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Interaction of Human T-cell Lymphotropic Virus Type I Tax, Ets1, and Sp1 in Transactivation of the PTHrP P2 Promoter*

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We have previously shown that the parathyroid hormone-related protein (PTHrP) promoter contains binding sites for transcription factors Ets1 and Sp1 and that human T-cell lymphotropic virus type I (HTLV-I) Tax cooperates with Ets1 to transactivate the PTHrP P2 promoter. Using the yeast two-hybrid interaction system, we now provide evidence that Tax interacts with Ets1. Moreover, a double mutation (D22A,C23S) in the Tax protein that abrogated the Tax/Ets1 interaction also inhibited the Tax/Ets1 cooperative effect, suggesting that the interaction between Tax and Ets1 is important for transactivation of the PTHrP promoter. In coimmunoprecipitation assays, we find that Tax facilitates the interaction between Ets1 and Sp1, forming a ternary complex. When the Sp1 site in the PTHrP promoter was mutated, the Tax/Ets1 cooperative effect was dramatically decreased. This suggests that Sp1 plays an important role in the Ets1-dependent Tax transactivation of the PTHrP P2 promoter. Finally, we demonstrate that Gal4-Tax is a strong activator of the Gal PTHrP promoter, implying that Tax contributes directly to the transcriptional activation of the promoter. We propose a model in which the Tax/Ets1 cooperative effect on the PTHrP P2 promoter is based on the ability of Tax, Ets1, and Sp1 to form a ternary complex on the template DNA. Tax facilitates the interaction of Ets1/Sp1 and participates directly in the transcription initiation process.

Human T-cell lymphotropic (leukemia) virus type I (HTLV-I)[1] is associated with adult T-cell leukemia and the neurological disease tropical spastic paraparesis/HTLV-I-associated myelopathy (1–7). The HTLV-I genome encodes a unique protein (Tax) that is required for the virus-induced transformation of T-lymphocytes (8–10). An important function of this protein is to deregulate certain cellular genes including interleukin-2, interleukin-2 receptor, c-*fos*, major histocompatibility class II, and parathyroid hormone-related protein (PTHrP) (11–17, 19–21).[2] Tax exerts its transactivating effect on promoters through its ability to interact with and activate certain transcription factors, like the cAMP-responsive element-binding protein (CREB), the serum response factor, NF-κB, and NF-Y[2] (22–32). Tax activates NF-κB by mediating its release from a complex with the inhibitor IκB (33–36). It increases the activity of CREB by facilitating its dimerization (25–28) and its binding to the cofactor CREB-binding protein (CBP) (24). Tethering to DNA through a transcription factor would allow Tax to communicate with the basal transcription machinery. Consistent with this hypothesis, Tax has been shown to be capable of activating promoters when fused to a DNA-binding domain (37–40). In addition, Tax has been reported to bind to the basal transcription factors TFIIA and TATA-binding protein (41, 42).

We have previously shown that the transcription factor Ets1 is important for Tax transactivation of the PTHrP P2 promoter (21). Ets1, a member of the Ets family of transcription factors (for review, see Ref. 43), is expressed only in certain cell types, including T-lymphocytes. It cooperates with Sp1 to transactivate certain promoters, including the PTHrP P2 promoter (44–46). PTHrP was initially described as the causative agent for humoral hypercalcemia of malignancy, a disease associated with a variety of tumors (for review, see Ref. 47). More recently, PTHrP has also been shown to play an important role in controlling proliferation and apoptosis in normal cells and cancer cells (48–58). PTHrP is expressed by most HTLV-I-transformed cells (20, 59–61). This study was undertaken to investigate the mechanism underlying the Tax/Ets1 cooperative effect on the PTHrP P2 promoter. We present evidence that Tax interacts with Ets1 in the yeast two-hybrid system. Moreover, a Tax mutant that disrupts the complex decreases the functional interaction between Tax and Ets1 on the PTHrP promoter. Moreover, the interaction between Tax and Ets1 is part of a multimeric complex composed of Tax, Ets1, and Sp1.

MATERIALS AND METHODS

Cell Culture and Transient Transfections—Osteosarcoma OsA-CL cells (62) were maintained in RPMI 1640 medium with 10% fetal calf serum. For transient transfections, cells were trypsinized, suspended in RPMI 1640 medium with 10% serum, centrifuged, and washed in RPMI 1640 medium. Cells were resuspended in RPMI 1640 medium (1×10^7 cells/250 μl), mixed with CsCl-purified plasmid DNA, and electroporated using a Life Technologies Cell-Porator (250 V, 800 microfarads). After incubation on ice for 30 min, cells were plated on tissue cultures dishes (100 × 20 mm) in 10 ml of RPMI 1640 medium with 10% serum and incubated at 37 °C either overnight (pCTax) or for 2 days (pSG424-Tax). Chloramphenicol acetyltransferase activity was determined using [^{14}C]chloramphenicol as described previously (63) or by chloramphenicol acetyltransferase enzyme-linked immunosorbent assay (Boehringer Mannheim).

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[1] The abbreviations used are: HTLV-I, human T-cell lymphotropic virus type I; PTHrP, parathyroid hormone-related protein; CREB, cAMP-responsive element-binding protein.

[2] Pise-Masison, C. A., Dittmer, J., Clemens, K. E., and Brady, J. N., (1997) *Mol. Cell. Biol.*

Plasmids—Plasmids for the expression of wild-type Ets1, Δexon VII mutant Ets1 and Tax, as well as the chloramphenicol acetyltransferase constructs containing the wild-type −328/+20 PTHrP fragment or the Ets1 or Sp1 mutant version of this promoter have been described earlier (21, 46, 64–66). The expression plasmid for the Gal4-Tax fusion protein was created as follows. First, an *Eco*RI/*Bam*HI DNA fragment of Tax encoding amino acids 2–353 was subcloned from pLex-Tax (41) into the pCRII vector (Invitrogen). Second, the Tax-pCRII plasmid was digested with *Eco*RI and *Sac*I, and the resulting Tax-containing fragment was cloned into the multiple cloning site of plasmid pSG424 (67), allowing the *Tax* gene to be in frame with the Gal4 DNA-binding domain located upstream of Tax. Gal4-Tax mutants Gal4-M5, Gal4-M6, and Gal4-M22 were produced in two steps by cloning the *Acc*I/*Xma*I fragments from M5, M6, or M22 (66), respectively, into Tax-pCRII followed by cloning the *Eco*RI/*Sac*I fragment from the M5-, M6-, or M22-pCRII plasmid into pSG424. In a similar way, Gal4-M3 was created, except that in the initial step, the *Pfl*MI/*Xma*I fragment of wild-type Tax was replaced by the corresponding fragment of M3 (66). The first 17 amino acids of Tax were deleted by digesting Tax-pCRII with *Acc*I and *Eco*RI, followed by blunt ending with Klenow polymerase and ligation. The resulting Tax mutant ΔN18 was cloned into pSG424 as described above. Tax mutant M17/20 (V17G,Y18G,F20D) was generated in Tax-pCRII as follows. First, an oligonucleotide (sense strand, 5′-TT GGA CAG AGT CTT-CTT TTC GGA TAC CCA GGA GGC *GTC GAC* GGA GAC TGT-3′; and antisense strand, 5′-G TAC ACA GTC TCC *GTC GAC* GCC TCC TGG-GTA TCC GAA AAG AAG ACT CTG TCC AAA CC-3′ (mutated nucleotides are underlined, and the created *Sal*I site is shown in italics)) was inserted between the *Pfl*MI site and the downstream *Bsr*GI site, followed by reinsertion of the Tax *Bsr*GI fragment. The mutant was then cloned into pSG424. By using polynucleotide chain reaction and the pCRII vector as described (21), the Gal4 mutant −328/+20 PTHrP promoter was created, which contains a single Gal4-binding site (CG-GAGGACAGTACTCCG) in place of the PTHrP sequence between nucleotides −63 and −47.

Western Blot Analysis—Extracts of OsA-CL cells were separated on a 10 or 12% SDS-polyacrylamide gel in a Bio-Rad Miniprotean II cell, and proteins were subsequently blotted onto Immobilon-P membrane in a Bio-Rad Trans-Blot cell. After blocking in 3% nonfat milk dissolved in blocking buffer (100 mM NaCl, 2 mM EDTA, and 10 mM Tris-HCl, pH 7.5) for 30 min, the membrane was probed with the primary antibody for 20 min, followed by three washings in blocking buffer for 5 min each. After a 20-min incubation with an anti-IgG-horseradish peroxidase conjugate (1:10,000 dilution in blocking buffer with 0.3% milk) that recognized either anti-rabbit or anti-mouse IgG depending on the type of primary antibody used, the filter was extensively washed in blocking buffer (for ~1 h) and incubated with ECL reagent (Amersham Corp.). Bands were visualized by using Kodak X-Omat AR film. The following primary antibodies were used: rabbit anti-Ets1 (C-20, Santa Cruz Biotechnology Inc.; 1:2000 dilution in blocking buffer with 1% milk), rabbit anti-Sp1 (PEP-2, Santa Cruz Biotechnology Inc.; 1:1000 dilution in blocking buffer with 1% bovine serum albumin), mouse anti-Tax (1:500 dilution in blocking buffer with 1% bovine serum albumin), and rabbit anti-Gal4 DNA-binding domain (Upstate Biotechnology, Inc.; 1:2000 dilution in blocking buffer with 1% bovine serum albumin).

Immunoprecipitation—Ets1 baculovirus extract (0.4 μl) was incubated with Tax baculovirus extract (0.4 μl) and/or 0.2 μl of purified Sp1 (Promega) in a total volume of 10 μl of immunoprecipitation buffer (0.65 mM dithiothreitol, 1 mM EDTA, 10 mM Tris, pH 7.5, 12.5 mM KCl, 1.8 mM HEPES, pH 7.3, 1 mM $MgCl_2$, 1 μM $ZnCl_2$, 1 mg/ml bovine serum albumin, 12% glycerol, and 0.06% Nonidet P-40) for 10 min on ice. After the addition of 6 μl of anti-Ets1-agarose conjugate suspension (Santa Cruz Biotechnology Inc.; 1:5.5 dilution in immunoprecipitation buffer) to each sample and incubation on ice for 30 min, the agarose beads were spun down and washed twice in immunoprecipitation buffer. Ten μl of 2 × standard protein sample buffer was added; the mixture was boiled in water for 5 min and centrifuged; and the supernatant was analyzed by Western blotting as described above. In control experiments, Ets1 was not added to the reaction mixture.

Yeast Two-hybrid Assay for Protein-Protein Interaction—An *Ets1* gene fragment encoding amino acids 2–441 and containing a *Eco*RI site and an *Xho*I site at the 5′- and 3′-ends, respectively, was synthesized by polymerase chain reaction using high-fidelity polymerase Vent (New England Biolabs Inc.). To generate an exon VII-deleted version of this fragment, the wild-type *Bgl*II/*Aat*II sequence was replaced by the corresponding fragment of Δexon VII mutant Ets1. The fragments were then cloned into the yeast shuttle vector pJG4-5 downstream of the DNA sequences that encode the B42 acidic activator domain, the influenza virus HA1 epitope, and the simian virus 40 nuclear localization signal (41). The vector allowing the expression of LexA-Tax has been described elsewhere (41). LexA-Tax was continuously expressed in yeast cells, whereas expression of the Ets1-acidic activator domain fusion protein or the acidic activator domain alone was induced when yeast cells were grown under galactose-dependent conditions. After 5–6 h of growth in galactose-containing medium, cells were harvested and assayed for β-galactosidase activity as described.[2] To determine expression of the fusion proteins, yeast cells were lysed as described,[2] and extracts were analyzed by Western blotting.

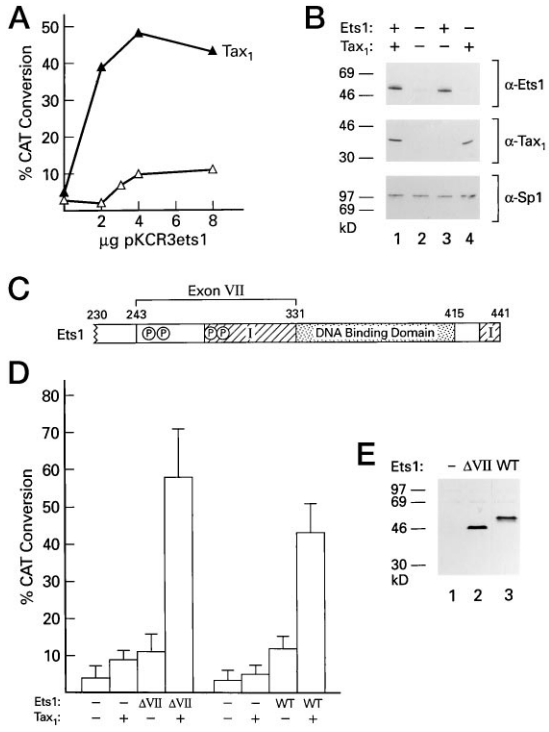

FIG. 1. **Cooperative effect of Tax and Ets1 on the −328/+20 PTHrP promoter fragment in OsA-CL cells.** *A*, chloramphenicol acetyltransferase (*CAT*) activity using increasing amounts of Ets1 expression vector pKCR3ets1 in the absence or presence of 8 μg of pCTax. *B*, Western blot analysis of OsA-CL cells transfected with 8 μg of pKCR3ets1 and 8 μg of pCTax, no DNA, 8 μg of pKCR3ets1, or 8 μg of pCTax. The blot was subsequently probed with anti-Ets1 and anti-Tax followed by reprobing with anti-Sp1 to show that the same amount of protein has been applied in all lanes. *C*, schematic representation of a C-terminal portion of Ets1 protein between amino acids 230 and 441. *Hatched* areas indicate sequences involved in autoinhibition (*I*) of Ets1. Calcium-dependent phosphorylation sites in the exon VII domain are marked (*P*). *D*, comparison of transactivation of the PTHrP promoter by Δexon VII mutant Ets1 *versus* wild-type (*WT*) Ets1 in the absence and presence of Tax. Eight μg of each expression plasmid was used. *Bars* represent the average value of three (Δexon VII mutant Ets1) or 11 (wild-type Ets1) independent experiments. *E*, Ets1-specific Western blot analysis of OsA-CL cells transfected with control, Δexon VII mutant Ets1, or wild-type Ets1 plasmid DNA.

RESULTS

The Exon VII Domain of Ets1 Is Dispensable for Tax/Ets1 Cooperation—To study the mechanism underlying the cooperative effect of Tax and Ets1 on the activity of the PTHrP P2 promoter, we utilized the human osteosarcoma cell line OsA-CL. In this cell line, Tax is not able to transactivate the P2 promoter containing the −328/+20 PTHrP gene fragment unless Ets1 is cotransfected with Tax (Fig. 1*A*). More important, Ets1 did not change the level of Tax expression, nor did Tax have an effect on the Ets1 level (Fig. 1*B*).

We also tested a Δexon VII mutant of Ets1 (Fig. 1, *C*, *D*, and *E*) for its ability to functionally interact with Tax. As shown in Fig. 1*D*, exon VII was dispensable for the Tax/Ets1 synergism. In addition, the removal of the exon VII domain did not increase the activity of Ets1 in the absence of Tax. These results

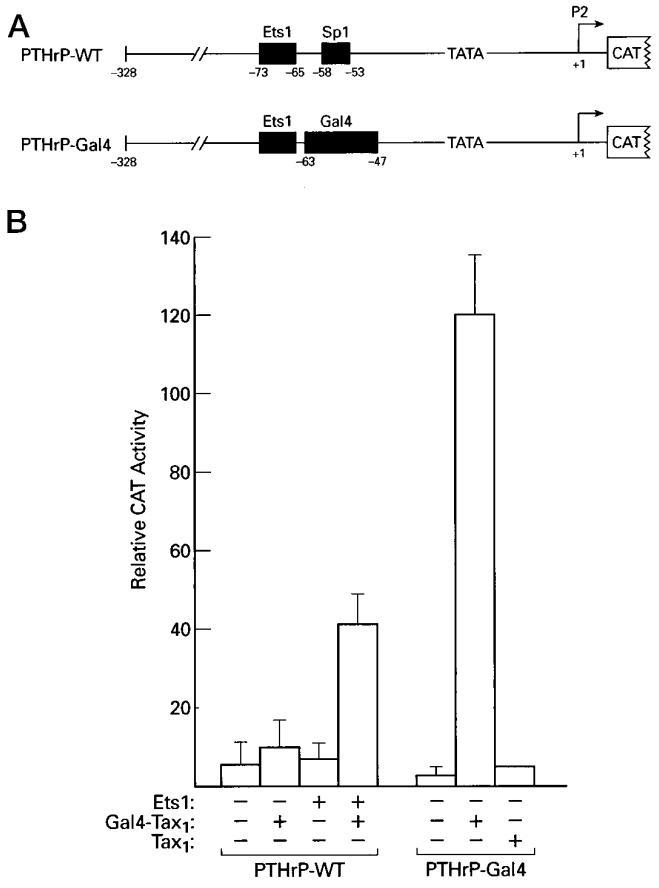

FIG. 2. *A*, schematic of the wild-type (*PTHrP-WT*) and Gal4 −328/+20 (*PTHrP-Gal4*) promoters; *B*, effect of Gal4-Tax on the wild-type −328/+20 PTHrP promoter fragment and a Gal4 mutant version of this promoter. OsA-CL cells were transfected with either 8 μg of Gal4-Tax expression plasmid or pSG424-Tax alone or together with 3 μg of pKCR3ets1 or with the Ets1 expression vector alone or with none of these plasmids. *Bars* represent the mean value of seven independent experiments. In two control experiments, 8 μg of pCTax was cotransfected with the Gal4 PTHrP chloramphenicol acetyltransferase (*CAT*) plasmid in place of pSG424-Tax.

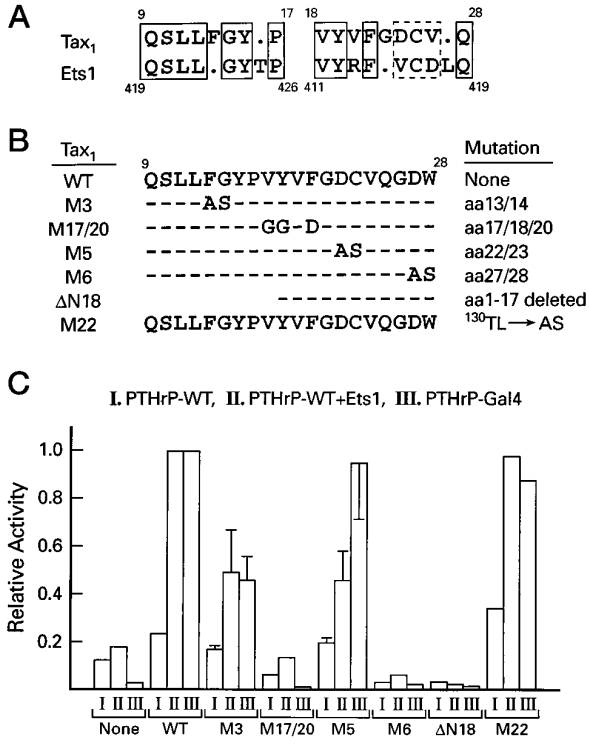

FIG. 3. **A double mutation at amino acids 22 and 23 interferes with the Tax/Ets1 cooperative effect.** *A*, alignment of the Tax amino acid sequence between positions 9 and 28 with two sequences in the C-terminal part of Ets1 (amino acids (*aa*) 411–419 and 419–426); *B*, schematic of the Gal4-Tax mutants used for transfections into OsA-CL cells; *C*, transfection experiments using either the wild-type PTHrP (*PTHrP-WT*) (*I* and *II*) or the Gal4 PTHrP (*PTHrP-Gal4*) (*III*) chloramphenicol acetyltransferase construct as a reporter plasmid. Eight μg of mutant or wild-type Gal4-Tax was transfected alone (*I* and *III*) or together with 3 μg of pKCR3ets1 (*II*). Tax mutant-induced activities of the wild-type PTHrP promoter were calculated relative to the activity of the wild-type promoter in the presence of wild-type Gal4-Tax and Ets1 together (*I* and *II*). Relative activities of the Gal4 PTHrP promoter resulted from a comparison with the activity of this promoter in the presence of Gal4-Tax alone (*III*).

suggest that exon VII-regulated DNA binding (65, 68, 69) is not a limiting factor for Ets1 transactivation of the PTHrP P2 promoter and that Tax does not exert its effect on Ets1 through an increase in its DNA binding activity.

Gal4-Tax Is Able to Activate a Gal4 PTHrP P2 Promoter—To investigate the possibility of whether Tax could directly activate the PTHrP P2 promoter, we tested a Gal4-Tax fusion protein for its ability to transactivate a Gal4 PTHrP promoter (Fig. 2*A*). As shown in Fig. 2*B*, Gal4-Tax increased the Gal4 PTHrP promoter activity by 38-fold. The Gal4-Tax fusion protein did not activate the PTHrP promoter in the absence of the Gal4-binding site, nor did Gal4-Tax significantly increase the activity of the wild-type promoter in the absence of Ets1. Furthermore, native Tax failed to activate the Gal4 PTHrP promoter. This demonstrates that the strong activation of the Gal4 PTHrP P2 promoter by Gal4-Tax was dependent on the Gal4 domain and the Gal4 DNA-binding site. It is noteworthy that of the Gal4 binding site-containing promoters we have analyzed, the Gal4 PTHrP P2 promoter was one of the strongest responders to Tax (data not shown). The results suggest that the Tax/Ets1 synergistic effect may be partly due to the ability of Tax to communicate with the basal transcription machinery of the PTHrP P2 promoter, increasing Ets1-dependent transcriptional activation of the wild-type promoter.

Mutations in the N terminus of Tax Reduce the Ability of Tax to Cooperate with Ets1—The data in Fig. 2 also show that, similar to native Tax, Gal4-Tax can cooperate with Ets1 to transactivate the wild-type PTHrP promoter. The requirement of Ets1 for Gal4-Tax-mediated transactivation of the wild-type promoter, but not the Gal4 promoter, allows screening of Tax mutants that fail to interact functionally with Ets1. Gal4-Tax mutants that fully activate the Gal4 promoter in the absence of Ets1, but that would be unable to transactivate the wild-type promoter in the presence of Ets1, could be considered as being specifically impaired in their ability to cooperate with Ets1. We tested four deletion mutants of Tax (ΔN18, ΔN60, Δ60–284, and ΔC284). None of these Tax mutants were able to transactivate either the Gal4 or the wild-type PTHrP promoter (Fig. 3*B* and data not shown). Subsequently, double or triple mutations were introduced into the *Tax* gene, focusing primarily on a stretch of N-terminal amino acids from positions 9 to 25 (Fig. 3*B*). This sequence contains homology to two adjacent C-terminal peptides in Ets1 (amino acids 411–419 and 419–426) that include the β-strand S4 (amino acids 411–414) of the DNA-binding domain and the linking region between the DNA-binding domain and the C-terminal inhibitory helix H4 (amino acids 427–432) (Fig. 3*A*). Of the mutations tested, only Gal4-M5 (D22A,C23S) Tax showed a differential activation pattern (Fig. 3*C*). This mutant was fully active on the Gal4 promoter, yet it showed a significantly reduced ability to transactivate the wild-type promoter in concert with Ets1. It is important to note that there were no differences in expression

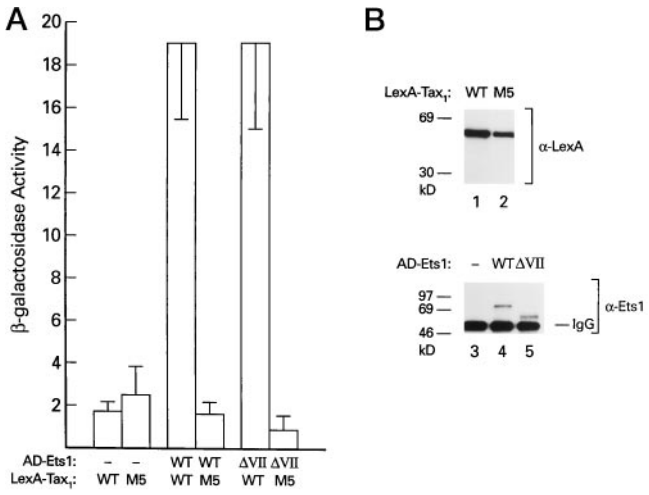

FIG. 4. **Tax binds to Ets1 *in vivo*.** *A*, for two-hybrid protein-protein binding analysis in yeast, wild-type (*WT*) or M5 mutant Tax was fused to the LexA DNA-binding domain and tested for its ability to transactivate a LexA/β-galactosidase gene reporter plasmid in the absence or presence of a wild-type Ets1 or a Δexon VII mutant Ets1 fusion protein, each containing an acidic activator domain (*AD*). β-Galactosidase activity was measured 5–6 h after culturing cells in galactose-enriched medium to induce expression of the Ets1-acidic activator domain fusion proteins or the acidic activator domain alone. *B*, Western blot analysis was performed on extracts from yeast cells incubated in galactose medium for 6 h. The expression of LexA-wild-type Tax or LexA-M5 Tax was determined by an anti-LexA antibody (*lanes 1* and *2*). The expression of wild-type or Δexon VII Ets1 was determined by an Ets1-specific antibody (*lanes 3–5*).

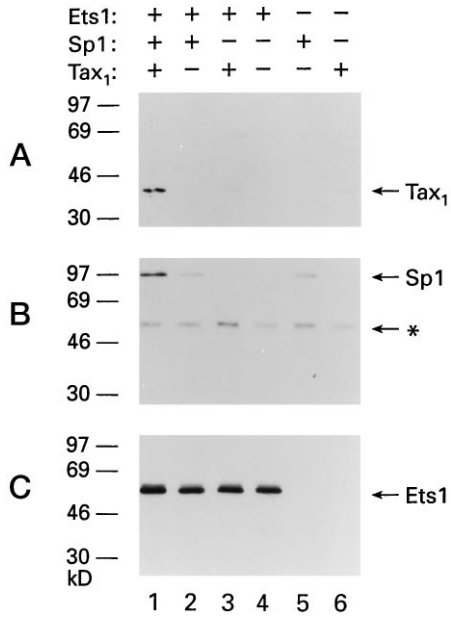

FIG. 5. **Tax forms a ternary complex with Ets1 and Sp1.** Equal amounts (~5 ng) of Tax and/or Sp1 and Ets1 were mixed and incubated with an anti-Ets1 antibody conjugate to immunoprecipitate Ets1 and associating proteins under conditions as described under "Materials and Methods." As a control, Tax or Sp1 was incubated with the antibody beads in the absence of Ets1 (*lanes 5* and *6*). The washed precipitates were analyzed for the presence of Tax, Ets1, and Sp1 by the Western blot technique. Bands indicated by an *asterisk* represent the heavy chain of the anti-Ets1 antibody.

levels of wild-type and Gal4-M5 Tax in the presence or absence of Ets1 (data not shown). Therefore, it is likely that the double mutation at positions 22 and 23 interfered specifically with the ability of Tax to cooperate with Ets1.

Tax Binds to Ets1 in the Yeast Two-hybrid System—To investigate the possibility that for functional cooperativity with Ets1, Tax must associate with Ets1, we chose the yeast two-hybrid system for the detection of protein-protein interaction *in vivo*. Tax was fused to the LexA DNA-binding domain and Ets1 to an acidic activator. After both proteins were expressed in yeast cells carrying a LexA-responsive β-galactosidase gene cassette, β-galactosidase activity was measured and compared with that of a control transfection with LexA-Tax and the acidic activator alone. As shown in Fig. 4*A*, β-galactosidase activity was increased by ~10-fold when Ets1 was coexpressed with Tax. Similar results were obtained with the Δexon VII splicing mutant Ets1 in place of wild-type Ets1. As a control for these studies, when *Drosophila* bicoid, human papilloma virus E6, or human immunodeficiency virus Tat was fused to LexA and expressed together with Ets1, no increase in β-galactosidase activity was observed (data not shown). These results suggest that Tax and Ets1 interact specifically in the yeast two-hybrid system.

Since the M5 Tax mutation impaired the ability of Tax to functionally cooperate with Ets1, we were interested in analyzing the effect of this mutation on Ets1 binding in the two-hybrid system. When we coexpressed a LexA-M5 Tax fusion protein together with either wild-type or Δexon VII Ets1, no increase in β-galactosidase activity was observed (Fig. 4*A*). As shown in Fig. 4*B*, LexA-M5 Tax was expressed in levels comparable to wild-type Tax. Therefore, the double mutation at positions 22 and 23 not only affected Tax/Ets1 synergism, but also interfered with the ability of Tax to interact with Ets1 in the two-hybrid system, suggesting that the cooperation with Ets1 and Tax requires interaction between the two proteins.

Tax Forms a Ternary Complex with Ets1 and Sp1 in Vitro—To analyze Tax/Ets1 binding *in vitro*, we performed immunoprecipitation experiments with an anti-Ets1 antibody that can recognize native Ets1 (70). We mixed equal volumes of Ets1 and Tax baculovirus extract with anti-Ets1-agarose conjugate and analyzed the washed immunoprecipitate for the presence of Tax by Western blot analysis. The amount of Tax in the immunoprecipitate was comparable to the background signal obtained in control reactions (Fig. 5*A*, *lanes 3* and *6*).

We have previously shown that Sp1 binds adjacent to Ets1 in the PTHrP P2 promoter and cooperates with Ets1 to activate this promoter (46). We were interested in analyzing whether the presence of Sp1 would affect the outcome of the immunoprecipitation experiment. When we added Sp1 to Tax and Ets1, we found that the amount of Tax that precipitated together with Ets1 was greatly increased (Fig. 5*A*, *lane 1*). When we reprobed the Western blot with anti-Sp1, a pattern was observed that was similar to that seen with anti-Tax. With Ets1 alone or in the absence of Ets1, little Sp1 was precipitated (Fig. 5*B*, *lanes 2* and *5*). In contrast, Sp1 binding was found to be significantly increased when both Ets1 and Tax were present (*lane 1*). More important, the amounts of antibody used in these experiments were similar as equal amounts of IgG heavy chain could be detected in all lanes (Fig. 5*B*). Also, similar amounts of Ets1 were precipitated with the Ets1 antibody beads (Fig. 5*C*, *lanes 1–4*). These results suggest that Sp1 stabilizes the interaction between Tax and Ets1, forming a stable ternary complex.

Tax Fails to Cooperate with Ets1 to Activate an Sp1 Mutant PTHrP P2 Promoter—Since Tax was able to form a ternary complex with Ets1 and Sp1, it was of interest to determine whether a mutation in the Sp1-binding site of the PTHrP P2 promoter would affect Ets1-dependent transactivation of the PTHrP P2 promoter by Tax. When tested in the presence of Ets1 alone, the Sp1 and Ets1 mutant PTHrP P2 promoters (Fig. 6*A*) showed a 3-fold decreased activity compared with the wild-type promoter (Fig. 6*B*). This is consistent with results

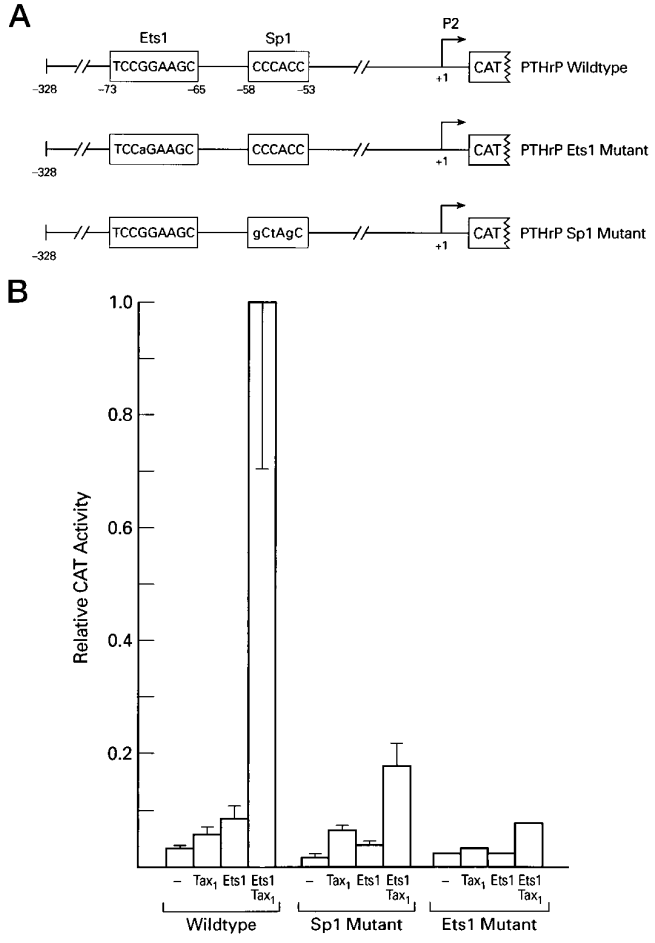

FIG. 6. **Tax fails to cooperate with Ets1 to transactivate an Sp1 mutant version of the −328/+20 PTHrP promoter.** An Sp1 and an Ets1 mutant PTHrP P2 promoter (*A*) were tested for their abilities to respond to Tax (8 μg of pCTax) and/or Ets1 (8 μg of pKCR3ets1) in transient transfection experiments (*B*) as described under "Material and Methods." *CAT*, chloramphenicol acetyltransferase.

obtained previously with Jurkat cells (46). When both Ets1 and Tax were expressed, these differences were more dramatic, as indicated by a 5.5- or 11-fold lower activity of the Sp1 or Ets1 mutant promoter, respectively, *versus* the wild-type promoter. These data show that not only the Ets1 site, but also the Sp1 site is required for the Tax/Ets1 cooperative effect.

DISCUSSION

Previous studies have demonstrated that the transcription factor Ets1 is important for Tax transactivation of the PTHrP promoter. Our present studies, using the yeast two-hybrid system, further demonstrate that Tax is able to form a complex with the transcription factor Ets1 *in vivo*. More important, mutation of Tax at amino acids 22 and 23 (Tax mutant M5) inhibited the interaction between Tax and Ets1 and the Tax/Ets1 cooperative effect. These results suggest that the interaction between Tax and Ets1 is important for transactivation of the PTHrP P2 promoter. It is interesting that, although the mutations are in close proximity, Tax mutants M17/20 and M6 did not show the differential activity pattern on the two PTHrP P2 promoters. The importance of specific amino acids for Tax transactivation through NF-κB (M22, amino acids 137 and 138) or CREB (M47, amino acids 319 and 320) has also been observed (66). It is of interest to note that Tax amino acids 22 and 23 are located within a sequence that shows homology to a C-terminal motif of the Ets1 protein. Based on preliminary studies, we speculate that this domain might be important for intra- and intermolecular interactions with the N-terminal 37 amino acids of Ets1. Consistent with this hypothesis, Ets2, which has homology to the C but not the N terminus of Ets1, fails to cooperate with Tax to transactivate the Ets1/Sp1-responsive HTLV-I long terminal repeat (71–73).

The immunoprecipitation results suggest that Tax, Ets, and Sp1 form a stable ternary complex on the DNA. The indirect binding of Tax to DNA would allow Tax to interact with basal transcription factors like TFIIA or TFIID (41, 42) and facilitate transcription initiation. Consistent with this hypothesis, we found that Tax is a strong activator of this promoter once it is directly tethered to the DNA through a Gal4 DNA-binding domain. In addition, Tax binding to Ets1 may superactivate Ets1 in that it may mimic Ras-dependent phosphorylation of Ets1 at threonine 38 (74). Such a pathway would resemble the CREB-dependent transactivation of the HTLV-I promoter, where the Tax-CREB complex binds the cofactor CREB-binding protein irrespective of CREB phosphorylation (24).

Removal of the exon VII domain of Ets1, which results in an increase in Ets1 DNA binding activity by 10- to 20-fold *in vitro* (75), did not affect Ets1 activity on the PTHrP P2 promoter. This suggests that exon VII-regulated DNA binding activity of Ets1 is not a limiting factor for the transactivation of the PTHrP P2 promoter, at least not under transient transfection conditions where Ets1 is overexpressed.

There may be several advantages for formation of the Tax-Ets-Sp1 complex. First, in complex with Tax, Ets1 and Sp1 might be protected from the action of specific inhibitor proteins. In myelomonocytic cells, Ets1 activity is controlled by an AP1-like protein (MafB) that directly binds to Ets1 (76). Similarly, nuclear protein p74 has been reported to bind specifically to Sp1 and to inhibit Sp1-mediated transactivation (77). Second, Tax association with Ets and Sp1 could reduce the activity of inhibitory kinases that phosphorylate and inactivate Ets1 or Sp1 (78–80). Third, as a complex, Ets1 and Sp1 may be less likely to be replaced by related proteins that recognize the Ets1- or Sp1-binding site (18, 81–84). Thus, Tax not only contributes directly to the activation of the PTHrP promoter, but by complexing with Sp1 and Ets1, assures optimal regulation of transcriptional activity.

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