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Transactivation of the P2 Promoter of Parathyroid Hormone-Related Protein by Human T-Cell Lymphotropic Virus Type I Tax_1: Evidence for the Involvement of Transcription Factor Ets1

JÜRGEN DITTMER, SCOTT D. GITLIN, ROBERT L. REID, AND JOHN N. BRADY*
Laboratory of Molecular Virology, National Cancer Institute, Bethesda, Maryland 20892

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Expression of the parathyroid hormone-related protein (PTHrP), a protein that plays a primary role in the development of the humoral hypercalcemia of malignancy, is regulated by two distinct promoters, P1 and P2. PTHrP is overexpressed in lymphocytes from adult T-cell leukemia patients. We now demonstrate that in the human T-cell lymphotropic virus type I-transformed cell line MT-2, RNA synthesis is initiated primarily at the P2 promoter. Furthermore, in cotransfection experiments, Tax_1 transactivates the P2 promoter 10- to 12-fold. By using deletion and site-specific point mutations, we have identified a promoter-proximal sequence (positions −72 to −40) which is important for Tax_1 transactivation. The PTHrP promoter-proximal element contains two potential overlapping Ets1 binding sites, EBS I and EBS II. Gel shift analysis demonstrated that Ets1 binds specifically to both EBS I and EBS II. Mutation of the consensus GGAA core motif in EBS I abolished binding and Tax_1 transactivation in Jurkat T lymphocytes. In Ets1-deficient cells, cotransfection of Tax_1 and Ets1 expression plasmids stimulates PTHrP promoter activity. In the absence of Ets1, minimal transactivation of the PTHrP promoter is observed. These data suggest that Ets1 binds to EBS I and cooperates with Tax_1 to transactivate the PTHrP P2 promoter.

Several types of human cancers, including adult T-cell leukemia induced by the human T-cell lymphotropic virus type I (HTLV-I), are frequently associated with hypercalcemia (22, 39, 40). Humoral hypercalcemia of malignancy is primarily caused by a protein, parathyroid hormone (PTH)-related protein (PTHrP), that is synthesized and secreted by tumor cells (6, 12, 23, 31, 43, 44, 65). This protein shares with PTH the ability to interact with the PTH-PTHrP receptor and, consequently, to induce bone absorption and increased calcium reabsorption in the kidney, which eventually results in an increased calcium level in the blood (32, 33, 58).

PTHrP expression is regulated by two distinct promoters, P1 and P2 (39, 61). Transcription initiated from either of these promoters gives rise to multiply spliced mRNAs that each contain the PTHrP coding sequence required for binding to the PTH-PTHrP receptor. Previously, HTLV-I Tax_1 has been found to be able to transactivate a 2.8-kbp-long fragment of the PTHrP gene containing elements of both P1 and P2 (65).

Tax_1 is a 40-kDa viral protein, encoded by the pX region of the HTLV-I genome, that induces viral transcription through the Tax_1-responsive elements TRE-1 and TRE-2 in the HTLV-I long terminal repeat (LTR) (11, 41, 48, 54, 56). Tax_1 is also able to transactivate a number of cellular genes, including those for PTHrP, interleukin-2, interleukin-2 receptor α, c-*fos* and *egr1* and *egr2* (1, 2, 20, 35, 52, 63). Several Tax_1-responsive elements have been found that confer Tax_1 responsiveness to promoters. For example, the NF-κB element and the cyclic AMP (cAMP) responsive element (CRE) are common Tax_1-responsive elements in several genes (1–3, 17, 35, 49, 52, 63). Other Tax_1-responsive elements include the serum response element (SRE [1, 2]), the CATTA(A/T) box (45), CLE1, CLE2, and the GC-rich box (42). In each case, Tax_1 activity depends upon regulation of cellular transcription factors. Consistent with this hypothesis, Tax_1 has been shown to physically interact with CREB (4, 59, 67, 68) and serum response factor (21), which bind to the CRE and SRE, respectively. In addition, Tax_1 interacts with p105 (30), the precursor for the NF-κB protein p50 that binds to the NF-κB element (18).

The data presented here suggest that, in HTLV-I transformed cells, PTHrP is preferentially expressed through the P2 promoter. Furthermore, Tax_1 can specifically transactivate the P2 promoter in the absence of other viral proteins. Interestingly, Tax_1 is able to synergistically cooperate with the transcription factor Ets1 to increase P2 promoter activity. An Ets1 binding site within the P2 promoter-proximal sequences was important for Tax_1 transactivation and the cooperation between Tax_1 and Ets1.

MATERIALS AND METHODS

Cell lines. Jurkat, MT-2, and OsA-CL cells were grown in RPMI medium supplemented with 10% fetal calf serum. CV-1 cells were cultured in Dulbecco's modified Eagle's medium and 10% fetal calf serum. The osteosarcoma cell line OsA-CL (47) was kindly provided by T. Look.

Plasmids. The chloramphenicol acetyltransferase (CAT) plasmid pCAT1.1kb/Bam-Hind that contains the PTHrP gene fragment between positions −625 and +485 (Fig. 1) was kindly provided by A. E. Broadus. For construction of the upstream and downstream deletions to positions −329 and +168, as well as for the internal deletion of the fragment between positions −552 and −418, the restriction enzymes *Avr*II, *Nsi*I, and *Apa*I, respectively, were used (Fig. 1). Further deletions and mutations were performed by synthesizing fragments of the PTHrP gene by polymerase chain reaction and cloning these fragments into the vector pCR II

* Corresponding author.

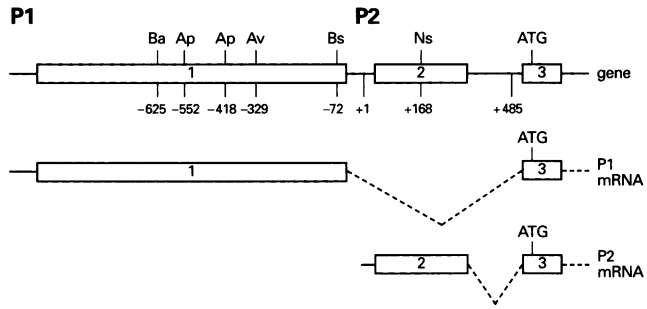

FIG. 1. Organization of the human PTHrP gene upstream of exon 4 (coding sequence). PTHrP expression is regulated by two promoters, P1 and P2. P1-driven transcription leads to RNA species containing exon 1 (P1 mRNA); P2 activity gives rise to an RNA species that contains exon 2 (P2 mRNA) (39). The initiation site of P1 has not yet been identified. The recognition sites of the restriction enzymes *Bam*HI (Ba), *Apa*I (Ap), *Avr*II (Av), *Bsp*EI (Bs), and *Nsi*I (Ns) in the PTHrP gene and their positions relative to the P2 initiation site (57, 61) are indicated. The numbers 1, 2, and 3 in the bars denote exons 1, 2, and 3, respectively.

(Invitrogen). The recombinant plasmid was cut by *Hin*dIII and *Pst*I, and the isolated fragment was ligated into the CAT vector, which had been cut by the same restriction enzymes. For expression of chicken Ets1 protein ($p54^{c\text{-}ets1}$), the plasmid pKCR3/ets was used (8). Plasmids containing wild-type Tax_1 ($pCTax_1$) and mutant Tax_1 (M5-M12, M22, M33, and M47) have been described previously (55). The plasmids pU3R-CAT (56) and pLTR-CAT were used to study the effect of Tax_1 on the HTLV-I LTR and human immunodeficiency virus (HIV) LTR, respectively. The plasmid pLTR-CAT was kindly provided by M. Martin.

Transfection and CAT assay. For transfection, Jurkat cells were centrifuged, washed twice with RPMI without fetal calf serum, and resuspended in RPMI at a density of approximately 10^7 cells per ml. Transfection was carried out by electroporation (Cell Porator at 800 μF, 250 V; GIBCO-BRL). For each sample, 250 μl of cell suspension was used. The amounts of plasmid DNA added to each transfection were 5 μg of PTHrP-CAT plasmid, 7.5 μg of pKCR3/ets, and/or 8 μg of Tax_1-expressing vector. Salmon sperm DNA was added to keep the total amount of DNA per sample constant. After 18 h of incubation, cells were harvested, lysed, and analyzed for CAT activity. To correct for variation in cell number, CAT assays were performed with the same amount of cellular protein per sample. CV-1 cells were transfected by the calcium phosphate technique as described previously (27). CAT assays were carried out according to the method of Gorman et al. (28).

EMSA. The electrophoretic mobility shift assay (EMSA) was performed as follows. The oligonucleotide used for Ets1 binding corresponded to the wild-type PTHrP P2-specific sequence between positions −94 and −34: 5′-CAGGGTGT GTGGAATCAACTTTCCGGAAGCAACCAGCCCACCA GAGGAGGTAGACAGACA-3′. It contains the two overlapping Ets1 binding sites, EBS I and EBS II, between positions −76 and −65 (underlined). The oligonucleotide was prepared by synthesizing sense and antisense strands of the oligonucleotide on a DNA synthesizer (Applied Biosystems). The strands were separated in a 10% polyacrylamide gel under denaturing conditions, purified, and annealed. The double-stranded PTHrP-specific probe was end labeled by T4 polynucleotide kinase to a specific activity of 10^8 cpm/μg of DNA by using [γ-^{32}P]ATP (>5,000 Ci/mmol; Amersham). The labeled oligonucleotide was purified by passing the reaction mixture through two Sephadex G-50 spin columns. The binding reaction was performed by mixing 0.5 ng of the labeled probe with 25 ng of an extract from *Spodoptera frugiperda* SF9 cells, infected with an *Autographa californica* nuclear polyhedrosis virus-Ets1 recombinant baculovirus (8), and incubated in a final volume of 12 μl of Ets binding buffer (19) for 30 min at room temperature. For competition experiments, oligonucleotides carrying a G-to-A mutation at position −70 (PTHrP-M2), −71 (PTHrP-M3), or at −70 and −71 (PTHrP-M1) as well as oligonucleotides 5′-ACTCGAGAAGGCTCTGACGTCTCCCCCGGA-3′ and 5′-TCGGGCTCGAGATAAACAGGAAGTGGTC-3′, which contain the sequence of the HTLV-I TRE-1 III (underlined) or a high-affinity Ets1 binding site (underlined), respectively, were used. The binding reaction mixture was loaded onto a 4% polyacrylamide gel and electrophoresed in 0.25× TBE (1× TBE is 89 mM Tris-boric acid [pH 8.3] and 1 mM EDTA) under nondenaturing conditions for 2 h at 150 V and room temperature. Bands were visualized by autoradiography with Kodak X-ray film.

Northern blot analysis. Total RNA from Jurkat cells was isolated as described previously (7). Total RNA from OsA-CL and MT-2 cells was prepared according to the method of Chirgwin et al. (13). For poly(A)$^+$ selection, poly(dT) cellulose from Collaborative Research Inc. (Waltham, Mass.) was used. RNA agarose gel electrophoresis and Northern (RNA) blot hybridization were carried out as described previously (15) with slight modifications. Briefly, 14 μg of poly(A)$^+$ RNA was dissolved in 3 μl of water, mixed with 12 μl of sample buffer {53% formamide, 1× MOPS [200 mM 3-(*N*-morpholino)propanesulfonic acid, 50 mM sodium acetate, 10 mM EDTA], 2.1 M formaldehyde, 6% glycerol, 0.1% bromophenol blue in water}, incubated at 65°C for 15 min, and loaded onto a 1.2% GTG agarose gel (Seakem) containing 1× MOPS and 0.66 M formaldehyde. RNA was run at 50 V in 1× MOPS buffer. The gel was treated with 0.05 M NaOH–1× SSC (1× SSC is 0.15 M NaCl plus 0.15 M sodium citrate) for 10 min and then was neutralized with 20× SSC. The RNA was blotted onto a Schleicher & Schuell Nytran membrane by using capillary force and was fixed on the membrane by UV light with a UV-Stratalinker 2400 (Stratagene) by following the manufacturer's protocol. As a probe for exon 4-specific DNA (coding region of the PTHrP gene), the *Sac*I fragment of pG.HHM-CR, a plasmid that was kindly provided by A. E. Broadus, was used. Exon 1- and exon 2-containing PTHrP RNAs were detected by hybridization with the oligonucleotide 5′-CTG TCTGTCTACCTCCTCTGGTGGGCTGGTTGCTTCCGG AAAGTTGATTCCACACACCCTG-3′ or the oligonucleotide 5′-TTCGTGGAACAGGGCTAACCGCCGCCTCCTAA AAGAAGAAAGTTTCCCCCTC-3′, respectively, which had similar GC contents (56% versus 54%). For the detection of β-actin RNA, an internal 40-base probe was used (60). The single-stranded oligonucleotides were end labeled by T4 polynucleotide kinase as described above. The exon 4-specific double-stranded DNA fragment was labeled by random primer extension reaction to a specific activity of 10^9 cpm/μg of DNA with a multiprime labeling kit (Amersham) and [α-^{32}P]CTP (6,000 Ci/mmol; Amersham). The Northern blot was incubated in prehybridization buffer (50% formamide, 4× SSC, 25 mM sodium phosphate [pH 6.0], 5× Denhardt's solution, 1% sodium dodecyl sulfate [SDS], and 200 μg of sheared salmon sperm DNA per ml) for 5 to 7 h at 42 or 39°C, depending on the temperature at which the hybridization was carried out. Hybridization was performed overnight in

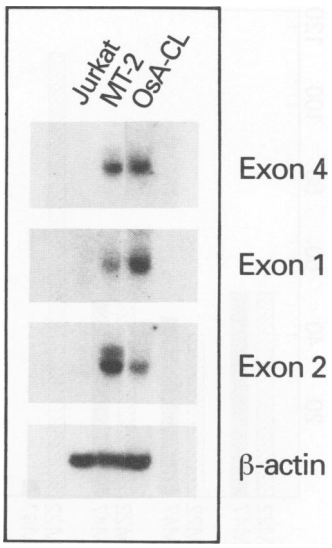

FIG. 2. Northern blot analysis of mRNA from MT-2, OsA-CL, and Jurkat cells with PTHrP-specific probes. Fourteen micrograms of poly(A)$^+$ RNA from each cell line, MT-2, OsA-CL and Jurkat, was separated on a 1% agarose gel and blotted onto a nylon membrane as described in Materials and Methods. The blot was sequentially hybridized with ^{32}P-labeled probes specific for PTHrP exon 4, exon 1, exon 2, and β-actin.

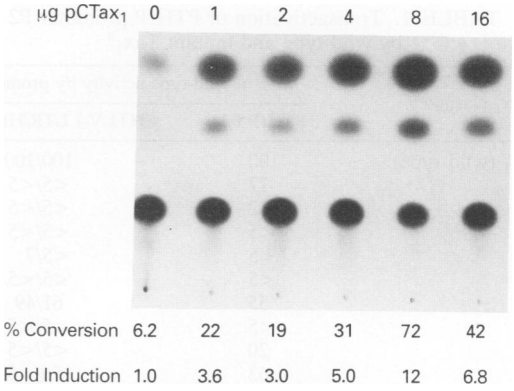

FIG. 3. Transactivation of the PTHrP gene fragment −625/+485 by Tax_1. Increasing amounts of Tax_1-expressing vector $pCTax_1$ were cotransfected along with 5 μg of the CAT plasmid pCAT 1.1kb/Bam-Hind (which contains the PTHrP gene fragment −625/+485) into Jurkat T cells by electroporation. After 18 h of incubation, cells were harvested and assayed for CAT activity. Electroporations and CAT assays were carried out as described in Materials and Methods.

hybridization buffer (50% formamide, 5× SSC, 20 mM sodium phosphate [pH 6.0], 1× Denhardt's solution, 0.15% SDS, and 100 μg of sheared salmon sperm DNA containing 1 ng of ^{32}P-labeled probe per ml of buffer at 42°C (exon 4-specific DNA fragment) or at 39°C (exon 1-, exon 2-, and β-actin-specific oligonucleotides). After hybridization, the blot was washed in 0.1× SSC–0.5% SDS at 57°C for 15 min twice (exon-4-specific DNA fragment) or 37°C for 5 min once (oligonucleotide probes).

RESULTS

Characterization of PTHrP P1 and P2 promoter activities in the HTLV-I-transformed MT-2 cell line. To evaluate the utilization of the P1 and P2 PTHrP promoter expression in HTLV-I-infected cells, we determined the relative expression of PTHrP exon 1 and exon 2 in MT-2 cells. Exon 1-containing RNAs are generated when transcription is driven by P1; exon 2-containing RNAs are produced when transcription is initiated from P2 (Fig. 1 [40]). The HTLV-I-transformed T-cell line MT-2 was chosen for this analysis since, similar to most ATL cells, MT-2 has been shown to synthesize PTHrP mRNA at high levels and to secrete PTHrP with PTH-like activities (23, 42). For comparison, we used the osteosarcoma cell line OsA-CL, which we found expresses PTHrP RNA (coding region is exon 4) to a similar extent as MT-2 (Fig. 2). Jurkat T cells served as a negative control for PTHrP expression. Northern blot analysis demonstrated that the relative levels of exon 1- and exon 2-containing RNAs were different in the MT-2 and OsA-CL cells (Fig. 2). As determined by densitometric measurement, the ratio of exon 2 to exon 1 expression was about 5:1 in MT-2 cells and 0.5:1 in OsA-CL cells. These results suggest that in the HTLV-I-transformed MT-2 cell line, the P2 promoter is preferentially utilized for PTHrP expression.

The PTHrP P2 promoter can be transactivated by Tax_1. In view of the results presented above, we were interested in determining whether the HTLV-I transactivator Tax_1 regulates P2 promoter expression. A PTHrP gene fragment spanning sequences from positions −625 to +485 relative to the P2 RNA initiation site (Fig. 1) was tested for Tax_1 responsiveness. The PTHrP-CAT reporter plasmid, pCAT 1.1kb/Bam-Hind, was cotransfected with increasing amounts of the Tax_1-expressing vector, $pCTax_1$, into Jurkat T cells. As shown in Fig. 3, up to a 12-fold increase in CAT activity was observed in the presence of Tax_1. In order to demonstrate that the Tax_1 protein was responsible for PTHrP transactivation, a series of Tax_1 protein mutants have been analyzed for their ability to induce PTHrP P2 promoter activity. These mutants were described in detail previously (55). Tax_1 mutants M5 through M12 and M33 have been shown to have a reduced ability to transactivate Tax_1-responsive promoters (Table 1 [55]). Similarly, these Tax_1 mutants showed a reduced ability to transactivate the PTHrP P2 promoter (Table 1). The ability of Tax_1 mutants M22 and M47 to transactivate a Tax_1-responsive promoter depends on the nature of the promoter. Tax_1 mutant M22 contains an alanine and serine at amino acid positions 137 and 138 instead of a glycine and leucine. The mutations in M22 do not alter the protein's ability to transactivate promoters whose Tax_1-responsive region contains a CRE. However, M22 fails to transactivate promoters, such as the HIV LTR, whose Tax_1 responsiveness is dependent upon NF-κB. In Tax_1 mutant M47, leucines at amino acid positions 319 and 320 have been mutated to arginine and serine, respectively. In contrast to M22, M47 induces transcription from promoters with a Tax_1-responsive NF-κB element, but not with a Tax_1-responsive CRE. CRE and NF-κB elements are the most common Tax_1-responsive elements found in Tax_1-responsive promoters (1–3, 17, 36, 49, 52, 63). Both M22 and M47 were able to transactivate the PTHrP P2 promoter (Table 1). These mutants were about 60% as efficient as wild-type Tax_1 in inducing PTHrP activity. These results suggest that Tax_1 may use elements other than NF-κB or CRE to transactivate the PTHrP P2 promoter.

The sequence between positions −72 and −40 is important for Tax_1 transactivation of the PTHrP P2 promoter. To

TABLE 1. Transactivation of PTHrP promoter P2 by wild-type and mutant Tax_1[a]

Tax_1 plasmid	% of wild-type activity by promoter:	
	PTHrP P2	HTLV-I LTR/HIV LTR[b]
$pCTax_1$ (wild type)	100	100/100
M5	17	<5/<5
M6	12	<5/<5
M7	<5	<5/<5
M8	<5	<5/7
M9	<5	<5/<5
M10	55	61/49
M11	<5	<5/<5
M12	20	<5/<5
M22	63	56/<5
M33	12	<5/<5
M47	60	<5/120

[a] Jurkat T cells were cotransfected with 5 μg of pCAT1.1kb/Bam-Hind, which contains the PTHrP promoter fragment −625/+485 and 8 μg of a wild-type or mutant (M) Tax_1 expression vector. Transfections and CAT assays were performed as described in Materials and Methods. The percentage of wild-type CAT activity was calculated for each mutant by comparing the ability of the mutant Tax_1 to induce P2 promoter activity with that of wild-type Tax_1.

[b] For comparison, data published in reference 55 showing the inducibility of the HTLV-I and HIV LTR by these Tax_1 mutants have been added.

identify promoter sequences that are required for Tax_1 transactivation of the P2 promoter, a series of 5′ and 3′ deletions of the P2 promoter were constructed. Deletion of the downstream region of the PTHrP gene fragment to position +168 decreased Tax_1 transactivation approximately twofold (Fig. 4B). 5′ deletions of sequences between positions −625 and −329 (Fig. 4C and D) or downstream deletions to position +20 (Fig. 4E) had little additional effect on Tax_1 transactivation. The minimal promoter construct which retained Tax_1 responsiveness contained sequences from positions −72 to +20 (Fig. 4F). A significant decrease in promoter activity was found when the upstream region

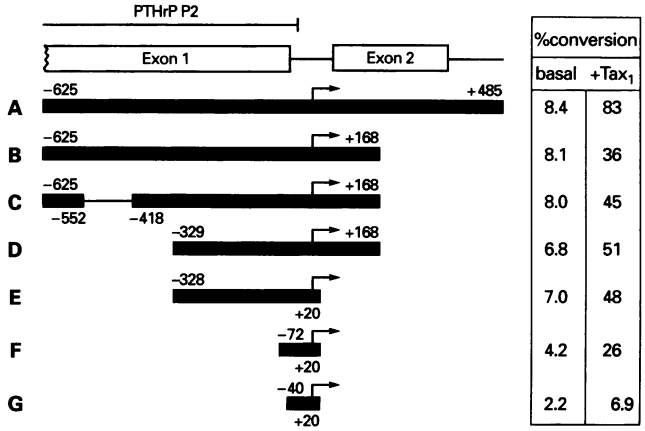

FIG. 4. Effect of 5′ and 3′ deletions on the responsiveness of the PTHrP P2 promoter to Tax_1. Jurkat T cells were transfected with 5 μg of CAT plasmid carrying the indicated PTHrP gene fragments (A to G) upstream of the CAT gene. Transfections were performed in the absence (basal) or presence (+Tax_1) of 8 μg of the Tax_1-expressing vector $pCTax_1$. After 18 h of incubation, cells were harvested and assayed for CAT activity as described in Materials and Methods. Each value represents the average value of three to seven independent experiments.

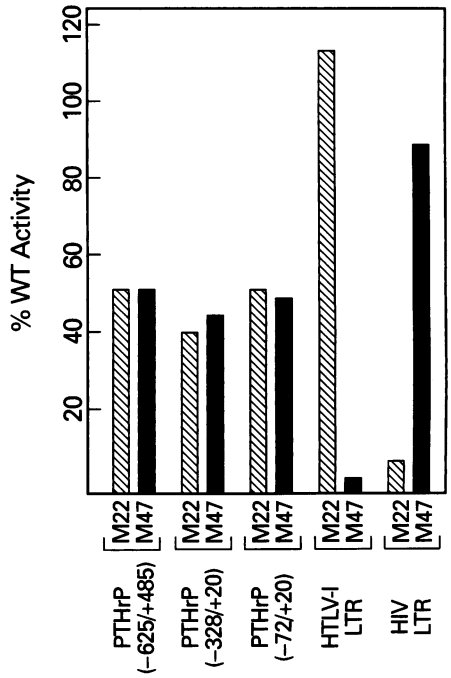

FIG. 5. Tax_1 mutants M22 and M47 are able to transactivate the PTHrP P2 promoter. Jurkat cells were transfected with 5 μg of PTHrP-CAT constructs, containing the PTHrP gene fragment −685/+485, −328/+20, or −72/+20, along with 8 μg of $pCTax_1$, or 8 μg of the vector that expresses the Tax_1 mutants M22 or M47. HTLV-I LTR (pU3R-CAT, 2.5 μg) and HIV LTR (pLTR-CAT, 3 μg) were used as control promoters for M22 and M47, respectively. The activities of M22 or M47 relative to wild-type (WT) Tax_1 were calculated by comparing the CAT activity in the presence of M22 or M47 with the CAT activity in the presence of wild-type Tax_1. Each value represents the average value of two to four independent experiments.

was further deleted from positions −72 to −40 (Fig. 4G). These results suggest that the sequences between positions −72 and −40 are important for Tax_1 transactivation of the PTHrP P2 promoter.

As shown in Fig. 5, Tax_1 mutants M22 and M47 were equally efficient in transactivating the P2 promoter fragments −625/+485, −328/+20, and −72/+20. The percent activities of the mutants, relative to wild-type Tax_1, were similar for all three fragments. Controls for these experiments demonstrated that M47, but not M22, transactivated the HIV LTR. Conversely, M22, but not M47, induced CAT expression from the HTLV-I LTR. These results suggest that, for the Tax_1 responsiveness of the P2 promoter deletion mutants, an NF-κB or CRE element is not required (also see above). No consensus NF-κB or CRE element was found in the sequence between positions −72 and −40 in the PTHrP P2 promoter.

The sequence between positions −72 and −40 contains an Ets1 binding site. Interestingly, the sequence between positions −72 and −40 shows a high level of homology to a sequence within the Tax_1- and Ets1-responsive element TRE-2/ERR1 of the HTLV-I LTR (Fig. 6) (26). In particular, these sequences share overlapping Ets1 consensus binding sites. We, therefore, examined whether Ets1 could bind to this site. An oligonucleotide corresponding to the PTHrP sequence between positions −94 and −34 was incubated with a baculovirus Ets1 extract. As shown by EMSA, an

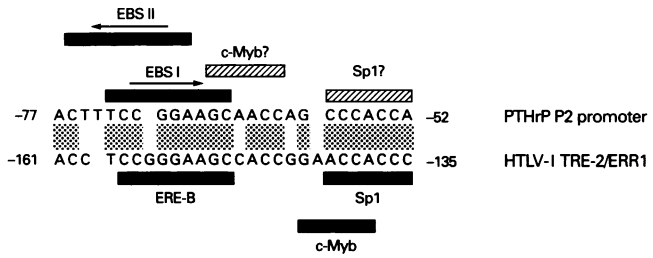

FIG. 6. Partial alignment of the sequence of the PTHrP P2 promoter with the sequence of the TRE-2/ERR1 of the HTLV-I LTR. The PTHrP sequence between positions −52 and −77 shows high homology to a sequence within the Tax_1- and Ets1-responsive element TRE-2/ERR1 of the HTLV-I LTR at positions −135 to −161. These sequences share potential binding sites for Ets1, Sp1, and c-Myb. For the sequence within the TRE-2/ERR1, Ets1 and Sp1 have been shown to specifically bind to the indicated Ets1-binding site ERE-B and the Sp1 binding site, respectively (24, 26). The location of the indicated c-Myb binding site within the TRE-2/ERR1 is based on data obtained from DNaseI footprinting (9).

Ets1-DNA complex was formed (Fig. 7B, lane 2). The formation of this complex was inhibited when a specific oligonucleotide competitor containing the Ets1 consensus binding site (lane 4) was added in 150-fold excess over the probe. In contrast, no change in Ets1 binding to the probe was observed upon addition of a 150-fold excess of a nonspecific competitor (HTLV-I 21-bp repeat, TRE-1) (lane 3).

As indicated above, the oligonucleotide used for EMSA contains two overlapping putative Ets1 binding sites. Ets1 binding site I (EBS I) is located between positions −73 and −65 (5′-TCCGGAAGC-3′), and Ets1 binding site II (EBS II) is in the opposite orientation between positions −68 and −76 (5′-TCCGGAAAG-3′) (Fig. 7A). EBS I has 89% identity and EBS II has 78% identity to the Ets1 consensus binding site A/GCCGGAA/TGT/C (46). To evaluate the importance of these binding sites for the physical interaction with Ets1, the core motif GGAA in either or both of Ets1 binding sites was mutated. Mutation of the core motif has been shown to abolish binding of Ets1 in vitro (19). When the core motifs of both Ets1 binding sites were mutated at positions −70 and −71 (PTHrP-M1), inhibition of Ets1 binding to the wild-type probe was abolished (Fig. 7B, lanes 7 and 8). The introduction of a single G-to-A mutation into the PTHrP oligonucleotide at position −70 of the EBS I core motif (PTHrP-M2) strongly reduced the ability of the oligonucleotide to compete with the wild-type probe (Fig. 7B, compare lanes 9 and 10). A mutation in the core motif of EBS II at position −71 (PTHrP-M3) resulted in a slight decrease in competition with the wild-type probe for Ets1 binding (lanes 11 and 12). The inhibitory effect of the −71 mutation was not as strong as that of the −70 mutation, suggesting that EBS I is a higher-affinity Ets1 binding site than EBS II.

The Ets1 binding site EBS I is required for Tax_1 transactivation of the PTHrP P2 promoter in Jurkat cells. According to the EMSA data, the core motif of EBS I constitutes a stronger Ets1 binding site than that of EBS II. To evaluate the importance of the EBS I for Tax_1 transactivation of the PTHrP P2 promoter, we introduced a single G-to-A mutation in the −328/+20 and −72/+20 PTHrP fragments in the core motif of EBS I at position −70. When these EBS I PTHrP P2 promoter mutants were cotransfected with the Tax_1 expression vector into Jurkat cells, a significant drop in basal activity and Tax_1 transactivation of the P2 promoter was

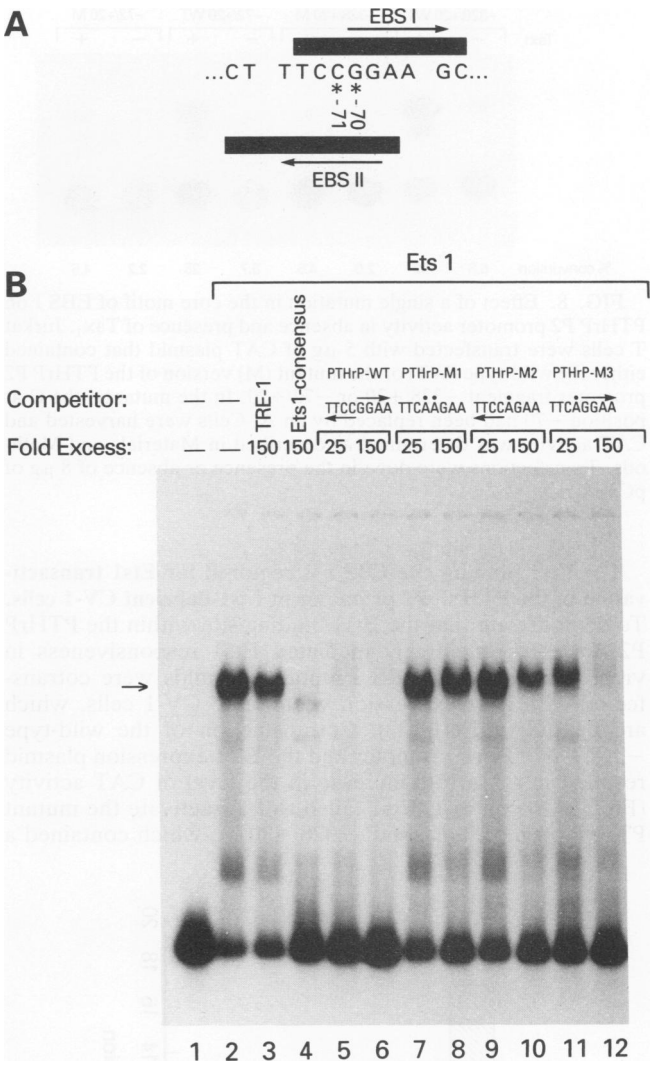

FIG. 7. Ets1 binding to the putative Ets1 binding sites EBS I and EBS II. (A) For EMSA, a probe was used that corresponded to the PTHrP-specific sequence between positions −94 and −34. This sequence contained the putative Ets1 binding sites EBS I and EBS II. Arrows indicate the orientations of EBS I and EBS II. Asterisks indicate the positions (−70 and −71) where mutations were made. G-to-A mutations at positions −70 and/or −71 were introduced to change the GGAA core motifs of EBS I and/or EBS II, respectively. (B) Baculovirus Ets1 was incubated with a ^{32}P-labeled oligonucleotide, which corresponded to the wild-type (WT) PTHrP sequence between positions −94 and −34, and complex formation was analyzed by EMSA as described in Materials and Methods. To study the importance of EBS I and EBS II for Ets1 binding, competition analyses were performed with mutated versions of the PTHrP oligonucleotide in which the core motifs of both EBS I and EBS II (PTHrP-M1, lanes 7 and 8), EBS I alone (PTHrP-M2, lanes 9 and 10), or EBS II alone (PTHrP-M3, lanes 11 and 12) had been changed.

observed (Fig. 8). For example, Tax_1 transactivated the wild-type −328/+20 promoter plasmid approximately 7.5-fold (Fig. 8, lanes 1 and 2). In contrast, only a twofold increase in Tax_1 transactivation was observed with the −328/+20 EBS I mutant plasmid (Fig. 8, lanes 3 and 4). Similar results were obtained with the −72/+20 PTHrP wild-type and mutant plasmids (Fig. 8, lanes 5 to 8).

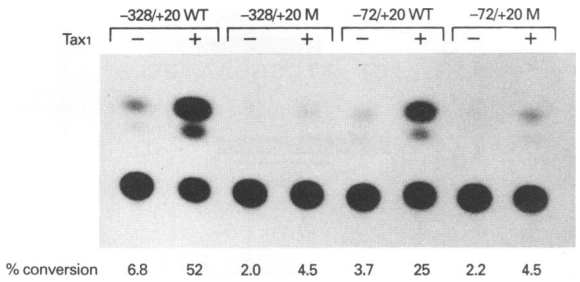

FIG. 8. Effect of a single mutation in the core motif of EBS I on PTHrP P2 promoter activity in absence and presence of Tax_1. Jurkat T cells were transfected with 5 μg of CAT plasmid that contained either the wild-type (WT) or the mutant (M) version of the PTHrP P2 promoter fragment −328/+20 or −72/+20. In the mutants, the G at position −70 had been replaced by an A. Cells were harvested and CAT activity was determined as described in Materials and Methods. Transfections were done in the presence or absence of 8 μg of $pCTax_1$.

The Ets1 binding site EBS I is required for Ets1 transactivation of the PTHrP P2 promoter in Ets1-deficient CV-1 cells. To demonstrate that the Ets1 binding site within the PTHrP P2 promoter effectively mediated Ets1 responsiveness in vivo, wild-type and EBS I mutant plasmids were cotransfected with Ets1 expression vector into CV-1 cells, which are devoid of Ets1 (27). Cotransfection of the wild-type −328/+20 PTHrP promoter and the Ets1 expression plasmid resulted in a fourfold increase in the level of CAT activity (Fig. 9). In contrast, Ets1 failed to transactivate the mutant PTHrP promoter plasmid, −328/+20 M, which contained a single base substitution in the EBS I Ets1 binding site. We have also tested the minimal PTHrP promoter in the CV-1 transfection assay. Cotransfection of the wild-type −72/+20 PTHrP promoter and the Ets1 expression plasmid resulted in a sevenfold increase in the level of CAT activity (Fig. 9). In contrast, the mutant PTHrP promoter (−72/+20 M) was not transactivated by Ets1.

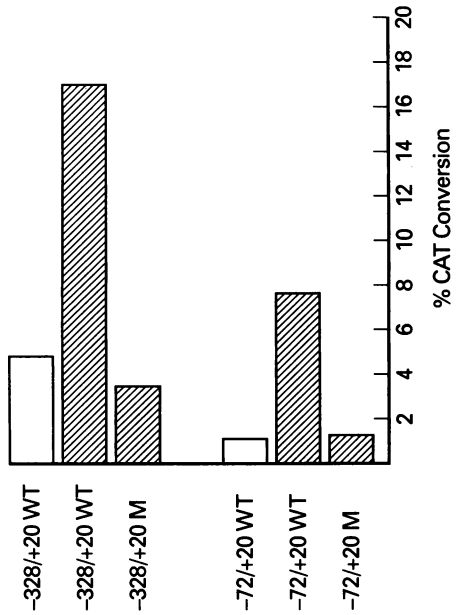

FIG. 9. The Ets1 binding site EBS I is required for Ets1 transactivation of the PTHrP promoter in Ets1-deficient CV-1 cells. CV-1 cells were transfected with 4 μg of CAT plasmid that contained either the wild-type (WT) or mutant (M) version of the PTHrP P2 promoter fragment −328/+20 or −72/+20. In the mutants, the G in the EBS I core motif at position −70 had been changed to an A. Transfections and CAT assays were performed as described previously (27). Transfections were carried out in the presence (hatched bars) or absence (open bars) of 7.5 μg of pKCR3/ets.

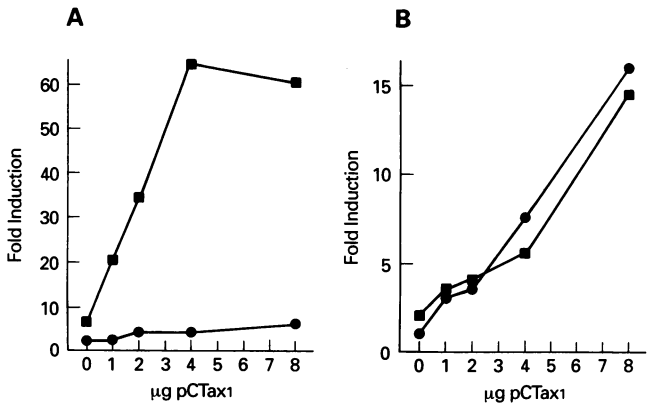

FIG. 10. Cooperative effect of Tax_1 and Ets1 on the PTHrP P2 promoter in Ets1-deficient CV-1 cells. Increasing amounts of $pCTax_1$, in the presence (■) or absence (●) of 7.5 μg of pKCR3/ets, were cotransfected along with 5 μg of pCAT 1.1kb/Bam-Hind, which contains the PTHrP gene fragment −625/+485, into Ets1-deficient CV-1 cells (A) or Jurkat T cells (B). Cells were harvested and assayed for CAT activity as described in Materials and Methods. Fold inductions were calculated by comparison with basal PTHrP CAT activity.

Tax_1 cooperates with Ets1 to transactivate the PTHrP P2 promoter in CV-1 cells. In view of the above results, it was of interest to analyze the combined effects of Tax_1 and Ets1 on PTHrP gene expression. We compared the relative activity of the −625/+485 PTHrP P2 promoter fragment in the presence of Tax_1, Ets1, or Tax_1 and Ets1 combined. Consistent with the results presented above, in the presence of Ets1 a seven- to eightfold increase in CAT activity was observed (Fig. 10A; 0 μg of Tax_1, 7.5 μg of Ets1). Similarly, in the presence of Tax_1 alone, a small but reproducible four- to fivefold transactivation of the PTHrP P2 promoter was observed. Interestingly, coexpression of Tax_1 and Ets1 together increased P2 promoter activity by 60-fold. These experiments were also performed with Jurkat cells, which express Ets1 at high levels like most other T cells (25, 53). In these cells, the addition of pKCR3/ets had no effect on Tax_1 transactivation of the P2 promoter (Fig. 10B).

To determine whether EBS I was important for the Tax_1-Ets1 cooperative effect in CV-1 cells, the minimal P2 promoter was transfected into CV-1 cells along with Tax_1 and/or Ets1 expression vectors. Similar to the results obtained with the larger PTHrP promoter fragment (−625/+485), the wild-type −72/+20 promoter was transactivated by Tax_1 alone approximately 3-fold, by Ets1 alone approximately 6-fold, and by Tax_1 and Ets1 together approximately 24-fold (Fig. 11). A mutation in the EBS I Ets1 binding site at position −70 decreased both Tax_1 and Ets1 transactivation and resulted in a sixfold decrease in activity of the −72/+20 promoter in the presence of both Tax_1 and Ets1. Thus, EBS I appears to be important for both the Tax_1 transactivation of the P2 promoter in Jurkat cells (Fig. 8) and the Tax_1-Ets1 cooperative effect in CV-1 cells (Fig. 11). We conclude that in Jurkat cells, in which addition of the Ets1

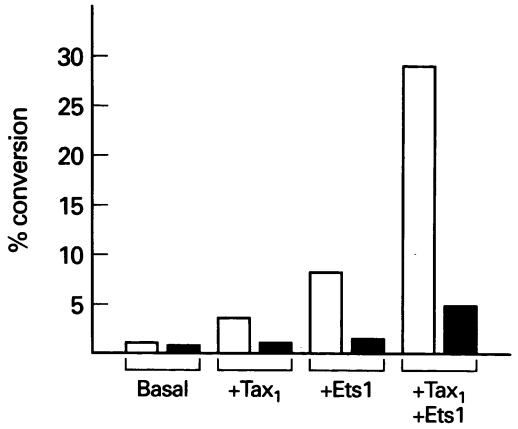

FIG. 11. Mutation of EBS I decreases the cooperative effect of Tax_1 and Ets1 on the minimal PTHrP P2 promoter in CV-1 cells. CV-1 cells were transfected with 4 μg of CAT plasmid that contained the wild-type (open bars) or mutant (solid bars) EBS I in the minimal −72/+20 PTHrP P2 promoter. In the mutant, the EBS I core motif was mutated at position −70 from GGAA to AGAA. Transfections were carried out in the presence or absence of 4 μg of $pCTax_1$, 7.5 μg of pKCR3/ets, or 4 μg of $pCTax_1$ and 7.5 μg of pKCR3/ets as indicated. Transfections and CAT assays were performed as described in Materials and Methods.

expression vector did not increase Tax_1 transactivation, endogenous Ets1 protein seems to cooperate with Tax_1 to transactivate the PTHrP P2 promoter.

DISCUSSION

The data presented demonstrate that Tax_1 can transactivate the PTHrP P2 promoter. A sequence between positions −72 and −40 in the PTHrP P2 promoter was required for full Tax_1 responsiveness of the promoter in Jurkat T cells. This sequence contains overlapping Ets1 binding sites, EBS I and EBS II. A single mutation in EBS I, which reduced Ets1 binding in vitro, also decreased Tax_1 transactivation of the PTHrP P2 promoter. Furthermore, we demonstrate that, in the Ets1-deficient CV-1 monkey kidney cell, Ets1 can induce PTHrP P2 promoter activity in the presence of Tax_1. For the cooperative effect of Tax_1 and Ets1 on P2, the EBS I was critical. The data suggest that Ets1 is required for maximal Tax_1 transactivation of the PTHrP P2 promoter and that, for this, Ets1 needs to bind to EBS I. The stronger effect of Tax_1 alone and the failure of Ets1 to increase Tax_1 transactivation in Jurkat cells suggest that, in these cells, endogenous Ets1 cooperated with Tax_1 to increase PTHrP P2 activity. The PTHrP P2 promoter sequence between positions −77 and −52, which includes EBS I, contains homology to a sequence in the Tax_1- and Ets1-responsive element TRE-2/ERR1 in the HTLV-I LTR. This sequence in the HTLV-I LTR contains an Ets1 binding site (26). Interestingly, the HTLV-I LTR can also be cooperatively transactivated by Tax_1 and Ets1. This cooperative effect depends on the presence of the Ets1 binding site and the adjacent 21-bp repeat (27).

What is the basis for this cooperative activity on both promoters? Tax_1 might physically interact with Ets1 in such a way that it facilitates Ets1 binding to its cognate DNA sequence. It has been suggested that Ets1 contains a repressor domain which physically blocks Ets1 DNA-binding activity (37). Tax_1 might increase Ets1 DNA-binding activity by interfering with this interaction. Alternatively, Tax_1 might facilitate the interaction of Ets1 with other transcription factors. Ets1 has been shown to cooperate with Sp1 (24). In addition, the highly related Ets2 can cooperate with Myb to activate transcription (16). Putative binding sites of both proteins, Sp1 and Myb, are found adjacent to the Ets1-binding site EBS I, in the PTHrP P2 promoter (Fig. 6). We are currently investigating whether Sp1 and/or Myb are involved in Tax_1-Ets1 transactivation of the PTHrP P2 promoter.

The level of PTHrP expression in different HTLV-I-transformed cells or in leukemic cell lines from adult T-cell leukemia patients varies from undetectable to highly detectable as judged by Northern blot analysis (31, 43). Consistent with this observation, we have found that, in contrast to MT-2 cells, HTLV-I-transformed MT-4 and C81 cells did not express detectable levels of PTHrP mRNA (data not shown). Interestingly, the PTHrP expression pattern among these cells paralleled that of Ets1 expression, which was high in MT-2 cells but very low in MT-4 and C81 cells (data not shown). The observation that Ets1 expression can be low in transformed T-cell lines is consistent with a previous report that compared Ets1 levels in cells from patients with different malignant T-cell leukemias (55). It is possible that the level of Ets1 in an HTLV-I-transformed T cell determines whether PTHrP is expressed at high or low levels.

Although we have presented evidence that Ets1 binds to EBS I, it is also possible that other members of the Ets family may bind to this sequence as well. These proteins include Elf-1 (62), Elk-1 (50), Erg 1 and Erg 2 (51), Ets2 (10, 66), E1A-F (29), Fli-1 (5), SAP-1 (14), and Spi-1/PU.1 (34). They share with Ets1 the Ets binding domain, allowing them to bind to the same sequence with similar affinity (for review, see reference 38). However, they are different in their transactivating properties (64) and their abundance in different cell types. This may be important with respect to the ability of other, non-Tax_1-producing tumor cells, such as the osteosarcoma cell line OsA-CL, to express PTHrP through the P2 promoter.

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