

# A Novel *ets*-related Transcription Factor, ERT/ESX/ESE-1, Regulates Expression of the Transforming Growth Factor- $\beta$ Type II Receptor\*

(Received for publication, August 18, 1997)

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**A 2.5-kilobase cDNA clone that encodes a 371-amino acid novel transcription factor was isolated from a human placenta cDNA library using a yeast one-hybrid system. The novel *ets*-related transcription factor (ERT) showed a homology with the ETS DNA-binding domain. Using constructs of the transforming growth factor- $\beta$  (TGF- $\beta$ ) type II receptor (RII) promoter linked to the luciferase gene, we have demonstrated that ERT activates transcription of the TGF- $\beta$  RII gene through the 5'-TTTCTGTTTCC-3' response element spanning nucleotides +13 to +24 and multiple additional ETS binding sites between -1816 and -82 of the TGF- $\beta$  RII promoter. A specific interaction between ERT and the ETS binding sites was also demonstrated using an electrophoretic mobility shift assay. Deletion mapping of ERT protein suggests that the transactivation domain resides in the amino terminus while the DNA-binding domain is localized to the carboxyl-terminal region. Our results suggest that ERT might be a major transcription factor involved in the transcriptional regulation of the TGF- $\beta$  RII gene.**

Transforming growth factor- $\beta$  (TGF- $\beta$ )<sup>1</sup> plays a critical role in many cellular processes, including regulation of the cell cycle, cell differentiation, and extracellular matrix synthesis (1, 2). Aberrant TGF- $\beta$  function has been implicated in the pathogenesis of many diseases, and it has also been suggested that diminished responsiveness to TGF- $\beta$  may contribute to the process of malignant transformation (1). This decreased responsiveness to TGF- $\beta$  could be caused by defects not only in TGF- $\beta$  expression or activation but also by defects in the regulation of TGF- $\beta$  receptors (3–6).

Much work has recently been directed toward characterizing the TGF- $\beta$  receptors and their intracellular signaling pathways. TGF- $\beta$  type II and type I receptors (TGF- $\beta$  RII and RI,

respectively) are transmembrane serine/threonine kinases that together are sufficient for signal transduction (7). Association between the type I and type II receptors is essential for signaling responses (8). It has been repeatedly demonstrated that a genetic alteration of either RI or RII resulting in dominant negative or loss of function can lead to loss of responsiveness (3–6, 9–12).

In a previous study, our laboratory described a series of gastric cancer cell lines in which resistance to TGF- $\beta$  is correlated with gross structural mutations in the TGF- $\beta$  RII gene (3). We have now studied several additional TGF- $\beta$ -resistant cell lines in which Southern analysis failed to show gross deletions or rearrangements, yet in which no TGF- $\beta$  RII protein or mRNA was produced. This suggested that abnormalities in transcriptional regulation of the type II receptor might also be found to underlie certain instances of escape from TGF- $\beta$ -mediated growth inhibition.

We have recently cloned and sequenced the promoter region of the TGF- $\beta$  RII gene, identified several positive and negative transcriptional regulatory elements, and reported the relevant target sequences for three putative novel transcriptional factor complexes (13, 14). Basal levels of transcription are determined by the core promoter element in cooperation with both PRE1 and PRE2 (positive regulatory elements 1 and 2). PRE1, consisting of nucleotides -219 to -172, contains two discrete target sequences that bind an AP1/CREB-like transcription factor in addition to an unidentified novel transcription factor complex. PRE2 is located between +1 and +35 and contains two overlapping target sequences, both of which appear to bind novel transcription factor complexes.

To identify potential transcriptional activators of the TGF- $\beta$  RII gene, we adapted the yeast one-hybrid system (15) to find proteins that recognize the PRE2 of the TGF- $\beta$  type II receptor gene. Screening a human placenta cDNA library fused to the GAL4 activation domain, we isolated a cDNA clone that induced greater LacZ activity. DNA sequencing analysis of a corresponding plasmid, pACT2ERT, revealed that the encoded gene belongs to a novel member of the *ets* transcription factor family (16–22). Comparison of the nucleotide sequence of ERT to the recently reported epithelial specific *ets*-family member, ESX/ESE-1 (23, 24) showed it to be identical, but the ERT cDNA revealed an additional 524 nucleotides in the 3'-UTR. Further, we demonstrate that the ERT protein specifically binds to the PRE2 region of the TGF- $\beta$  RII gene and activates its transcription.

## MATERIALS AND METHODS

**Reporter Constructs for Library Screen**—The following oligonucleotides, 5'-GAGGAGTTTCTGTTTCCCGC-3' and 5'-GCGGGG-GAAACAGGTAACTCCTC-3', containing the previously described PRE2 binding site were synthesized and annealed (13). The PRE2 mutant oligonucleotides were constructed by replacing the underlined sequences with 5'-AAGTG-3' and 5'-CACTT-3', respectively. The oligo-

\* This work was supported in part by HAN Project of Korean Ministry of Science and Technology (MOST 8-1-10). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF017307.

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<sup>1</sup> The abbreviations used are: TGF- $\beta$ , transforming growth factor- $\beta$ ; PRE, positive regulatory element; ESX, epithelium-restricted with serine box; ESE-1, epithelium-specific ETS; ERT, *ets*-related transcription factor; EBS, ETS binding site; TGF- $\beta$  RI and RII, TGF- $\beta$  type I and II receptors, respectively; UTR, untranslated region; X-gal, 5-bromo-4-chloro-3-indolyl  $\beta$ -D-galactopyranoside; kb, kilobase; TCR $\alpha$ , T-cell receptor  $\alpha$ ; EMSA, electrophoretic mobility shift assay; ONPG, *O*-nitrophenyl  $\beta$ -D-galactopyranoside.

## A Reporter genes

Wild type (PRE2) 5'GAGGAGTTTCCTGTTCCCCCGC3'  
 Mutant(PRE2M) 5'GAGGAGTTTAAGTGTCCCCCGC3'

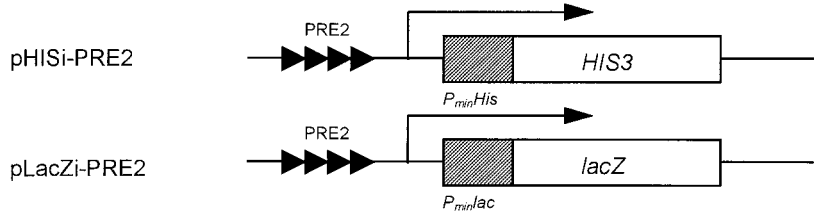
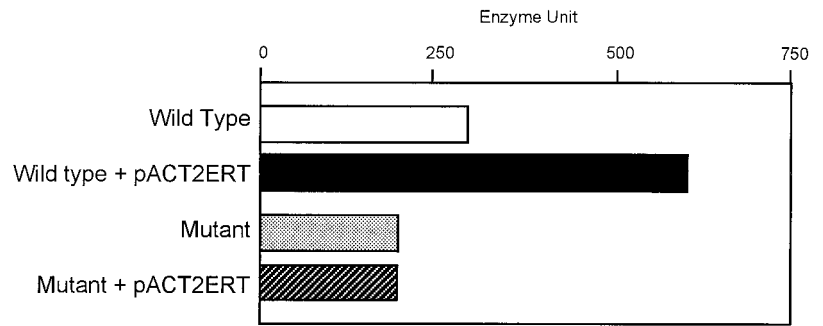


FIG. 1. Approach used to isolate cDNA encoding the PRE2 binding protein of the TGF- $\beta$  type II receptor promoter. A, structure of the reporter genes (see "Materials and Methods" for details). B,  $\beta$ -galactosidase activities of wild-type PRE2- and mutant PRE2-LacZ in the presence or absence of pACT2-ERT. One unit of  $\beta$ -galactosidase is defined as the amount that hydrolyzes 1  $\mu$ mol of ONPG to *O*-nitrophenol and *D*-galactose per min. The results are means of three independent experiments.

B  $\beta$ -galactosidase activities

nucleotides were ligated and subcloned into the *Bam*HI site of pUC18. A fragment corresponding to a four tandem repeat was subcloned into the yeast reporter plasmids, pHISi and pLacZi (CLONTECH). The reporter constructs were subsequently integrated into the yeast strain YM4271 yielding YM4271::PRE2::His3 (or *lacZ*) and YM4271::PRE2M::His3 (or *lacZ*). These yeast strains were used as host strains for the library screen.

**Bacterial and Yeast Strains**—*Saccharomyces cerevisiae* YM4271 (*MATa*, *ura3-52*, *his3-200*, *ade2-101*, *lys2-801*, *leu2-3*, *112*, *trp1-903*, *tyr1-501*) was purchased from CLONTECH and used for yeast transformation. *Escherichia coli* strains DH5 $\alpha$  and DH10B (Life Technologies, Inc.) were used for subcloning and electroporation experiments.

**Screening of the cDNA Library**—The histidine yeast reporter strain YM4271-PRE2-*His3* was transformed with a MATCHMAKER human placenta cDNA library (CLONTECH) by the LiAc/polyethylene glycol method. Approximately  $5 \times 10^4$  transformants were plated per 150-mm dish containing his<sup>-</sup>leu<sup>-</sup> minimal selective medium supplemented with 45 mM 3-aminotriazole. Approximately  $2 \times 10^6$  cDNA plasmids were screened in three different transformations. Based on large colony size and rapid growth, a total of 30 histidine positive clones were selected. Plasmids were recovered and electroporated into the *E. coli* strain DH10B. Plasmids were rescreened by transforming YM4271-PRE2-*lacZ* and plated on leu<sup>-</sup> ura<sup>-</sup> minimal medium. The filter replica method using X-gal (40  $\mu$ g/ml) was used to confirm  $\beta$ -galactosidase activities. One plasmid, pACT2ERT, showed the strongest blue color. The specific DNA binding of pACT2ERT was confirmed by comparing the  $\beta$ -galactosidase activities of wild-type and mutant reporter strains using both the filter replica method and *O*-nitrophenyl  $\beta$ -D-galactopyranoside (ONPG) liquid method (CLONTECH).

**Plasmid Constructions**—The plasmid pcDNA3.1-ERT was generated by subcloning a 2.5-kb *Eco*RI-*Hind*III fragment containing the entire ERT coding sequence into the *Eco*RI-*Hind*III sites of pcDNA3.1(-) (Invitrogen). The plasmid pGL2-pro derivatives including wild-type and mutant PRE2 sequences were constructed by inserting the *Kpn*I-*Xba*I fragment of pUC18/PRE2 and pUC18/PRE2M into the *Kpn*I-*Nhe*I site of pGL2-pro (Promega). The construction of plasmid -219/+35pTBPII-luc is explained elsewhere (13). TGF- $\beta$  RII promoter-luciferase constructs were generated by polymerase chain amplification using genomic DNA containing the 5'-untranslated region of TGF- $\beta$  RII as a template. Amplified DNA fragments were cloned into a promoterless

luciferase expression plasmid, pGL2 (Promega) using *Bgl*III and *Sst*I restriction sites built into the oligonucleotides used for amplification. The sequences of the polymerase chain reaction-generated portions of all constructs were verified by DNA sequencing.

Synthetic wild-type interleukin-2 receptor  $\beta$ -chain (25),  $\beta$ 2 integrin CD18 (26), HTLV-ILTR (27), HIV-2 LTR (28), polyomavirus PEA3 (29), and T-cell receptor  $\alpha$  (TCR $\alpha$ ) enhancer T $\alpha$ 2 (30) *ets* site oligonucleotides containing *Hind*III and *Sst*I ends were inserted into the *Hind*III/*Sst*I sites of the pGL2-promoter plasmid (Promega). All GAL4-ERT fusion plasmids were constructed by inserting the appropriate ERT DNA fragment in-frame to the GAL4 (1-147) sequence in the vector pSG424 (31). ERT DNA fragments were produced by polymerase chain reaction. For the construction of AU-tagged ERT expression plasmids, polymerase chain reaction products were ligated into elongation factor-1 promoter, pCEFL-AU5, using standard methods to generate pCEFL-AU-ERT (1-371, 1-301, 1-200, 1-120, 120-371, 200-371, and 301-371).

**In Vitro Transcription and Translation**—One  $\mu$ g of DNA of pcDNA3.1(-)-ERT constructs was used as the DNA template for *in vitro* RNA transcription using T7 RNA polymerase (Promega). The RNAs were translated *in vitro* using rabbit reticulocyte lysate (Promega) and [<sup>35</sup>S]methionine. Expressed proteins were electrophoresed on a 4-20% SDS-polyacrylamide gradient gel, dried, and autoradiographed.

**Electrophoretic Mobility Shift Assay (EMSA)**—*In vitro* translated protein-DNA complexes were formed by incubating at room temperature for 20 min with 10,000 cpm of <sup>32</sup>P-labeled probe, 50 mM Tris, pH 7.5, 50 mM NaCl, 5 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, 1 mM EDTA, 5% glycerol, bovine serum albumin (300  $\mu$ g/ml) in 20  $\mu$ l of binding mixture.

**Transient Transfection and Luciferase Assays**—HepG2 human hepatoblastoma cell line was maintained in minimal essential medium supplemented with 10% fetal bovine serum. For the transient expression assays, cells were transfected using the Lipofectin-mediated transfection method (Life Technologies, Inc.). Following incubation with Lipofectin for 15 min, cells were incubated for 48 h. The cells were then harvested, and luciferase activity was measured.  $\beta$ -Galactosidase activity was used to correct for transfection efficiencies.

**Northern Blot analysis**—SNU620 human gastric cancer cells, HeLa229 human cervical cancer cells, and SK-BR3 breast cancer cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum. Total RNA was isolated from cells using guanidium

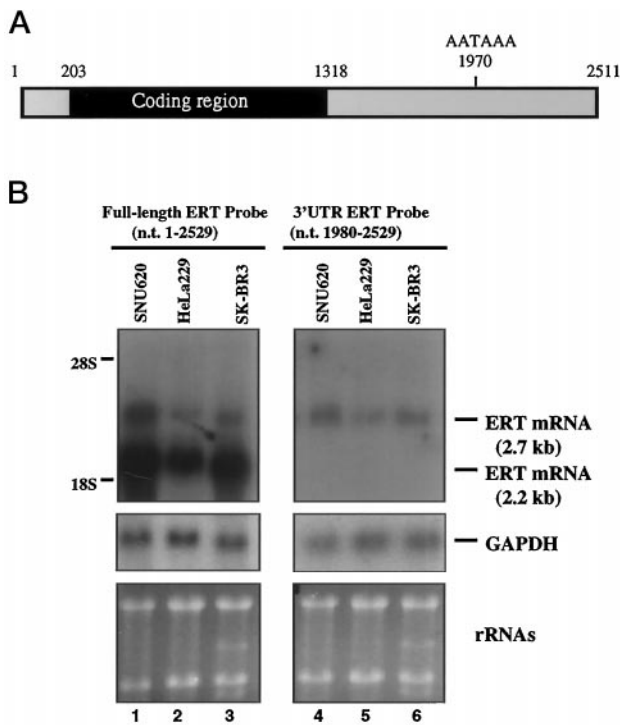


FIG. 2. Expression of ERT in human cancer cell lines. A, diagram of the ERT cDNA. The putative polyadenylation sequence, AATAAA, is shown at nucleotide 1970. B, RNA blot analysis of ERT expression in human cancer cell lines. Total RNA (10  $\mu$ g) from SNU620, HeLa229, and SK-BR3 cells was probed with either the full-length ERT cDNA (lanes 1–3) or 3'-UTR region of the ERT cDNA (lanes 4–6) and the glyceraldehyde-3-phosphate dehydrogenase probe.

isothiocyanate/phenol/chloroform. 10  $\mu$ g of RNA was electrophoresed on a 1.0% agarose gel containing 0.66 M formaldehyde, transferred to a Duralon-UV membrane, and cross-linked with a UV Stratilinker (Stratagene). Blots were prehybridized and hybridized in 1% bovine serum albumin, 7% (w/v) SDS, 0.5 M sodium phosphate, 1 mM EDTA at 65  $^{\circ}$ C. RNA blots were hybridized with  $^{32}$ P-labeled cDNA probes for ERT/ESX/ESE-1 (23, 24). A probe for glyceraldehyde-3-phosphate dehydrogenase was used to assess sample loading.

## RESULTS

**Isolation of the cDNA Clone Encoding a TGF- $\beta$  RII Promoter-binding Protein**—We are interested in isolating novel transcription factors that regulate expression of the TGF- $\beta$  RII gene. The “yeast one-hybrid” strategy (15) was used to screen for human placenta cDNAs encoding for proteins which bind to the TGF- $\beta$  RII receptor promoter PRE2. Four tandem copies of the PRE2 were ligated together and subcloned into the upstream region of the minimal promoter of either the pHISi or pLacZi reporter plasmids and integrated into the yeast genome of YM4271 (Fig. 1A). The nucleotide sequence of the ligated PRE2 was verified by DNA sequencing. With the strategy described under “Materials and Methods,” we isolated several plasmids that induced greater LacZ activity in YM4271 (PRE2wt). One cDNA clone, pACT2ERT, showed the strongest blue color on  $\beta$ -galactosidase assay utilizing the filter replica method. Liquid assay of  $\beta$ -galactosidase activity using ONPG as a substrate confirmed the specific transcriptional activation of pACT2ERT. While the presence of the pACT2ERT plasmid doubled  $\beta$ -galactosidase activity in the wild-type strain, it did not increase enzymatic activity in the mutant strain (Fig. 1B).

**Deduced Amino Acid Sequences of ERT and Sequence Comparison in the ETS Domain between ERT and Other ets Family Members**—The complete sequence of a 2.5-kb cDNA insert was determined by the dideoxy sequencing method. The 2.5-kb ERT cDNA contains a 1116-base pair open reading frame that en-

codes a 371-amino acid protein with a predicted molecular mass of 41,000 daltons. Comparison of the nucleotide sequence of ERT to the recently reported epithelial specific *ets*-family member, ESX/ESE-1 (23, 24), was identical, but the ERT cDNA revealed an additional 524 nucleotides in the 3'-UTR. Comparison of the amino acid sequences of ERT and other *ets* family members revealed a high degree of homology in the ETS domain, located at the carboxyl-terminal region of the molecule. Approximately 40% of the ETS domain amino acid sequence is shared by ERT and other *ets* family members such as *ets-1* (16), *ets-2* (16), ERGB (17), Spi-1 (18), E1A-F (19), ER81 (20), and ERM (21) in the ETS domain. There is no significant homology between ERT and any other *ets* family members outside the carboxyl-terminal domain.

Northern blot analysis of various cancer cell lines showed a major 2.2-kb and a minor 2.7-kb message when probed with the cloned 2.5-kb ERT cDNA insert (Fig. 2). Since the major 2.2-kb ERT mRNA is smaller than the cloned 2.5-kb ERT cDNA insert, we probed the same blot with the 3'-UTR sequences from nucleotides 1980 to 2529. This 3'-UTR probe recognized only the 2.7-kb ERT transcript (Fig. 2, lanes 4–6), suggesting that the two different ERT transcripts resulted from alternative poly(A) signals.

**Transcriptional Activation of the TGF- $\beta$  RII Promoter by ERT**—Most of the *ets* family members are known to be potent transcriptional activators when tested in transient transfection assays (22). To analyze the ability of ERT to activate the TGF- $\beta$  type II receptor promoter, the TGF- $\beta$  RII promoter-luciferase constructs were cotransfected with an expression vector for ERT into HepG2 human hepatoblastoma cells. While ERT did not induce luciferase expression in the control reporter construct, we found ERT to be a potent transcriptional activator of the TGF- $\beta$  type II receptor promoter construct. As seen in Fig. 3,  $-1,670/+36$ pTBPII-luc construct was induced 5-fold by ERT. The  $-1,670/+36$ mtpTBPII-luc construct, in which the second positive regulatory element was mutated, was also activated more than 2.5-fold, suggesting that the fragment between  $-1,670$  and  $+2$  contains multiple ERT regulatory sequences (Fig. 3). In the previous study (13), we reported that at least two distinct nuclear DNA binding proteins shared a common recognition sequence from  $+11$  to  $+29$  in the PRE2 of the TGF- $\beta$  type II receptor promoter. This sequence contains two putative target sequences for protein-binding of *ets* family members in a reverse-orientation (5'-GGAAACAGGAAACT-3'). Competitive inhibition for DNA binding to the  $+1/+50$  sequence was abolished by mutation of nucleotides  $+16$  to  $+20$  (see Fig. 6B).

To characterize the putative ERT response element in the second positive regulatory element, we generated chimeric constructs containing four tandemly linked copies of the PRE2 sequence of the TGF- $\beta$  RII promoter between  $+1$  and  $+36$  linked to a pGL2-promoter. Mutated forms of the putative ERT responsive elements (shown in *bold*) were generated by nucleotide substitution as described in Fig. 4A. In cotransfection assays, ERT induced luciferase expression by the wild-type construct (WT-luc) approximately 3.5-fold over that of the control. Mutation of nucleotides  $+16$  to  $+20$  of the ERT response element in construct MT1-luc resulted in significant reduction in transcriptional activation (Fig. 4B). Therefore, the sequence 5'-TTTCCTGTTTTC-3' (GGAAACAGGAAA in a reverse orientation) located between  $+13$  and  $+24$  appears to be responsible for ERT-mediated activation of the TGF- $\beta$  type II receptor gene. Next, we mutated the individual ETS binding site (EBS) in the second positive regulatory element to see whether both EBSs are required for the maximum induction of TGF- $\beta$  type II receptor promoter activity by ERT. MT2, in which the first EBS



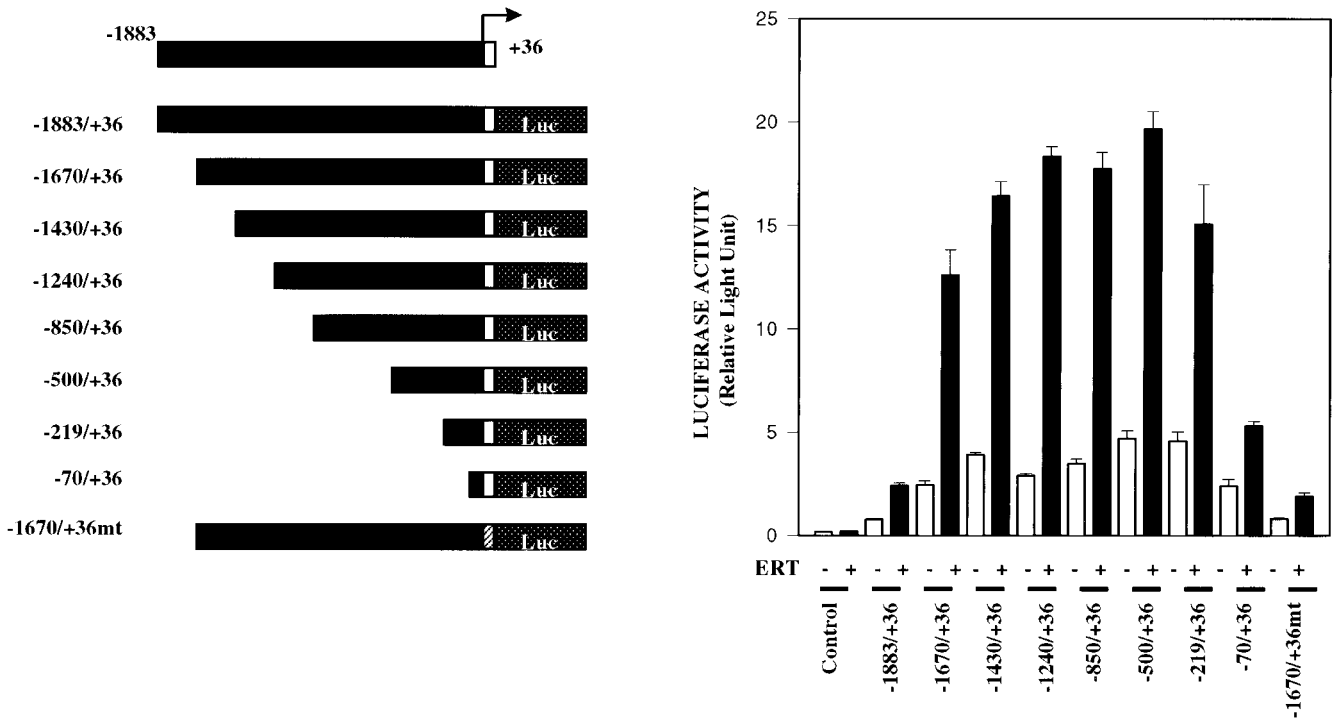


FIG. 3. **Transactivation of the TGF- $\beta$  type II receptor promoter by ERT.** Deletion analysis of TGF- $\beta$  type II receptor promoter constructs for ERT activation is shown. Progressively shorter fragments of the 5'-flanking region of the TGF- $\beta$  type II gene were ligated to the luciferase gene. Numbers represent position relative to the transcription start site. Constructs were transfected into HepG2 cells, which were then harvested after 48 h and assayed for luciferase. Since ERT activates the viral promoter, results are the mean and standard deviation of the three transfections.

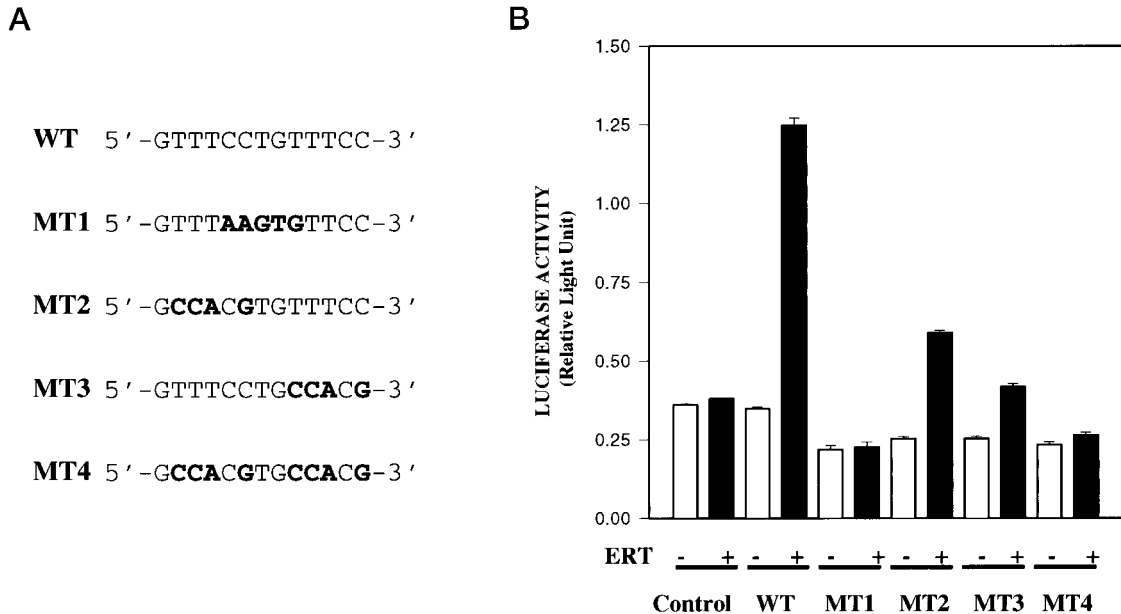


FIG. 4. **Evidence that ERT regulates the PRE2 of the TGF- $\beta$  type II receptor promoter through the two EBSs.** A, diagram of the mutations introduced into the two EBSs contained within PRE2. Either the 5', 3', or both EBSs was changed in *MT1*, *MT2*, *MT3*, and *MT4*, respectively. B, effect of ERT on transcription of the EBS mutants in PRE2 in HepG2 cells. The wild-type (*WT*) PRE2 and the four mutants were cotransfected with either a control vector or a ERT expression vector into HepG2 cells, which were then harvested after 48 h and assayed for luciferase. The level of ERT-mediated activation is shown.

is mutated (Fig. 4A), was induced 2-fold by ERT, and MT3, in which the second EBS was mutated, was also induced 1.5-fold, whereas MT4, in which both EBSs were mutated, showed little induction (Fig. 4B). These results suggest that ERT can activate the TGF- $\beta$  type II receptor promoter through either EBS but that both sites are required for the maximal induction.

We next examined whether additional EBS might be identified in the entire TGF- $\beta$  RII promoter. A computer search revealed multiple copies of EBS between -1816 and +25 (Fig.

5). As seen in Fig. 3, the activity of TGF- $\beta$  type II receptor promoter-luciferase constructs was induced 2- to 4-fold by ERT. The activity of construct -70/+36 was induced 2-fold by ERT, suggesting that this induction was due to EBSs located in +8/+36 promoter region since no EBS is present in the sequences between -70/-1. Upon mutating the two EBSs between +8/+25 (-1670/+36mt), basal levels of activity dropped 2-fold suggesting that these sites are required for the basal expression of the TGF- $\beta$  type II receptor. In the presence of

ERT, the mutated construct induced activity 2-fold, indicating that other upstream EBSs are also responsive to ERT.

**ERT Binds to the PRE2 of the TGF- $\beta$  RII Promoter**—To analyze the ability of ERT to bind to the TGF- $\beta$  RII promoter element PRE2, *in vitro* translated ERT protein was made by subcloning the *EcoRI*-*Bgl*II portion of the insert into the *EcoRI*-*Bam*HI site of pcDNA3.1. This 41-kDa *in vitro* translated ERT protein was consistent with the predicted size of the open reading frame and was used for the electrophoretic mobility

-1816/-1806 5' -GAGGAAGGAAG-3'  
 -1625/-1615 5' -GGGGAAGCCAT-3'  
 -1489/-1479 5' -TGGAAGACTC-3'  
 -1435/-1425 5' -AGGGAAATAGGG-3'  
 -1417/-1407 5' -TTGGAACTCAA-3'  
 -1399/-1409 3' -GAGGAAAGATTG-5'  
 -1356/-1346 5' -TGGAAGAAAGAA-3'  
 -1293/-1283 5' -TGGAAGTGGC-3'  
 -1200/-1190 5' -CAGGAAAGATT-3'  
 -1148/-1158 5' -AGGGAAATAAG-3'  
 -1009/-1019 5' -GAGGAAAGATTG-3'  
 -1001/-1091 5' -AAGGAATTCAT-3'  
 -1028/-1018 5' -TGGAAGTAGG-3'  
 -934/-924 5' -CAGGAATGTCT-3'  
 -895/-885 5' -AAGGAATTTG-3'  
 -806/-796 5' -AGGGAAATAAAA-3'  
 -710/-720 3' -TGGAATATGTA-5'  
 -658/-648 5' -CGGAAGAGGC-3'  
 -542/-552 3' -CTGGAAATCTT-5'  
 -385/-375 5' -TTGGAAAGAAA-3'  
 -244/-234 5' -AGGGAAACGT-3'  
 -167/-157 5' -GAGGAACTCCT-3'  
 -92/-82 5' -TAGGAAACATG-3'  
 +18/+8 3' -CAGGAAACTCC-5'  
 +25/+15 3' -GGGGAACAGG-5'

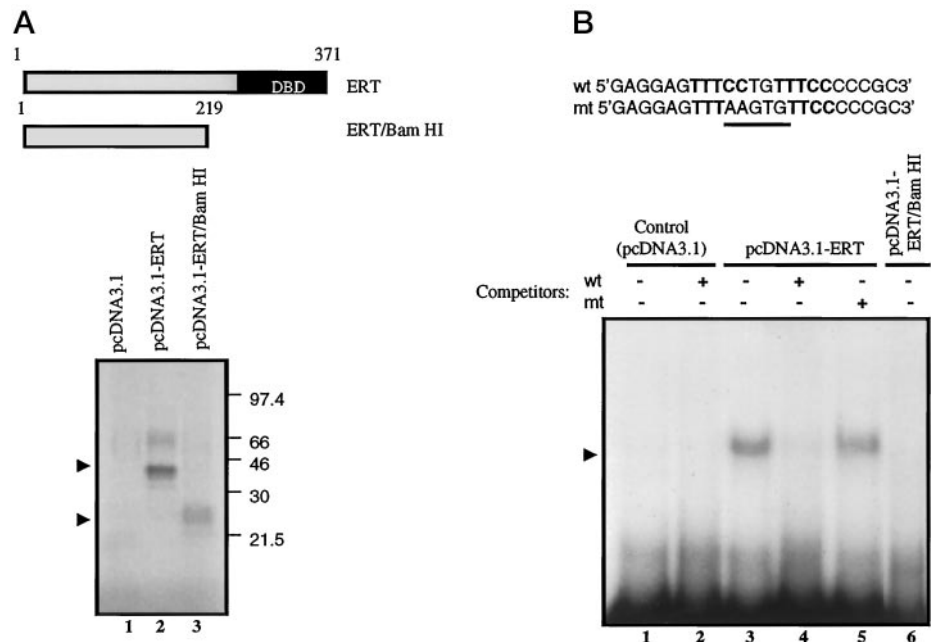
FIG. 5. A computer analysis of a common recognition sequence, 5'-GGAA-3' for the *ets* family in the region between -1 and -1883 of the TGF- $\beta$  type II receptor promoter.

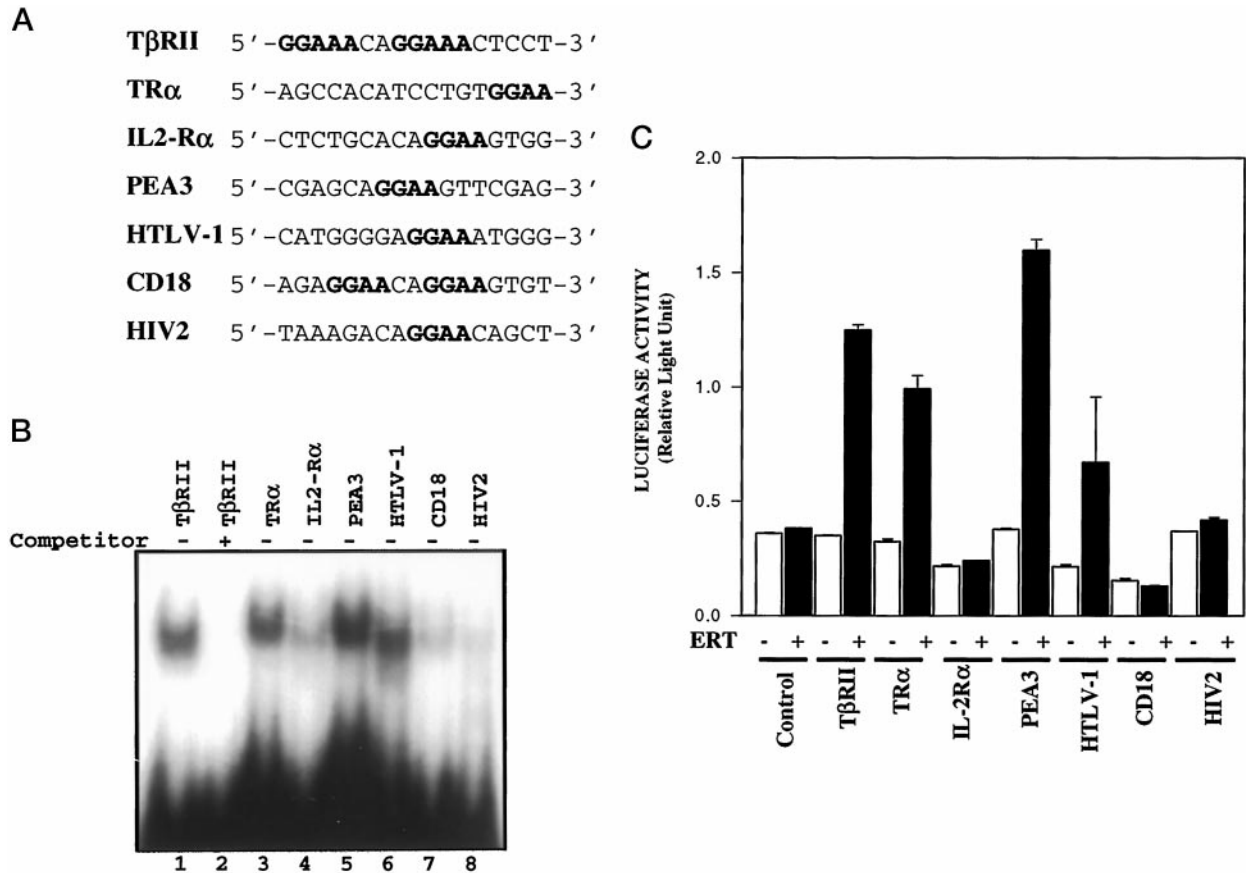
shift assay (Fig. 6A). A radiolabeled PRE2 (+1/+50) probe was incubated with *in vitro* translated ERT protein in competition with unlabeled PRE2 and mutant PRE2 oligonucleotides. While specific unlabeled competitors competed for labeled protein-DNA complexes (Fig. 6B, lane 4), oligonucleotides mutated in nucleotides +16 to +20 comprising the ETS-binding site (lane 5) did not compete for binding to ERT. Deletion of the carboxyl-terminal ETS-domain from the ERT protein produced a smaller protein of approximately 30 kDa (Fig. 6A, lane 3) that failed to interact with the wtPRE2 (Fig. 6B, lane 6).

**ERT Binds to Functionally Important ETS-related Binding Sites in a Variety of Genes**—To analyze the DNA sequence requirements for the binding of ERT and the relative binding affinities, we designed oligonucleotides encoding a whole spectrum of different functionally relevant binding sites for *ets*-related factors, including the site in the interleukin-2 receptor  $\beta$ -chain gene (Fig. 7A). The relative binding affinities of ERT for these sites were compared with its affinity for the human TGF- $\beta$  RII promoter ETS sites. Equivalent amounts of wild-type labeled oligonucleotides were used as probes in EMSAs with equal amounts of full-length ERT *in vitro* translated protein (Fig. 7B). The *in vitro* translated ERT formed a complex with all the *ets*-related binding site oligonucleotides tested but with different affinities (Fig. 7B). EBS oligonucleotides in the TCR $\alpha$  enhancer T $\alpha$ 2, polyomavirus PEA3, and HTLV-1 LTR promoters bound to the ERT with strong affinity, whereas ERT interacted with IL-2-receptor  $\beta$  chain,  $\beta$ 2 integrin CD18, and HIV2 LTR *ets* binding site oligonucleotides with weaker affinities. To determine if ERT binding correlates to the transcriptional induction by ERT, we cotransfected HepG2 cells with these oligonucleotides linked to a luciferase reporter gene and an ERT expression vector or a control pGL2-pro. A direct correlation was found between the relative strengths of the binding affinities and the level of activity induced by ERT (Fig. 7C). PEA3 was induced 5-fold, TR $\alpha$  and HTLV-1 were both induced 3-fold, while the other weak ERT affinity binding oligonucleotides showed no appreciable induction, suggesting that binding is necessary for ERT-mediated transcriptional induction.

**Mapping of Transactivation Domain**—Transcription factors often display a modular structure with domains being responsible for DNA binding, transactivation, or protein-protein interaction. To identify potential transactivation domains within

**FIG. 6. Electrophoretic mobility shift assay of *in vitro* translated ERT.** A, intact ERT and the ERT deletion mutant were transcribed *in vitro* with T7 polymerase and translated in the rabbit reticulocyte system in the presence of [<sup>35</sup>S]methionine (arrowheads). The *Bam*HI restriction enzyme was used to generate a construct in which the carboxyl-terminal region common to the *ets* family members was deleted. B, gel shift assays demonstrating that ERT binds to the PRE2 of the TGF- $\beta$  type II receptor promoter (arrowhead). The PRE2 probe used in all reactions was a <sup>32</sup>P-labeled dimer of the oligonucleotide +1/+50, a wild-type PRE2. Cold competitor oligonucleotides were present at 100-fold molar excess. The sequences of the competitors used in this experiment are shown at the top. The underlined bases show the mutated nucleotides.





**FIG. 7. Interaction of ERT with functionally relevant regulatory sites in different genes and induction of these genes by ERT.** *A*, oligonucleotides used for ERT binding and construction of the luciferase reporter. *B*, comparison of the relative binding affinities of ERT for *ets*-binding sites in the transcriptional regulatory regions of various genes that have been shown to bind *ets*-related factors or that correspond to the consensus *ets*-binding site, including TCR $\alpha$  enhancer T $\alpha$ 2 (30), IL2-receptor  $\alpha$  chain promoter (25), polyomavirus PEA3 (29), HTLV-1 LTR (27),  $\beta$ 2 intergrin CD18 promoter (26), and HIV2 LTR (28) are shown. *In vitro*-translated ERT was incubated with the labeled oligonucleotide probes. *C*, transcriptional activation of *ets*-binding sites described in panel *A* by the ERT. HepG2 cells were cotransfected with the ERT expression vector construct and luciferase construct containing one copy of wild-type *ets*-binding sites subcloned into pGL2-pro vector (Promega). Luciferase activity in the lysates was determined 48 h after transfection. Data shown are means of triplicate measurements from one representative transfection. The experiment was repeated at least three times with different plasmid preparations, with comparable results.

ERT, several truncations of ERT were tested in transient transfection assays (Fig. 8). Deletion of the first 200 amino acids led to a severe reduction of ERT-mediated transcription, implicating the existence of an amino-terminal transactivation domain. Interestingly, the ERT DNA binding domain alone (200/371 or 301/371) suppressed basal activity of TGF- $\beta$  type II receptor promoter, suggesting that it acts as a dominant negative mutant.

To analyze the location of the transactivation domain in ERT, fusions of ERT amino acids to the DNA binding domain of the yeast transcription factor GAL4 were constructed. These plasmids were transfected into the HepG2 cells with a luciferase reporter (G5BLuc) containing five GAL4 binding sites upstream from the E1B TATA box (Fig. 9). Comparable expression of the GAL4-ERT fusion proteins was investigated by Western blot analyses using anti-GAL4 antibodies (data not shown). Significant increases of basal transcription were observed with the GAL4-ERT-(1-371). Transcriptional activity was diminished with deletion of the amino terminal region of ERT (Fig. 9B). Basal level of transcription was significantly elevated with the GAL4-ERT-(1-200) fusion protein. In addition, the GAL4-ERT-(1-300) is less active than GAL4-ERT-(1-200), suggesting that amino acids 200 to 300 exert an inhibitory effect on transactivation (Fig. 9B). These data are in agreement with the identification of a transactivation domain at the amino terminus of ERT.

## DISCUSSION

We have isolated a new member of the *ets* family of transcription factors, which is a potent transcriptional activator of the TGF- $\beta$  RII gene, by using a novel genetic approach based on the yeast one-hybrid system. Using this genetic selection to screen a human placenta cDNA library for sequences encoding DNA-binding domains that can recognize the PRE2 of the human TGF- $\beta$  RII gene in yeast, we isolated a human cDNA that codes for a protein, ERT, that binds to the PRE2 of the TGF- $\beta$  RII promoter in a sequence-specific manner and activates transcription. The deduced amino acid sequence shows high homology with the ETS-domain, the DNA binding region in the *ets* family genes. Comparison of the nucleotide sequence of ERT to the recently reported epithelial specific *ets*-family member, ESX/ESE-1 (23, 24) was identical, but the ERT cDNA revealed an additional 524 nucleotides in the 3'-UTR.

In a previous study, we demonstrated that PRE2 contained at least one nuclear protein recognition sequence from +11 to +29 (13). This region contains two direct repeats of the purine-rich sequences (GGAAAC) in a reverse-orientation. Competition for binding to ERT was abolished by mutation of this sequence, suggesting that these purine-rich sequences are the binding sites for ERT. Expression of exogenous ERT increases the level of transcription from the TGF- $\beta$  RII promoter, implying an activating role for ERT in TGF- $\beta$  RII expression. We

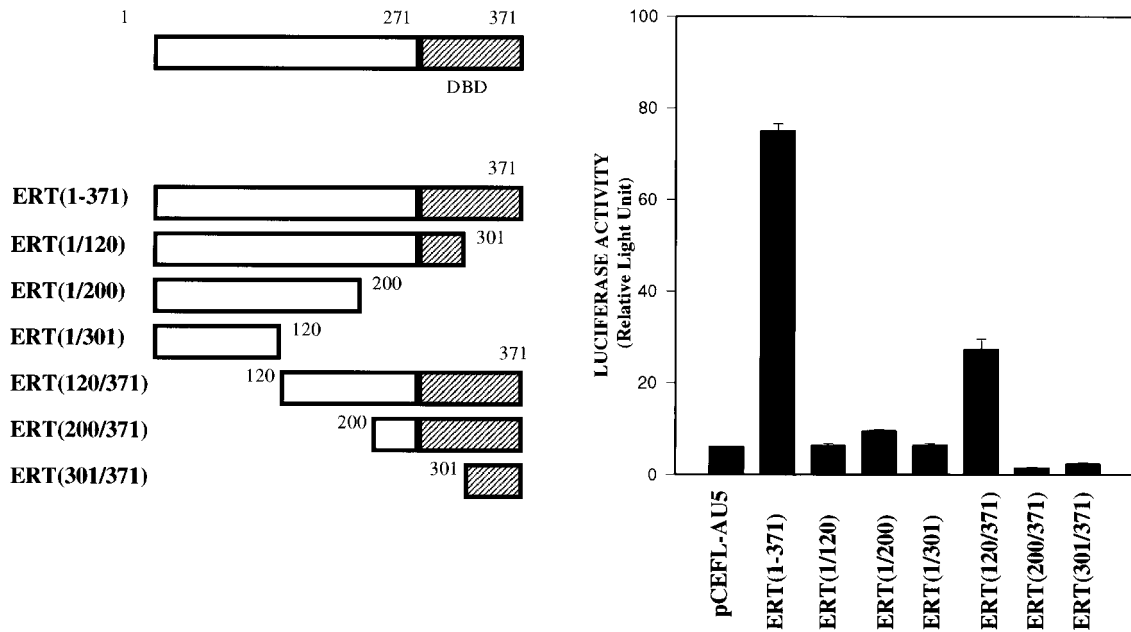


FIG. 8. Mapping of the ERT domains for the DNA binding and transactivation. The structure of human wild-type and deletion ERT expression constructs is shown in the left panel. Target reporter plasmids (1  $\mu$ g) containing four copies of the TGF- $\beta$  type II receptor promoter PRE2 from position +1 to +50 inserted into a pGL2-promoter (wtTP2-Luc) (Promega) was cotransfected into HepG2 cells together with 1  $\mu$ g of either a control expression plasmid (pCEFL-AU5) or 1  $\mu$ g of the same vector expressing the wild-type or deletion ERT proteins and assayed for luciferase activity. Data shown are means of triplicate measurements from one representative transfection. The experiment was repeated at least three times with different plasmid preparations, with comparable results.

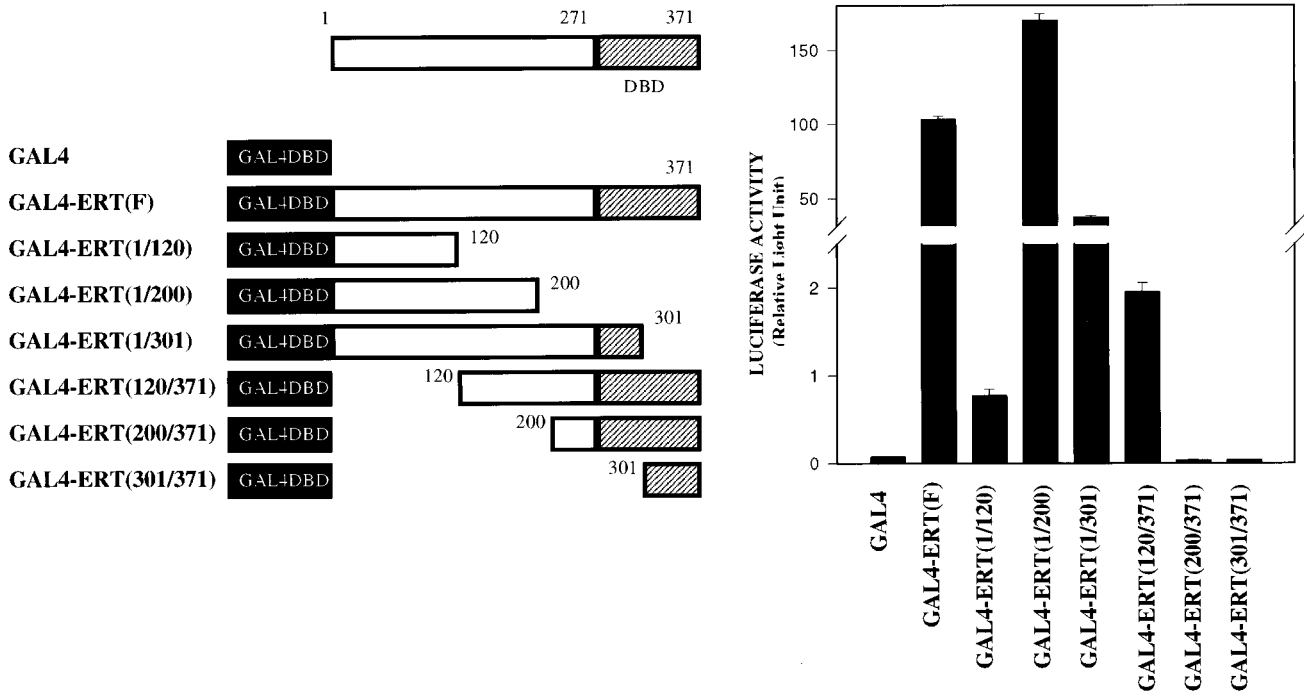


FIG. 9. Delineation of protein domain involved in activation of ERT. Schematic representation of the deletion mutant of ERT fused to GAL4 binding domain is shown at the left panel. HepG2 cells were cotransfected with 1  $\mu$ g each of the G5Luc reporter plasmid and the indicated GAL4 constructs. Data shown are means of triplicate measurements from one representative transfection. The experiment was repeated at least three times with different plasmid preparations, with comparable results.

have detected high-affinity binding sites for ERT in the regulatory regions of various genes, and we have demonstrated that ERT can transactivate the isolated ETS sites of these promoters.

Proteins of the *ets* gene family members have a conserved DNA-binding domain (the ETS domain) and regulate transcriptional initiation from a variety of cellular and viral gene promoter and enhancer elements, including the human interleu-

kin-2 receptor  $\beta$ -chain gene promoter (25) and the human  $\beta$ 2 intergrin CD18 promoter (26). A combination of EMSA and methylation interference studies on the binding of ETS proteins to the target sequences has shown that the GGAA purine core is essential for the specific binding of ETS-related proteins. All members of the *ets* family share a common recognition sequence, whereas the flanking sequences are divergent for different members of the *ets* family. Differences in ERT



binding to and ERT-mediated transactivation of a variety of genes containing EBS examined in this study could therefore reflect the precise recognition sequence for ERT. We identified multiple EBS-like sequences in the region between -1816 and +36 by a computer analysis. The TGF- $\beta$  RII promoter PRE2 contains two copies of the consensus core sequence GGAA located at +14 to +17 and +21 to +24 which are between +1 and +50. This region is essential for TGF- $\beta$  RII promoter and enhancer activities. It is noteworthy, however, that these ETS-binding sites by themselves are not sufficient to fully activate the promoter since mutational analysis reveals that the first positive regulatory element (PRE1) of the TGF- $\beta$  RII promoter cooperates with the second positive regulatory element (PRE2) to sustain basal levels of promoter activity (13). Since the sequence between -1 and -1883 is also responsive to ERT, it is possible that these ETS-binding sites functionally interact with the first positive regulatory element to achieve the full promoter activity of the TGF- $\beta$  RII gene.

The expression patterns of different members of the *ets* gene family vary between tissues (22). Many members of this family are expressed in hematopoietic cells, suggesting a role for these members of the *ets* family in hematopoietic cell growth and differentiation (22). All the *ets* family genes, with the exception of *yan* (32) and *ERF* (33), are known to be potent transcriptional transactivators. Recently, Chang *et al.* (23) and Oettgen *et al.* (24) reported the identification of a new epithelium-restricted ETS, ESX/ESE-1, based on a search of expressed sequence tags. The sequence of the ESX gene is identical to the ERT nucleotide sequence; however, our ERT cDNA is 524 nucleotides longer in the 3'-untranslated region and 150 nucleotides longer in the 5'-untranslated region compared with the published ESX sequence (23, 24). Our preliminary results show that other *ets* family members such as ETS-1 and ETS-2 also induce TGF- $\beta$  RII promoter activity, suggesting that *ets* family members may be one of the major transcription factors involved in regulation of TGF- $\beta$  RII gene expression. Since most cells express the TGF- $\beta$  receptors, it is possible that expression of the TGF- $\beta$  RII gene may be regulated by distinct *ets* family members in different tissues.

TGF- $\beta$  plays a critical role in many cellular processes, including regulation of cell cycle and cell differentiation, and it has now been demonstrated that aberrant expression of TGF- $\beta$  receptors may play a role in a wide variety of human pathologies. We have recently identified a subset of human gastric cancer cell lines that are resistant to TGF- $\beta$  and that lack TGF- $\beta$  RII mRNA expression despite evidence of a normal gene, suggesting that transcriptional regulation may play an important role in controlling TGF- $\beta$  RII expression. We demonstrate that there is a strong correlation between expression patterns of TGF- $\beta$  RII mRNA and ERT mRNA in human gastric cancer cell lines.<sup>2</sup> Examination of the mechanisms underlying loss of TGF- $\beta$  RII expression in different human gastric cancer cell lines suggests a complex interplay between mutational events and transcriptional regulation. Given the importance of the *ets* family genes in regulating TGF- $\beta$  RII gene

expression, it is quite likely that regulation of this family of transcription factors, including ERT, will emerge as a key mechanism controlling cellular responsiveness to TGF- $\beta$ .

**Acknowledgments**—We thank Drs. J. Silvio Gutkind for pCEFL-AU5 expression vector and Anita B. Roberts for helpful discussion and critical review of the manuscript.

## REFERENCES

1. Roberts, A. B., and Sporn, M. B. (1990) *Peptide Growth Factors and Their Receptors. Handbook of Experimental Pharmacology*. pp.419–472, Springer-Verlag, Heidelberg
2. Massagué, J., Attisano, L., and Wrana, J. (1994) *Trends Cell Biol.* **4**, 172–178
3. Park, K., Kim, S.-J., Bang, Y.-J., Park, J.-G., Kim, N., K. Roberts, A. B., and Sporn, M. B. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 8772–8776
4. Kadin, M., Cavaille-Coll, M. W., Gertz, R., Massagué, J., Cheifetz, S., and George, D. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **9**, 6002–6006
5. Kim, D. H. and Kim, S.-J. (1996) *J. Biomed. Sci.* **3**, 143–158
6. Markowitz, S., and Roberts, A. B. (1996) *Cytokine & Growth Factor Reviews*, Vol. 7, pp. 93–102, Elsevier Science, Cambridge
7. Lin, H., Wang, X.-F., Ng-Eaton, E., Weinberg, R., and Lodish, H. (1992) *Cell* **68**, 775–785
8. Wrana, J. L., Attisano, L., Wieser, R., Ventura, F., and Massagué, J. (1994) *Nature* **370**, 341–347
9. Markowitz, S., Wang, J., Myeroff, L., Parsons, R., Sun, L. Z., Lutterbaugh, J., Fan, R. S., Zborowska, E., Kinzler, K. W., Vogelstein, B., Brattain, M. G., and Willson, J. K. V. (1995) *Science* **268**, 1336–1338
10. Myeroff, L. L., Parsons, R., Kim, S.-J., Hedrick, L., Cho, K. R., Orth, K., Mathis, M., Kinzler, K. W., Lutterbaugh, J., Park, K., Bang, Y.-J., Lee, H. Y., Park, J.-G., Lynch, H. T., Roberts, A. B., Vogelstein, B., and Markowitz, S. D. (1995) *Cancer Res.* **55**, 5545–5547
11. Parsons, R., Myeroff, L. L., Liu, B. L., Willson, J. K. V., Markowitz, S. D., Kinzler, K. W., and Vogelstein, B. (1995) *Cancer Res.* **55**, 5548–5550
12. Sun, L., Wu, G., Willson, J. K. V., Zborowska, E., Yang, J., Rajkarunanyake, I., Wang, J., Gentry, L. E., Wang, X.-F., and Brattain, M. G. (1994) *J. Biol. Chem.* **269**, 26449–26455
13. Bae, H. W., Geiser, A. G., Kim, D. H., Chung, M. T., Burmester, J. K., Sporn, M. B., Roberts, A. B., and Kim, S.-J. (1995) *J. Biol. Chem.* **270**, 29460–29468
14. Kim, D. H., Chang, J. H., Lee, K. W., Lee, H. Y., and Kim, S.-J. (1997) *J. Biol. Chem.* **272**, 688–694
15. Li, J. J., and Herkowitz, I. (1993) *Science* **262**, 1870–1874
16. Watson, D. K., McWilliam, M. J., Lapis, P., Lautenberger, J. A., Schweinfest, C. W., and Papas, T. S. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85**, 7862–7866
17. Watson, D. K., Smyth, F. E., Thompson, D. M., Cheng, J. Q., Testa, J. R., Papas, T. S., and Seth, A. (1992) *Cell Growth Differ.* **3**, 705–713
18. Ray, D., Bosselut, R., Ghysdael, J., Mattei, M. G., Tavittian, A., and Moreau-Gachelin, F. (1992) *Mol. Cell. Biol.* **12**, 4297–4304
19. Higashino, F., Yoshida, K., Fujinaga, Y., Kamio, K., and Fujinaga, K. *Nucleic Acids Res.* **21**, 547–553
20. Monte, D., Baert, J. L., Defossez, P. A., de Launoit, Y., and Stehelin, D. (1994) *Oncogene* **9**, 1397–1406, 1994
21. Monte, D., Coutte, L., Baert, J. L., Angeli, I., Stehelin, D., and de Launoit, Y. (1995) *Oncogene* **11**, 771–779
22. Macleod, K., LePrince, D., and Stehelin, D. (1992) *Trends Biochem. Sci.* **17**, 251–256
23. Chang, C.-H., Scott, G. H., Kuo, W.-L., Xiong, X., Suzdaltseva, Y., Park, J. W., Sayre, P., Erny, K., Collins, C., Gray, J. W., and Benz, C. C. (1997) *Oncogene* **14**, 1617–1622
24. Oettgen, P., Alani, R. M., Barcinski, M. A., Brown, L., Akbarali, Y., Boltax, J., Kunsch, C., Munger, K., and Libermann, T. A. (1997) *Mol. Cell. Biol.* **17**, 4419–4433
25. Lin, J.-X., Bhat, N. K., John, S., Queale, W. S., and Leonard, W. J. (1993) *Mol. Cell. Biol.* **13**, 6201–6210
26. Böttinger, E. P., Shelley, C. S., Farokhzad, O. C., and Arnaout, M. A. (1994) *Mol. Cell. Biol.* **14**, 2604–2615
27. Gitlin, S. D., Bosselut, R., Geronne, A., Ghysdael, J., and Brady, J. N. (1991) *J. Virol.* **65**, 5513–5523
28. Leiden, J. M., Wang, C.-Y., Petryniak, B., Markovitz, D. M., Nabel, G. J., and Thompson, C. B. (1992) *J. Virol.* **66**, 5890–5897
29. Xin, J.-H., Cowie, A., Lachance, P., and Hassell, J. A. (1992) *Genes & Dev.* **6**, 481–496
30. Ho, I. C., Bhat, N. K., Gottschalk, L. R., Lindsten, T., Thompson, C. B., Papas, T. S., and Leiden, J. M. (1990) *Science* **250**, 814–818
31. Sadowski, I., Ma, J., Triszenberg, S., and Ptashne, M. (1988) *Nature* **335**, 563–564
32. Lai, Z.-C., and Rubin, G. M. (1992) *Cell* **70**, 609–620
33. Sgouras, D. N., Athanasiou, M. A., Beal, G. J., Jr., Fisher, R. J., Blair, D. G., and Mavrothalassitis, G. J. (1995) *EMBO J.* **14**, 4781–4793

<sup>2</sup> S.-G. Choi, M. Kato, J. Chang, Y.-J. Bang, and S.-J. Kim, manuscript in preparation.