The human c-sis proto-oncogene promoter is transactivated by the human T-cell leukemia virus type 1 (HTLV-1) tax protein in human Jurkat T-cells. Transactivation was >7-fold in Jurkat cells stably expressing the Tax protein (Jurkat-Tax) than in Jurkat E6.1 cells and was further enhanced in Jurkat-Tax cells stimulated with 12-O-tetradecanoylphorbol-13-acetate and the calcium ionophore, ionomycin. Deletion analysis showed that a 167-base pair promoter fragment retained full Tax responsiveness. Insertion of this minimal Tax-responsive region into a heterologous, minimal promoter renders it responsive. Insertion of this minimal Tax-responsive region resulted in approximately a 7-fold increase of transcriptional activation in the presence of Tax. Linker-scanning insertion analysis of this region identified Tax-responsive elements at nucleotides −64 to −45 (TRE1) and −34 to −15 (TATA box region). TRE1 contains a consensus binding site for the Sp family of transcription factors. The TATA box region corresponds to the TATA box and its 3′-neighboring sequence. Gel-shift and antibody supershift analysis of TRE1-binding proteins in unstimulated Jurkat E6.1 and Jurkat-Tax nuclear extracts identified Sp1 and Sp3 as the main TRE1 binding factors. Nuclear extracts from stimulated Jurkat E6.1 and Jurkat-Tax cells identified an additional TRE1 binding factor, Egr-1. These studies define a novel mechanism whereby Tax transactivates the c-sis promoter.

Human T-cell leukemia virus type 1 (HTLV-1) is the etiologic agent of adult T-cell lymphoma/leukemia (1) and transforms normal human T lymphocytes in vitro (2, 3). The cellular events whereby HTLV-1 infection leads to T-cell transformation are not clearly understood. Unlike many other acutely transforming retroviruses, the HTLV-1 genome does not encode an oncogene homologous to a cellular sequence (4). This suggests that HTLV-1 transformation occurs through another mechanism mediated by a virus-produced regulatory protein(s) which may transactivate some cellular genes involved in cell proliferation. Indeed, the HTLV-1 regulatory protein Tax is a potent transactivator of the HTLV-1 long terminal repeat (LTR) and numerous cellular genes, including IL-2 (5), IL-2R-α (6), granulocyte macrophage colony-stimulating factor (7), transforming growth factor β (5), c-fos (5), and c-sis (6). Tax does not appear to bind DNA directly (7, 8). Recent evidence indicates that Tax activates transcription by inducing or modifying the activity of certain host transcription factors, including members of the activating transcription factor/cAMP response element binding protein (ATF/CREB) family of proteins, serum response factor, fos-J un, and NF-κB (9–16).

T-cells infected with HTLV-1 express high levels of trans-activators for the c-sis proto-oncogene (17, 18), which encodes the B-chain of platelet-derived growth factor (PDGF) (19, 20). Expression of the c-sis gene is restricted to selected cell types, which include activated monocytes (21) and megakaryocytes (22), developing placenta (23), and vascular smooth muscle cells (24). It is not normally expressed, however, in lymphocytes. PDGF is a potent mitogen for cells of mesenchymal origin (25). Biologically active PDGF is a dimeric protein consisting of homo- and heterodimeric combinations of two polypeptide chains, A and B (26). 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 α receptor, which binds both A and B chains with high affinity, and the β receptor, which binds only the B chain (27). PDGF was first implicated in the process of transformation when one of its peptide chains, the B-chain/c-sis, was found to be homologous to the viral sis oncogene (v-sis) (19, 20). Expression of a recombinant, wild-type human c-sis/PDGF-B gene in mouse 3T3 cells, which express both the α and β PDGF receptors, resulted in the transformation of these cells (28). Interestingly, it has also been demonstrated that HTLV-1-infected T-cells 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 heterodimer (17). These findings raise the possibility that cells which constitutively synthesize both a mitogenic growth factor and its receptor might acquire an autostimulatory mechanism that does not necessarily require secretion of the mitogenic ligand (29, 30).

With regard to the regulatory mechanisms that underlie c-sis/PDGF-B expression in HTLV-1-infected T-cells, a previous study provided preliminary deletion analysis of the 5′-flanking region and demonstrated that activation was due to the HTLV-1 regulatory protein Tax (6). In the current study, we have analyzed the Tax-mediated transactivation of the c-sis/PDGF-B promoter in human Jurkat T-cells and a Jurkat T-cell line stably expressing the Tax protein (Jurkat-Tax) (31). We prepared a series of 5′-promoter deletion mutants and a series of linker-scanning insertion mutants and used them to identify two sites, Tax-responsive element 1 (TRE1) and the TATA box region, essential for Tax-mediated transactivation. Gel-shift and antibody supershift analysis of Jurkat E6.1 and Jurkat-
Tax cells, along with the HTLV-1-infected T-cell line, HUT102, showed preferential binding of three nuclear proteins to a site within the TRE1 element.

MATERIALS AND METHODS

Cell Culture—The T-cell lines, J urkat E6.1, J urkat-Tax (gift from Warner C. Greene) (31) and the HTLV-1-infected T-cell line, HUT102, were grown at 37°C in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 100 units of penicillin per ml, and 100 μg of streptomycin per ml. Stimulation of cells was performed by treatment for 6 to 8 h (unless otherwise specified) with 12-O-tetradecanoylphorbol-13-acetate (TPA) (10 ng/ml) and ionomycin (0.4 μg/ml).

Plasmids—pSISCAT has been described previously (32). 5′-pSISCAT promoter-deletion mutants were constructed by polymerase chain reaction amplification of the designated promoter regions using specific 5′ (forward) XbaI-linked oligonucleotides and a common 3′ (reverse) PstI-linked oligonucleotide as primers. The resulting promoter fragments were digested with both XbaI and PstI and cloned into the XbaI and PstI sites of pSISCAT. ptSmFNCAT minimal promoter constructs were made by polymerase chain reaction amplification of the designated promoter regions using specific 5′ (forward) XbaI-linked oligonucleotides and a common 3′ (reverse) XbaI-linked oligonucleotide as primers. The resulting promoter fragments were digested with XbaI and cloned into the forward and reverse orientations into the XbaI site of pTAF-FN-CAT (a generous gift of Douglas C. Dean) (33). pSfHFNCAT minimal promoter constructs were made by cloning annealed oligonucleotides containing the minimal fibronectin promoter region −280 to +8 (5′-atacttccatatagccccgctccgcagccggctgcag-3′ (sense strand), 5′-ccgggctcgagcgctggctcattata-tggtgat-3′ (antisense strand)) into the EcoRV and PstI sites of the ptSmFNCAT minimal promoter constructs (terminal nucleotides shown in lowercase type were added to create EcoRV and PstI sites). pRLuc and pRLuc linker-scanning mutants have been described previously (34, 35). The Tax expression plasmid, pCTax, was a generous gift from Warner C. Greene (36). pHTLV1CAT (6) and pCAT (37) have been described previously. All mutants were sequenced by the dideoxy chain termination method (U.S. Biochemical).

Transfections, CAT Assays, and Luciferase Assays—5 × 106 Jurkat-Tax cells were transfected with either 20 μg of chloramphenicol acetyltransferase (CAT) reporter plasmid or 20 μg of luciferase reporter plasmid, plus, where indicated, 20 μg of a Tax expression plasmid, using the DEAE-dextran method as described previously (38). When stimulated, 36 h post-transfection the cells were divided equally into two flasks; one flask was supplemented with TPA (10 ng/ml) and ionomycin (0.4 μg/ml), and the other flask received the same volume of solvent. Thirty-six hours after stimulation, the cells were harvested by centrifugation, washed with phosphate-buffered saline, and cell lysates were prepared by three cycles of freeze-thawing in an ethanethiol/water bath at 37°C. Cell extracts were normalized for protein content by a commercially available kit (Bio-Rad). Equal amounts of protein were used in CAT assays (32) and luciferase assays (39, 40) as described previously.

Preparation of Nuclear Extracts—Nuclear extracts were prepared from HUT102 cells, along with unstimulated and stimulated J urkat E6.1 and J urkat-Tax cells as described by Lodden et al. (41). 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 (DTT), 5% glycerol, 8 μg of aprotinin/ml, 2 μg of leupeptin/ml, 0.5 mM phenylmethylsulfonyl fluoride, and 0.2% Nonidet P-40 (v/v). Nuclei were resuspended in a solution of 20 mM HEPES (pH 7.9), 0.4 M KCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 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 6–24 h at 4°C against buffer containing 20 mM HEPES (pH 7.9), 0.1 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, and 20% (v/v) glycerol and were then dialyzed in aliquots at −70°C. Protein concentrations were determined with a commercially available kit (Bio-Rad).

Eletrophoretic Mobility Shift Assays (EMSAs)—The following double-stranded oligonucleotide probes and competitors corresponding to the cis-sis/PGDF-B promoter region were prepared by annealing complementary, single-stranded oligonucleotides (Life Technologies, Inc.) in a thermostable 7.5 min, 95°C, and 20°C (0.2 μg/ml): wt-83/45, 5′-GGCCAGAAAGGAGGGAAGGCTGTCCTCCACCCACCTCTGGAGC-3′; wt-57/42, 5′-CTGTCCTCACACCTTCTGACCT-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 5 μg of nuclear extract, 1 μg of poly(d-d-dC) (Amersham), 1 × Superdex buffer (9) (25 mM HEPES (pH 7.9), 125 mM MgCl2, 10 mM 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 (15–30 × 106 cpm). To reduce nonspecific binding, nuclear extracts were preincubated with poly(d-d-dC) in 1 × Superdex buffer for 10 min at room temperature; probe was then added, and the mixture was incubated for an additional 20 min at room temperature. The DNA-protein complexes were then resolved by electrophoresis on a 5% nondenaturing, 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 glycerine, 1 mM EDTA). In reactions that included cold (unlabeled) oligonucleotide competitors, the nuclear extracts were allowed to incubate with the cold oligonucleotide probes, along with poly(d-d-dC) in 1 × Superdex buffer, for 10 min at room temperature before the addition of the labeled DNA probe. In supershift reactions, 2 μl of antiserum directed against either Sp1, Sp3, or Egr-1 (Santa Cruz Biotechnology) was incubated with the nuclear extract, along with poly(d-d-dC) in 1 × Superdex buffer, for 1 h at 4°C prior to the addition of the labeled DNA probe.

RESULTS

Identification of the Minimal Sequence Necessary for Tax Responsiveness in the c-sis/PGDF-B Promoter—A previous study provided preliminary deletion analysis of the cis-sis/PGDF-B promoter in human J urkat T-cells and showed that a 406-bp fragment (containing 386 bp 5′ to the mRNA initiation site, as well as 16 bp 3′ to the mRNA initiation site) fused to the chloramphenicol acetyltransferase (CAT) reporter gene (psISCAT) retained full Tax responsiveness (6). As shown in Fig. 1, when psISCAT was transiently transfected into J urkat E6.1 cells either alone or cotransfected with a Tax expression plasmid, CAT activity was increased >7-fold in the presence of Tax. A >7-fold increase in CAT activity was also observed when

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Tax Activation of c-sis

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pSISCAT was transiently transfected into Jurkat-Tax cells compared with that observed with Jurkat E6.1 cells in the absence of Tax. Similar results were obtained with the positive control vector pHTLV/ICAT. (Fig. 1). As a result, instead of cotransfектing Jurkat E6.1 cells with a Tax-expression plasmid, we decided to use Jurkat-Tax cells to further investigate the Tax-mediated transactivation of the c-sis/PDGF-B promoter. To define more precisely the minimal sequence required for Tax-mediated transactivation, a series of 5′-promoter deletion mutants, generated from pSISCAT, was constructed (Fig. 2). These constructs were transfected into either Jurkat E6.1 or Jurkat-Tax cells and CAT activity was measured. Constructs d-242 and d-151 retained full Tax-responsiveness. Deletions downstream of d-151, however, markedly attenuated Tax responsiveness (Fig. 2). These data identify the minimal Tax-responsive region as that spanned by d-151.

Enhancement of pTA-FN-CAT Expression by the Minimal Tax-responsive Region of the c-sis/PDGF-B Promoter—To investigate whether the region we identified as the minimal Tax-responsive region was capable of conferring Tax-responsiveness onto a heterologous, minimal promoter, the region spanning nucleotides −151 to −46 (just upstream of the c-sis/PDGF-B promoter TATA box) was inserted into the CAT expression plasmid pTA-FN-CAT, in both the forward and reverse orientations (Fig. 3A). In its minimal promoter, the pTA-FN-CAT expression plasmid contains only the fibronectin gene promoter TATA box and RNA initiator sequence. The distance between nucleotide −46 of the c-sis/PDGF-B promoter and the fibronectin gene promoter TATA box is identical with the distance between nucleotide −46 and the TATA box of the wild-type c-sis/PDGF-B promoter. Thus, pSISCAT activity in Jurkat-Tax cells was increased by approximately 7-fold in the presence of Tax. When inserted in the reverse orientation (phSmFN/151-46), the wild-type c-sis/PDGF-B promoter oligonucleotide −151 to −46 increased the level of activity approximately 7-fold in the presence of Tax. When inserted in the reverse orientation (phSmFN/151-46), the level of activity was increased approximately 6-fold in the presence of Tax. Activity was further enhanced in the presence of Tax when the cells were stimulated with TPA and the calcium ionophore, ionomycin. In contrast, when the distance between nucleotide −46 of the c-sis/PDGF-B promoter and the fibronectin gene promoter TATA box was increased by 41 nucleotides compared with that of nucleotide −46 and the TATA box of the wild-type c-sis/PDGF-B promoter, Tax responsiveness was completely abolished (data not shown). Thus, it appears that the observed Tax responsiveness is distance/spacing-dependent. These results indicated that the −151 to −46 region of the c-sis/PDGF-B promoter served the function of a Tax-responsive element.

Fig. 2. Relative CAT activity of the c-sis/PDGF-B 5′-promoter deletion constructs in Jurkat E6.1 cells versus Jurkat-Tax cells. 20 μg of each reporter construct was transiently transfected into either Jurkat E6.1 cells or Jurkat-Tax cells. The cells were subsequently lysed and assayed for CAT activity as described under “Materials and Methods.” After subtraction of background pCAT activity from all of the reporter constructs, pSISCAT activity in Jurkat-Tax cells was arbitrarily given a value of 100, 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. The length of the promoter in each construct is indicated as the number of base pairs upstream of the mRNA initiation site.
were measured. As shown in Fig. 5, we observed two regions that were essential for Tax-mediated transactivation. The first region, ablated by mutants −64/−55 and −54/−45, was designated Tax-responsive element 1 (TRE1). The second region, ablated by mutants −34/−25 and −24/−15, included the TATA box and its 3′-neighboring sequence. When compared with wild-type level of activity, mutation of either TRE1 or the TATA box region resulted in substantial reductions in Tax-mediated luciferase expression (Fig. 5). Similar results were obtained using stimulated cells (data not shown). The level of Tax-mediated luciferase expression did not differ significantly from wild-type when linker-scanning mutants corresponding to the region located between nucleotides −154 and −94 were tested (data not shown).

TRE1 Binds Nuclear Factors in Human Leukemic T-cell Lines—Identification of the TRE1 site in the c-sis/PDGFB promoter prompted us to investigate whether this element bound nuclear factors. A set of double-stranded oligonucleotides (corresponding to nucleotides −83 to −45) containing the TRE1 site was prepared. The TRE1 oligonucleotide was labeled and used as a probe for in vitro electrophoretic mobility shift assays (EMSAs). Four major EMSA complexes were observed after the TRE1 probe was incubated with nuclear extracts prepared from Jurkat E6.1 (Fig. 6, lanes 2 and 3) and Jurkat-Tax cells (lanes 4 and 5). Three of the major EMSA complexes (C1, C2, and C4) were seen with nuclear extracts prepared from both unstimulated Jurkat E6.1 (lane 2) and Jurkat-Tax cells (lane 4) and with extracts prepared from TPA/ionomycin-treated Jurkat E6.1 (lane 3) and Jurkat-Tax cells (lane 5). However, the major remaining EMSA complex observed (C3) with extracts from Jurkat E6.1 and Jurkat-Tax cells (lanes 3 and 5), appeared only upon stimulation with TPA and ionomycin. Interestingly, when nuclear extracts prepared from unstimulated HUT102 cells were incubated with the TRE1 probe, the same four major EMSA complexes were observed (C1–C4) as with unstimulated and stimulated extracts from Jurkat E6.1 and Jurkat-Tax cells (lane 6). In addition, a fifth major EMSA complex (C5), which migrated between C3 and C4, was observed only with extract prepared from HUT102 cells (lane 6). These EMSA complexes were specific for the probe, since their formation was blocked by unlabeled TRE1 probe using extract prepared from stimulated Jurkat-Tax cells (Fig. 7, compare lane 3 versus lane 2). Similar results were obtained using extracts prepared from unstimulated Jurkat E6.1, Jurkat-Tax, and HUT102 cells, as well as stimulated Jurkat E6.1 cells (data not shown).

Due to the fact that the TRE1 probe contained 19 nucleotides upstream of the −64 to −45 TRE1 region itself, it was possible that one or more of the EMSA complexes were binding to this region. To investigate this possibility, we conducted a competition experiment using an unlabeled probe containing only the TRE1 region itself (−67 to −42). As shown in Fig. 7, formation of the EMSA complexes was blocked when extract prepared from stimulated Jurkat-Tax cells was incubated with unlabeled −67/−42 probe (compare lane 4 versus lane 2). Similar results were obtained using extracts prepared from unstimulated Jurkat E6.1, Jurkat-Tax, and HUT102 cells, along with stimulated Jurkat E6.1 cells (data not shown). These results indicated that the major EMSA complexes were indeed binding.
to the TRE1 region itself.

Identification of Sp1, Sp3, and Egr-1 as the Main TRE1 Binding Factors in Human Leukemic T-cell Line Nuclear Extracts—Analysis of the nucleotide sequence within TRE1 revealed the presence of a CACCC regulatory motif. Previous work has demonstrated that this motif represents a cis-acting element for the Sp family of transcription factors (42-45). To investigate whether members of the Sp family of transcription factors were present in any of the EMSA complexes obtained with the TRE1 probe mentioned above, we performed a competition experiment using an unlabeled consensus Sp family probe. As shown in Fig. 7, formation of complexes C1, C2, and C4, but not C3, was blocked when extract prepared from stimulated Jurkat-Tax cells was incubated with unlabeled consensus Sp family probe (compare lane 5 versus lane 2). Similar results were obtained using extracts prepared from unstimulated Jurkat E6.1, Jurkat-Tax, and HUT102 cells, along with stimulated Jurkat E6.1 cells (data not shown). We next used antibodies raised against the Sp family members, Sp1 and Sp3, in EMSA-antibody supershift assays. A monoclonal antibody specific for human Sp1 was able to supershift complex C1 when extract prepared from stimulated Jurkat-Tax cells was pre-

![Image](http://www.jbc.org/)

**Fig. 5.** Relative luciferase activity of the c-sis/PDGFB promoter linker-scanning insertion constructs in Jurkat E6.1 cells versus Jurkat-Tax cells. Twenty μg of each reporter construct was transiently transfected into either Jurkat E6.1 or Jurkat-Tax cells. The cells were subsequently lysed and assayed for luciferase activity as described under “Materials and Methods.” After subtraction of background pLUC activity from all of the reporter constructs, pRALuc activity in Jurkat-Tax cells was arbitrarily given a value of 1, and the activities of the other transfections were adjusted relative to this activity. The region of linker-scanning insertion within the promoter is represented by a black shaded box within each construct. The precise location of each linker-scanning insertion is indicated as the number of base pairs upstream of the mRNA initiation site. Error bars represent 1 S.D. calculated from at least two independent experiments.

**Fig. 6.** EMSA showing binding of nuclear proteins prepared from either unstimulated Jurkat E6.1 (lane 2), Jurkat-Tax (lane 4), and HUT102 (lane 6) cells or stimulated Jurkat E6.1 (lane 3) and Jurkat-Tax (lane 5) cells to an oligonucleotide containing the c-sis/PDGFB promoter TRE1 (−83 to −45). Lane 1 contains probe alone. The positions of complexes C1, C2, C3, C4, and C5 are indicated by arrows to the left of the gel. Stimulation of the cells was performed as described under “Materials and Methods.”

![Image](http://www.jbc.org/)

**Fig. 7.** Specificity and identification of nuclear factors binding to TRE1 of the c-sis/PDGFB promoter using a nuclear extract prepared from stimulated Jurkat-Tax cells. An oligonucleotide containing the c-sis/PDGFB promoter TRE1 (−83 to −45) was used as the probe. Unlabeled TRE1 probe (−83 to −45) (lane 3), an oligonucleotide corresponding to the c-sis/PDGFB promoter region −67 to −42 (lane 4), and an Sp family consensus oligonucleotide (lane 5) were used as competitors. Concentrations of competitor oligonucleotides are shown as molar excesses as compared with the concentration of the TRE1 probe (−83 to −45). The positions of complexes C1, C2, C3, and C4 are indicated by arrows to the left of the gel. Lanes 6–8 indicate antibody supershift assays using antibodies specific for human Sp1 (lane 6), human Sp3 (lane 7), and human Egr-1 (lane 8). Lane 1 contains probe alone. Positions of the supershifted complexes are indicated to the right of the gel. Open arrowheads represent an Sp3 supershifted complex, and the arrow represents an Sp1 supershifted complex. The cells was performed as described under “Materials and Methods.”

- The relative luciferase activity of the c-sis/PDGFB promoter linker-scanning insertion constructs in Jurkat E6.1 cells versus Jurkat-Tax cells was analyzed.
- Antibodies specific for human Sp1 and Sp3 were used in EMSA-antibody supershift assays to identify nuclear factors binding to TRE1.
- The positions of complexes C1, C2, C3, C4, and C5 were indicated by arrows to the left of the gel.
- Stimulation of the cells was performed as described under “Materials and Methods.”
- Specificity and identification of nuclear factors binding to TRE1 were performed using antibodies specific for human Sp1, Sp3, and Egr-1.

![Image](http://www.jbc.org/)

**Relative Luciferase Activity**

![Image](http://www.jbc.org/)

**Tax Activation of c-sis**

![Image](http://www.jbc.org/)

**c-sis Promoter/Regulatory Region**

- Jurkat E6.1 Cells
- Jurkat-Tax Cells

![Image](http://www.jbc.org/)

**Competitor (1000X)**

- Free Probe
- Jurkat E6.1 NE
- Jurkat-Tax NE
- HUT102 NE

![Image](http://www.jbc.org/)

**Antibody**

- pRLuc
- pLUC

![Image](http://www.jbc.org/)

**C1**

- Sp1 supershifted complex

![Image](http://www.jbc.org/)

**C2**

- Egr-1 supershifted complex

![Image](http://www.jbc.org/)

**C3**

- Sp3 supershifted complex
DISCUSSION

In this study, we have examined the HTLV-1 Tax-mediated transactivation of the c-sis/PDGFB-B promoter in human J urkat T-cells by deletion, linker-scanning substitution, and gel shift analysis. By transient transfection analysis of 5′-promoter deletion mutants, a sequence consisting of 151 bp upstream and 16 bp downstream of the mRNA initiation site was found to retain full Tax responsiveness (Fig. 2). Insertion of this minimal Tax-responsive region, both in the forward and reverse orientations, into a heterologous, minimal expression vector resulted in a >6-fold increase in transcription in the presence of Tax. Tax responsiveness was further enhanced upon stimulation with TPA and ionomycin (Figs. 3 and 4).

To further examine the minimal Tax-responsive region of the c-sis/PDGFB-B promoter, we constructed a series of linker-scanning mutants in which each plasmid received a 10-bp substitution linker sequence (containing a Pvul restriction site) at a single site within this region (Fig. 5). There is a 2-fold advantage in using linker-scanning mutants to identify regulatory elements. First, wild-type DNA sequence and promoter architecture are preserved throughout the entire promoter region being analyzed except for the 10-bp substitution site, leaving 5′-regions intact. Second, since the substitution linker was the same in each mutant, this served to minimize any extrinsic effect(s) attributable to the substitution linker sequence.

When we analyzed the linker-scanning mutants for Tax responsiveness, we identified two cis-acting elements necessary for Tax-mediated transactivation. The first element, which we named TRE1, was located between nucleotides −64 and −45. TRE1 was shown to contain a consensus binding sequence for the Sp family of transcription factors, CACCC. This same 20-nucleotide sequence was also identified (34) as essential for the activation of the c-sis/PDGFB-B promoter that is observed in TPA-treated K562 cells as they differentiate into megakaryocytes. The second element, located between nucleotides −34 and −15, corresponded to the TATA box and its 3′-neighboring sequence of the c-sis/PDGFB-B promoter. This result indicated that the Tax-mediated increase of transcription in J urkat cells was initiated within the initiator region of the c-sis/PDGFB-B promoter and was dependent upon the TATA-binding-RNA initiation complex.

It is interesting to note that when promoter elements previously reported to be critical for c-sis/PDGFB-B expression in bovine aortic endothelial cells and human umbilical vein endothelial cells (51) were mutated by linker-scanning substitution mutants −93 to −84, −84 to −75, and −74 to −65, Tax-mediated transactivation in J urkat cells was not substantially decreased (Fig. 5). The −93 to −84 region contains an AP-1-like consensus binding sequence (−92 to −86), while the −84 to −65 region contains a consensus binding site for the Ets family of transcription factors (−78 to −68). The results observed with the −84 to −65 region were surprising in light of the fact that several reports have demonstrated the importance of Ets consensus binding sites for the Tax-mediated transactivation of the HTLV-1 LTR (11, 52, 53). In addition, it has been shown that multiple Ets family members are present in resting and activated human T-cells (54–57).

EMSA and antibody supershift analysis showed preferential binding of three nuclear proteins, Sp1, Sp3, and Egr-1, to the TRE1 element (Fig. 7, lanes 6–8). Since TRE1 was shown to contain a consensus binding sequence for the Sp family of transcription factors, it was not a surprise that the Sp family members, Sp1 and Sp3, were identified as two of the three main TRE1 binding factors. This same CACCC regulatory motif has also been identified in a Tax-responsive element of the HTLV-1 LTR (52). The binding of Egr-1 to TRE1, upon stimulation, was also not unexpected due to the fact that, in many cases, binding sites for the transcription factor Sp1 also contain overlapping, cryptic binding sites for Egr-1 (46–50). In addition, the fact that binding of Egr-1 was shown to occur only upon stimulation, is consistent with induction of Egr-1 synthesis in lymphocytes in response to mitogenic stimulation (58). Furthermore, the binding of Egr-1 to TRE1 using extracts prepared from unstimulated HUT102 cells (data not shown) was consistent with the finding that HTLV-1 Tax induces the expression of various immediate early serum response genes, including Egr-1 (59). Thus, Tax may replace/bypass growth signals, at least in part, in this manner.

These results represent the first demonstration of an Egr-1/Sp family cis-acting element mediating Tax responsiveness of a cellular gene. The cis elements mediating Tax responsiveness of various other cellular genes are diverse. The CAR box in the 5′-flanking sequence of c-fos is a Tax-responsive element (60). p67SRF, a CAI binding factor which is constitutively localized in the nucleus, mediates transcriptional activation by Tax through direct interaction with Tax. Tax transactivation of the IL-2Rα gene is mediated through NF-κB, which is induced by Tax and subsequently interacts with the NF-κB motif (GGG-GAATTC/CC) in the IL-2Rα promoter (61, 62). Tax responsiveness of the human granulocyte macrophage/colony-stimulating factor promoter is mediated through a 22-bp sequence, containing CATT(ATT) repeats, which is also required for mitogen inducibility of the same promoter (63, 64). The critical region in the transforming growth factor β promoter which is required for Tax-mediated induction contains a sequence which is similar to an AP-1 binding site (65).

It remains to be seen how Tax mediates the transactivation of the c-sis/PDGFB-B promoter. One clue stems from the recent findings that Tax transactivation may involve enhancement in the DNA binding activity of target transcriptional regulatory proteins (9). These findings demonstrated that Tax was able to enhance the site-specific DNA binding activity of serum response factor and Fos-J un and modestly enhanced the binding of the NF-κB subunits, p50 and p65. In addition, they also showed that Tax was able to increase the DNA binding activity of the eukaryotic transcription factors ATF-1, Sp1, and GALL. In accordance, recent evidence suggests that Tax may stimulate HTLV-1 transactivation, at least in part, through enhanced binding of AFT/CREB proteins to their recognition elements within the Tax-responsive 21-bp repeats of the viral LTR (13). It may be that one possible mechanism of Tax-mediated c-sis/ PDGFB-B promoter transactivation involves enhancing the DNA binding activity of Sp1 by Tax. With regard to Egr-1, in addition to up-regulating its expression, Tax may also have an effect on its DNA binding activity. Cooperative versus antagonistic binding between Sp1, Sp3, and Egr-1 will also need to be addressed, since all three are present in unstimulated nuclear extracts prepared from HUT102 cells (Fig. 6, lane 6). In addition, further insight into the mechanism of c-sis/PDGFB-B promoter transactivation by Tax may be gained by identification of the protein(s) comprising the major EMSA complex C5, observed with nuclear extracts prepared from HUT102 cells (Fig. 6, lane 6). Future efforts should determine what functional role(s) each of the TRE1 binding proteins play in supporting Tax-mediated c-sis/PDGFB-B promoter transactivation.
c-sis/PDGF-B Promoter Transactivation by the Tax Protein of Human T-cell Leukemia Virus Type 1
Samuel R. Trejo, William E. Fahl and Lee Ratner

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