-227.
26. Jeang, K. T., C. Z. Giam, M. Nerenberg, and G. Khoury. 1987. Abundant synthesis of functional human T-cell leukemia virus type I p40^x protein in eucaryotic cells by using a baculovirus expression vector. *J. Virol.* **61**:708-713.
27. Jeang, K. T., P. R. Shank, and A. Kumar. 1988. Transcriptional activation of homologous viral long term repeats by the human immunodeficiency virus type I or the human T-cell leukemia virus type I tat proteins occurs in the absence of *de novo* protein synthesis. *Proc. Natl. Acad. Sci. USA* **85**:8291-8295.
28. Kalyanaraman, V. S., M. G. Sarangaharan, Y. Nakao, Y. Ito, T. Aoki, and R. C. Gallo. 1982. Natural antibodies to the structural core protein (p24) of the human T-cell leukemia (lymphoma) retrovirus found in the sera of leukemia patients in Japan. *Proc. Natl. Acad. Sci. USA* **79**:1653-1657.
29. Koeffler, H., I. Chen, and D. Golde. 1984. Characterization of a novel HTLV-I infected cell line. *Blood* **64**:482-490.
30. Lee, K. A. W., T.-Y. Hai, L. SivaRaman, B. Thimmappaya, H. C. Hurst, N. C. Jones, and M. R. Green. 1987. A cellular protein, activating transcription factor, activates transcription of multiple E1a inducible adenovirus early promoters. *Proc. Natl. Acad. Sci. USA* **84**:8355-8459.
31. Leung, K., and G. J. Nabel. 1988. HTLV-I transactivator induces interleukin-2 receptor expression through an NF- κ B like factor. *Nature (London)* **333**:776-778.
32. Lilienbaum, A., M. D. Dodon, C. Alexandre, L. Gazzolo, and D. Paulin. 1990. Effect of human T-cell leukemia virus type I tax protein on activation of the human vimentin gene. *J. Virol.* **64**:256-263.
33. Lillie, J. W., and M. R. Green. 1989. Transcription activation by the adenovirus E1a protein. *Nature (London)* **338**:39-44.
34. Lin, Y.-S., and M. Green. 1991. Mechanism of activation of an acidic transcriptional activator in vitro. *Cell* **64**:971-981.
35. Lindholm, P., S. Marriott, S. Gitlin, C. Bohan, and J. Brady. 1991. Induction of nuclear factor NF- κ B DNA binding activity after exposure of lymphoid cells to soluble Tax₁ protein. *New Biol.* **2**:1034-1043.
36. Liu, F., and M. R. Green. 1990. A specific member of the ATF transcription factor family can mediate transcription activation by the adenovirus E1a protein. *Cell* **61**:1217-1224.
37. Maekawa, T., S. Matsuda, J. Fujisawa, M. Yoshida, and S. Ishii. 1991. Cyclic AMP response element-binding protein, CRE-BP1, mediates the E1A-induced but not the Tax-induced trans-activation. *Oncogene* **6**:627-632.
38. Maekawa, T., H. Sakura, C. Kanei-Ishii, T. Sudo, T. Yoshimura, J. Fujisawa, M. Yoshida, and I. Ishii. 1989. Leucine zipper structure of the protein CRE-BP1 binding to the cyclic AMP response element in the brain. *EMBO J.* **8**:2023-2028.
39. McGuire, H. F., J. P. Hoefler, and A. Siddiqui. 1991. HBV X protein alters the DNA binding specificity of CREB and ATF-2 by protein-protein interactions. *Science* **252**:842-844.
40. Maguire, K., X.-P. Shi, N. Horikoshi, J. Rappaport, M. Rosenberg, and R. Weinmann. 1991. Interactions between adenovirus E1a and members of the AP-1 family of cellular transcription factors. *Oncogene* **6**:1417-1422.
41. Markowitz, R.-B., and W. Dynan. 1988. Binding of cellular proteins to the regulatory region of BK virus DNA. *J. Virol.* **62**:1388-1398.
42. Marriott, S. J., I. Boros, J. F. Duvall, and J. N. Brady. 1989. Indirect binding of human T-cell leukemia virus type I Tax₁ to a responsive element in the viral long terminal repeat. *Mol. Cell. Biol.* **9**:4152-4160.
43. Marriott, S. J., P. F. Lindholm, K. M. Brown, S. D. Gitlin, J. F. Duvall, M. F. Radonovich, and J. N. Brady. 1990. A 36-kilodalton cellular transcription factor mediates an indirect interaction of human T-cell leukemia/lymphoma virus type I TAX₁ with a responsive element in the viral long terminal repeat. *Mol. Cell. Biol.* **10**:4192-4201.
44. Miyatake, S., M. Seiki, M. Yoshida, and K.-I. Arai. 1988. T-cell activation signals and the human T-cell leukemia virus type-I encoded p40^x protein activate the mouse granulocyte-macrophage colony-stimulating factor gene through a common DNA element. *Mol. Cell. Biol.* **8**:5581-5587.
45. Montagne, J., C. Beraud, I. Crenon, G. Lombard-Platet, L. Gazzolo, A. Sergeant, and P. Jalinot. 1990. Tax I induction of the HTLV-I 21 bp enhancer requires cooperation between two cellular DNA-binding proteins. *EMBO J.* **9**:957-964.
46. Montminy, M. R., K. A. Sevarino, J. A. Wagner, G. Mandel, and R. H. Goodman. 1986. Identification of a cyclic-AMP responsive element in the rat somatostatin gene. *Proc. Natl. Acad. Sci. USA* **83**:6682-6686.
47. Nagata, K., K. Ohtani, M. Nakamura, and K. Sugamura. 1988. Activation of endogenous *c-fos* proto-oncogene expression by human T-cell leukemia virus type I-encoded p40^{tax} protein in the human T-cell line Jurkat. *J. Virol.* **63**:3220-3226.
48. Nerenberg, M. Biologic and molecular biologic aspects of the HTLV-I-associated diseases. In R. Roos (ed.), *Molecular biological approaches to the study of CNS viral disease*, in press.
49. Nerenberg, M., S. Hinrichs, R. K. Reynolds, G. Khoury, and G. Jay. 1987. The Tat gene of human T-lymphotropic virus type I

- induces mesenchymal tumors in transgenic mice. *Science* **237**: 1324–1329.
50. Nerenberg, M. I. 1990. An HTLV-I transgenic mouse model: role of the *tax* gene in pathogenesis in multiple organ systems. *Curr. Top. Microbiol. Immunol.* **160**:121–128.
 51. Nerenberg, M. I., T. Minor, J. Price, D. N. Ernst, T. Shinohara, and H. Schwarz. 1991. Transgenic thymocytes are refractory to transformation by the human T-cell leukemia virus type I *tax* gene. *J. Virol.* **65**:3349–3353.
 52. Nimer, S. D., J. C. Gasson, K. Hu, I. Smalberg, J. L. Williams, I. S. Y. Chen, and J. D. Rosenblatt. 1989. Activation of the GM-CSF promoter by HTLV-I and -II *tax* proteins. *Oncogene* **4**:671–676.
 53. Nyborg, J. K., and W. S. Dynan. 1990. Interaction of cellular proteins with the human T-cell leukemia virus type I transcriptional control region: Purification of cellular proteins that bind the 21-base pair repeat elements. *J. Biol. Chem.* **265**:8230–8236.
 54. Nyborg, J. K., W. S. Dynan, I. S. Y. Chen, and W. Wachsman. 1988. Multiple host cell protein binding sites on the HTLV-I LTR: implications for transcriptional regulation. *Proc. Natl. Acad. Sci. USA* **85**:1457–1461.
 55. Nyborg, J. K., M.-A. H. Matthews, J. Yucel, L. Walls, W. T. Golde, W. S. Dynan, and W. Wachsman. 1990. Interaction of host cell proteins with the human T-cell leukemia virus type I transcriptional control region. II. A comprehensive map of protein-binding sites facilitates construction of a simple chimeric promoter responsive to the viral *tax*₂ gene product. *J. Biol. Chem.* **265**:8237–8242.
 56. Park, R. E., W. A. Haseltine, and C. Rosen. 1988. A nuclear factor is required for transactivation of HTLV-I gene expression. *Oncogene* **3**:275–279.
 57. Polesz, B. J., F. W. Ruscetti, A. F. Aazdar, P. A. Bunn, J. D. Minna, and R. C. Gallo. 1980. Detection and isolation of type C retrovirus particles from fresh and cultured lymphocytes of a patient with cutaneous T-cell lymphoma. *Proc. Natl. Acad. Sci. USA* **77**:7415–7419.
 58. Poteat, H. T., F. Y. Chen, P. Kaidson, J. G. Sodroski, and W. Haseltine. 1990. Protein kinase A-dependent binding of a nuclear factor to the 21-base-pair repeat of the human T-cell leukemia virus type I long terminal repeat. *J. Virol.* **64**:1264–1270.
 59. Poteat, H. T., P. Kaidson, K. McGuire, L. Park, J. Sodroski, and W. Haseltine. 1989. Response of the human T-cell leukemia virus type I long terminal repeat to cyclic AMP. *J. Virol.* **63**:1604–1611.
 60. Pozzatti, R., J. Vogel, and G. Jay. 1990. The human T-lymphotropic virus type I *tax* gene can cooperate with the *ras* oncogene to induce neoplastic transformation of cells. *Mol. Cell. Biol.* **10**:413–417.
 61. Ruben, S., H. Poteat, T. Tan, K. Kawakami, R. Roeder, W. Haseltine, and C. Rosen. 1988. Cellular transcription factors and the regulation of IL-2 receptor gene expression by the HTLV-I *tax* gene product. *Science* **241**:89–92.
 62. Ruben, S. M., A. Perkins, and C. A. Rosen. 1989. Activation of NF- κ B by the HTLV-I trans-activator protein Tax requires an additional factor present in lymphoid cells. *New Biol.* **1**:275–284.
 63. Sadowski, I., J. Ma, S. Triezenberg, and M. Ptashne. 1988. GAL4-VP16 is an unusually potent transcriptional activator. *Nature (London)* **335**:563–564.
 64. Saito, S., M. Nakamura, K. Ohtani, M. Ichijo, K. Sugamura, and Y. Hinuma. 1988. *trans*-activation of the simian virus 40 enhancer by a pX product of human T-cell leukemia virus type I. *J. Virol.* **62**:644–648.
 65. Seiki, M., J.-I. Inoue, T. Takeda, and M. Yoshida. 1986. Direct evidence that p40* of human T-cell leukemia virus type I is a trans-acting transcriptional activator. *EMBO J.* **5**:561–565.
 66. Shimotohno, K., M. Takano, T. Teruuchi, and M. Miwa. 1986. Requirement of multiple copies of a 21-nucleotide sequence in the U3 region of human T-cell leukemia virus type I and type II long terminal repeats for trans-acting activation of transcription. *Proc. Natl. Acad. Sci. USA* **83**:8112–8116.
 67. Siekevitz, M., M. B. Feinberg, N. Holbrook, F. Wong-Staal, and W. C. Green. 1987. Activation of the interleukin-2 and interleukin-2 receptor (Tac) promoter expression by the trans-activator (tat) gene product of human T-cell leukemia virus, type I. *Proc. Natl. Acad. Sci. USA* **84**:5389–5393.
 68. Siekevitz, M., S. F. Josephs, M. Dukovich, N. Pfeffer, F. Wong-Staal, and W. C. Greene. 1987. Activation of the HIV-I LTR by T cell mitogens and the trans-activator protein of HTLV-I. *Science* **238**:1575–1578.
 69. Slamon, D. J., M. F. Press, L. M. Souza, D. C. Murdock, M. J. Cline, D. Golde, J. C. Gasson, and I. S. Y. Chen. 1985. Studies of the putative transforming protein of the type I human T-cell leukemia virus. *Science* **228**:1427–1430.
 70. Smith, M. R., and W. C. Green. 1991. Identification of HTLV-I *tax* trans-activator mutants exhibiting novel transcriptional phenotypes. *Genes Dev.* **4**:1875–1885.
 71. Sodroski, J. G., C. Rosen, W. C. Goh, and W. Haseltine. 1985. A transcriptional activator protein encoded by the x-lor region of the human T-cell leukemia virus. *Science* **228**:1430–1434.
 72. Stern, S., M. Tanaka, and W. Herr. 1989. The oct-1 homeo-domain directs formation of a multiprotein-DNA complex with the HSV transactivator, VP16. *Nature (London)* **341**:624–630.
 73. Stringer, K. F., J. C. Ingles, and J. Greenblatt. 1990. Direct and selective binding of an acidic transcriptional activation domain to the TATA-box factor TFIID. *Nature (London)* **345**:783–786.
 74. Summers, M. D., and G. E. Smith. 1987. A manual of methods for baculovirus vectors and insect cell culture procedures. Texas Agricultural Experimental Station Bulletin no. 1555. College Station, Tex.
 75. Tan, T., M. Horikoshi, and R. G. Roeder. 1989. Purification and characterization of multiple nuclear factors that bind to the TAX-inducible enhancer within the human T-cell leukemia virus type I long terminal repeat. *Mol. Cell. Biol.* **9**:1733–1745.
 76. Tan, T., R. Jia, and R. G. Roeder. 1989. Utilization of signal transduction pathway by the human T-cell leukemia virus type I transcriptional activator *tax*. *J. Virol.* **63**:3761–3768.
 77. Tanaka, A., C. Takahashi, S. Yamaoka, T. Nosaka, M. Maki, and M. Hatanaka. 1990. Oncogenic transformation by the *tax* gene of human T-cell leukemia virus type I *in vitro*. *Proc. Natl. Acad. Sci. USA* **87**:1071–1075.
 78. Tsujimoto, A., H. Nyunoya, T. Morita, T. Sato, and K. Shimotohno. 1991. Isolation of cDNAs for DNA-binding proteins which specifically bind to a *tax*-responsive enhancer element in the long terminal repeat of human T-cell leukemia virus type I. *J. Virol.* **65**:1420–1426.
 79. Unger, T., and Y. Shaul. 1990. The X protein of the hepatitis B virus acts as a transcription factor when targeted to its responsive element. *EMBO J.* **9**:1889–1895.
 80. Weiss, R. A., and T. F. Schulz. 1990. Transforming properties of the HTLV-I *tax* gene. *Cancer Cells* **2**:281–283.
 81. Yoshimura, T., J.-I. Fujisawa, and M. Yoshida. 1990. Multiple cDNA clones encoding nuclear proteins that bind to the tax-dependent enhancer of HTLV-I: all contain a leucine zipper structure and a basic amino acid domain. *EMBO J.* **9**:2537–2524.
 82. Zhao, L.-J., and C.-Z. Giam. 1991. Interaction of the human T-cell lymphotropic virus type I (HTLV-I) transcriptional activator Tax with cellular factors that bind specifically to the 21-base-pair repeats in the HTLV-I enhancer. *Proc. Natl. Acad. Sci. USA* **88**:11445–11449.