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A New Regulatory Element That Augments the Tax-Dependent Enhancer of Human T-Cell Leukemia Virus Type 1 and Cloning of cDNAs Encoding Its Binding Proteins

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The Tax protein of human T-cell leukemia virus type 1 (HTLV-1) *trans* activates the 21-bp enhancer of HTLV-1. A sequence of more than two copies of the 21-bp enhancer is efficiently activated by Tax, but one copy is not activated extensively. Another sequence (TRE-2, positions −163 to −117) adjacent to the 21-bp enhancer in the long terminal repeat of HTLV-1 can enhance a single copy of the 21-bp enhancer activity in *trans* activation by Tax. This sequence contains motifs related to the Ets- and NF-κB-binding sequences, but mutations at these sites indicated that neither is responsive to cooperation with the 21-bp enhancer. A deletion mutation of TRE-2 identified 25 bases at positions −158 to −134 (TRE-2S) as an essential sequence, and TRE-2S was sufficient to give maximum cooperation with one copy of the 21-bp enhancer in *trans* activation by Tax protein. Using TRE-2S as a probe, we screened a cDNA library of HUT102 cells by the Southwestern (DNA-protein) procedure and isolated two cDNA clones, THP-1 and -2. These two clones encode TRE-2S-binding proteins, and they differ by only an extra 17 amino acids in THP-2. Both THP proteins contain five zinc finger motifs which are strikingly similar to those of the GLI family, an amplified gene product in glyoma cells. The binding site of THP-1 and -2 was GAACCACCCA in TRE-2S, which is highly homologous to the GLI-binding site. These results suggest that binding of THP to TRE-2S may be involved in cooperation with one copy of the 21-bp enhancer in responding to Tax *trans* activation.

Human T-cell leukemia virus type 1 (HTLV-1) (34, 43) is an etiologic agent of adult T-cell leukemia (44). Transcription from the viral long terminal repeat (LTR) is activated in *trans* by its own gene product, Tax protein (7, 11, 40, 41). For *trans* activation of the LTR of HTLV-1, direct repeats of a 21-bp sequence are responsive (12, 33, 37). The Tax protein also activates the expression of several cellular genes, such as interleukin-2 receptor α subunit (IL-2Rα) (6, 26, 38), granulocyte macrophage–colony-stimulating factor (28), c-*fos* (9), and c-*jun* (8). The NF-κB-binding sequence in the IL-2Rα gene and CArG boxes in the c-*fos* gene are also responsive to *trans* activation by Tax (10, 21, 22). Thus, three structurally unrelated enhancers, the LTR 21-bp sequence, the NF-κB site, and the CArG boxes, are *trans* activated by Tax protein. But Tax protein does not bind directly to these enhancer DNAs, suggesting the participation of host cell factors (1, 14, 30, 32, 45, 47). In fact, Tax protein was previously shown to bind to two 21-bp enhancer-binding proteins, CREB (cyclic AMP-responsive element-binding protein) and CREM (cyclic AMP responsive element-modulator), and to associate with the enhancer DNA (42, 48). Tax was also shown to interact with serum-responsive factor, which binds to the CArG box (10).

The 21-bp enhancer contains a *tax*-responsive element (TRE-1) (13), and at least two copies of the 21-bp enhancer are *trans* activated by Tax. However, one copy of the 21-bp enhancer was not activated extensively (13, 37). This is consistent with findings on many other enhancers that have repeating units. Brady et al. (5) reported that a deletion mutant of the LTR containing one copy of the 21-bp enhancer was strongly activated by Tax protein and that the sequence (TRE-2) located at positions −163 to −117 between two 21-bp sequences enhances one copy of the 21-bp enhancer in *trans* activation by Tax. But this sequence had no homology to that of the 21-bp enhancer. Binding of protein factors to the TRE-2 region have been analyzed, and Ets-1, Ets-2 (3, 16), TIF-1 (25), and NF-κB (29) have previously been proposed to be associated with *trans* activation by Tax protein. However, no further studies on their functional significance in *trans* activation have been reported.

We have focused on the cooperative sequence TRE-2, since the cooperation of two unrelated sequences in Tax *trans* activation may represent a new mechanism of *trans* activation by Tax protein. In this report, we show that the Ets-1-, Ets-2-, and NF-κB-binding sites were dispensable but that a new element, a 25-bp sequence (TRE-2S), is required for cooperation with the 21-bp enhancer. We isolated two human cDNA clones for TRE-2S-binding proteins (THP-1 and -2). These two proteins contain five zinc finger motifs which are strikingly similar to those of GLI family proteins (19). The TRE-2 holding protein (THP) binds to TRE-2S at the site homologous to the GLI binding site (20), suggesting that THP-1 and/or THP-2 is responsive to cooperative enhancement of one copy of the 21-bp enhancer in the LTR in responding to Tax-mediated *trans* activation.

MATERIALS AND METHODS

Cells and plasmids. FL cells, a human amnion cell line, were maintained in Dulbecco modified Eagle medium with 5% fetal calf serum. A human T-cell line, Jurkat; an HTLV-1-infected T-cell line, HUT102; and the human B-cell lines Raji, Namalva, and BJAB were maintained in RPMI 1640 with 10% fetal calf serum.

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To construct reporter plasmids for testing enhancer activity, we inserted synthesized oligonucleotides upstream of the promoter in pUCdN55-CAT (12) or pUCdN111-CAT (13). pUCdN55-CAT contains the enhancerless promoter of HTLV-1 linked to the CAT gene and a polylinker sequence at the 5′ end of the promoter (−55 bp from the mRNA start site of the HTLV-1 LTR). pUCdN111-CAT is analogous to pUCdN55-CAT but contains the sequence up to position −111 carrying one copy of the 21-bp enhancer. Each plasmid was cleaved by *Xba*I and *Hin*dIII or *Sal*I at the polylinker and blunted with the Klenow fragment of DNA polymerase I, and a monomeric or multimeric oligonucleotide was inserted. cDNAs of Ets-1 and -2 were amplified by polymerase chain reaction from a HUT102 cDNA library and were inserted into the pCG expression vector (14). The constructed plasmids, pCG-Ets-1 and pCG-Ets-2, were used for expression of Ets-1 and -2, respectively. For expression of Tax protein, pCG-Tax (14) was used. The expression plasmids of the fusion proteins GST-THP-1 and -2 were constructed by inserting cDNA coding 1 to 300 and 1 to 317 amino acids, respectively, into a vector, pGEX (39).

Transfection and CAT assay. The calcium phosphate precipitation technique was used for transfection of 5×10^5 FL cells with 10 μg of DNA, and the DEAE-dextran procedure was applied for transfection of 10^6 Jurkat, Raji, Namalva, or BJAB cells with 5 μg of DNA as described previously (11, 12). The total DNA concentration was adjusted with salmon sperm DNA. For expression of Tax or Ets protein, 0.1 μg each of pCG-Tax or pCG-Ets-1 or -2 was used. 12-*O*-Tetradecanoylphorbol-13-acetate (TPA) was added at a final concentration of 40 ng/ml, and the calcium ionophore A23187 was added at a final concentration of 0.5 μM 25 h after transfection (that is, 15 h before cell harvesting). The cells were harvested 40 h after transfection and assayed for chloramphenicol acetyltransferase (CAT) activity as described previously (11, 12). Under the conditions used, the activity was linearly proportional to the incubation time and the protein concentration. The assays were repeated at least three times to confirm reproducibility. CAT activity was defined as percent acetylation of chloramphenicol per 100 μg of protein in 30 min at 37°C.

Gel retardation assay. Whole-cell extracts of HUT102 cells were prepared by the method of Manley et al. (23). Samples of 5 μg of protein were preincubated in a total volume of 10 μl of buffer containing 12 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) (pH 7.5), 0.6 mM EDTA, 0.6 mM dithiothreitol, 60 mM KCl, 12% glycerol, and 2.5 μg of poly(dI-dC) with or without competitor DNA at 25°C for 10 min. Then radiolabelled oligonucleotide probe (5×10^4 cpm; 1 ng) was added, and the mixtures were incubated for 15 min at 25°C and then analyzed by electrophoresis in a 4% nondenaturing polyacrylamide gel.

Isolation of THP cDNA and sequencing. An amplified λgt11 cDNA library prepared from HUT102 cells (45) was infected into *Escherichia coli* Y1090, and β-galactosidase fusion proteins were induced by treating with isopropyl thiogalactoside as described by Huynh et al. (17). Recombinant phages (2×10^6) were screened with a ^{32}P-labelled DNA probe, seven repeats of the double-stranded TRE-2S sequence, CCGGGAAGCCACCGGGAACCACCCA. The recombinant DNA from positive clones was cleaved by *Eco*RI, and the insert was subcloned into pBluescript KS$^+$ and sequenced by the dideoxy method (45).

Nucleotide sequence accession numbers. The sequences of THP-1 and -2 have been submitted to DDBJ under accession numbers D14827 and D14828.

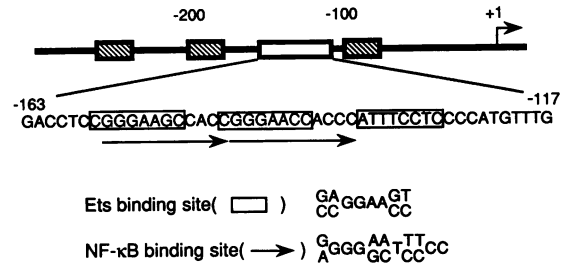

FIG. 1. Region responsive to cooperative activation of one copy of 21-bp enhancer in responding to Tax protein. The diagram shows an alignment of motifs in the LTR of HTLV-1: three direct repeats of the 21-bp enhancer (hatched boxes) and the TRE-2 sequence (open box). Motifs homologous to Ets- and NF-κB-binding sites are marked with boxes and arrows on the sequence, respectively. The consensus sequence for each binding site is shown.

RESULTS

Sequence essential for cooperation with one copy of the 21-bp enhancer. The region between positions −163 and −117 (TRE-2) located between two 21-bp enhancers in the LTR of HTLV-1 cooperates synergistically with one copy of the 21-bp sequence in responding to Tax *trans* activation (Fig. 1) (5, 24). This sequence has no similarity to the 21-bp sequence but has several motifs homologous to Ets- and NF-κB-binding sites. To understand the mechanism of cooperation of this sequence with one copy of the 21-bp enhancer, we tried to identify the minimum sequence essential for cooperative function. For this, we prepared a series of external deletion mutants from the 5′ or 3′ terminus. The fragments of these deletion mutants were inserted upstream of the promoter containing one copy of the 21-bp enhancer in pUCdN111-CAT, which contained the sequence from positions −111 to +320 (the CAP site is position +1) of the LTR. These mutant sequences were also inserted into the HTLV-1 promoter in pUCdN55-CAT, which did not contain any 21-bp enhancer. Each plasmid was transfected into the human cell line FL with or without Tax expression plasmid pCG-Tax, and CAT activity was assayed on day 2 after transfection (Fig. 2).

As described previously (5, 12, 24, 37), one copy of the 21-bp enhancer alone showed only 11- to 12-fold activation by Tax, and TRE-2 by itself showed no activation (Fig. 2A, rows a and b). But insertions of these two sequences together augmented CAT expression 97-fold in the presence of Tax (Fig. 2A, row c). Therefore, TRE-2 was confirmed to cooperatively activate one copy of the 21-bp enhancer. The 3′ deletion of this fragment up to position −134 did not reduce its enhancing activity, but further deletion of 7 nucleotides up to position −140 abolished its activity completely (Fig. 2B). Therefore, the 3′ boundary of the responsive element should be located at positions −140 to −134. On the other hand, a 5′ deletion up to position −158 reduced the activity only slightly, while further deletion to position −134 resulted in complete inactivation, confirming the results of 3′ deletions. These results indicate that at least the region between positions −158 and −134 is essential. In fact, a fragment containing positions −158 to −134 was found to be almost as active as the original TRE-2 (Fig. 2A, row d). Therefore, the 25-bp sequence (TRE-2S) of positions −158 to −134 was concluded to be essential and sufficient for cooperative activation of the 21-bp enhancer in responding to Tax.

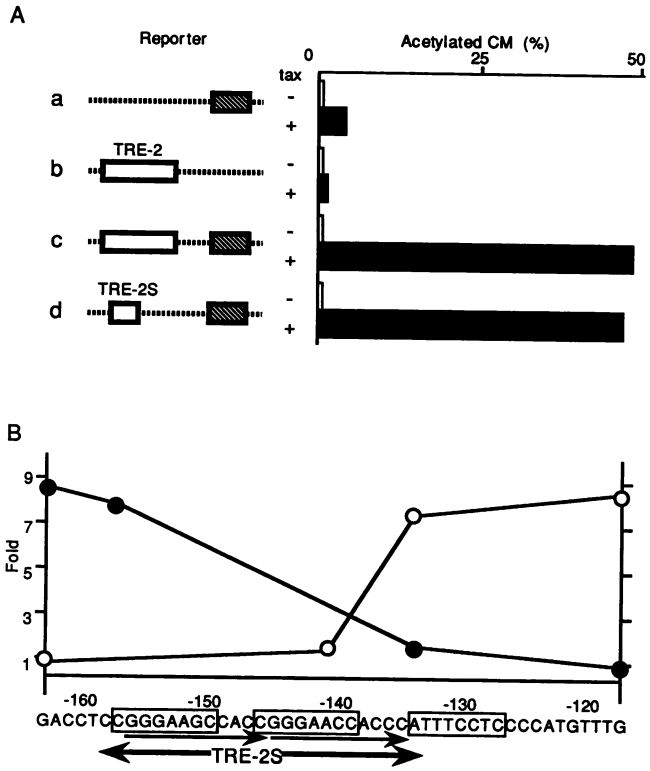

FIG. 2. Identification of minimum element (TRE-2S) responsive to cooperative activation of one copy of 21-bp enhancer. (A) Enhancing activity of TRE-2 and TRE-2S. CAT constructs containing one copy of the 21-bp enhancer (a), TRE-2 (b), TRE-2 plus the 21-bp enhancer (c), and TRE-2S plus the 21-bp enhancer (d) were transfected into FL cells with (+) or without (−) Tax expression plasmid. CAT activity was assayed 2 days after transfection. CM, chloramphenicol. (B) Summary of deletion mutants of TRE-2. Each deletion mutant was inserted upstream of the 21-bp enhancer of pUCdN111-CAT and was cotransfected with Tax expression plasmid. Open circles represent deletions (map positions, −117, −134, −141, and −163) from the 3′ end, and closed circles represent deletions (map points, −163, −158, −134, and −117) from the 5′ end.

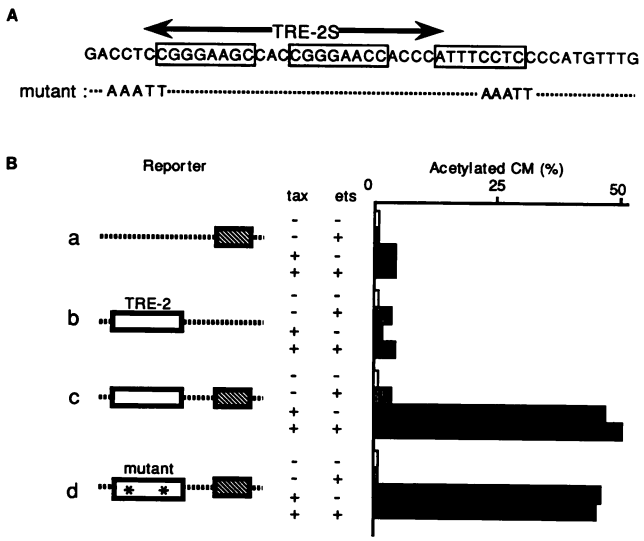

FIG. 3. Effects of mutations at the Ets-binding sites on enhancing activity of TRE-2. The asterisks in panel B show the locations of the mutations illustrated in panel A. The construct was cotransfected with or without Tax and/or Ets expression plasmid into FL cells, and CAT activity was assayed. CM, chloramphenicol.

The c-Ets-binding site in TRE-2S is dispensable for the activity. Bosselut et al. (3) reported that both Ets-1 and Ets-2 bound specifically to two sites in TRE-2 (the first and last sites in Fig. 1 and 3A). The newly defined TRE-2S sequence contains one of the functional binding sequences, so we tested the possible contribution of Ets protein binding to Tax-mediated *trans* activation. The same mutations used by Bosselut et al. (3) were introduced into TRE-2 to destroy the Ets-binding activities: two repeats of CCTCC at positions −130 to −126 and −161 to −157, which partly overlap the Ets-binding sites, were replaced by AAATT (Fig. 3). The wild and mutant types of the fragment were inserted upstream of the 21-bp enhancer in pUCdN111-CAT, and their effects on CAT expression were assayed. Ets-1 expression caused three- to fivefold stimulation of the wild-type TRE-2 but had no effect on the mutant. Therefore, Ets-1 contributes to the activation of TRE-2 to some extent, as previously described (3); however, a similar effect was observed without the 21-bp sequence (Fig. 3B, row b), indicating that the effect of Ets-1 protein was independent of the 21-bp enhancer. The mutant fragment was effectively activated by Tax protein in a manner similar to that for the wild-type TRE-2 in cooperation with one copy of the 21-bp sequence, but coexpression of Ets-1 did not affect the *trans* activation, even with increasing doses up to 10-fold that of the standard assay (see Materials and Methods). Similar results were obtained with Ets-2 (data not shown). Therefore, it was concluded that neither Ets-1 nor Ets-2 plays a critical role in the cooperative activation of one copy of the 21-bp enhancer in response to the Tax protein.

The NF-κB sequence is not involved in TRE-2 function. TRE-2S also contains two imperfect repeats of a sequence homologous to the NF-κB-binding motif (Fig. 1). Its 3′ region overlaps the 3′ boundary of the sequence essential for the cooperative function of TRE-2S (Fig. 2B). In fact, these two direct repeats were shown by Numata et al. (29) to bind the NF-κB protein and to contribute to the activation of transcription. To confirm their results, we compared the responses of TRE-2S and the authentic NF-κB-binding site to Tax *trans* activation and TPA stimulation. TRE-2S was multimerized seven times and inserted upstream of the promoter into pUCdN55-CAT, which has no 21-bp enhancer. As a control experiment, the NF-κB-binding sequence of the IL-2Rα gene was multimerized and inserted into the same vector. The heptamer of TRE-2S was not significantly activated either by Tax protein or by TPA and the calcium ionophore A23187 (Fig. 4). In contrast, the NF-κB-binding sequence was strongly activated by Tax and also responded efficiently to TPA treatment, as previously reported (6, 18, 21, 22, 26). Therefore, TRE-2S did not function in a manner similar to that of the NF-κB-binding sequence of the IL-2Rα gene. These observations are not consistent with those reported by Numata et al. (29).

Protein binding to TRE-2S. The DNA sequence of TRE-2 gave several bands in a gel retardation assay with a nuclear extract from HUT102 cells in which Tax *trans* activation is taking place (Fig. 5A). To identify the band that is correlated with the function of TRE-2, we examined a series of deletion mutants by gel retardation assay. Each fragment gave sev-

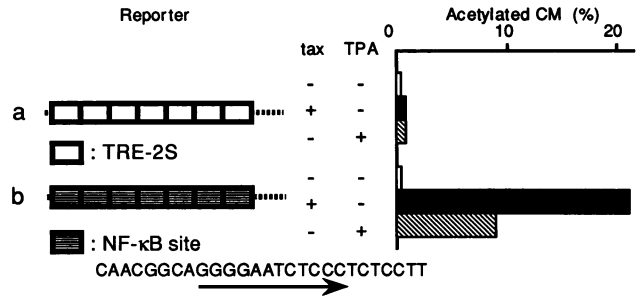

FIG. 4. Comparison of enhancer activities of TRE-2S and NF-κB-binding sequences. A heptamer of TRE-2S (row a) or the NF-κB-binding site of the IL-2Rα promoter (row b) was inserted upstream of a promoter without the 21-bp enhancer (pUCdN55-CAT). The arrow represents the consensus of the NF-κB-binding site. In experiments with TPA, transfected cells were treated with (TPA+) or without (TPA−) TPA (40 ng/ml) and A23187 (0.5 μM) as described in Materials and Methods. The effects of Tax and TPA on CAT expression were assayed with Jurkat cells. CM, chloramphenicol.

eral shifted bands, and some of them were cancelled by the same unlabelled sequence, indicating specific protein binding. One of these bands was observed to be specific to the mutant fragments a, b, d, and f containing TRE-2S (Fig. 5A), which were all active in cooperative activation of one copy of the 21-bp enhancer (Fig. 2B). Moreover, this specific band was not affected by the excess of the 21-bp sequence or Sp1-binding site (Fig. 5B), thus indicating that the Sp1-like binding site in TRE-2 was not involved. To confirm that the

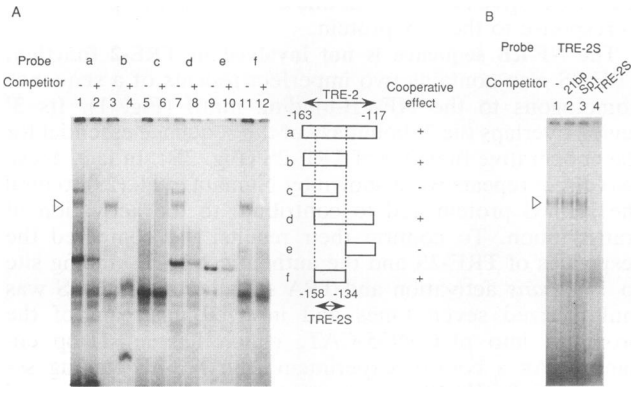

FIG. 5. Gel retardation assay of TRE-2S-binding protein. (A) Gel retardation with various fragments of TRE-2. At the right, open boxes represent probes and pluses and minuses represent their cooperative activity with one copy of the 21-bp enhancer. The double-headed arrows at the top and bottom represent the region of the TRE-2 and the minimum region (TRE-2S) for cooperative activation, respectively. At the left, each ^{32}P-radiolabelled mutant probe was incubated with HUT102 nuclear extract in the absence (−) or presence (+) of a 20-fold excess of the same fragment as competitor and analyzed by gel retardation assay. The arrowhead represents the band observed only with the cooperatively responsive fragment. (B) Competition of TRE-2S and Sp1 or 21-bp sequence. TRE-2S was incubated with HUT102 nuclear extracts in the absence (lane 1) or presence (lane 2) of a 20-fold excess of the 21-bp enhancer, the Sp1-binding site (GGGCGGGGCGGGT-TAGAC [15]) (lane 3), and TRE-2S (lane 4). ▷, bands that correspond to those marked similarly in panel A.

specific protein binding is detected in cells used for the transfection assays, we tested nuclear extracts of the human cell lines FL and Jurkat and obtained results similar to those in Fig. 5A. These results suggest that a specific protein(s) binds to the TRE-2S sequence in various cell lines and possibly mediates the cooperative activation of one copy of the 21-bp enhancer in the presence of Tax protein. We call this protein(s) THP (TRE-2 holding protein).

Isolation and sequencing of cDNA clones of THP proteins. To isolate cDNA clones encoding TRE-2S-binding proteins, a randomly primed λgt11 cDNA library prepared from HUT102 cells was screened with a double-stranded oligonucleotide of TRE-2S by the Southwestern (DNA-protein) procedure (45). The TRE-2S sequence was multimerized seven times and was used a probe. Eleven clones were isolated from about 2×10^6 recombinant phages. By partial sequencing, clones were classified into two groups: six clones in type 1 and five clones in type 2. Sequence analysis of the longest clones in each group showed that both types were identical except for an insertion of 51 bp in type 2 in the coding sequence (Fig. 6A).

By using the 5′ and 3′ fragments of the longest clone, cDNA clones covering about 4 kbp were isolated. The size of THP mRNA was about 4.3 kb; thus, these cDNA clones should cover nearly the full length of the mRNA sequence. The general features of the cDNAs are illustrated in Fig. 6A, although the poly(A) site is still missing. The initiation codon for an open reading frame was identified by a preceding stop codon at position −197 in frame. The sequences of these clones allow us to identify two types of THP cDNA: THP-1 has an open reading frame coding for 506 amino acids, and THP-2 has one coding for 523 amino acids and having an insertion of 51 bp at position +196 (Fig. 6B and C). These proteins are calculated to have molecular masses of 55.6 and 57.4 kDa, respectively. In addition to this, THP-2 has a deletion of 155 bp at position −316 (Fig. 6A).

The deduced amino acid sequences of THP contained five repeats of the zinc finger motif, Cys-Cys–His-His (C_2H_2) (2, 27). These zinc finger motifs are very similar to those of the human GLI gene (20) and the GLI3 gene (35), with the similarity greater than 80% (Fig. 7). They were also similar to those in *Drosophila* cubitus interruptus Dominant (ci^D) (31) and the *tra-1* gene (46). However, sequence other than the zinc finger motifs showed almost no homology, indicating that THP-1 and -2 are new DNA binding proteins of the GLI family.

THP-binding site in TRE-2S sequence. The sequence conservation of the zinc finger motifs between THP and GLI suggested that their binding sequences may be similar to each other. In fact, the sequence GAACCACCCA of TRE-2S at the 3′ boundary is highly homologous to the GLI-binding site GGACCACCCA (Fig. 8A). A synthetic nucleotide, GACCACCCA (GLI-B1), which has been previously identified as a binding sequence of GLI (20), competed efficiently with TRE-2S in binding to a nuclear factor(s) (Fig. 8B). Conversely, TRE-2S competed with the GLI-B1 sequence, indicating that the GLI-binding motif in TRE-2S is responsive to the specific protein binding. THP-1 and -2 proteins were expressed in *E. coli* in the fusion form with GST and purified for testing DNA binding capability. Both GST-THP-1 and -2 were demonstrated to bind to TRE-2S and GLI-B1 (Fig. 8C). Moreover, these proteins did not bind to a mutant (TRE-2S-M) that was inactive in cooperative *trans* activation of the 21-bp enhancer. These observations suggest that THP-1 and -2 are the nuclear factors showing specific binding to TRE-2S and are involved in cooperative

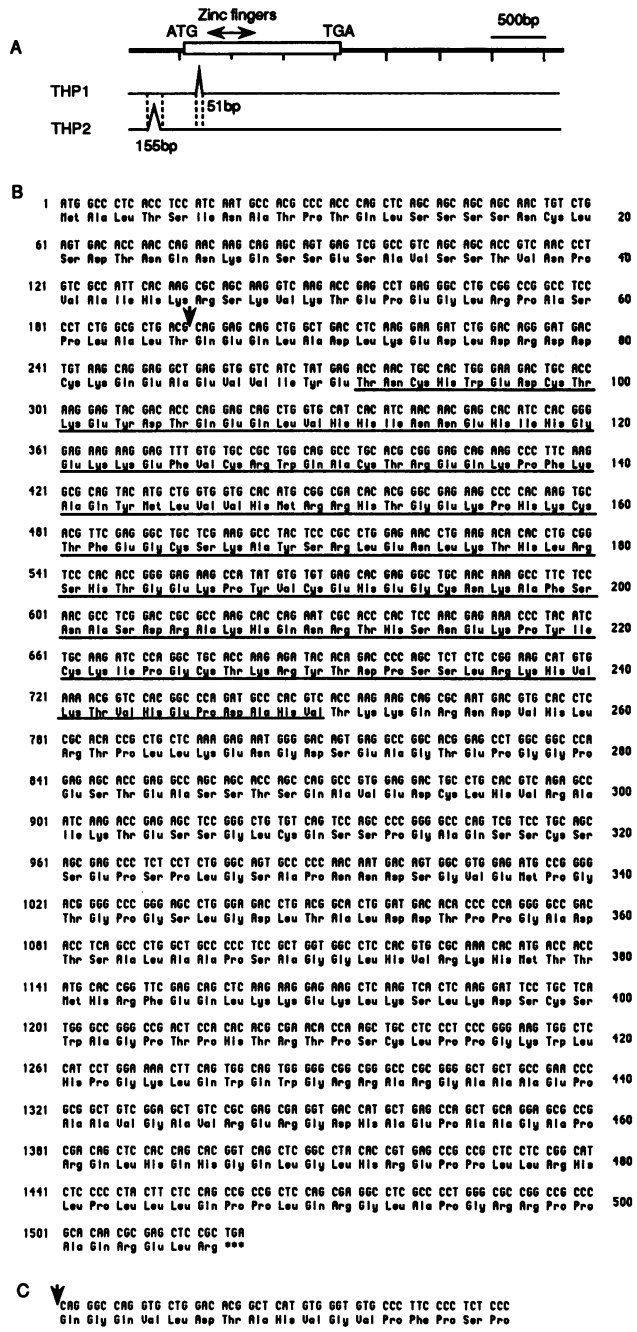

FIG. 6. cDNA and amino acid sequences of THP-1 and THP-2. (A) Illustration of THP-1 and -2 cDNAs. (B) Sequence of THP-1. The zinc finger domain is underlined. (C) Sequence unique to the THP-2 that is inserted at the site marked with the arrow in panel B.

trans activation of one copy of the 21-bp enhancer by Tax protein.

Expression of THP in various cells. As described in the previous section, TRE-2 cooperatively enhanced Tax *trans* activation of one copy of the 21-bp enhancer in various cell lines. To determine how the expression of THP and transcriptional enhancement correspond, we examined the expression of THP in mRNAs in HUT102, Jurkat, and FL cells (Fig. 9). Northern (RNA) blot analysis detected a single

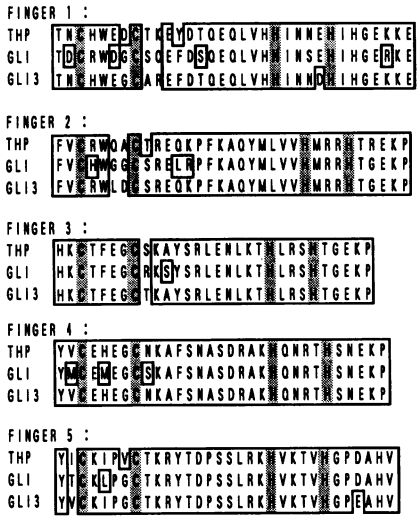

FIG. 7. Comparison of zinc finger motifs of THP with those of GLI and GLI-3. Identical residues are boxed. Cysteine and histidine, which are essential for the zinc finger motifs, are shaded.

major band of 4.3-kb mRNA in various types of cell lines. The size of the mRNA indicated that our cDNA clones covered most of the region of the mRNA, and ubiquitous expression is consistent with Tax *trans* activation of one

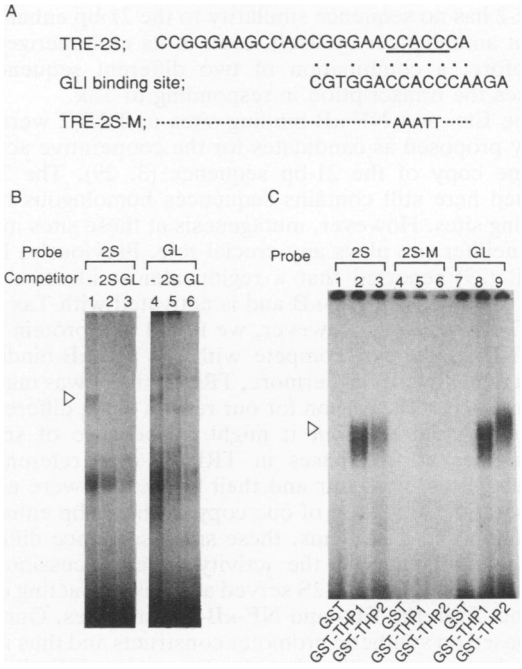

FIG. 8. Competition of TRE-2S and GLI-binding sequences in gel retardation assay. (A) Comparison of TRE-2S and GLI-binding site. Nucleotides identical to those in TRE-2S are indicated by dots on the sequence. TRE-2S-M has a mutated sequence of AAATT at the CCACC sequence. (B) TRE-2S (2S) (lanes 1 to 3) or the GLI-binding sequence (GL) (lanes 4 to 6) was incubated with HUT102 nuclear extract in the absence (lanes 1 and 4) or presence (lanes 2, 3, 5, and 6) of competitors in 20-fold excess. (C) DNA binding activities of GST-THP fusion proteins. GST, glutathione *S*-transferase. ▷, bands showing the specific binding.

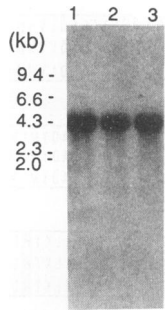

FIG. 9. Expression of THP in various cell lines. Total RNA from different cell lines was analyzed by RNA blotting with cDNA of THP-1 as a probe. Lanes: 1, HUT102; 2, Jurkat; 3, FL. Molecular sizes are on the left.

copy of the 21-bp enhancer cooperating with TRE-2 in various types of cell lines.

DISCUSSION

In this article, we have defined a new *cis*-acting transcriptional element, TRE-2S, in the LTR of HTLV-1. TRE-2 responds to Tax *trans* activation cooperatively with one copy of the 21-bp enhancer; however, it is almost inactive by itself. Therefore, TRE-2 can substitute the second copy of the direct repeat of the 21-bp enhancer, which is efficiently activated by Tax. Generally, multicopies of an enhancer sequence result in activation of transcription. However, TRE-2 has no sequence similarity to the 21-bp enhancer and is not an enhancer by itself, even in its multimerized form; therefore, a combination of two different sequences enhances the transcription in responding to Tax.

The Ets- and NF-κB-binding sites in TRE-2 were previously proposed as candidates for the cooperative activation of one copy of the 21-bp sequence (3, 29). The TRE-2S defined here still contains sequences homologous to these binding sites. However, mutagenesis at these sites indicated that neither site plays any crucial role. Previously, Numata et al. (29) reported that a region almost identical to our TRE-2S binds the NF-κB and is activated with Tax protein and TPA by itself. However, we found that protein binding to TRE-2S did not compete with the NF-κB-binding site (data not shown); furthermore, TRE-2S itself was inactive as an enhancer. The reason for our results being different from theirs is unknown, but it might be because of sequence differences at two bases in TRE-2S (see reference 29). Nevertheless, both our and their sequences were active in cooperative activation of one copy of the 21-bp enhancer in responding to Tax. Thus, these small sequence differences had no significance in the activity under discussion. Thus, we conclude that TRE-2S served as a new *cis*-acting element distinct from the Ets- and NF-κB-binding sites. Our results are based on synthetic promoter constructs and thus may not exclude the possibility that the Ets and/or NF-κB protein contributes to the control of the LTR activity.

We isolated two types of cDNA clones, THP-1 and -2, encoding the TRE-2S-binding proteins. Although TRE-2S contains motifs related to the NF-κB- and Myb-binding sites and the binding of Myb protein to this sequence has been reported previously (4), no cDNA clones related to these factors were isolated. This result might reflect the fact that THPs were the main components of TRE-2S-binding proteins in HeLa cells. Sequence analysis of these clones showed that THPs have five zinc fingers, which are conserved in GLI family proteins. A GLI is an oncogene amplified in glyoma cell lines (19) that transforms rat embryo fibroblasts in cooperating with adenovirus E1A (36) and binds to the DNA sequence GACCACCCA (20). A highly homologous sequence, GAACCACCCA, is present in TRE-2S for the binding of GLI family proteins (20, 36). In fact, a synthetic oligonucleotide with this sequence competed with THP binding to TRE-2S. Moreover, the mutant of TRE-2S that cannot bind to THP-1 and -2 was inactive to activate the transcription. These binding properties were identical to those observed in the gel shift assay with a nuclear extract of HUT102 cells, thus strongly suggesting that THP proteins are identical to those detected in the nuclear extract.

The binding properties of THP proteins to the mutant probes suggest that THP binding to the GLI-binding site is important to activate one copy of the 21-bp enhancer in responding to Tax *trans* activation. We have tried to evaluate the effect of THP-1 and -2 on Tax *trans* activation by cotransfection with Tax expression plasmid; however, the maximum *trans* activation was obtained by Tax alone, and expression of THP-1 or -2 reduced the *trans* activation (data not shown). These results were expected, since various cell lines used for this assay expressed the THP gene constitutively; thus, the exogenously supplied THP proteins would not be able to give rise to additional activation and instead might result in a suppressive effect through squelching of an essential factor(s).

We and others have previously shown that Tax binds to the CREB or CREM protein, which binds to multicopies of the 21-bp enhancer (42, 47). Therefore, it is possible that THP-1 and/or -2 binds to TRE-2S and might interact with Tax or CREB and CREM proteins for cooperative activation of one copy of the 21-bp enhancer. This is an interesting hypothesis to be studied. In this regard, Marriott et al. reported a protein (TIF) of 36 kDa that binds to the sequence containing TRE-2 (25). Interestingly, they also showed that Tax protein binds to this TIF protein. However, the difference in molecular mass shows that THPs are not the TIF.

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