The Tax Protein of Human T-cell Leukemia Virus Type 1 Mediates the Transactivation of the c-sis/Platelet-derived Growth Factor-B Promoter through Interactions with the Zinc Finger Transcription Factors Sp1 and NGFI-A/Egr-1*

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Transcriptional up-regulation of the c-sis/platelet-derived growth factor-B (PDGF-B) proto-oncogene by the Tax protein of human T-cell leukemia virus type 1 has been implicated as one possible mechanism of cellular transformation by human T-cell leukemia virus type 1. In previous work, we identified an essential site in the c-sis/PDGF-B promoter, Tax-responsive element 1 (TRE1), necessary for transactivation by Tax. We also identified Sp1, Sp3, and NGFI-A/Egr-1 as the primary nuclear transcription factors binding to TRE1 which mediate Tax responsiveness. In the present work, we have investigated the mechanism(s) whereby Tax trans-activates the c-sis/PDGF-B proto-oncogene. In vitro transcription assays showed that Tax was able to significantly increase the transcriptional activity of a template containing the −257 to +74 region of the c-sis/PDGF-B promoter. Electrophoretic mobility shift assay analysis showed that Tax increased the DNA binding activity of both Sp1 and NGFI-A/Egr-1 using a TRE1 probe. Analysis of Tax mutants showed that two mutants, IEXC29S and IEXL320G, were unable to significantly transactivate the c-sis/PDGF-B promoter. Finally, co-immunoprecipitation analysis revealed that Tax is able to stably bind to both Sp1 and NGFI-A/Egr-1. Interestingly, co-immunoprecipitation analysis also revealed that Tax mutant IEXC29S is unable to interact with NGFI-A/Egr-1, whereas Tax mutant IEXL320G is able to interact with NGFI-A/Egr-1.

Infection by human T-cell leukemia virus type 1 (HTLV-1) is associated with a highly aggressive and fatal malignancy of mature and T-helper lymphocytes, adult T-cell leukemia/lymphoma (1), and the degenerative neuromuscular disease, tropical spastic paraparesis/HTLV-1 associated myelopathy, in humans (2, 3). Replication of the virus is strongly dependent upon expression of the virally encoded Tax protein, a potent trans-activator of the HTLV-1 long terminal repeat (4–8). Tax is highly pleiotropic, as it has been shown to transcriptionally activate a wide variety of cellular genes, including IL-2 (9), IL-2Ra (9), granulocyte macrophage colony-stimulating factor (9), transforming growth factor β (9, c-fos (9), and c-sis (10). Tax does not bind DNA directly (11, 12), but appears to stimulate RNA synthesis mediated through several structurally unrelated cellular transcriptional activator proteins. These include members of the cAMP response element binding proteins and activating transcription factor (CREB/ATF) family, serum response factor, Fos-Jun, and the NF-κB family of transcription regulatory proteins (13–20). The expression of only a single gene, the β-polymerease gene, has been shown to be repressed by Tax (21, 22).

The c-sis proto-oncogene, which encodes the B-chain of platelet-derived growth factor (PDGF) (23, 24), is actively transcribed in T-cells infected with HTLV-1. The transcript has been cloned and sequenced, and is apparently normal (25–27). Expression of c-sis is normally tightly regulated in a cell type- and developmental stage-specific manner and is thought to play a role in wound healing and early development (28). It is not normally expressed, however, in lymphocytes. PDGF is a potent mitogen and chemoattractant for cells of mesenchymal origin (29). Biologically active PDGF is a dimeric protein consisting of homo- and heterodimeric combinations of two polypeptide chains, A and B (30). The major function of PDGF is to induce mitosis in quiescent target cells. PDGF exerts its effects through binding to two types of receptors, the a receptor, which binds both A and B chains with high affinity, and the β receptor, which binds only the B chain (31). PDGF was first implicated in the process of transformation when one of its polypeptide chains, the B-chain/c-sis, was found to be homologous to the viral sis oncogene (v-sis) (23, 24). Indeed, expression of a recombinant, wild-type human c-sis/PDGF-B gene in mouse 3T3 cells, which express both the a and β PDGF receptors, resulted in the transformation of these cells (32).

Because lymphocytes in general were thought to have no receptors for PDGF, it was previously postulated that the c-sis/PDGF-B expression in HTLV-1 infected T-cells could possibly provide a paracrine function, perhaps stimulating stromal cells, or other nonlymphocytic cells known to have PDGF receptors. More recently, however, it has been demonstrated that HTLV-1 infected T-cells also express high levels of PDGF-β receptor transcripts and synthesize protein that can be immunoprecipitated with antibodies specific for the PDGF receptor that binds the PDGF-B homodimer and the PDGF-AB het-
eredimer (33). These findings suggest the possibility that HTLV-1 infected T-cells might acquire an autocrine mechanism of cell proliferation that involves PDGF.

With regard to the regulatory mechanism(s) that underlies c-sis/PDGFB expression in HTLV-1 infected T-cells, previous work from our laboratory (34), and others (35–39), identified a regulatory site at −64 to −45 within the c-sis/PDGFB-B promoter, that we named Tax-responsive element 1 (TRE1). This regulatory site was shown to be essential for transactivation by Tax. In addition, electrophoretic mobility shift assay (EMSA) analysis and antibody supershift analysis of TRE1-binding proteins in Jurkat-E6.1 and Jurkat-Tax cell nuclear extracts, along with nuclear extracts prepared from the HTLV-1 infected T-cell line, HUT102, identified the Sp family members, Sp1 and Sp3, along with a member of the immediate early response gene family, NGFI-A/Egr-1 (for the sake of simplicity, NGFI-A/Egr-1 from this point on will be referred to as Egr-1), as the main TRE1-binding factors mediating Tax-responsiveness.

In the current study, we have investigated the mechanism(s) whereby Tax transactivates the c-sis/PDGFB proto-oncogene promoter. In vitro transcription analysis showed that Tax was able to markedly increase RNA synthesis from a template containing the −257 to +74 region of the c-sis/PDGFB-B promoter. Site-directed mutagenesis of the TRE1 region was used to identify a CAACCC and GNGGGGNG motif essential for transactivation by Tax. EMSA analysis hinted at a possible mechanism of Tax transactivation in that Tax was able to substantially increase the DNA binding activity of both Sp1 and Egr-1 to their DNA recognition sites contained within TRE1. In addition, EMSA analysis also indicated the possibility of ternary complex formation consisting of Sp1 or Egr-1, Tax, and DNA, suggesting that Tax might stably interact with both Sp1 and Egr-1 through protein-protein contacts. We show by co-immunoprecipitation analysis that Tax does indeed stably interact with both Sp1 and Egr-1 in both the absence of DNA and from intact cells. Finally, we identified two Tax mutants, C29S and L320G, that were unable to transactivate the c-sis/PDGFB-B promoter by more than 2-fold. Interestingly, co-immunoprecipitation analysis also revealed that Tax mutant C29S was unable to interact with Egr-1, whereas, Tax mutant L320G was able to interact with Egr-1. The results presented in this article support the possibility of the existence of an additional, as yet uncharacterized, pathway of transactivation by Tax involving members of the zinc finger family of transcription factors.

**MATERIALS AND METHODS**

**Cell Culture**—The T-cell lines, Jurkat E6.1 and Jurkat-Tax (J-tax-19; a generous gift from Warner C. Greene) (40), and the HTLV-1 infected T-cell line, MT2, were grown at 37 °C in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 100 units of penicillin/ml, and 100 µg of streptomycin/ml. COS-7 cells were grown at 37 °C in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum, 110 µg/ml sodium pyruvate, 100 units of penicillin/ml, and 100 µg of streptomycin/ml. Stimulation of cells was performed by treatment for 6–8 h (unless otherwise specified) with 12-O-tetradecanoylphorbol-13-acetate (TPA) (10 ng/ml) and ionomycin (0.4 µg/ml).

**Plasmids**—The c-sis/PDGFB luciferase reporter plasmid, pRALuc, the pRALuc linker-scanning mutants, −64/−55, −54/−45, and −34/−25 and the pRALuc site-directed mutant constructs, YL0–YL7, have been described previously (38, 39). The wild-type Tax expression plasmid IEX, and the Tax mutant expression plasmids, IEXS10A (serine at position 257 changed to alanine), IEXS258A (serine at position 258 changed to alanine), IEXH43Q (histidine at position 43 changed to glutamine), IEXS258A (serine at position 258 changed to alanine), and IEXL320G (leucine at position 320 changed to glycine), have been described previously (41). The NGFI-A/Egr-1 expression plasmid, pJDM1731, used for the Ni2⁺ chelate chromatography purification of Tax, and the NGFI-A/Egr-1 expression plasmid, pJDM1731, used for the Ni2⁺ chelate chromatography purification of NGFI-A/Egr-1, have been described previously (42, 43). All mutants were sequenced by the DNA sequencing method (44). Briefly, nuclei were isolated by centrifugation at 14,000 × g for 2 min following cell lysis with 40 mM KCl, 10 mM HEPES (pH 7.0), 3 mM MgCl₂, 1 mM dithiothreitol, 5% glycerol, 8 µg of aprotinin/ml, 2 µg of leupeptin/ml, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and 0.2% Nonidet P-40 (v/v).

**Preparation of Nuclear Extracts**—Nuclear extracts were prepared from Jurkat E6.1 cells treated with 10 ng/ml TPA and 0.4 µg/ml ionomycin for 1–2 h as described by Leiden et al. (44). Briefly, nuclei were isolated by centrifugation at 14,000 × g for 2 min following cell lysis with 40 mM KCl, 10 mM HEPES (pH 7.0), 3 mM MgCl₂, 1 mM dithiothreitol, 5% glycerol, 8 µg of aprotinin/ml, 2 µg of leupeptin/ml, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and 0.2% Nonidet P-40 (v/v). Nuclei were resuspended in a solution of 20 mM HEPES (pH 7.9), 0.42 M KCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM PMSF, and 25% (v/v) glycerol for 30 min at 4 °C. Extracts were cleared by centrifugation at 14,000 × g for 10 min at 4 °C. The resulting supernatants were dialyzed for 6–10 h at 4 °C against 1 × in vitro transcription buffer (buffer A) containing 20 mM HEPES (pH 7.9), 0.1 M KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM PMSF, and 2% (v/v) glycerol and frozen in aliquots at −70 °C. Protein concentrations were determined with a commercially available kit (Bio-Rad).

**Bacterial Expression and Purification of Recombinant TaxH6 and Egr-1**—Proceded as follows. Escherichia coli BL21(DE3) cells transformed with pTaxH6 were grown at 37 °C in 1 liter of Terrific Broth medium containing 100 µg/ml ampicillin until A₅₀₀ = 1.0–1.5, then induced for TaxH6 expression with 40 µl isopropyl-1-thio-β-D-galactopyranoside at room temperature overnight. Cells were harvested and resuspended in 20 ml of ice-cold PBS containing 300 mM NaCl, 0.25 mM PMSF, 0.5 mM 2-mercaptoethanol, and 10 mM imidazole. The cells were sonicated for 1 min at 4 °C, and the supernatant fraction carried out a ruptured by sonication carried out at 60% duty cycle for 4 × 1 min duty cycle. After centrifugation at 16,000 × g for 30 min at 4 °C, the supernatant was mixed with 2 ml of Ni²⁺-NTA-agarose (Qiagen) at 4 °C for at least 2 h. The protein bound gel matrix was then packed into a column (1.5 cm × 10 cm) and washed with 40 ml of the same buffer containing 40 µM imidazole. TaxH6 protein was then eluted with a 50-ml gradient of 50–500 mM imidazole. A negative control bacterial extract (prepared in the same manner from bacteria that were transformed with a vector containing no insert DNA) was also prepared. Purification of NGFI-A/Egr-1H6 proceeded as follows: E. coli BL21(DE3)pLysS cells transformed with pJDM1731 were grown at 37 °C in 1 liter of Terrific Broth medium containing 100 µg/ml ampicillin until A₅₀₀ = 0.7–1.0, then induced for TaxH6 expression with 40 µl isopropyl-1-thio-β-D-galactopyranoside at room temperature overnight. Cells were harvested and resuspended in 20 ml of ice-cold PBS containing 300 mM NaCl, 0.25 mM PMSF, 0.5 mM 2-mercaptoethanol, and 10 mM imidazole. The cells were sonicated for 1 min at 4 °C, and the supernatant fraction carried out a ruptured by sonication carried out at 60% duty cycle for 4 × 1 min duty cycle. After centrifugation at 16,000 × g for 30 min at 4 °C, the supernatant was mixed with 2 ml of Ni²⁺-NTA-agarose (Qiagen) at 4 °C for at least 2 h. The protein bound gel matrix was then packed into a column (1.5 cm × 10 cm) and washed with 40 ml of the same buffer containing 40 µM imidazole. NGFI-A/Egr-1H6 protein was then eluted with a 50-ml gradient of 50–500 mM imidazole. Fractions collected from both the TaxH6 purification and the NGFI-A/Egr-1H6 purification were analyzed by SDS-7.5% polyacrylamide gel electrophoresis, followed by Coomassie Brilliant Blue staining. Purity of each was determined to be approxi-
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Immunoprecipitation Analysis of Tax Mutants in COS-7 Cells—

5 μg of either the wild type Tax expression plasmid, IEX, or the Tax mutant expression plasmids, IEX29S and IEXL320G, were transfected into 40–60% confluent COS-7 cells in a 100-mm tissue culture dish, either alone or with 5 μg of the Egr-1 expression plasmid, pMDM485, using the Lipofectamine reagent (Life Technologies, Inc.). 5 μg of pMDM485 was also transfected alone into 40–60% confluent COS-7 cells in a 100-mm tissue culture dish as a control for Egr-1 expression. Each of the transfections was performed in triplicate. Forty-eight hours post-transfection, the cells were washed with PBS, harvested by trypsinization, and pelleted by centrifugation at 14,000 × g for 5 min at 4 °C. The cells from all samples, except the Egr-1 alone sample, were then lysed by resuspending the cell pellet in 0.5 ml of RIPA buffer for 5 min at room temperature and clarified by spinning at 14,000 × g for 5 min at 4 °C. For the Egr-1 alone sample, a whole cell extract was prepared by three cycles of freeze-thawing in an ethanol/dry ice bath and 37 °C water bath. 5 μl of pooled αTax mAbs was added to each sample, except the Egr-1 alone sample, and the samples were then incubated for 1 h on a rotator at 4 °C. 50 μl of a solution of immobilized rProtein A-Sepharose (Pharmacia Corp.) was then added to each sample. The reaction mixtures were incubated for 18–24 h on a rotator at 4 °C. The beads were then gently washed 5–8 times with 500 μl of RIPA buffer. The bound proteins were eluted in SDS sample loading buffer, subjected to electrophoresis on a 7.5% denaturing polyacrylamide gel, transferred to nitrocellulose, and Western blotted for Tax (a generous gift from W. C. Greene), and NGFI-A/Egr-1 (Santa Cruz Biotechnology). The fractions containing either purified TaxH1 or NGFI-A/Egr-1 were, or a mutated Sp1 CC motif region, respectively, were prepared by annealing complementary, single-stranded oligonucleotides (Life Technologies, Inc.) in a thermocycler (95 °C for 5 min, cool to 25 °C at 1 °C/min). TRE1, 5′-GCCAGAAGAG-GAAAAAGCTGCTCATCCACCTGCGAC-3′; TRE2, 5′-GCCAAGAGGAAAAGCTGCTGATCGAACCTCTCGCAC-3′. Each probe was end-labeled with γ-32P-ATP (ICN Biomedicals, Inc.) and T4 polynucleotide kinase (New England Biolabs). Typical in vitro binding reactions (20 μl) contained approximately 1 ng of either purified Sp1 (Promega) or purified NGFI-A/Egr-1 (Amer- sham), 1 × Superdex buffer (13) (25 mM HEPES (pH 7.9), 12.5 mM MgCl2, 10 μM ZnSO4, 150 mM KCl, 4 mM 2-mercaptoethanol, 20% (v/v) glycerol, 0.1% Nonidet P-40), and 10–20 fmol of 32P-labeled probe (150−400 cpm). In samples containing TaxH1, approximately 400 ng of purified TaxH1 (NGRI), 200 ng of poly(dI-dC) (Amer- sham), 1 × Superdex buffer, and 70 ng of poly(dI-dC) (Am- sham), was also used in each sample. The co-immunoprecipitations were then resolved by electrophoresis on a 5% nondenatur- ing, polyacrylamide gel (acrylamide/N,N'-methylenebisacrylamide weight ratio, 49:1) at 165 V for 4 h at 4 °C in 1 × TGE buffer (25 mM Tris-HCl (pH 8.5), 190 mM glyoxyl, 1 mM EDTA). In reactions that included cold (unlabeled) oligonucleotide competitors, the samples were allowed to incubate with the cold oligonucleotide probes, along with poly(dI-dC) in 1 × Superdex buffer, for 10 min at room temperature prior to the addition of the labeled DNA probe. In reactions containing αTax Ab, 2 μl of 10 μg/ml antibody was added to the appropriate samples and incubated for 1 h at 4 °C, along with poly(dI-dC) in 1 × Superdex buffer, for 1 h at 4 °C prior to the addition of the labeled DNA probe. The gels were then dried under vacuum at 80 °C for 1 h and exposed to XAR-film.

Immunoprecipitation—Co-immunoprecipitation reactions (50 μl), using purified proteins, contained approximately 200 ng of either purified Sp1 (Promega) or purified NGFI-A/Egr-1 (Amer- sham), and approximately 400 ng of purified TaxH1 in 1 × Superdex buffer. The samples, containing the various purified proteins, were allowed to incubate for 15 min at room temperature prior to the addition of 5 μl of pooled Tax monochoncal antibodies (αTax) (Santa Cruz Biotechnology). After each Western blot, the blot was stripped by submerging the membrane in stripping buffer and incubating at 50 °C for 30 min. The same blot (membrane) was used for both Western blots.

In Vitro Transcription Reactions—Typical in vitro transcription reactions (25 μl) contained 50 μg of stimulated Jurkat E6.1 nuclear extract, 500 ng of glass-bead purified template DNA (the 954-bp NcoI–NotI fragment of either the wild type Tax expression plasmid, IEX, or the Tax mutant expression plasmids, IEX29S and IEXL320G, were transfected into 40–60% confluent COS-7 cells in a 100-mm tissue culture dish, either alone or with 5 μg of the Egr-1 expression plasmid, pMDM485, using the Lipofectamine reagent (Life Technologies, Inc.). Each of the transfections was performed in triplicate. Forty-eight hours post-transfection, the cells were washed with PBS, harvested by trypsinization, and pelleted by centrifugation at 14,000 × g for 5 min at 4 °C. The cells from all samples, except the Egr-1 alone sample, were then lysed by resuspending the cell pellet in 0.5 ml of RIPA buffer for 5 min at room temperature and clarified by spinning at 14,000 × g for 5 min at 4 °C. For the Egr-1 alone sample, a whole cell extract was prepared by three cycles of freeze-thawing in an ethanol/dry ice bath and 37 °C water bath. 5 μl of pooled αTax mAbs was added to each sample, except the Egr-1 alone sample, and the samples were then incubated for 1 h on a rotator at 4 °C. 50 μl of a solution of immobilized rProtein A-Sepharose (Pharmacia Corp.) was then added to each sample. The reaction mixtures were incubated for 18–24 h on a rotator at 4 °C. The beads were then gently washed 5–8 times with 500 μl of RIPA buffer. The bound proteins were eluted in SDS sample loading buffer, subjected to electrophoresis on a 7.5% denaturing polyacrylamide gel, transferred to nitrocellulose, and Western blotted for Tax (a generous gift from W. C. Greene), and NGFI-A/Egr-1 (Santa Cruz Biotechnology). The fractions containing either purified TaxH1 or NGFI-A/Egr-1 were, or a mutated Sp1 CC motif region, respectively, were prepared by annealing complementary, single-stranded oligonucleotides (Life Technologies, Inc.) in a thermocycler (95 °C for 5 min, cool to 25 °C at 1 °C/min). TRE1, 5′-GCCAGAAGAG-GAAAAAGCTGCTCATCCACCTGCGAC-3′; TRE2, 5′-GCCAAGAGGAAAAGCTGCTGATCGAACCTCTCGCAC-3′. Each probe was end-labeled with γ-32P-ATP (ICN Biomedicals, Inc.) and T4 polynucleotide kinase (New England Biolabs). Typical in vitro binding reactions (20 μl) contained approximately 1 ng of either purified Sp1 (Promega) or purified NGFI-A/Egr-1 (Amer- sham), 1 × Superdex buffer (13) (25 mM HEPES (pH 7.9), 12.5 mM MgCl2, 10 μM ZnSO4, 150 mM KCl, 4 mM 2-mercaptoethanol, 20% (v/v) glycerol, 0.1% Nonidet P-40), and 10–20 fmol of 32P-labeled probe (150−400 cpm). In samples containing TaxH1, approximately 400 ng of purified TaxH1 (NGRI), 200 ng of poly(dI-dC) (Am- sham), 1 × Superdex buffer, and 70 ng of poly(dI-dC) (Am- sham), was also used in each sample. The co-immunoprecipitations were then resolved by electrophoresis on a 5% nondenatur- ing, polyacrylamide gel (acrylamide/N,N'-methylenebisacrylamide weight ratio, 49:1) at 165 V for 4 h at 4 °C in 1 × TGE buffer (25 mM Tris-HCl (pH 8.5), 190 mM glyoxyl, 1 mM EDTA). In reactions that included cold (unlabeled) oligonucleotide competitors, the samples were allowed to incubate with the cold oligonucleotide probes, along with poly(dI-dC) in 1 × Superdex buffer, for 10 min at room temperature prior to the addition of the labeled DNA probe. In reactions containing αTax Ab, 2 μl of 10 μg/ml antibody was added to the appropriate samples and incubated for 1 h at 4 °C, along with poly(dI-dC) in 1 × Superdex buffer, for 1 h at 4 °C prior to the addition of the labeled DNA probe. The gels were then dried under vacuum at 80 °C for 1 h and exposed to XAR-film.
To demonstrate that the transcription observed in our in vitro run-off transcription assays was mediated by RNA polymerase II (pol II), the pol II inhibitor, α-amanitin (a bicyclic octapeptide from the mushroom Amanita phalloides), was added to the reactions. At low concentrations, α-amanitin selectively inhibits pol II-dependent transcription through binding to the largest subunit of pol II and blocking transcription elongation (46–49). Transcription from the c-sis/PDGF-B promoter was completely abolished upon addition of α-amanitin to 5 mg/ml (Fig. 1B, lane 5), as well as transactivation by Tax (Fig. 1B, lane 6).

To demonstrate the necessity of the TRE1 region for both basal transcription and transactivation by Tax, in vitro run-off transcription reactions were performed using the pRALuc linker-insertion mutants 264/255, 254/245, and 234/225 as templates. Each mutant contains a 10-bp substitution linker sequence at the designated nucleotides. Mutants 264/255 and 254/245 disrupt TRE1 while mutant 234/225 disrupts the TATA box/initiator region. As shown in Fig. 1B, transcription from the mutant templates −64/−55 and −54/−45 is significantly reduced both in the absence and presence of Tax (compare lane 7 versus lanes 1 and 8, and lane 9 versus lanes 1 and 10, respectively). When the TATA box/initiator region is disrupted by mutant −34/−25, transcription from this template is completely abolished in both the absence and presence of Tax (Fig. 1B, lanes 11 and 12). These data demonstrate Tax's ability to enhance pol II-dependent transcription from the c-sis/PDGF-B promoter in an in vitro run-off transcription assay along with the relative importance of the TRE1 region for both basal transcription and Tax-mediated transactivation.

Site-directed Mutagenesis of TRE1—To further investigate the regulatory region contained within TRE1, a series of c-sis/PDGF-B promoter mutants containing high-resolution mutations (Fig. 2) within TRE1, driving expression of the firefly luciferase reporter gene, was constructed. These site-directed mutants were transiently transfected into Jurkat-Tax cells, which were then treated with 10 ng/ml TPA and 0.4 μg/ml ionomycin. The cells were subsequently lysed and assayed for luciferase activity as described under “Materials and Methods.” After subtraction of background activity from all of the reporter constructs, pRALuc was arbitrarily given a value of 1 and the activities of the other transfections were adjusted relative to this activity. The individual 2–3 base substitutions (YL1-YL-7) are indicated. The span of the 264/255 and 254/245 linker-scanning substitution mutants are also indicated. Error bars represent 1 S.D. calculated from at least three independent experiments. WT, wild type.
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Ternary Complex Formation between Either Sp1 or Egr-1, Tax, and DNA—The observation that the addition of Tax to the binding reaction mixtures resulted in the formation of additional, slower migrating, shifted complexes (Fig. 4 and Fig. 5, Sp1 C2 and Egr-1H C2) prompted us to investigate the possibility that Tax itself, was stably incorporated into the complex. We addressed this possibility by antibody supershift analysis utilizing the TRE1 probe mentioned above. As shown in Fig. 5, when an antibody directed against Tax was included in either the Sp1 (compare lane 4 versus lanes 2 and 3) or Egr-1 (compare lane 7 versus lanes 5 and 6) samples containing Tax, not only was Tax-mediated enhancement of binding abolished, but also, basal binding of Sp1 and Egr-1 was completely abolished. Surprisingly, the addition of anti-Tax antibody did not give rise to the formation of antibody supershifted complexes, but, rather, completely blocked the formation of all shifted complexes. This effect was not due to the antibody directly interfering with the binding activity of Sp1 or Egr-1, since their binding was not inhibited by the antibody in the absence of Tax (lane 8 and lane 9, respectively). In addition, this effect of the antibody was specific for Tax, since preimmune serum did not affect Tax-mediated enhancement of either Sp1 or Egr-1 binding (lane 10.
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Fig. 4. Enhancement of Sp1 and Egr-1 DNA binding activity by Tax. DNA binding activities of Sp1 and Egr-1 were assayed by EMSA analysis in the presence and absence of Tax. Purified Sp1 (~1 ng) (lanes 2–4), or purified Egr-1H6 (~1 ng) (lanes 6–8) were incubated with a probe containing the TRE1 site of the c-sis/PDGF-B promoter. Binding reactions were carried out in the absence (lanes 2 and 6, respectively) or presence of TaxH6 (lanes 3 and 7, respectively). To demonstrate specificity of binding, Sp1 (lane 4) and Egr-1H6 (lane 8) were incubated in the presence of a 1000-fold molar excess of unlabeled TRE1 probe. As a control, the TRE1 probe was incubated by itself (lane 1) or with TaxH6 only (lane 5). Binding reactions were carried out as described under “Materials and Methods.” Similar results were observed using dilutions of TaxH6 down to 25 ng. Sp1 shifted complexes, Sp1 C1 and Sp1 C2, are indicated on the left side of the gel by arrows. Egr-1H6 shifted complexes, Egr-1H6 C1 and Egr-1H6 C2, are indicated on the right side of the gel by arrows. Lanes containing TRE1 probe, or the mutated TRE1 probe, TRE1-mSp1, are indicated.

Fig. 5. Inhibition of Tax-mediated enhancement of Sp1 and Egr-1 DNA binding activity by Tax-specific antisera. Sp1 and Egr-1 Tax-mediated supershift complex formation was assayed by EMSA analysis using Tax-specific antisera. Purified Sp1 (~1 ng) was incubated with a probe containing the TRE1 site of the c-sis/PDGF-B promoter either alone (lane 2), in the presence of TaxH6 (lane 3), or in the presence of TaxH6 and 2 μl of Tax-antisera (aTaxC Ab) (lane 4). Purified Egr-1H6 (~1 ng) was incubated with a probe containing the TRE1 site of the c-sis/PDGF-B promoter alone (lane 5), in the presence of TaxH6 (lane 6), or in the presence of TaxH6 and 2 μl of aTaxC Ab (lane 7). To demonstrate that the aTaxC Ab did not effect Sp1 or Egr-1H6 binding in the absence of TaxH6, both Sp1 (lane 8) and Egr-1H6 (lane 9) were incubated in the presence of aTaxC Ab alone. Specificity of aTaxC Ab is demonstrated by incubation of either Sp1 (lane 10) or Egr-1H6 (lane 11), with preimmune sera in the presence of TaxH6. As a control, the TRE1 probe was incubated by itself (lane 1), with aTaxC Ab only (lane 12), or with preimmune sera only (lane 13). Binding reactions were carried out as described under “Materials and Methods.” Sp1 shifted complexes, Sp1 C1 and Sp1 C2, are indicated on the left side of the gel by arrows. Egr-1H6 shifted complexes, Egr-1H6 C1 and Egr-1H6 C2, are indicated on the right side of the gel by arrows. *nsp indicates a nonspecific shifted complex.
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To further demonstrate the relevance of these interactions, co-immunoprecipitation reactions were performed using whole cell extracts prepared from the HTLV-1 infected T-cell line, MT2. As shown in Fig. 7A, for co-immunoprecipitation reactions involving Sp1, a band of approximately 95 kDa (corresponding to Sp1) was detected in the sample incubated in the presence of α-Tax monoclonal antibodies (lane 1), whereas it was not detected in the sample incubated in the absence of α-Tax monoclonal antibodies (lane 2). This band also comigrated with the bands detected in the positive control samples using nuclear extract prepared from mitogen-stimulated Jurkat E6.1 cells and purified Sp1 (Fig. 7B, lanes 4 and 5). Similarly, for co-immunoprecipitation reactions involving Egr-1, a band of approximately 85 kDa (corresponding to Egr-1) was detected in the sample incubated in the presence of α-Tax monoclonal antibodies (Fig. 7B, lane 1), whereas it was not detected in the sample incubated in the absence of α-Tax monoclonal antibodies (Fig. 7B, lane 2). This band also comigrated with the band detected in the positive control sample.

Each sample (Fig. 6C, lanes 2–4).

To further demonstrate the relevance of these interactions, co-immunoprecipitation reactions were performed using whole cell extracts prepared from the HTLV-1 infected T-cell line, MT2. As shown in Fig. 7A, for co-immunoprecipitation reactions involving Sp1, a band of approximately 95 kDa (corresponding to Sp1) was detected in the sample incubated in the presence of α-Tax monoclonal antibodies (lane 1), whereas it was not detected in the sample incubated in the absence of α-Tax monoclonal antibodies (lane 2). This band also comigrated with the bands detected in the positive control samples using nuclear extract prepared from mitogen-stimulated Jurkat E6.1 cells and purified Sp1 (Fig. 7B, lanes 4 and 5, respectively). Similarly, for co-immunoprecipitation reactions involving Egr-1, a band of approximately 85 kDa (corresponding to Egr-1) was detected in the sample incubated in the presence of α-Tax monoclonal antibodies (Fig. 7B, lane 1), whereas it was not detected in the sample incubated in the absence of α-Tax monoclonal antibodies (Fig. 7B, lane 2). This band also comigrated with the band detected in the positive control sample.
using nuclear extract prepared from mitogen-stimulated Jurkat E6.1 cells (Fig. 7B, lane 4). Taken together, these findings demonstrate that Tax physically interacts with both Sp1 and Egr-1, through protein-protein contacts.

**Ability of HTLV-1 Tax Mutants to Transactivate the c-sis/ PDGF-B Promoter**—To examine the transactivation activities of Tax mutants on the c-sis/PDGF-B promoter, Jurkat E6.1 cells were transiently transfected with the pRALuc luciferase reporter plasmid either alone or together with the wild-type or mutant Tax expression plasmids, IEIXS10A, IEIXC29S, IEIXH43Q, IEIXS258A, and IEIXL320G. The cells were then treated with RNA polymerase II transcription inhibitor, actinomycin D, and assayed for luciferase activity as described under “Materials and Methods.” After subtraction of background activity from all of the reporter constructs, pRALuc was arbitrarily designated as 1-fold induction and the activities of the other transfections were adjusted relative to this activity. Error bars represent 1 S.D. calculated from at least two independent experiments.

**Analysis of HTLV-1 Tax Mutants IEIXC29S and IEIXL320G to Interact with Egr-1**—With the knowledge that the HTLV-1 Tax mutants IEIXC29S and IEIXL320G were unable to significantly transactivate the c-sis/PDGF-B promoter, we reasoned that this might be a result of their inability to interact with Egr-1. The idea of focusing on the interaction between Tax and Egr-1, and not Tax and Sp1, is based on the observations that Sp1 and Egr-1, through protein-protein contacts, interact prepared from COS-7 cells either alone or cotransfected with the Egr-1 expression plasmid, pJDM948. (lanes 5-7, respectively). Whole cell extracts were prepared and co-immunoprecipitation reactions were carried out as described under “Materials and Methods.” Five μg of pJDM948 was also transfected alone into COS-7 cells as a control for Egr-1 expression (lane 8). For the Egr-1 alone sample, a whole cell extract was prepared by three cycles of freeze-thawing in an ethanol/dry ice bath and 37 °C water bath. Western blots (WB) for Egr-1 and Tax are indicated to the left of each blot. The Egr-1 and Tax bands are indicated by arrows to the right of the each gel. Molecular weight markers are indicated in kilodaltons (kDa) to the left of each individual Western blot.

**DISCUSSION**

In this study, we have investigated the mechanism of HTLV-1 Tax-mediated transactivation of the c-sis/PDGF-B promoter by *in vitro* transcription assay and site-directed mutation analysis, along with EMSA and co-immunoprecipitation analysis. We have shown by *in vitro* transcription assay analysis that addition of Tax to the *in vitro* transcription reaction led to an 11-fold increase in RNA synthesis (Fig. 1B, compare lane 3 versus lane 2). It has previously been reported that Tax-mediated *in vitro* transcription from the HTLV-1 promoter is resistant to low levels of α-amanitin (at low concentrations, α-amanitin selectively inhibits RNA pol II) in reactions containing either HeLa or CEM T-cell extract. This is surprising in light of the fact that the HTLV-1 promoter contains all of the structural features of a typical RNA pol II transcription template: a TATA box ~30 bp upstream of the transcription initiation site, binding sites for several pol II transcription factors, and long poly(A)+ RNA is synthesized from the integrated HTLV-1 proviral DNA *in vivo*. In addition, *in vitro* transcription experiments, supported by either HeLa or CEM T-cell extracts, showed that the HTLV-1 promoter contains overlapping transcription units which utilize the same transcription initiation site as that of pol II-dependent transcription. Further...
thermore, neutralization and depletion experiments with pol II antibodies strongly suggested that classical pol II is not involved in the HTLV-1 overlapping transcription unit (61, 62).

Similarly, the c-sis/PDGF-B promoter contains all of the structural features of a typical RNA pol II transcription template: a TATA box -30 bp upstream of the transcription initiation site, binding sites for several pol II transcription factors, and poly(A)^+ RNA transcripts are synthesized from the promoter. Given the above mentioned data and the fact that our in vitro run-off transcription assays were supported by nuclear extracts prepared from stimulated Jurkat E6.1 T-cells, we tested our in vitro transcription assays for pol II-dependent transcription. We showed that both transcription in the absence of Tax, and Tax-mediated transactivation, was completely inhibited at a low concentration of α-amanitin (Fig. 1B, compare lanes 5 and 6 versus lanes 2 and 3). In addition, we also demonstrated the relevant importance of the TRE1 region for Tax-mediated transactivation by using DNA templates containing linker-insertion substitution mutations within TRE1 (Fig. 1B, lanes 7–10). This would suggest that the binding of Sp1 or Egr-1 to TRE1 is essential for transactivation mediated by Tax. Together, these results indicated that Tax was able to greatly stimulate RNA synthesis in vitro from the c-sis/PDGF-B promoter, and that this Tax-mediated transactivation was dependent not only on RNA pol II, but also on the binding of either Sp1 or Egr-1 to the TRE1 region.

When the SIS-Luc TRE1 site-directed mutants were analyzed for Tax-responsiveness, a CCACC motif (mutants YL1-YL3) and a GNGNGGGNG motif (mutants YL1-YL5) were clearly identified as being essential for conferring Tax responsiveness in stimulated Jurkat-Tax cells (Fig. 2). That the results observed in this experiment were indeed due to Tax and not a result of the effects of TPA and ionomycin stimulation is evidenced by the fact that we have previously (34) analyzed a series of linker-scanning substitution mutants within the TRE1 region using unstimulated Jurkat E6.1 cells versus unstimulated Jurkat-Tax cells and observed the same results as those presented in this article. It should also be noted that stimulation in the presence of Tax results in a synergistic activation of the c-sis/PDGF-B promoter. Stimulation is necessary to transiently activate the expression of Egr-1.

The CCACC motif has been previously reported to be a positive regulatory element in the promoters of several genes, capable of binding members of the Sp family of zinc finger transcription factors (50–53). Indeed, the nucleotides CCACC also comprise the core nucleotide sequence of the DNA recognition sequence for the Sp family member, Sp1 (Fig. 3A) (57). This same CCACC motif has also been demonstrated to be critical for transcription of the c-sis/PDGF-B gene in the human osteosarcoma cell line, U2-OS, involving the Sp family members, Sp1 and Sp3 (35, 38). Likewise, the GNGNGGGNG motif has also been previously reported to be a positive cis-acting regulatory element found in the promoters of several genes, capable of binding the zinc finger transcription factor Egr-1, which is a member of the immediate-early transcription factor gene family (52, 55, 56). Previous work from our laboratory (34) demonstrated that the main nuclear factors binding to TRE1 from the stimulated T-cell lines, Jurkat E6.1 and Jurkat-Tax, along with the HTLV-1 infected T-cell line, HUT102, included the Sp family members Sp1 and Sp3, along with the immediate-early transcription factor gene family member, Egr-1. Furthermore, it should be noted that since the TRE1 region of the c-sis/PDGF-B promoter contains overlapping binding sites for the Sp family members, Sp1 and Sp3, and the immediate-early transcription factor gene family member, Egr-1, these factors cannot associate together and simultaneously bind to TRE1. Indeed, it has recently been shown that in unstimulated cells, Sp1 occupies this element and is displaced by increasing amounts of Egr-1 upon stimulation (37).

Both the CCACC motif, and the GNGNGGGNG motif, are also found in the promoters of two genes previously shown to be up-regulated and transactivated by Tax. These include the PTHrP gene and the IL-2 gene (Fig. 3B) (58, 59). These motifs represent binding sites for members of the zinc finger family of transcription factors. This proves to be interesting in light of previous experiments carried out to investigate the mechanism(s) of Tax-mediated transactivation. Site-directed mutational analysis of Tax has allowed for an approximate demarcation of regions within the Tax protein itself necessary for transactivation (41, 63). These analyses indicated that Tax transactivation of viral and cellular promoters occurs through at least two distinct cellular transcription pathways: 1) the CREB/ATF family and 2) the NFp65/Rel protein family of transcription factors. It now appears that an additional Tax-mediated transactivation pathway exists involving members of the zinc finger family of transcription factors.

EMSA analysis showed that Tax dramatically enhanced the site-specific DNA binding activity of the zinc finger transcription factors Sp1 (Fig. 4, compare lane 2 versus lane 3) and Egr-1 (Fig. 4, compare lane 6 versus lane 7) to a probe containing the TRE1 site. This is consistent with previous reports which suggested that Tax may deregulate target genes by enhancing the DNA binding activity of the cellular transcription factors that recognize the Tax-responsive promoter elements (19, 20, 64). Indeed, Tax has been shown to increase the DNA binding activity of a wide variety of eukaryotic transcription factors, including, members of the CREB/ATF family of proteins, serum response factor, Fos-Jun, and the NFkB subunits, p50 and p65 (13, 14). Mutation of the CCACC and GNGNGGGNG motifs prevented the binding of Sp1 and Egr-1, respectively, to the TRE1 probe, in both the presence and absence of Tax (Fig. 4, lanes 9–13). This further demonstrated the importance of each of these motifs for conferring Tax responsiveness.

EMSA analysis also indicated that Tax altered the mobility of both the Sp1-DNA complex (Fig. 4, lane 3, Sp1 C2) and the Egr-1-DNA complex (Fig. 4, lane 7, Egr-1H6 C2), suggesting that the observed increase in DNA binding activity for Sp1 and Egr-1 (mediated by Tax) involves Tax entering into the complex, leading to the formation of ternary complexes. It is possible that the Sp1 C2 and Egr-1H6 C2 complexes are the result of Tax increasing the affinity of a less abundant complex that was previously not visible in the Tax-minus samples (Fig. 4, lanes 2 and 6, respectively). However, this was not the case since these C2 complexes were not observed upon overexposure of the autoradiogram (data not shown). This was further substantiated by the finding that an antibody directed against the C-terminal 13 amino acids of Tax was able to completely abolish the formation of the Sp1-DNA complexes Sp1 C1 and Sp1 C2 (Fig. 5, lane 4), and the Egr-1H6 complexes, Egr-1H6 C1 and Egr-1H6 C2 (Fig. 5, lane 7), when added to the samples containing Tax. This is indicative of a potentially strong interaction between Tax and both Sp1 and Egr-1, in which binding of the antibody to Tax sterically interferes with their ability to bind DNA. As a result, if all of the Sp1 and Egr-1 in the sample is complexed with Tax, then, no binding whatsoever should occur.

Further investigation of the physical interaction between Tax and both Sp1 and Egr-1, by co-immunoprecipitation analysis, showed that Tax did, indeed, stably interact with, and bind to, both purified Sp1 (Fig. 6A, lane 3) and purified Egr-1 (Fig. 6B, lane 4). In addition, it was also demonstrated by co-immunoprecipitation analysis that Tax interacts with both...
The fact that Tax mutant IEXL320G still binds to Egr-1, but failure to transactivate the c-sis/PDG-F-B promoter is, not expected. The defect in IEXL320G most likely results from a mutation in the transactivation domain of Tax. Indeed, the presence of such a domain in Tax has been previously demonstrated (65). It was shown that amino acid residues 284 to 322 in the carboxyl-terminal region of Tax constituted a functional transactivation domain. Similar mutations of the same amino acid residues in Tax have been reported to be defective in transactivation of the HTLV-1 long terminal repeat by Tax, but have been shown to still be able to interact with CREB and the HTLV-1 21-bp repeats to assemble ternary Tax-CREB-DNA complexes (66).

As mentioned above, Tax is able to increase the DNA binding activity of a wide variety of eukaryotic transcription factors. Perhaps the best characterized of these is the basic region-leucine zipper (bZIP) DNA-binding domain family, which includes members of the CREB/ATF protein family. Dimerization is essential for bZIP proteins to bind DNA. Consequently, it was demonstrated that Tax stimulated the DNA binding activities of CREB/ATF proteins both quantitatively and qualitatively (67–69). First, it was shown that Tax enhanced dimerization, thereby increasing the overall level of DNA binding. Second, Tax altered DNA-binding site selectivity. Several reports then demonstrated that Tax was able to physically interact with several members of the CREB/ATF protein family (66, 70, 71). A more recent report demonstrated that the physical interaction between the bZIP protein, CREB, and Tax, to form a Tax-CREB-DNA ternary complex involves the incorporation of Tax into the ternary complex as a dimer. In addition, the ability of Tax to form a dimer was shown to be necessary for its interaction with CREB (72). It has not escaped our attention that Tax’s interactions with bZIP proteins deals exclusively with the ability of both Tax and the bZIP protein, to dimerize.

The results presented in this article support the possibility of the existence of an additional, as yet uncharacterized, pathway of transactivation by Tax involving members of the zinc finger family of transcription factors. Several questions as to the exact nature of this interaction between Tax and zinc finger family transcription factors do not normally dimerize prior to DNA binding, or that, dimerization is not essential for their DNA binding activity, immediately becomes apparent.

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