ETS-1 Transcription Factor Binds Cooperatively to the Palindromic Head to Head ETS-binding Sites of the Stromelysin-1 Promoter by Counteracting Autoinhibition*

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David Baillat‡, Agnès Bègue, Dominique Stéhelin, and Marc Aumercier§

From the CNRS Unité Mixte de Recherche 8526, Institut de Biologie de Lille, Institut Pasteur de Lille, B.P. 447, 1 Rue Calmette, 59021 Lille Cedex, France

Stromelysin-1 (matrix metalloproteinase-3) is a member of the matrix metalloproteinase family. Regulation of its gene expression is critical for tissue homeostasis. Patterns of increased co-expression of stromelysin-1 and ETS-1 genes have been observed in pathological processes. Stromelysin-1 promoter is transactivated by ETS proteins through two palindromic head to head ETS-binding sites, an unusual configuration among metalloproteinase promoters. By using surface plasmon resonance, electrophoretic mobility shift assay, and photocross-linking, we showed that full-length human ETS-1 (p51) binds cooperatively to the ETS-binding site palindrome of the human stromelysin-1 promoter, with facilitated binding of the second ETS-1 molecule to form an ETS-1-DNA-ETS-1 ternary complex. The study of N-terminal deletion mutants allowed us to conclude that cooperative binding implied autoinhibition counteraction, requiring the 245-330-residue region of the protein that is encoded by exon VII of the gene. This region was deleted in the natural p42 isoform of ETS-1, which was unable to bind cooperatively to the palindrome. Transient transfection experiments showed a good correlation between DNA binding and promoter transactivation for p51. In contrast, p42 showed a poorer transactivation, reinforcing the significance of cooperative binding for full transactivation. It is the first time that ETS-1 was shown to be able to counteract its own autoinhibition.

Stromelysin-1 (matrix metalloproteinase-3) is a member of the matrix metalloproteinase family with a wide spectrum of substrates. It plays a crucial role in extracellular matrix remodeling during normal processes such as tissue morphogenesis, growth, and wound repair (1, 2). A tight regulation of its gene expression is critical for tissue homeostasis. Indeed, its misregulation is associated with pathologic conditions such as rheumatoid and osteoarthritis (3, 4), Alzheimer’s disease (5), tumor invasiveness, and metastasis (6–8). In addition, it was also shown that ETS-1 was shown to be able to counteract its own autoinhibition.

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‡ To whom correspondence should be addressed. Tel.: 33-3-20-87-10-97; Fax: 33-3-20-87-11-11; E-mail: marc.aumercier@ibl.fr.
§ To whom correspondence should be addressed. Tel.: 33-3-20-87-10-97; Fax: 33-3-20-87-11-11; E-mail: marc.aumercier@ibl.fr.

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**ETS-1 Binding to Stromelysin-1 Promoter**

**TABLE I**

Sequences of stromelysin-1 promoter, wild type, and mutants

| Name     | Sequences          | EBS topology |
|----------|--------------------|--------------|
| WT       | 5'-ACCAAGACAGGAAGCACATTCCCTGAGGATTA-3' | ← ←            |
| M1       | 5'-ACCAAGACAAAAGACATTCCCTGAGGATTA-3' | × ←            |
| M2       | 5'-ACCAAGACAGGAAGCACATTCCCTGAGGATTA-3' | × ×            |
| DR       | 5'-ACCAAGACAGGAAGCACATTCCCTGAGGATTA-3' | × ←            |
| IP       | 5'-ACCAAGACAGGAAGCACATTCCCTGAGGATTA-3' | ← →            |
| WT+4     | 5'-ACCAAGACAGGAAGCACATTCCCTGAGGATTA-3' | ← ←            |
| M1M2     | 5'-ACCAAGACAGGAAGCACATTCCCTGAGGATTA-3' | × ×            |

The EBS core sequences are represented as boldface letters in the sequence. ← and ← represent the EBS in both possible orientations, and × designates a mutated EBS.

**Materials and Methods**

**Site-directed Mutagenesis**—Mutant forms of the human stromelysin-1 promoter corresponding to M1, M2, M1M2, DR, and IP were generated by site-directed mutagenesis using appropriate oligonucleotides (Table I) and the QuickChange Site-directed Mutagenesis kit (Stratagene®) with a pSG5 plasmid (HGH-TGES, Nichols Institute Diagnostics) containing the −1303/+4 region of the wild type human stromelysin-1 promoter as a template (13). The human ETS-1 isoform p42 inserted in a pSG5 vector already existed (42). The ETS-1 p42 isoform cDNA cloned in pSG5 vector was obtained by the same way as for the pTYb2 cloning.

**Generation of Biotinylated Oligonucleotides for SPR Experiments**—The 99-bp biotinylated double-stranded DNA fragments were obtained by PCR amplification using 5'-biotinyl-GAATTCCAGTCAATTTTTCGAG-3' as a forward primer and 5'-CAACGGTGGCACTGATTTATG-3' as a reverse primer. Amplified fragments were purified on QiAquick columns (Qiagen) and checked for sequence before immobilization on the Sensor Chip.

**Expression and Purification of ETS-1 Proteins**—Proteins were purified using the T7-Impact System (New England Biolabs). Escherichia coli (ER2566) was transformed with the appropriate recombinant plasmid. Fresh overnight cultures were diluted 1:25 in 250 ml of Luria-Bertani medium and incubated at 37 °C with shaking at 250 rpm. When culture density reached an OD600 = 0.7, isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.3 mM. The culture was incubated at 30 °C with shaking for 3–4 h. Cultures were harvested, and pellets were washed with phosphate-buffered saline and suspended in 10 ml of lysis buffer (50 mM Tris, pH 8, 150 mM NaCl, 1 mM EDTA, 0.1% (v/v) Triton X-100, complete protease inhibitors mixture (Complete tablets, Roche Molecular Biochemicals)). Bacteria were lysed with a French press using a 1000 pounds/square inch pressure. Lysates were clarified by a 5-min centrifugation at 20,000 × g at 4 °C followed by a subsequent 15-min centrifugation at 20,000 × g at 4 °C. Each clarified lysate was applied to a 5-ml chitin bead column (New England Biolabs). Columns were washed with 20 volumes of column buffer (lysis buffer without protease inhibitors) and rapidly flushed with 3 volumes of elution buffer (column buffer without Triton X-100) containing 50 mM dithiothreitol (DTT) (Roche Molecular Biochemicals). Columns were then stored 16 h at 4 °C for peptide cleavage. Proteins were eluted by 15 ml of elution buffer in 1-mI fractions. Fractions with proteins were pooled, and for further purification were diluted and chromatographed on a Mono S HR 5/5 column (Amersham Biosciences) equilibrated with 10 mM Tris, pH 8, 50 mM NaCl, 1 mM DTT, and 0.5 mM phenylmethylsulfonyl fluoride using a Bio-Rad Basic Chromatography System (Bio-Rad). Proteins were eluted by a NaCl gradient (0.05–1 M). Fractions containing the protein were quick-frozen by immersion in liquid nitrogen and stored at −80 °C after dialysis against HBS-EP (0.01 M Hepes, pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% (v/v) polyborate 20). Yields were measured by colorimetry (Bio-Rad protein assay) and corrected by comparison against known protein standards (bovine serum albumin) on SDS-PAGE after Coomassie Blue staining.

**Electrophoretic Mobility Shift Assay**—Double-stranded synthetic oligonucleotides corresponding to the WT, M1, M2, WT + 4, and M1M2 mutants of the stromelysin-1 (−223/−194) promoter region (Table I) were end-labeled using T4 polynucleotide kinase and γ-[32P]ATP and were subsequently purified by electrophoresis on a 20% polyacrylamide gel in TBE buffer (90 mM Tris borate, 1 mM EDTA). Recombinant proteins (4 pmol) were incubated with 0.5 ng of probe in 20 μl of binding reaction buffer (20 mM Tris, pH 7.9, 80 mM NaCl, 1 mM EDTA, 2 mM...
PCR primers used for cloning the human ETS-1 N-terminal deletion mutants

| PCR Primersa | 5′-AGATCATCATATGATGTCTCAAGCCATATTAAAGC-3′ |
|-------------|-----------------------------------------------|
| ΔN45       | 5′-AGATCATCATATTGGAGTCACCACGCTTATCC-3′       |
| ΔN144      | 5′-AGATCATATGGAACTCGGAGGCGACAGGC-3′          |
| ΔN245      | 5′-AGATCATATGGGTCCCTCTCATTACAGCTTCC-3′       |
| ΔN280      | 5′-AGATCATATGGCTGATGACATCTTCAAGGC-3′         |
| ΔN301      | 5′-AGATCATATGGATGGCAGCATTACAGC-3′            |
| ΔN331      | 5′-AGATCATATGGGCAGTGGACCAATCCAG-3′           |

a Forward primers were designed in frame with NdeI site (bold letters). The six additional 5′ bases were introduced to enable NdeI digestion.

b The same reverse primer was used for all PCR amplifications.

### Table II

**Schematic representation of the kinetic models and the related differential equations**

A corresponds to the analyte (ETS-1) and B to the ligand (DNA). A,B depicts the same ternary complex but in a rearranged form. The concentration of X is represented by [X].

### Table III

% of complexes formed by the specific interaction between streptavidin and biotinylated double-stranded oligonucleotide (99 bp long) immobilized on a streptavidin-coupled CM5 Sensor Chip using standard protocol as indicated in the Amine Coupling Kit (Biacore®). HBS-EP BIA certified buffer (0.01 M Heps, pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% (v/v) polystyrene 20, Biacore®) was used as a running buffer. Briefly, flow rate was fixed at 10 μl/min. Streptavidin was injected at 500 ng/μl in 10 mM sodium acetate, pH 3.5, for 12 min. Biotinylated double-stranded oligonucleotides were injected through each flow cell at 200 ng/ml onto the Sensor Chip until a suitable equilibrium was reached. The integrity and quantity of fixed DNA was checked by a 120-s injection of 1.6 RU signal was obtained. The integrity and quantity of fixed DNA was checked by a 120-s injection of 1.6 RU signal was obtained.

| Forward Primes | Reverse Primes |
|----------------|----------------|
| ΔN45           | 5′-CCCCTGTCGGCATCTGCTTGAC-3′ |
| ΔN144          | 5′-AGATCATATGGATGGCAGCATTACAGC-3′ |
| ΔN245          | 5′-AGATCATATGGCTGATGACATCTTCAAGGC-3′ |
| ΔN280          | 5′-AGATCATATGGGTCCCTCTCATTACAGCTTCC-3′ |
| ΔN301          | 5′-AGATCATATGGATGGCAGCATTACAGC-3′ |
| ΔN331          | 5′-AGATCATATGGGCAGTGGACCAATCCAG-3′ |

DTT, 10% glycerol) for 30 min on ice. Complexes formed were resolved on a 5% polyacrylamide (acrylamide/bisacrylamide 29:1, Euromex) non-denaturing gel in 0.25× TBE buffer at room temperature. Gels were dried and autoradiographed at –80 °C.

**Kination Assay**—Recombinant human ETS-1 p51 (4 pmol) was phosphorylated by incubation with 150 units of calmodulin-dependent protein kinase II (CaMKII, rat truncated recombinant protein, New England Biolabs) at 30 °C for various times in 20 μl of kinase buffer (20 mM Tris, pH 7.5, 10 μM MgCl₂, 0.5 mM DTT, 0.1 mM EDTA, 2 mM CaCl₂, 2.4 μM calmodulin, 100 μM ATP) in the presence of [γ-32P]ATP. The reaction was stopped by addition of 20 μl of 2× gel loading buffer (0.1 μl Tris, 4% SDS, 1.44 μM β-mercaptoethanol, 20% glycerol, 0.2% xylene cyanol, and 0.2% bromphenol blue) and subsequent boiling for 2 min. Samples were resolved by SDS-PAGE, and gels were dried and quantified using a PhosphorImager (Amersham Biosciences). For gel shift assays, the recombinant proteins (4 pmol) were phosphorylated under the same conditions for 90 min in absence of radioactive ATP. Subsequent binding reactions were performed with 0.5 ng of [32P]-labeled probe. The volume was adjusted to 30 μl with binding reaction buffer.

**Cross-linking Experiments**—Cross-linking reactions were carried out in a total volume of 20 μl in phosphate-buffered saline solution (Invitrogen). 4 pmol of ETS-1 were incubated with various amounts of [32P]-labeled oligonucleotides (0.125 to 2 ng) for 30 min on ice. Ru(bpy)₃Cl₂ (Aldrich) and ammonium peroxide were added to 150 μM and 2.5 μM, respectively, just before illumination by standard flash light at a distance of 10 cm. Then the samples were immediately quenched with 20 μl of 2× gel loading buffer and heated to 100 °C for 2 min. Complexes were resolved by electrophoresis through a 10% SDS-polyacrylamide gel. Gel were dried and autoradiographed at –80 °C.

**SPR Binding Assay**—SPR measurements were carried out using a Biacore 2000 apparatus (Biacore®). Double-stranded biotinylated oligonucleotides (99 bp long) were immobilized on a streptavidin-coupled CM5 Sensor Chip using standard protocol as indicated in the Amine Coupling Kit (Biacore®). HBS-EP BIA certified buffer (0.01 M Heps, pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% (v/v) polystyrene 20, Biacore®) was used as a running buffer. Briefly, flow rate was fixed at 10 μl/min. Streptavidin was injected at 500 ng/μl in 10 mM sodium acetate, pH 3.5, for 12 min. Biotinylated double-stranded oligonucleotides were injected through each flow cell at 200 ng/ml onto the Sensor Chip until a suitable stable RU signal was obtained. The integrity and quantity of fixed DNA was checked by a 120-s injection of 1.6 RU signal was obtained. The integrity and quantity of fixed DNA was checked by a 120-s injection of 1.6 RU signal was obtained.

**SPR Kinetic Assay**—Kinetic experiments were carried out at 15 °C at a flow rate of 30 μl/min on a CM5 Sensor Chip prepared as described above with a 100-RU stable fixation of each biotinylated double-stranded oligonucleotide. ETS-1 p51 isoform was injected using the KINJECT procedure for 90 s at 7.25, 14.5, and 29, and 58 nM concentrations. Dissociation of the complex was then monitored for 300 s before regen-
eration by a 60-s injection of 0.03% SDS in distilled water. Each injection was repeated three times to obtain a complete data set of 12 curves. Raw data were corrected by subtraction of the blank curve corresponding to M1M2 oligonucleotide. Each data set was globally fitted with Biaeval software. The schematic representation of the models that we edited and used for the data analysis and their related set of differential rate equations are listed in Table III. For each model, the kinetic parameters as well as the maximum binding capacity of the immobilized ligand were considered as global parameters for a given data set. Moreover, two local parameters were added for each curve to take into account the refractive index changes at the beginning of the wash-on and wash-off phase.

FIG. 1. ETS-1 specifically binds to the head to head EBS palindrome in a cooperative way. A, sensorgrams of a 200 nM ETS-1 injection over a Sensor Chip functionalized with 280 RU of WT, M1, and M2 oligonucleotides. The dotted line represents the sum of M1 and M2 sensorgrams. B, sensorgrams of a 200 nM ETS-1 injection over a Sensor Chip functionalized with 260 RU of WT, DR, IP, and M1 oligonucleotides (IP sensorgram comes from another Sensor Chip with the same amount of immobilized DNA). A and B, a flow cell functionalized with M1M2 oligonucleotides was used as a reference for nonspecific binding. C, gel shift assay. ETS-1 (4 pmol) was incubated with WT (lanes 1–4) or M1 (lane 5) 32P-labeled DNA probe (0.5 ng) in the absence (lane 1) or in presence of WT (200×, lane 2), M1M2 (200×, lane 3), and (M1 400×, lane 4) unlabeled competitors, WT (lane 6), and M1 (lane 7) free probes were loaded. D, photo-cross-linking assay resolved by SDS-PAGE. The same amount of ETS-1 (4 pmol) was incubated with increasing amounts (0.125, 0.25, 0.5, 1, and 2 ng) of WT (lanes 2–6) or M1 (lanes 8–12) 32P-labeled DNA probe prior addition of photo-cross-linker (Ru(bpy)3Cl2, ammonium persulfate) and illumination. Lanes 1 and 7 are controls containing 2 ng of WT or M1 probe, respectively, cross-linked in the absence of ETS-1. E, same experiment as D, realized with an equimolar mixture of 32P-labeled 15-mer oligonucleotides (referred to as 15-mer) corresponding to −223/−209 and −208/−194 sequences of the stromelysin-1 promoter as a probe (0.5, 1, and 2 ng, lanes 1–3). Lane 4 is a control containing 2 ng of probe in absence of ETS-1. The arrow 1 indicates binary ETS-1/DNA complex. The arrow 2 indicates ternary ETS-1/DNA:ETS-1 complex.
ETS-1 Binding to Stromelysin-1 Promoter

Transfection and Reporter Gene Assay—HEK293 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum in 12-well plates (2 ml per well) to reach 60–80% confluence at the time of transfection. Efficiency of transfection was tested with a β-galactosidase encoding expression vector under the control of the cytomegalovirus promoter after coloration of the transfected cells with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) (Roche Molecular Biochemicals). Before transfection, Exgen 500 Transfection Reagent (1.5 μl per well, Euromedex) was incubated with 250 ng of each respective reporter (pGL3) and expression vector (pSG5) for 10 min at room temperature in a volume of 50 μl of a 150 mM NaCl solution. Cell medium was changed for 500 μl of Opti-MEM (Invitrogen), and DNA-Exgen 500 mixture was added. After 16 h, medium was changed for 2 ml of Dulbecco’s modified Eagle’s medium. Cells were harvested 48 h after transfection with 250 μl of cell lysis buffer (1% Triton X-100, 25 mM glycylglycine, pH 7.8, 15 mM MgSO4, 4 mM EGTA, 1 mM DTT). 20-μl aliquots of each supernatant were tested for luciferase activity (luciferase assay kit, Promega) using a Lumat LB 9501 (Berthold). The expression of the proteins of interest (ETS-1 p51 or p42) was tested by Western blot analysis on total cell lysates using a primary antibody directed against the ETS-1 DBD (C-20, Santa Cruz Biotechnology).

RESULTS

SPR Measurement of the Cooperative Binding of ETS-1 to the EBS Palindrome of the Stromelysin-1 Promoter—To investigate the characteristics of the interaction between ETS-1 and the head to head EBS palindromic stromelysin-1 promoter (referred to as wild type (WT) in the following), several mutants of the EBS repeat were produced. Either one (M1 or M2) or both (M1M2) EBS were inactivated by directed mutagenesis exchanging the 5′-GGAAA-3′ core consensus with 5′-AAAAA-3′ (Table I). Biotinylated PCR fragments of the −276/−177 region were used as a ligand for SPR measurements of the interaction between ETS-1 and the EBS palindrome (Fig. 1A). We observed a positive cooperative binding to the WT site in comparison to M1 or M2 mutants. Indeed, the measured RU signal corresponding to the ETS-1 binding to the WT site was higher than the sum of the RU signal for M1 and M2 mutants (dotted line on Fig. 1A). To determine whether the cooperative binding was dependent on the mutual orientation of the EBS, we produced mutants in which one (direct repeat, referred to as DR) or both (inverted palindromic, referred to as IP) EBS orientations were changed, taking care to preserve their flanking sequence (Table I). SPR measurements showed a complete disappearance of the cooperative binding to the IP and DR sites as binding to these mutants was approximately twice the binding to the M1 site (Fig. 1B).

This quantitatively confirms the observation that ETS-1 or ETS-2 tends to bind head to head EBS palindromes, such as those encountered in GATA-1 (38) or p53 (39) promoters, better than a single EBS or other topology of EBS repeats (44). The fact that ETS-1 could be able to discriminate between different topologies of EBS repetitions tends to prove that this effect could not be driven by structural effects like cooperative bending of the DNA facilitated by the proximity of the EBS. Indeed structural studies showed that ETS DNA binding domain (DBD) binding to DNA induces moderate bending (45, 46). If such a phenomenon was the unique strength of the cooperativity, binding to IP and DR mutant sites would also be enhanced by the DNA bending generated by the first ETS-1 molecule. Then other mechanisms, like protein-protein interactions, should be envisaged.

Visualization of ETS-1-DNA Complexes by EMSA and Photo-cross-linking—The major drawback of BIAcore® technology is its inability to identify the complexes formed between the ligand and the analyte during the injection time. So we confirmed our observations by visualizing the protein-DNA complexes at the equilibrium by EMSA and photo-cross-linking.

EMSA showed the formation of a single major complex between ETS-1 and the WT probe (Fig. 1C, lane 1). Whereas a 200× molar excess of WT non-labeled probe was able to disrupt this complex (Fig. 1C, lane 2), a 400× molar excess of M1 probe, corresponding to the same amount of binding sites, was unable to do so (Fig. 1C, lane 4). Moreover, the association of ETS-1 with M1 probe only led to a weak fading signal (Fig. 1C, lane 5) in comparison to the WT complex, and thus with the same protein and probe amounts (compare lanes 1 and 5 on Fig. 1C). Similar results were obtained with M2 probe (data not shown). This experiment confirms the cooperative binding of ETS-1 to the head to head EBS palindrome present in the stromelysin-1 promoter.

In order to visualize the complex formed with M1 probe, which did not seem to resist migration through the polyacrylamide gel used under our experimental conditions, we performed cross-linking with Ru(bpy)3Cl2. This reagent enabled instant high yield cross-linking with no bridging agent (47). At low probe concentrations, only a weak signal was visible with WT probe (Fig. 1D, lane 3), and no signal was observed with M1 (Fig. 1D, lanes 8–10). This signal, migrating at −120 kDa, corresponds to two ETS-1 molecules linked to the WT probe. Increasing the probe concentration results for the M1 probe (Fig. 1D, lanes 11 and 12) in the formation of a weak complex at about 60 kDa corresponded to a single ETS-1 molecule linked to the probe. This signal appeared as the concentration of WT probe increased (Fig. 1D, lane 6) but remained less intense than the 120-kDa signal. Interestingly, no signal was observed at 120 kDa in an experience using an equimolar mixture of 32P-labeled oligonucleotides corresponding to −229/−209 and −209/−194 sequences of the stromelysin-1 promoter as a probe (referred to as EBS 15-mer in Fig. 1E). We obtained a pattern similar to M1, although we could have hoped to restore a trimeric complex by protein-protein interactions. This suggests that if such contacts exist, they are dependent on the strict vicinity and positioning provided by the topology of the palindromic binding sites.

Kinetic Study of the Interaction between ETS-1 and the Stromelysin-1 Promoter—We characterized the binding cooperativity by modeling the interaction between ETS-1 and the EBS palindrome of the stromelysin-1 promoter. New data sets were produced under conditions more favorable for kinetic studies as follows: (i) lower binding capacity (100 RU of immobilized DNA) and higher flow rate (30 μl/min) to reduce mass transfer effects at the surface of the Sensor Chip; (ii) lower temperature to reduce kinetic rates; and (iii) addition of bovine serum albumin to 100 μg/ml in the running buffer to prevent excessive refractive index changes during injections. A typical quadruplet of curves for each oligonucleotide (M1, M2, and WT) is shown in Fig. 2. The kinetic models used to fit the data sets and the related differential rate equations are represented in Table III. Kinetic and equilibrium parameters obtained for each model are listed in Table IV.

ETS-1 binding to M1 or M2 oligonucleotides was fitted according to the simplest kinetic model provided, a one to one interaction. ETS-1 binding to the WT oligonucleotide was fitted with our own edited models of increasing complexity, representing the possible interactions of two ETS-1 molecules with DNA. We tried to keep to a minimum the number of kinetic parameters in order to preserve the significance of the model. We considered models in which binding of the two ETS-1 molecules could be independent (models A and B) or sequential (models C and D). We also introduced the possibility of a conformational change of the ternary complex after binding to the DNA (models B and D). In addition to the kinetic parameters, for each of these models we calculated in Table IV an apparent binding affinity corresponding to the affinity of two independent and equivalent EBS, referred to as $K_{\text{B(app)}}$ giving...
the same global affinity. This apparent affinity enabled us to determine, in each case, the binding cooperativity in comparison to the M1 and M2 oligonucleotides (see Table IV for results and equations).

The fitting data show that, in the presence of two binding sites, kinetics seem to be faster (compare $k_{d1}$ and $k_{d2}$ with M1 or M2 and the WT models in general) but without considerably altering the overall equilibrium constants (compare $K_d$ values). This can be due to a difference of accessibility to the DNA or more likely to the difference in binding mechanisms between both DNA configurations (M1 or M2 in comparison to WT). Considering the $\chi^2$ values of the models, it is obvious that simple model depicting independent binding sites (model A) is insufficient to describe the overall binding mechanism. Nevertheless, adding a conformational change after DNA binding (model B) improves fitting ($\chi^2$ from 3.4 to 2.77). In that case, $K_d$ values ($K_{d1}$ and $K_{d2}$) are similar to the $K_d$ values obtained for M1 and M2, and the conformational change provides the 6.7-fold cooperativity for the ternary complex formation.

In order to describe better a cooperative mechanism, a sequential binding (models C and D), where the binding of the first molecule is able to influence the binding of the second one, seems to be more accurate. Evidence of this is the fact that the calculated cooperativity fold increases according to the $\chi^2$ value. A simple sequential model (model C) provides a better $\chi^2$ value than model A (2.65 compared with 3.4), which uses the same number of parameters but with independent binding sites. It is noteworthy that $K_{d1}$ between model A and C is not greatly affected, but $K_{d2}$ is 3-fold lower, in accordance with the definition of a cooperative binding mechanism. Indeed the reduction of $K_{d2}$ is mainly due to an increase of $k_{a2}$, reflecting a better association of the second protein to the complex. The last model (model D), comprising a sequential binding followed by a conformational change, provides the better $\chi^2$ of the series (0.996) and so the best description of the cooperative phenomenon. As for model C, but to a greater extent, the $K_{d2}$ value of the second step ($K_{d2}$) is reduced compared with the first one (about 50 times lower than $K_{d1}$) or to the second step of model B (about 20 times lower) which uses the same number of parameters but with independent binding sites. In both cases this reduction is due to the increase in $K_{a1}$ values. The $K_{d2}$ value of 0.95 indicates that at the equilibrium the ternary complex exchanges freely between two conformations. The cooperativity fold is then maximum for this model reaching 19.7-fold for a $K_{d(app)}$ of 18.7 nM.

This kinetic study confirms the cooperative binding of two ETS-1 molecules to the EBS palindrome. The cooperativity observed seems to be driven by the facilitated binding of the second ETS-1 molecule to form the ternary complex rather than by the formation of a ternary complex in a locked conformation after a conformational change as $k_{d2}$, $k_{a2}$, and $k_{a3}$ gave no evidence of it. Indeed, in that case we would have encountered slower dissociation steps for the ternary complex, with lower $k_{a2}$ and $k_{a3}$ values.

Mapping of the Protein Region Responsible for the Cooperative Binding by N-terminal Deletion Mutants of ETS-1 Protein—In order to determine whether a particular protein region was responsible for the observed cooperative behavior, we produced N-terminal deletion mutants of ETS-1 by PCR amplification and cloning into pTyb2 vector for bacterial expression (Fig. 3A). Recombinant proteins were purified as under “Materials and Methods.” A SDS-PAGE of the various mutants was silver-stained to assess purity (Fig. 3C).

The ability of the different deletion mutants to cooperate for binding to the EBS palindrome was evaluated by SPR measurement at the equilibrium (Fig. 4A). The same concentration (200 nM) of each protein was injected over a Sensor Chip functionalized with WT, M1, and M2 oligonucleotides. At the end of the injection, when binding equilibrium was reached, and after blank correction using M1M2 oligonucleotide, RU signal for each flow cell was measured, and the following ratio was calculated: WT/M1 = RU(WT)/RU(M1). Results are displayed in Fig. 4B. The WT/M2 and WT/(M1 + M2) ratios, also representative of the cooperative binding of the proteins to DNA, showed similar variations (data not shown) and so will not be mentioned in this study. We observed a rapid increase of the WT/M1 ratio from ΔN331, which represents a DBD without its N-terminal inhibitory region, to ΔN245. For deletion mutants beyond amino acid 245, it stabilized at a value corresponding to the ratio obtained with the full-length protein.

RU variation is representative of a mass variation at the surface of the Sensor Chip with the correlation that 1000 RU
TABLE IV
Kinetic and equilibrium parameters for ETS-1 interaction with the EBS palindrome of the stromelysin-1 promoter, as determined by surface plasmon resonance.

All the notations for the parameters are consistent with the ones used in Table III.

| Ligand | $k_{a1}$ | $k_{d1}$ | $K_{d1}$ | $k_{a2}$ | $K_{d2}$ | $k_{d2}$ | $K_{d2}$ | $K_{d(app)}$ | Cooperativity fold | $\chi^2$ |
|--------|---------|---------|---------|---------|---------|---------|---------|------------|-----------------|---------|
| M1     | 2.00 ± 0.02 | 2.10 ± 0.04 | 105 ± 2 | 5.90 ± 0.3 | 54.1 ± 5.7 | 2800 ± 400 | 450 ± 60 | 0.16 ± 0.03 | | 2.02 ± 0.22 | 3.4 |
| M2     | 2.00 ± 0.02 | 1.90 ± 0.01 | 65.5 ± 0.6 | 54.1 ± 5.7 | 2800 ± 400 | 450 ± 60 | 0.16 ± 0.03 | | | |
| WT, model A | 32.0 ± 0.8 | 20.1 ± 0.1 | 62.8 ± 1.6 | 10.9 ± 1.0 | 5.90 ± 0.3 | 2800 ± 400 | 450 ± 60 | 0.16 ± 0.03 | | 2.02 ± 0.22 | 3.4 |
| WT, model B | 18.9 ± 1.7 | 20.4 ± 1.6 | 108 ± 13 | 24.9 ± 1.8 | 14.8 ± 0.5 | 59.7 ± 4.7 | 2800 ± 400 | 450 ± 60 | 0.16 ± 0.03 | | 2.02 ± 0.22 | 3.4 |
| WT, model C | 46.0 ± 0.7 | 20.0 ± 0.1 | 43.5 ± 0.7 | 36 ± 1 | 6.5 ± 0.1 | 18.1 ± 0.6 | 2800 ± 400 | 450 ± 60 | 0.16 ± 0.03 | | 2.02 ± 0.22 | 3.4 |
| WT, model D | 27.0 ± 0.1 | 36.0 ± 0.7 | 133 ± 3 | 1300 ± 20 | 36 ± 1 | 2.80 ± 0.9 | 7.3 ± 0.2 | 6.9 ± 0.1 | 0.95 ± 0.03 | 18.7 ± 0.5 | 19.7 ± 1.1 | 0.996 |

a Incertitude is calculated using the equation:

$\Delta y^2 = \sum_{i=1}^{n} \left( \frac{y_i - \hat{y}_i}{\sigma_i} \right)^2$.

b Apparent binding affinity of a single EBS of the WT palindrom considered as the juxtaposition of two independent and equivalent binding sites (same $K_d$ and no cooperativity) calculated using the equation:

$K_{d(app)} = \prod_{i=1}^{n} K_{d_i}$.

c Cooperativity fold observed between the two EBS of the WT palindrom calculated using the equation:

$\alpha = \frac{K_{d1}^{WT} \cdot K_{d2}^{WT}}{K_{d1}^{WT} \cdot K_{d2}^{WT}} = \prod_{i=1}^{n} K_{d_i}^{WT}$.

Incertitude is calculated using the equation:

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Apparent binding affinity of a single EBS of the WT palindrom considered as the juxtaposition of two independent and equivalent binding sites (same $K_d$ and no cooperativity) calculated using the equation:

$K_{d(app)} = \prod_{i=1}^{n} K_{d_i}$.

c Cooperativity fold observed between the two EBS of the WT palindrom calculated using the equation:

$\alpha = \frac{K_{d1}^{WT} \cdot K_{d2}^{WT}}{K_{d1}^{WT} \cdot K_{d2}^{WT}} = \prod_{i=1}^{n} K_{d_i}^{WT}$.
ternary complex made of two proteins bound to the WT probe (lane 4). The binary complex, which was observed with M1, was also present with the WT probe but weaker. ΔN280 binds weakly to the M1 probe (Fig. 4D, lane 5) and only formed with the WT probe a slower migrating ternary complex (lane 6). Binding patterns for ΔN245, ΔN144, ΔN45, and ETS-1 were identical, e.g. no distinct complex formed with M1 probe (Fig. 4D, lanes 7, 9, 11, and 13) and only a strong slower migrating ternary complex formed with WT probe (lanes 8, 10, 12, and 14).

These observations tend to designate the region located between amino acid 245 and 331 as responsible for the cooperative binding of two ETS-1 molecules to the EBS palindrome. The mechanism implied is the suppression of the DNA-binding autoinhibition. It seems possible that two ETS-1 molecules overcome DNA binding autoinhibition by a protein-protein interaction in the 245–331-residue region or another mechanism implying this region, during the ternary complex formation, that would favor an unpressed form with an HI-1 inhibitory helix unwound (see Fig. 3B for schematic representation of the structural elements of the ETS-1 DBD and inhibitory regions). Such mechanism has already been described for ETS-1, which

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**Fig. 4. Localization of the ETS-1 peptidic region implicated in the stromelysin-1 promoter binding cooperativity.** As for Fig. 1 and Fig. 2, M1M2 oligonucleotide was used as a reference for nonspecific DNA binding. A, amount of protein bound to WT and M1 oligonucleotides at the equilibrium phase during a 200 nM injection of ETS-1 or its N-terminal deletion mutants, expressed in RU. B, representation of the ratio between the different proteins bound to WT oligonucleotide and M1 oligonucleotide for a 200 nM protein injection. RU for calculation were measured at the end of the injection at equilibrium phase. C, protein binding to WT and M1 oligonucleotides, expressed in fmol/mm². D, gel shift assay. Same amount of ETS-1 or its N-terminal deletion mutants (4 pmol) was incubated with the WT or M1 ³²P-labeled probe (0.5 ng).
was able to interact with a binding partner in order to stabilize its unpressed conformation (29, 31). It would account for the easier and faster formation of the ternary complex emphasized by the kinetic study.

**Binding Pattern of the p42 Isoform of ETS-1 to the EBS Palindrome of the Stromelysin-1 Promoter**

**ETS-1 physiologically exists under two different isoforms: p51, the full-length protein, and p42, a shorter isoform that lacks the 245–331-residue region encoded by exon VII of the gene (Fig. 5A). These two proteins, although sharing similar characteristics due to their common DBD and pointed domain, also have distinct properties (48, 49). It was particularly interesting to study the binding of the ETS-1 p42 isoform to the EBS palindrome of the stromelysin-1 promoter. Its cDNA was cloned in a pTyb2 vector and the recombinant protein was expressed and purified (see “Material and Methods”). By using EMSA, we compared DNA binding to WT and M1 probes for the ETS-1 p51 and p42 isoforms. Surprisingly, p42

**FIG. 5.** Comparison between binding of ETS-1 p51 and p42 isoforms to the EBS palindrome of the stromelysin-1 promoter. A, schematic organization of p51 and p42 isoforms of ETS-1. P represents a serine susceptible to be phosphorylated by CaMKII. B, gel shift assay. Equal amounts (4 pmol) of ETS-1 p51 (lanes 1–6) and ETS-1 p42 (lanes 7–12) isoforms are incubated with WT (lanes 1–3 and 7–9) or M1 (lanes 4–6 and 10–12) 32P-labeled oligonucleotides (0.5 ng) in the presence of increasing amounts of unlabeled competitor (50× lanes 2, 5, 8, and 11, and 200× lanes 3, 6, 9, and 12). C, gel shift assay. The same amount (4 pmol) of ETS-1 p42 was incubated with WT (lane 1), M1 (lane 2), WT and M2 labeled probes (0.5 ng). D, SPR experiment. Ratio of protein bound to WT and M1 or WT and M2 oligonucleotides during the equilibrium phase of a 100 nM p51 or p42 injection. E, variation of the previous ratios as a function of the analyte (Δ, p51, or ■, p42) concentration (75, 100, 150, and 200 nM). The arrow 1 corresponds to the binary p42-DNA complex, and arrow 2 to the ternary p42-DNA-p42 complex, and the arrow * to a complex formed with a minor contaminant.
to form a ternary complex with the WT oligonucleotide. The influence of protein concentration on the cooperative binding mechanism was evaluated by measuring the previous ratios for different concentrations (Fig. 5E). Their variation for p51, increasing for low concentrations where the cooperative factor is high, is in agreement with a cooperative behavior. On the contrary, for p42 isoform, the near 1 ratio value is independent on the protein concentration. All together, these data demonstrate the implication of the 245–331-residue region encoded by exon VII in the human ETS-1 cooperative binding to the head to head EBS palindrome present in the stromelysin-1 promoter.

**Effects of ETS-1 Phosphorylation by CaMKII on Cooperative Binding to the EBS Palindrome of the Stromelysin-1 Promoter**—Calcium-dependent phosphorylation of ETS-1 on residues Ser251, Ser275, Ser282, and Ser285 was reported to negatively regulate ETS-1 DNA binding *in vitro* and *in vivo* (50, 51). The proposed mechanism is a reinforced autoinhibition by stabilization of the inhibitory conformation through electrostatic interactions between phosphoserines and basic residues of the inhibitory module.

In order to extend our model and to determine the influence of calcium-dependent phosphorylation on the ETS-1 cooperative binding, we realized *in vitro* phosphorylation assays using CaMKII. First, we realized phosphorylation kinetics using ETS-1 as a substrate to determine time conditions under which phosphorylation was maximum for the amount of substrate and enzyme considered (Fig. 6A). We further used a 90-min incubation corresponding to the stationary phase of the reaction.

Phosphorylation effects were tested by EMSA, comparing binding to the WT probe of ETS-1 p51 and p42 isoforms incubated in the presence or absence of CaMKII. We also tested phosphorylation effects on ∆N331, which lacks calcium-dependent phosphorylation sites, and ∆N245, which both have a similar binding behavior to p42 and p51, respectively. For ETS-1 p51 isoform and ∆N245, hardly any binding of the phosphorylated proteins was observed with the WT probe (Fig. 6B, lanes 3 and 7). For ∆N331 or ETS-1 p42 isoform no significant difference in amounts of bound protein between the CaMKII-treated sample and the control (Fig. 6B, lanes 11 and 15) was observed. These results confirm the role of autoinhibition and the implication of the 245–331-residue region in the cooperative binding to the EBS palindrome. It seems that reinforcing autoinhibition by phosphorylation of the serine residues reduces dramatically the cooperative binding. This can be explained either by the stabilization of the inhibitory conformation of ETS-1, which reduces affinity for DNA, or by a possible electrostatic repulsion of the negatively charged phosphoserines that would prevent protein-protein interaction in the 245–331-residue region.

**Activation of the Stromelysin-1 Promoter by ETS-1 p51 and p42 Isoforms**—It was interesting to investigate whether the difference of DNA binding of the p51 and p42 isoforms was correlated with their transcriptional activation. The −478/4+4 region of the WT, M1, M2, and M1M2 stromelysin-1 promoters was cloned in the pGL3 basic vector (Fig. 7A). These vectors were co-transfected with either p51 or p42 eukaryotic expression vectors in HEK293 cells. Culture lysates were tested for luciferase activity. For p51, mutation of one of both EBS sites had dramatic effect on transactivation, resulting in an ~90% loss of activity. Double mutation readily abolished activation by p51. These results, obtained with ETS-1, were similar to those obtained with ETS-2 protein on the human stromelysin-1 promoter (13). This experiment provides a direct link between cooperative DNA binding and functional transactivation. Interestingly, the ETS-1 p42 isoform did not activate the stromely-
ETS-1 Binding to Stromelysin-1 Promoter

The human stromelysin-1 promoter has been shown to be differently regulated by various members of the ETS family through the EBS palindrome located −216/−201. ETS-1, ETS-2 (13, 14), and PEA-3 (52) are transcriptional activators, whereas TEL is reported to repress the gene (53). In addition, ETS-related gene (ERG) is able to inhibit the transcriptional activation mediated by ETS-2 (40). In this study, we investigated the mechanism of ETS-1 binding to this palindrome. We confirmed that DNA binding is cooperative, requiring the head to head orientation of the EBS and their strict vicinity (Fig. 1), which is fully consistent with results obtained with other EBS palindromes (39, 44). An anterior report showed cooperative transactivation, whereas no cooperative binding to the EBS palindrome was observed (14). By working with purified proteins and a homologous system, we showed cooperative binding with no ambiguity in comparison to this former study realized in a heterologous system. Kinetic analysis of ETS-1 binding to the WT promoter (Fig. 2 and Tables III and IV) confirms this hypothesis and enables us to give a plausible mechanism for the cooperativity. The binary complex formed between one ETS-1 molecule and an EBS facilitates the fast binding of the second ETS-1 molecule to form the ternary complex. The high rate of this step could be explained by a protein-protein contact between ETS-1 molecules that would give the right orientation, positioning, and conformation for the second ETS-1 molecule to recognize the EBS. By using deletion analysis of the ETS-1 protein, we further demonstrated that the 245–331-residue region, encoded by exon VII of the gene, was responsible for the observed cooperativity (Figs. 3 and 4), and this strongly suggests that the alleviation of the DNA binding autoinhibition is implicated in the cooperative binding. The sum of the kinetic and equilibrium SPR analyses causes us to propose the following binding model (Fig. 8) in which the first ETS-1 molecule bound to DNA facilitates the binding of a second ETS-1 molecule through an interaction implying the exon VII-encoded region. The second ETS-1 molecule would be presented in the right orientation and in an uninhibited conformation, explaining the binding cooperativity observed. This molecular event is represented in Fig. 8 with square brackets. The ETS-1 molecules contact through their exon VII-encoded region, and the DNA-bound ETS-1 molecule helps the second one to acquire its open uninhibited conformation in the right orientation to bind DNA, according to the palindromic topology of the binding sites. After formation of the ternary complex, the ETS-1 molecules flip between two conformations on DNA corresponding to the last equilibrium of the figure. According to the model, we proposed it to be a conformational change between the exon VII-encoded regions which were implicated in the previous step of the mechanism and could then adopt a relaxed conformation after the ternary complex was formed.

This may have biological significance because the natural ETS-1 p42 isoform, lacking the exon VII-encoded region, fails to bind cooperatively to the EBS palindrome and allows the binding of only one p42 molecule (Fig. 5). The absence of the inhibitory helices HI-1 and HI-2, preventing the formation of the postulated compact inhibitory bundle, could generate steric hindrance and account for this result (see Figs. 3, A and B, and 5A and Ref. 21 for structural elements of ETS-1). This phenomenon was also encountered with the shorter ΔN331 deletion mutant. Therefore, the C-terminal region of p42 or ΔN331, spanning amino acids 416–441 and comprising the H4 inhibi-

**DISCUSSION**

**FIG. 7.** Activation of the stromelysin-1 promoter by ETS-1 p51 and p42 isoforms. A, reporter gene constructs used for the transient transactivation assay. The −478/+4 region of the WT and mutant stromelysin-1 promoters was cloned into a pGL3basic vector, and LUC represents the luciferase reporter gene. B, transient transfection assays are realized using HEK293 cells at 60–80% confluence. 250 ng of each luciferase reporter vector were co-transfected with 250 ng of either p51 or p42 eukaryotic expression vector. Luciferase activities obtained were expressed as a percentage of the WT promoter activity co-transfected with the p51 expression vector (3.9-fold). Exact percentages are indicated on top of the bars. Results are the average of two representative experiments done in triplicate. C, Western blot analysis of HEK293 cell lysates after transfection with pSG5 (lane 1), pSG5-p51 (lane 2), and pSG5-p42 (lane 3). The primary antibody used is directed against ETS-1 DNA binding domain (C-20, Santa Cruz Biotechnology).
tory helix, could be responsible for the observed binding inhibition of a second p42 or ΔN331 molecule to the DNA because, except for the DBD, it is the only region they share in common. Such a mechanism remains hypothetical because recent co-crystallization of ETS-1 amino acids 331–440 with Pax5 (54) shows no evidence of a C-terminal region (comprising H4) extending away from the DBD. Other possibilities could be envisaged independently or in conjunction. Indeed, differential DNA bending between p51 and p42, which we rejected to explain the binding cooperativity of p51 because it was dependent on the EBS orientation, could contribute in this case to prevent a second p42 molecule from entering into the major groove of the DNA. A way to link destructuring of the inhibitory module with the differential DNA bending is the recently elucidated mechanism of interaction between the N-terminal end of helix H1 and the phosphate backbone of the DNA (55). A difference of H1 conformation between p42 and p51, linked to the absence of an inhibitory module, would influence DNA bending through a different neutralization of the phosphate backbone charge by H1.

Our results appear of interest in the context of the complex regulation of stromelysin-1 expression by factors of the ETS family. Previous studies showed that on the one hand, in given experimental conditions, the AP-1 complex was required for ETS-1 transcriptional activity (56) and on the other hand that the recruitment by direct protein-protein interactions of the AP-1 complex by ETS-2 was increased in the presence of the EBS palindrome of the stromelysin-1 promoter (41). Hence, the observed binding cooperativity, mediated by a structural rearrangement implying the exon VII-encoded region, could play a crucial role in the optimum presentation of the molecule to its partners. These spatial and molecular events could lead to the unmasking or the formation of a specific interaction surface. This does not only concern transcriptional partners like AP-1 or CBFα2 but also general transcriptional coactivators. Indeed, CBP/p300 was shown to interact directly with ETS-1 and ETS-2 and particularly in the case of transactivation of the stromelysin-1 promoter (42, 57). More recently, Sp100 was also shown to be able to interact with ETS-1 and stimulate its transcriptional activity (58). It is also noteworthy that not only the domain comprising HI-1 and HI-2 inhibitory helices is implicated but rather the entire exon VII-encoded region. The latter includes the four serines responsible for the CaMKII-dependent phosphorylation, regulating DNA binding (50, 51), which we proved to also play a role in the cooperative binding to the EBS palindrome (Fig. 6). It also contains an SCRLTQG motif located 261–267 in the protein sequence. This motif, according to computer-predicted secondary structure of ETS-1, forms an α-helix and also contains an arginine that is resistant to tryptic digestion (60). This suggests a stable secondary structure that could be involved in the autoinhibition regulation or in protein-protein contacts.

The observed cooperative binding can be easily linked to the strategy of combinatorial transcription regulation by ETS proteins. Various works studied cooperative binding of the ETS-1 protein with transcriptional partners such as CBFα2 (30, 31), USF-1 (27), or TFE3 (28) where intermolecular contacts raise autoinhibition. In the particular topology of the head to head EBS palindrome, we propose that the two ETS-1 molecules play the role of reciprocal transcriptional partners. In addition, this view can account for the observation that ERG inhibits the ETS-2 transcriptional activation of the stromelysin-1 promoter in transient co-transfection (40). ETS-2, like ETS-1, requires the presence of both vacant EBS to overcome autoinhibition and bind efficiently to the DNA. Thus, an ERG molecule would be able to occupy one of the two EBS long enough to prevent the cooperative fixation of two ETS-2 molecules. This also suggests a differential regulation of the promoter by the two isoforms of ETS-1, p42 and p51. The latter binds cooperatively to the palindrome and achieves maximum transactivation (Fig. 7) but binds poorly to M1 or M2 mutant due to DNA binding autoinhibition. In contrast, the p42 isoform can bind efficiently to a single EBS and thus prevent access to the second EBS of the palindrome, acting as a dominant negative for the activation of the stromelysin-1 promoter or other promoters where EBS are organized in the palindrome.

We postulated that ETS-1 overcomes its autoinhibition via intermolecular contacts involving the exon VII-encoding region which would favor the rapid formation of a ternary complex. The organization of two head to head EBS distant by 4 bp on a regular B-shaped double-stranded DNA in conjunction with the spatial structure of an ETS DBD bound to DNA indicates that both ETS-1 molecules are located on the same side of the DNA, and that the inhibitory modules are facing each other. However, we were not able to evidence a direct protein-protein contact. Various techniques including cross-linking, in vitro GST fusion pull-down assays, or SPR experiments failed to reveal a direct protein-protein interaction (data not shown). These difficulties may have two major causes. First, the supposed interaction is DNA-dependent, requiring strict proximity and proper arrangement of the molecules. Second, the inhibitory module does not exist on its own, resulting from the packing of four distinct partners: inhibitory helixes HI-1 and HI-2 located at the N terminus of the DBD, inhibitory helix H4 at the C terminus of the DBD, and helix H1 in the DBD (see Fig. 3B). To use an isolated exon VII peptide for in vitro interaction assays appears useless because its proper structure would not be obtained in absence of the C-terminal part of the protein. In this context, it is noteworthy that such an involve-
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mental of the exon VII-encoded region of ETS-1 in a cooperative binding to a transcriptional partner was already encountered with CBP/e2 and resulted with the same difficulty to prove direct protein-protein interaction even with such a sensitive tool as SPR (31).

The EBS palindrome with 4-bp interval between ETS-binding cores present in the stromelysin-1 promoter is not found in other known promoters of matrix metalloproteinases. Its ability to recruit, in a specific way, two ETS-1 p51 molecules to form a ETS-1-DNA:ETS-1 ternary complex via the exon VII-encoded region is inherent to the autoinhibition binding mechanism of p51. To our knowledge, it is the first time that ETS-1 complex remains to be checked in cells expressing both stromelysin-1 and ETS-1. Given that such palindromes are found in other promoters regulated by ETS proteins, these EBS palindromes might represent a hot spot to recruit autoinhibited ETS proteins and lead to specific transcriptional complexes.

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