The transcription factors Fos, Jun, and Ets regulate the expression of human stromelysin-1 and collagenase-1 genes. Recently, we found that ERG, an Ets family member, activates collagenase-1 gene but not stromelysin-1 by physically interacting with c-Fos/c-Jun. Interestingly, ERG binds to stromelysin-1 promoter and represses its activation by ETS2. Here, to investigate the molecular mechanism of this regulation, we have used an in vitro protein-protein interaction assay and studied the transcription factor interactions of ETS2. We found that ETS2 could weakly associate with in vitro synthesized ETS1, c-Fos, and c-Jun and strongly with c-Fosc-Jun complex and ERG via several distinct ETS2 domains including the C-terminal region that contains the DNA-binding domain. Strikingly, these interactions were stabilized in vitro by DNA as they were inhibited by ethidium bromide. Both the N-terminal region, comprising the transactivation domain, and the C-terminal region of ETS2 associated with ERG and, interestingly, the interaction of ERG through the transactivation domain of ETS2 was DNA-independent. The DNA-dependent interaction of ETS2 with c-Fos/c-Jun was enhanced by specific DNA fragments requiring two Ets-binding sites of the stromelysin-1 promoter. Using the two hybrid system, we also demonstrated that ETS2 interacts with c-Jun or ERG in vivo.

The transcription of genes is controlled positively or negatively by a variety of extracellular stimuli, yielding to a finely tuned regulation of gene expression. Gene transcription requires the assembly of RNA polymerase II with a multiprotein preinitiation complex at specific DNA sequences including the TATA promoter box. Interactions of general transcription factors with basal promoter elements are generally essential for basal transcription but cannot be sufficient to increase or decrease its rate. Activation and repression of gene transcription depend on the binding of several transcription factors to DNA-specific motifs that, when bound to their promoter elements, stimulate or inhibit transcription through protein-protein interactions with the basal transcription machinery (for review see Goodrich et al. (1)).

An extensive number of transcription activators and repressors that regulate cellular and viral genes have been characterized including the Fos, Jun, and Ets families of transcription factors. Generally, transcription factors contain distinct domains with different functions. For instance, Fos and Jun contain a leucine zipper domain that allows the formation of homodimeric complexes and a basic region, via which the c-Fos/c-Jun heterodimer binds to the 5'-TGATGCA-3' DNA sequence or AP-1 site (2). Similarly, the ETS family of transcription factors is composed of two main domains, the transactivation domain and the DNA-binding domain via which they bind to the 5'-GGA(A/T)-3' core sequence (3–5). The DNA-binding domain (DBD),1 also known as the ETS domain, is well conserved among the different members of the Ets family. Thus, Ets1, Ets2, Erg, and FliI share 98% homology in the ETS domain but have divergent transactivation (TA) domains (6, 7). Additional domains other than DBD and TA have been identified for Ets1, Ets2, and Erg. These domains include regulatory regions that modulate positively or negatively the transactivation property of the ETS proteins and inhibitory domains (ID) that interfere with the binding of the Ets proteins to their DNA motifs (8–10). It has been suggested that accessory factors would relieve the inhibitory effect of DNA binding by interacting with the ID domains (8, 11). However, supporting evidence for such interactions remains to be established.

The Fos, Jun, and Ets transcription factors can regulate the same set of cellular genes that contain composite target motifs (12, 13). The cellular genes controlled by Fos, Jun, and Ets include the well studied stromelysin-1 and collagenase-1 genes that encode two matrix metalloproteinases implicated in normal growth and development, as well as in tumor invasion and metastasis. The functional DNA motifs by which Fos, Jun, and Ets transcription factors regulate basal and induced transcription activities have been characterized (14–18). Although the AP-1 site is a well conserved sequence motif in collagenase-1 and stromelysin-1 genes and binds the Fos and Jun heterodimer, the Ets-binding sites (EBS) differ by sequence and position between the two genes, and they display different binding affinities for the Ets transcription factors. Recently, we reported that the adjacent EBS and AP-1 motifs of collagenase-1 promoter are both necessary for collagenase-1 activation by ERG. We have shown that ERG in defined conditions does not bind to the collagenase-1 EBS unless it is recruited, via physical interaction, by the c-Fos/c-Jun complex that binds to the adjacent AP-1 site. In contrast to the collagenase-1 promoter, ERG binds to the two linked EBS of the stromelysin-1 promoter but does not activate this gene. In transiently transfected cells, ETS2 activates stromelysin-1 gene; however, when ERG is coexpressed with ETS2, ERG inhibits stromelysin-1

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1 The abbreviations used are: DBD, DNA-binding domain; TA, transactivation; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; EtBr, ethidium bromide; ID, inhibitory domain(s); EBS, ETS-binding sites; PCR, polymerase chain reaction.
activation by ETS2, suggesting a possible mechanism of interaction between ETS2 and ERG.

In this report, we demonstrate that the ETS2 transcription factor physically interacts with the c-Fos/c-Jun heterodimer and with other Ets family members. We found that different domains of ETS2 make contacts with the Fos/Jun complex and with other members of the Ets family to form stable heterodimeric ETS2/Fos/Jun and heterodimeric ETS2/ERG and ETS2/Ets2 complexes. Also, we found that these complexes are stabilized by DNA and that stromelysin-1 promoter DNA specifically stabilized the ETS2/Fos/Jun complex, dependent on the two linked EBS. We discuss the implications of these results for combinatorial gene regulation and for stromelysin-1 gene regulation by the Ets family of transcription factors.

MATERIALS AND METHODS

Recombinant DNA Plasmids—The recombinant GST-ETS2-(1–469) plasmid containing the entire coding region of ETS2 was cloned as follows: an EcoRI fragment containing ETS2 cDNA was transferred from pSVKETS2 (17) to the EcoRI site of the pGEX1AT. This construct (pGEX ETS2) was digested with BamHI and SacI, which cut in the vector linker and in the coding region of ETS2, respectively. The digestion removed the 5′ end untranslated region and part of the ETS2 cDNA. This was replaced by an EcoRI-Sacl ETS2 fragment after treatment of the EcoRI end with mung bean nuclease and ligation into the filled end of BamHI and SacI. The EcoRI-Sacl fragment was obtained from a plasmid construct pSVKETS2-(1–469) that contains an EcoRI site, introduced by PCR, 9 nucleotides upstream of the ATG.

The GST-ETS2-(1–290) was derived from GST-ETS2-(1–469) digested with SacI and EcoRI (in the linker) to remove the 3′ part of the ETS2 gene. The SacI and EcoRI ends were treated with mung bean nuclease and the plasmid ligated back. The GST-ETS2-(1–129) plasmid was constructed by cloning a BamHI-BglII fragment, encoding the N-terminal 129 amino acids of ETS2, from an intermediate plasmid pGADETS2-(1–129) into the BamHI site of the pGEX1AT vector. The intermediate plasmid pGADETS2-(1–129) was generated by inserting a BamHI-PstI fragment, from the GST-ETS2-(1–469) into the BamHI and PstI sites of the pGAD 424 vector (CLONTECH) which contained a BglII site downstream of the PstI site.

The ETS2-DNA binding domain constructs were made by PCR amplification using the appropriate primers. The 5′ primer was always designed in-frame with BamHI site. The amplified fragments were then cloned into the Smal site of pUC18 (Sure Cut kit from Pharmacia) to create the intermediate constructs pUC18-ETS2-(289–469), pUC18-ETS2-(289–439), pUC18-ETS2-(359–439), pUC18-ETS2-(359–469), pUC18-ETS2-(289–359), and pUC18-ETS2-(443–469). The orientation and frame of the fragments were verified by double strand sequences. The fragments were then released from the pUC18 vector by BamHI and EcoRI digestion and cloned into the corresponding sites of the pGEX1T vector to generate the following GST-ETS2 binding domain fusion proteins: GST-ETS2-(289–469), GST-ETS2-(289–439), GST-ETS2-(359–439), GST-ETS2-(359–469), GST-ETS2-(289–359), and GST-ETS2-(443–469).

To construct the plasmids for the yeast two hybrid assay, plasmid vectors expressing Gal-4 binding (B) domain (pGBK79) and activation (A) domain (pGAD424) were purchased from CLONTECH. To generate the B-ETS2(289–469) plasmid the ETS2-BD fragment (residues 289–469) was made by PCR amplification and cloned into the Smal site of pGBK79 vector. The A-ERG construct was made by cloning the human ERG cDNA (residues 1–334) (19) into the Smal site of pGAD424. The cloning of A-Jun was done in two steps. First, an EcoRI-Smal fragment of c-Jun (residues 1–179) was amplified by PCR and cloned into the corresponding sites of pUC18 vector. A Smal-PstI fragment (residues 179–334) was released from mouse pSVc-Jun (20), purified by low melting point agarose gel, and cloned into the previous construct to generate pUC-Jun(1–334). This fragment was then released by digestion with EcoRI and PstI and cloned into the corresponding sites of pGAD424 vector.

Expression and Purification of Glutathione S-Transferase-ETS2 Fusion Proteins—Glutathione S-transferase fusion protein expression and purification were essentially as described by Smith and Johnson (21). E. coli DH5a was transformed with the full-length GST-ETS2-(1–469) or one of the recombinant plasmids described above. Fresh overnight cultures were diluted 1:20 in LB containing ampicillin (100 µg/ml) and incubated for about 4 h at 30 °C with shaking. When the culture density reached A600 of 0.6, isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.1 µM and induced for additional 4 h, at 30 °C.

To assay the level of expression of the fusion protein, aliquots of each bacterial culture were pelleted in a microcentrifuge, boiled in SDS buffer (Tris-HCl) 250 mM, pH 7.9, 1% β-mercaptoethanol, 2% SDS), and loaded into an SDS-polyacrylamide gel. Proteins were visualized by Coomassie Blue staining. For fusion protein recovery, bacterial culture were pelleted in microcentrifuge and resuspended in 1/20 volume of phosphate-buffered saline (140 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, pH 7.3). Aliquots of 500 µl of bacteria were then lysed.

**Fig. 1.** ETS2 interacts with the c-Fos/c-Jun complex. A, the figure shows an *in vitro* protein-protein interaction assay (pull down) for the GST-ETS2 fusion protein. GST-ETS2 (residues 1–469) or GST alone (62 pmol) was immobilized on glutathione-Sepharose beads and incubated with 0.03 pmol of 35S-labeled *in vitro* synthesized c-Fos, c-Jun, or c-Fos/c-Jun. The input lanes show the in *vitro* synthesized products before incubation with the beads. After washing, the bound proteins were analyzed by SDS-PAGE and visualized by autoradiography (3-h exposure), and the amount of bound proteins was quantitated by PhosphorImager analysis. Molecular size markers are shown in kilodaltons. B, the graph shows the amount of c-Fos, c-Jun, or c-Fos/c-Jun bound to GST or GST-ETS2 expressed as percentage of the total input. The bars indicate the standard error from three independent experiments. C, schematic representation of the GST-ETS2 fusion protein and c-Fos and c-Jun. Depicted are the TA domain, inhibitory domains 1 and 2 (ID1 and ID2), and the DNA-binding domain (DBD) of ETS2. The leucine zipper and basic domain of c-Fos and c-Jun are also indicated.
on ice by mild sonication (10 s) and centrifuged at 12,000 rpm for 20 min at 4 °C. Bacterial supernatant was applied to an affinity column glutathione-Sepharose 4B (RediPack purification module, Pharmacia Biotech Inc.), and the fusion proteins were eluted by the addition of 1 ml of Elution Buffer (20 mM reduced glutathione, 50 mM Tris, pH 8). Eluted proteins were dialyzed against incubation buffer at 4 °C overnight. Protease inhibitors (Complete, Boehringer Mannheim) and 25% glycerol were added before storage at −20 °C. Purified proteins were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and were estimated to be 50% pure by Coomassie Blue staining. Protein yields were measured by colorimetric reaction and approximated by comparison against known protein standards (bovine serum albumin).

**In Vitro Synthesis of Oncoproteins—** Rat cDNAs for c-Fos and c-Jun (22) were kindly provided by Tom Curran. The constructs pSVK3-ETS2 and pSG5-ERG containing the human ETS2 and ERG (isoform p55) cDNAs were previously described (17, 19). The translation and transcription of the cDNAs were simultaneously performed in the transcription and translation-coupled reticulocyte lysate system and [35S]methionine, according to the instructions of the manufacturer (Promega). The translation products were visualized in SDS-minigel and quantified by the PhosphorImager (Molecular Dynamics).

**Interaction Binding Assay—** A 50-μl volume of a 50% slurry of glutathione-Sepharose 4B beads (Pharmacia) was incubated with 62 pmol of GST or GST-ETS2 of fusion protein (5 μg) for 20 min in the incubation buffer (12 mM Hepes, pH 7.9, 4 mM Tris-Cl, pH 7.9, 50 mM NaCl, 10 mM EDTA, 1 mM DTT). The beads were washed three times with 0.2 ml of incubation buffer and centrifuged for 1 min at 500 × g. The GST or GST-ETS2 fusion proteins bound to the beads were then incubated with the indicated quantity of [35S]methionine-labeled in vitro translated protein, in a total volume of 25 μl of incubation buffer for 30 min at room temperature. The beads were then washed five times with 0.2 ml of NETN (0.5% Nonidet P-40, 1 mM EDTA, 20 mM Tris-Cl, pH 8, 100 mM NaCl) and incubated for 10 min at room temperature in 25 μl of elution buffer (50 mM Tris-Cl, pH 8, 15 mM reduced glutathione). The eluted labeled protein(s) were resolved by SDS-polyacrylamide gel electrophoresis. The gel was treated with Amplify (Amersham Corp.), dried, and subjected to autoradiography. The signals were quantified with the PhosphorImager.

**Stromelysin Promoter Fragments—** Restriction fragments from stromelysin-1 promoter (−1303 to +4) (15, 23) were isolated by agarose gel electrophoresis. The stromelysin wild-type fragment (−274 to −11, EcoRI-AvaI) (see Fig. 5c, fragment 5) carries two EBS motifs, with the GGAA (−214) and TTCC (−206) containing sequences, and an AP-1 site TGAGTCA (−70). The fragments containing mutant EBS or AP-1 sites have been described previously (15, 17). Briefly, to create the promoter mutants in the EBS and AP-1 sites, the GGAA sequence was mutated to AAAA (G hatch), and the TTCC to CTTT (C hatch). Two stromelysin promoter fragments (−755 to −479, KpnI-XbaI and −478 to −275, XhoI-EcoRI) (fragments 3 and 4) were used as unrelated fragments in pull down assay. These fragments contain no functional EBS or AP-1 sites as shown by transfection assay (17).

**In Vivo Interaction Analysis Using the Yeast Two Hybrid System—** Growth and maintenance of yeast strains and two hybrid assays (24) were essentially performed using standard protocols from CLONTECH. The Saccharomyces cerevisiae yeast strain SFY526 was transfected with plasmid expressing the Gal-4 binding domain fused to the DBD of ETS2 and plasmid expressing the Gal-4 activation domain fused to ERG or Jun. Transformants were plated on a synthetic medium lacking leucine and tryptophan. The plates were incubated at 30 °C for 3 days. To monitor the interactions, the β-galactosidase activity was estimated by colony lift assay and measured quantitatively from liquid culture. Several independent transformants were isolated and grown in liquid culture at 30 °C until A600 reached 0.8–1.0. β-Galactosidase activity was measured as described previously (25).

**RESULTS**

ETS2 Interacts with the Fos/Jun Heterodimer in Vitro—We have shown that the ETS2 transcription factor activates genes that contain EBS adjacent to AP-1 sites such as human collagenase-1 and stromelysin-1. To study the molecular mechanism(s) of this regulation, we first investigated possible protein-protein interactions of ETS2 and Fos/Jun using the GST pull down system. The E. coli expressed GST-ETS2 fusion protein was first immobilized on glutathione-Sepharose beads and incubated with in vitro translated [35S]labeled c-Fos, c-Jun, and c-Fos/c-Jun (Fig. 1C). The nonspecifically bound proteins were removed by extensive washing, and the bound proteins were eluted, subjected to SDS-PAGE, and detected by autoradiography. As shown in Fig. 1A, Fos or Jun proteins were specifically retained by the GST-ETS2 fusion protein (lanes 2, 4, and 6) but not by the beads carrying only GST (lanes 1, 3, and 5). Interaction of Jun with ETS2 was also detected in vivo, using the yeast two hybrid system (Fig. 3). These results suggest that Fos or Jun alone specifically associates, although weakly, with ETS2. By contrast, the Fos/Jun heterodimer bound strongly to the GST-ETS2 fusion protein (Fig. 1A, lane 6). The amount of Fos/Jun heterodimer retained by the GST-ETS2, quantitated by PhosphorImager analysis, was four times more than the amount of Fos or Jun alone (Fig. 1B).

ETS2 Physically Interacts with Other Ets Family Members— Recently, we found that, in transiently transfected cells, the

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**Fig. 2.** ETS2 interacts with other Ets family members. A, in vitro synthesized [35S]labeled ETS1, ETS2, ERG, or luciferase (as a negative control) were incubated with 62 pmol of E. coli-produced GST-ETS2 fusion protein (lanes 2, 4, 6, and 8) or GST protein alone (lanes 1, 3, 5, and 7) immobilized on glutathione-Sepharose beads. Bound proteins were analyzed in SDS-PAGE, visualized by autoradiography, and quantitated by PhosphorImager analysis. B, the graph shows the amount of luciferase (Luc), ETS1, ETS2, or ERG bound to GST or GST-ETS2 expressed as percentage of the total input. The bars indicate the standard error from five independent experiments. C, schematic diagram of the Ets family members used in in vitro synthesis reactions.
Ets family members and their combinations differentially regulate the human stromelysin-1 and collagenase-1 promoter activity in an antagonistic or synergistic fashion. For instance, ERG, when coexpressed with ETS2, strongly inhibits stromelysin-1 promoter activation by ETS2 (18). Therefore we investigated the role of protein interactions of ETS2 and ERG as possible mechanisms of the antagonistic effect on stromelysin-1 promoter. The immobilized GST-ETS2 fusion protein or the GST control was incubated with 35S-labeled ERG, ETS1, ETS2, or an unrelated protein, luciferase, as a negative control. Among the Ets family members tested (Fig. 2C), ERG and ETS1, but not ETS2, specifically interact with GST-ETS2 (Fig. 2, A and B). ERG interacts strongly, by reaching 29.8% (Fig. 2B) of the total input, whereas the percentage of ETS1 bound proteins was only 5.9% in the experimental conditions used. We also note that a weak but reproducible in vivo interaction between ETS2 and ERG was detected using the yeast two hybrid system (Fig. 3). These data suggest that ETS2 physically associates with other members of the Ets family to form ETS heterodimers. The data also suggest that ETS2 does not associate with itself as a homodimer. This result is consistent with previous reports showing that ETS2 binds to EBS as a monomer (11).

Different ETS2 Domains Are Involved in the Interaction with c-Fos/c-Jun, ERG, and ETS1—The ETS2 transcription factor has been shown to contain two main domains, the TA domain and the DBD which is flanked by two smaller domains called inhibitory domains (ID1 and ID2). To identify the ETS2 domains necessary for interaction with the Fos/Jun complex, ERG and ETS1, a series of N- and C-terminal ETS2 deletions were constructed as GST fusion proteins (Fig. 4). The Fos, Jun, ERG, or ETS1 proteins were synthesized and radiolabeled in vitro in reticulocyte lysate and incubated with glutathione-Sepharose beads carrying the various GST-ETS2 fusions. Fig. 4 shows that the full-length GST-ETS2-(1–469) but neither the N-terminal GST-ETS2-(1–129) nor the GST-ETS2-(1–290) containing the transactivation domain can bind the Fos/Jun complex (Fig. 4D, lanes 2, 3, and 10). This result indicates that the ETS2 domain involved in the interaction with Fos/Jun complex lies within residues 290 and 469 that contains the DBD and two inhibitory domains (ID1 and ID2). In support of this, binding assay using the fusion protein GST-ETS2-(289–469) shows that the Fos/Jun complex associates strongly with this domain (Fig. 4D, lane 9). Further mapping of this region indicates that the DBD (GST-ETS2-(359–439)) or the ID1 (GST-ETS2-(289–358)) and the ID2 (GST-ETS2-(440–469)) are independently unable to bind to the Fos/Jun complex (lanes 4, 6, and 8). The fusion protein GST-ETS2-(289–439) that contains the ID1 and the DBD was also unable to bind Fos/Jun, and the GST-ETS2-(359–469) containing the DBD and ID2 associates much stronger. These differences in binding were not due to a variation in the amount of GST fusion proteins since the quantity and integrity of all fusion proteins were found to be similar by Coomassie staining (data not shown). Therefore, these results suggest that ID2, which contains the C-terminal 29 residues of ETS2, enhances the interaction of the DBD with Fos/Jun. However, both ID1 and ID2 were required for better contacts of DBD with Fos/Jun. Similarly, the weak binding of Fos to ETS2 requires the C-terminal part of ETS2 that includes the DBD with the two inhibitory domains. In contrast, the weak binding of Jun to ETS2 appears to require all ETS2 domains. However, at the conditions employed here, the interactions are very weak compared with that observed with the Fos/Jun heterodimer (compare B, C, and D). Of interest is that, among the Ets family members, ETS1 gives results similar to those obtained with Fos/Jun by binding only with the C-terminal domain of ETS2 (Fig. 4E, lanes 10, 9, and 7). Notably, ERG interacts with additional ETS2 domains. For instance, ERG binds equally well to most of the ETS2 domains (Fig. 4F, lanes 2, 3, 5, 7, 9, and 10) including the TA domain (Fig. 4F, lanes 2 and 3). As for Fos/Jun, ERG does not associate with ID1, DBD, or ID2 alone (lanes 4, 6, and 8). ERG, however, interacts strongly with the C-terminal regions containing the DBD and one of the inhibitory domains ID1 or ID2 (Fig. 4F, lanes 5 and 7).

Protein-Protein Interactions of the DNA Binding Domain of ETS2 with Fos/Jun Is Enhanced by Stromelysin-1 Promoter DNA—Previously, we have shown that Fos/Jun and ETS2 transcription factors bind to the AP-1 and EBS motifs of the human stromelysin-1 and collagenase-1 promoters and activated these genes (17). Here, we have demonstrated that ETS2 associates

\*J. P. Basuyaux and G. Buttice, unpublished data.
in vitro with the Fos/Jun complex and that this interaction involves the C-terminal part of ETS2. To establish the importance of the stromelysin-1 promoter in the interaction of the trimeric complex ETS2/Fos/Jun, we performed an interaction assay between GST-ETS2 and Fos/Jun in the presence of stromelysin-1 promoter fragments. For these experiments, we used promoter fragments containing native or mutated EBS and AP-1 sites (Fig. 5C). Promoter fragments were simultaneously incubated with the Fos/Jun complex and immobilized GST-ETS2-(1–469). The amount of bound Fos/Jun proteins was then compared in the absence or presence of different DNA promoter fragments. PhosphorImager analysis of three independent experiments indicated that the stromelysin-1 promoter fragment (2274 to 211) containing two tail-to-tail EBS and one AP-1 site augmented 2-fold the amount of Fos/Jun proteins bound to ETS2 (Fig. 5B, compare lanes 2 and 5). This effect was specific since two unrelated stromelysin-1 promoter fragments did not increase or diminish the amount of Fos/Jun bound to GST-ETS2 (Fig. 5B, lanes 2–4). Interestingly, mutation of the AP-1 site did not significantly affect the amount of Fos/Jun bound to the GST-ETS2 (Fig. 5B, lanes 6 and 7). However, mutation of the upstream or downstream or both EBS significantly decreased the amount of Fos/Jun bound to GST-ETS2 (Fig. 5B, lanes 8–10). This suggests that the binding of ETS2 to the EBS of the stromelysin-1 promoter is necessary for the formation of the trimeric complex ETS2/Fos/Jun. However, the ETS2/Fos/Jun interaction is not a result of simultaneous binding to the EBS and AP-1 motifs of the same DNA molecule. If this would be the case, addition of DNA fragments with mutated AP-1 sites, which do not bind Fos/Jun (15), should result in a decrease of Fos/Jun protein recovered. Conversely, we found an increase of 2-fold in the amount of Fos/Jun protein recovered equal to that observed with the wild-type fragment (compare lanes 5–7). Control experiments were also performed with the addition in the reaction of different concentrations of ethidium bromide (EtBr) (Fig. 5A) which has been shown to disrupt DNA-mediated protein-protein interactions (26). Of interest is that the direct interaction between GST-ETS2 and Fos/Jun was inhibited by the addition of EtBr (Fig. 5A, lanes 2 and 4–6) and enhanced by addition of stromelysin promoter fragments (Fig. 5A, lane 3). Taken together these data suggest that stromelysin-1 promoter DNA can enhance the ETS2/Fos/Jun interaction, perhaps by stabilizing protein contacts, and that this depends on the integrity of
the EBS of the stromelysin-1 promoter. However, this DNA-dependent interaction does not require intact AP-1 site.

**Protein Interactions of the Transactivation Domain of ETS2**

**Are Not Influenced by the Presence of DNA**—As shown here, although ETS1 and the Fos/Jun complex bind only to the C-terminal part of ETS2, ERG also interacts with the N-terminal part that includes the transactivation domain of ETS2. To determine whether this type of interaction is DNA-dependent, we tested the effect of EtBr in the reaction. As expected, in the absence of EtBr, Fos/Jun, ETS1, and ERG bound to the entire ETS2 protein as well as to the C-terminal part that includes the DBD and the two IDs (Fig. 6, A-b and B-b). In the presence of EtBr, the recovery of bound Fos/Jun and ETS1 proteins to the full-length GST-ETS2-(1–469) and to the C-terminal part, GST-ETS2-(290–469), was dramatically reduced (Fig. 6). In contrast, the recovery of ERG-bound protein to the GST-ETS2-(1–469) was only marginally affected by the presence of EtBr (Fig. 6, A-c and B-c). Similar to Fos/Jun and ETS1, the amount of ERG-bound protein to the C-terminal part, GST-ETS2-(290–469), strongly decreased in the presence of EtBr (Fig. 6B-c). Interestingly, the percentage of ERG-bound protein to the N-terminal part of ETS2 was not affected by the addition of EtBr (Fig. 6C). These data suggest that although the C-terminal part of ETS2, which includes the DBD and the two IDs, is DNA-dependent in the interactions with other proteins, the transactivation domain physically associates with other proteins independently of DNA (Fig. 7). Indeed, the amount of ERG-bound protein to the transactivation domain of ETS2 (GST-ETS2-(1–290)) was totally recovered in the presence of EtBr (Fig. 6C) even at concentration of 50 μg/ml (data not shown). Moreover, addition of exogenous DNA from stromelysin-1 and collagenase-1 promoters did not further increase the percentage of ERG-bound protein to the TA of ETS2 (data not shown). These data suggest that ERG physically interacts with the TA of ETS2 in a DNA-independent fashion (Fig. 7).

**DISCUSSION**

Synergistic repression or activation of gene expression plays an important role in gene regulation. Combinatorial protein-protein interactions between multifunctional modular domains of transcription factors can provide the necessary complexity required to achieve appropriate gene regulation. In this study, using in vitro protein binding assay, we showed that the transcription factor ETS2 binds to the c-Fos/c-Jun complex and members of the Ets family such as ETS1 and ERG. We characterized the ETS2 binding domain and found that binding to c-Fos/c-Jun and ETS1 occurred through the C-terminal end (residues 289–469), which contains the inhibitory domain ID1-(289–358), the DNA binding domain (residues 359–439), and the inhibitory domain ID2-(440–469). None of these domains could separately bind to c-Fos/c-Jun and ETS1 although significant binding occurred through the combined DNA binding and ID domains. ETS2 binding to ERG through its C-terminal end occurred to the C-terminal end (residues 289–469), which contains the inhibitory domain ID1-(289–358), the DNA binding domain (residues 359–439), and the inhibitory domain ID2-(440–469). None of these domains could separately bind to c-Fos/c-Jun and ETS1 although significant binding occurred through the combined DNA binding and ID domains. ETS2 binding to ERG through its C-terminal end was similar to that observed for c-Fos/c-Jun and ETS1. In addition, ERG interacted with the N-terminal region (residues 1–290) containing the TA of ETS2 and significant binding also occurred through the N-terminal residues 1–129. This region constitutes the first half of the TA domain of ETS2. In comparison with the full-length ETS2, this region retained 30% of the capacity to bind to ERG.

**Significance of Interactions between DNA-binding Domains**—The major findings of our studies are that (i) only the C-terminal part of ETS2, which contains the DBD and the two C- and N-terminal IDs, makes contacts with the Fos/Jun heterodimer, and that (ii) this interaction is enhanced by specific DNA motifs suggesting diverse possible mechanisms of gene regulation by Fos, Jun, and Ets transcription factors. While this work was in progress, Leiden and co-workers (27) demonstrated that ELF1, an Ets family member, associates with the basic domain of Jun and thus can form the heterotrimeric complex ELF1/Jun/Fos. Therefore, it appears here that the ETS2/Fos/Jun/ protein-protein interactions are mediated via the DBD or basic domain of Jun, as demonstrated by Bassuk and Leiden (27) and via the C-terminal domain of ETS2 that includes DBD and the two IDs, as shown in this study. It is relevant to note that DNA binding domains have been found to be implicated in protein-protein interactions with other transcription factors (28). For instance, the p202, an interferon-inducible nuclear protein, interacts with the DNA binding domain of Fos and Jun and inhibits the expression of reporter genes activated by c-Fos and c-Jun (29). Moreover, recent results from Graf and co-workers (30) demonstrate that MafB, an AP-1 like protein, interacts via its basic region with the DBD of ETS1 and represses ETS1-mediated activation of the transferrin receptor gene. It is therefore easy to imagine that transcription factors that interact via their DBDs can interfere with
Transcription Factor Interactions of ETS2

Each other in DNA binding and as a result reciprocally inhibit gene transcription. Although the use of a DBD for a protein-protein interaction region would explain, in a simplistic way, interference and antagonistic effects in gene regulation, it might appear paradoxical to explain synergistic activation. It is intriguing, however, to note that protein-protein interactions involving the DBD are dependent on the presence of DNA as demonstrated by us and observed by few others (27, 31). Remarkably, we found that the amount of ETS2/Fos/Jun complex is enhanced by the presence of specific DNA promoter fragments, suggesting that DNA binding possibly increases the protein area of contact, and as a result the quaternary complex ETS2/Fos/Jun/DNA is more stable. In support of this view, the x-ray crystal structure of the heterodimer c-Fos/c-JUN bound to the DNA recently identified (32) shows that the FOS/JUN/DNA complex exhibits a certain asymmetry in its coiled-coil and flexibility in its protein fork. The authors suggest that this special feature would allow the heterodimer to recognize disparate binding surfaces presented on other transcription factors bound at adjacent sites on the DNA. As more information of protein-DNA interactions will clearly come from x-ray crystal structures, it will be very interesting to reveal in more detail the interaction we have detected in this study with the stromelysin-1 promoter DNA complexed with Fos, Jun, and Ets transcription factors.

Implications of the Role of DNA in Transcription Factor Interactions—It is intriguing that the protein-protein interactions of DNA-binding domains of several transcription factors often are DNA-dependent. As originally described by Lai and Herr (26), the DNA intercalator EtBr provides a simple tool for distinguishing genuine protein-protein interactions (DNA-independent) from those that are DNA-dependent. Although the protein associations stabilized by DNA (which is a contaminant, e.g., in samples of in vitro synthesized proteins) may be specific or nonspecific, DNA may play a significant biological role in the protein-protein interactions of transcription factors. DNA, by making weak (i.e. nonspecific) contacts with transcription factors, can significantly increase the probability for transcription factor interactions and, at the same time, generate stable DNA-transcription factor complexes. Similarly, although the protein-protein interactions of transcription factors can be weak, so weak that they may not last in solution, however, because of additional contacts with DNA, the transcription factor complex should be stabilized. Indeed, it makes sense that transcription factor interactions should preferentially occur on DNA rather than in solution. The significance of physical interactions between transcription factors such as Ets- and c-Fos/c-Jun-families members is that it can effectively link different promoter elements such as EBS and AP-1 in gene regulation. Our results give significance not only for the stromelysin-1 and collagenase-1 regulation but also for other genes regulated by Ets and AP-1 transcription factors.

Inhibitory Domains of ETS2 Enhance Protein-Protein Interactions—As shown here the c-Fos/c-Jun complex, as well as ETS1 and ERG, binds to the C-terminal region of ETS2. This region comprises not only the DBD but also the two inhibitory domains ID1 and ID2. These domains interact with each other and with the DBD via intramolecular contacts thus preventing the DBD to interact with the DNA. It has been suggested that association of the two IDs with other factors can release the DBD from their negative influence (10, 33). It is intriguing, however, that the inhibitory domains alone do not bind to any of the transcription factors tested, c-Fos, c-Jun, c-Fos/c-Jun, ETS1, and ERG (Fig. 4). In contrast when ID2 or both ID1 and ID2 are coupled to the DBD of ETS2, we detected increased binding of the transcription factors. Of interest is that ID2, which corresponds to the C-terminal end of 29 residues of...
ETS2, contributed largely to the increase of proteins bound to the DBD. Also, the C-terminal segment of 37 amino acids adjacent to the DBD (ETS domain) of GA binding protein a was found to mediate protein-protein interaction with the GA binding protein β subunit (34).

Repression of Transcription by Masking Transactivation Domains—The Ets family members ETS2, ETS1, FLI1, and ERG and their combinations differentially regulate collagenase-1 and stromelysin-1 promoter activity, in an additive or antagonistic fashion in HepG2 cells (18). Interestingly, while ETS2 enhanced the stromelysin-1 promoter activity, ERG inhibited the ETS2-mediated activation of stromelysin-1 promoter. Therefore, we hypothesized a protein-protein interaction between ERG and ETS2 as a possible mechanism of the antagonistic effect on the stromelysin-1 regulation. Our results here demonstrate that ETS2 physically interacts with ERG via several domains including the TA domain of ETS2, suggesting that ERG represses the ETS2-mediated activation in part by blocking the TA of ETS2. Transactivation domains of several other transcription factors are implicated in protein contacts leading to repression or activation of transcription. Thus, the protein product of the retinoblastoma gene represses transcription by interacting directly with E2F and blocking its transactivation domain (35). PU.1, a lymphoid-specific transcription factor, can directly bind to both TFIID and retinoblastoma protein via its activation domain (36). Our results show that not only the TA of ETS2 associates with ERG but also the C-terminal part of ETS2. However, the latter interaction is sensitive to EtBr treatment, suggesting that it is DNA-dependent. By contrast, the interaction of ERG to the TA of ETS2 is a direct protein-protein interaction apparently not affected by the presence of DNA. Interestingly, these results raise two possible mechanisms of gene regulation by ERG and ETS2, one of which involves the TA of ETS2 and the other one the DBD. In one possibility, ERG would inhibit the ETS2 effect by masking its TA and therefore cancelling out the activation potential of ETS2. In another possibility, ERG, by interacting with the DBD of ETS2, would prevent it from binding to the DNA.

To our knowledge, this is the first evidence of the formation of Ets heterodimers, ETS2/ETS1 and ETS2/ERG, in vitro and in vivo in yeast. Whether they exist as heterodimer or as a multiprotein complex with other cofactors in the cell still needs to be explored. Recently, only a few other groups (30, 31, 37) have provided evidence that the Ets family of transcription factors interacts with other transcription factors. Thus, the region of ETS1 (residues 123–240) that covers TA and ID1 domains interacts with the Runt domain of polyomavirus enhancer binding protein 2α (37), whereas the DBD of ETS1 associates with PAX-5 in a DNA-dependent manner (31) and with MafB (30).

Finally, we would like to propose that the C-terminal region of ETS2 is multifunctional, as it is capable not only of DNA sequence recognition but also of protein-protein interaction with other transcription factors. Here, we provided novel evidence demonstrating that the TA domain of ETS2 is implicated in protein-protein interaction with another member of the same family, ERG, and that this interaction might have functional relevance in the repression of gene transcription such as that of the stromelysin-1 promoter. In summary, our results provide a molecular basis to a better understanding of the synergistic or antagonistic gene regulation by the different members of the Ets- and AP-1 (c-Fos/c-Jun) families.

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