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FAST TRACK

Upregulation of Human Heme Oxygenase Gene Expression by Ets-Family Proteins

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Abstract Overexpression of human heme oxygenase-1 has been shown to have the potential to promote EC proliferation and angiogenesis. Since Ets-family proteins have been shown to play an important role in angiogenesis, we investigated the presence of ETS binding sites (EBS), GGAA/T, and ETS protein contributing to human HO-1 gene expression. Several chloramphenicol acetyltransferase constructs were examined in order to analyze the effect of ETS family proteins on the transduction of HO-1 in *Xenopus* oocytes and in microvessel endothelial cells. Heme oxygenase promoter activity was up-regulated by FLI-1ERGETS-1 protein(s). Chloramphenicol acetyltransferase (CAT) assays demonstrated that the promoter region (−1500 to +19) contains positive and negative control elements and that all three members of the ETS protein family were responsible for the up-regulation of HHO-1. Electrophoretic mobility shift assays (EMSA), performed with nuclear extracts from endothelial cells overexpressing HHO-1 gene, and specific HHO-1 oligonucleotides probes containing putative EBS resulted in a specific and marked bandshift. Synergistic binding was observed in EMSA between AP-1 on the one hand, FLI-1, ERG, and ETS-1 protein on the other. Moreover, 5′-deletion analysis demonstrated the existence of a negative control element of HHO-1 expression located between positions −1500 and −120 on the HHO-1 promoter. The presence of regulatory sequences for transcription factors such as ETS-1, FLI-1, or ERG, whose activity is associated with cell proliferation, endothelial cell differentiation, and matrix metalloproteinase transduction, may be an indication of the important role that HO-1 may play in coronary collateral circulation, tumor growth, angiogenesis, and hemoglobin-induced endothelial cell injuries. J. Cell. Biochem. 72:311–321, 1999.

Key words: Ets family proteins; EC cells; HO-1 gene expression; regulatory sequences

HO-1 is transcriptionally activated through several regulatory mechanisms by a variety of chemical as well as physical stresses [Wagner et al., 1997; Abraham et al, 1996], as well as its substrate heme. Studies in our laboratory, and in others, of the promoter region of HO-1 show the presence of several transcriptional elements, such as AP-1, AP-2, NF-κB, and IL-6 response elements [Alam et al., 1994; Lavrovsky et al., 1993, 1994a,b; Mitani et al., 1992]. Our finding that NF-κB, AP-1, and AP-2 binding sites [Lavrovsky et al., 1994a,b] are involved in HO-1 gene expression suggests the involvement of oxidative stress and reactive oxygen intermediates, including H_2O_2. Furthermore, several transcriptional factors often use H_2O_2 as a second messenger of their activation [Schenk et al., 1991, 1994]. NF-κB, AP-2, and AP-1 are involved in oxidative stress by H_2O_2 [Puri et al., 1995]. NF-κB and AP-1 are thought to play a central role in the regulation of a number of immune and inflammatory response genes and NF-κB is the first eukaryotic transcription factor shown to respond directly to oxidative stress [Grimm and Baeuerle, 1993]. That NF-κB activation and, to a lesser extent, AP-1, occurs shortly after addition of heme to cells, and the HO-1 promoter region contains an NF-κB and AP-1 binding site, suggests a role for these transcription factors in the regulation of HO-1 expression. Functional AP-2 binding sites have been identified in the enhancer regions of viral and cellular genes. Phorbol esters,

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cyclic adenosine monophosphate (cAMP), and retinoic acid, have been shown to induce AP-2 activity, which is believed to play a role in regulating the expression of genes involved in cellular differentiation [Lavrovsky et al., 1994a,b; Buettner et al., 1993; Snape et al., 1991].

The Ets-family proteins are transcription factors that bind to the regulatory control region of certain genes via the ETS binding site (EBS), GGAA/T [Wasylyk et al., 1993]. The Ets-family comprises a large number of genes that encode transcription factors/oncoproteins, which are expressed widely during development and cell proliferation. Ets-1 and fli are expressed largely in several cell types and are particularly abundant in EC [Meyer et al., 1997, 1995; Wernert et al., 1992]. Fli overexpression during *Xenopus* embryogenesis results in the frequent absence of circulating red blood cells, which may be accompanied by ectopic differentiation of mature erythrocytes in vesicles not connected to the vascular system [Remy et al., 1996]. It is therefore likely that Fli plays an important role both in vascularization and in hematopoiesis. Recently, we showed that overexpression of HO-1 activity by gene transfer into EC potentially promotes angiogenesis [Deramaudt et al., 1998]. Since Ets-family proteins such as ERG, FLI have been shown to increase angiogenesis [Remy et al., 1996] (also unpublished results), we examined HO-1 for the presence of ETS core sequences and the activation of HO-1 gene expression. We investigated the role of ETS family on activation of HO gene expression, using rabbit coronary microvessel endothelial cells and *Xenopus* oocytes.

MATERIALS AND METHODS

Oocyte Preparation

In order to avoid possible oocyte maturation due to contacts with tricaine anesthetics [Smith, 1989], female *Xenopus laevis* were anesthetized by hypothermia upon 30-min immersion in ice water. They were then kept on a bed of ice for subsequent surgery. A piece of ovary was withdrawn and stage V oocytes manually dissected, using two pairs of No. 5 Drummond forceps. Oocytes were kept in Barth's medium [Barth and Barth, 1959].

Oocyte Injections

Stage V oocytes were placed in Barth's medium, in Lucite plates, and injected in the cytoplasm with 2 ng of synthetic *ets*-1, *fli*, and *erg* mRNAs, in a volume of 36.8 nl of RNase free water, with a Drummond Nanoject injector (Drummond Scientific, Broomall, PA). They were then kept in Barth's medium at 19°C. They were injected in the nucleus, 4 h later, with a mixture of reporter DNA in water (18.4 nl/nucleus). The solution contained 0.17 μg/L of a construct expressing the chloramphenicol acetyltransferase (CAT) gene under the control of either the full-length heme oxygenase promoter (clone A, −1500/+1) or a minimal promoter (clone B, −120/+1), and 0.33 μg/L of an internal standard pCH110 vector (Pharmacia, Upsala, Sweden), containing a β-galactosidase reporter. For the determination of the basal transcriptional levels, control oocytes were used which were not injected with the synthetic mRNAs. Blank CAT and β-galactosidase activities were determined using extracts from noninjected oocytes and were subtracted from experimental values. Oocytes were kept at 19°C for 18 h and protein extracts were then prepared. Batches of 10–12 oocytes were used for a single CAT or β-galactosidase determination. All measurements were carried out in duplicate.

Preparation of the Synthetic Transcripts

The coding sequences of *X. laevis ets*-1, *fli*, and *erg* genes were subcloned, respectively, in pRN3 [Lemaire et al., 1995], pSP64T [Krieg and Melton, 1984], and *myc*-pNKS2 [Gloor et al., 1995]. The first two vectors allow the synthesis of a synthetic transcript containing the 5′- and 3′-UTRs of the globin gene, ensuring an efficient translation of the synthetic mRNA. The last one allows the synthesis of a transcript encoding a fusion protein containing a *myc*-tag, which can easily be detected with an anti-myc mab like 9E10. All the synthetic transcripts were capped upon addition of 4 mM m7G(5′) ppp(5′)G in the transcription mix. The Megascript transcription kits from Ambion (Woodward, TX) were used.

Preparation of Oocyte Extracts: Measurements of CAT and β-Galactosidase Activities

In this investigation, 10–12 oocytes were ground manually with a plastic homogenizer in an Eppendorf tube, in 200 μl of buffer (20 mM Tris-HCl, pH 7.6, 100 mM NaCl, 1 mM phenylmethylsulfonyl fluoride [PMSF], 1% Triton X 100). Extracts were centrifuged for 15 min at 14,000*g* and 4°C.

CAT activities were measured on 40-µl aliquots of the extracts, using the CAT-ELISA kit (Boehringer, Mannheim, Germany), under the conditions recommended by the manufacturer. β-Galactosidase activities were measured to correct for all experimental variations, using a colorimetric assay; 20 µl of oocyte extract was added to 650 µl of reaction buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol). After 5 min at 37°C, 140 µl of an o-nitrophenyl-D-galactopyranoside solution (4 mg/ml in reaction buffer) was added, and the color was allowed to develop for at least 90 min. Absorbances were read at 420 nm, against a control in which the oocyte extract had been omitted.

Control of Synthetic mRNA Translation

The efficiency of synthetic transcripts translation in the oocyte was checked by Western blotting. After injection of a *fli* synthetic mRNA, batches of five oocytes were taken out after 4 and 15 h. Oocytes were homogenized in 50 µl of 50 mM Tris-HCl buffer, pH 6.8, 120 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 2 mM PMSF. A total of 100 µl of 1,1,2 trichlorotrifluoroethane (Aldrich, Steinheim, Germany) was added to solubilize the lipids. After a 2-min a or b centrifugation at 14,000*g*, 6 µl of 350 mM Tris-HCl pH 6.8, 10% sodium dodecyl sulfate (SDS), 30% glycerol, 60 mM dTT were added to the samples, which were immediately boiled for 5 min. Proteins were then separated by SDS-polyacrylamide gel electrophoresis (PAGE), on a 10% acrylamide gel on a Miniprotean II apparatus (BioRad, Richmond, CA), according to the specifications of the manufacturer. Proteins were then transferred to nitrocellulose, in a Mini Trans-blot electrophoretic transfer cell from Bio-Rad, for 12 h at 14 V. The nitrocellulose membrane was then revealed upon incubation with a rabbit antiserum raised against the FLI protein and a peroxydase-conjugated goat:anti-rabbit secondary antibody (Sigma Chemical Co., St. Louis, MO), under the conditions recommended by Ausubel et al. [1987].

Use of *Xenopus* Oocytes to Study HO-1 Transcriptional Regulation

This system has been used as a complementary system to EC transfection assays. It is ideal to test the biological activity of any specific transcriptional factor, in the controlled microenvironment. *Xenopus* oocytes have been widely used for transcription analysis [for review, see Gurdon and Wickens, 1983; Soreq and Seidman, 1992]. We therefore examined the regulation of HO-1 transcription by ETS-1, FLI, and ERG proteins in *X. laevis* oocytes, as schematically depicted in Figure 1.

Transfection of RCME Cell Culture

Cells were isolated from the midportion of the rabbit myocardium by collagenase digestion, filtration, homogenization, and centrifugation as described [Abraham et al., 1995]. Transient transfection and CAT assays were performed as previously described [Lavrovsky et al., 1994a]. Alternatively, CAT enzyme-

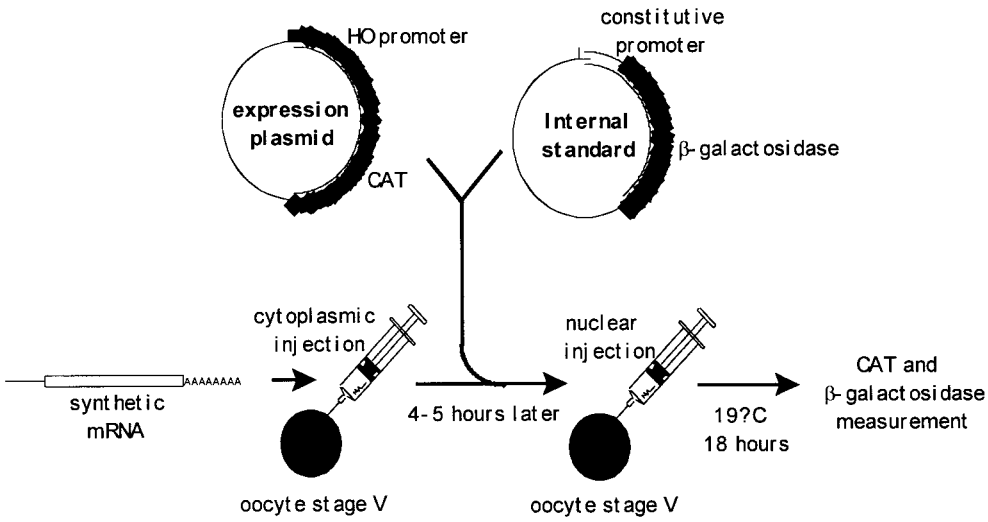

Fig. 1. Schematic representation of the *Xenopus* transactivation assay.

linked immunoassay (ELISA) was performed using the ELISA Kit (Boehringer-Mannheim, Indianapolis, IN). Data were analyzed using Statistica software (Microsoft, Tulsa, OK). In each set of experiments, CAT activity in extract prepared from cells transfected with the promoterless test plasmid pCAT-basic was subtracted from that in the extract with each test plasmid. For analysis of the basal promoter activity of the human HO gene, each test plasmid was introduced into EC by electroporation together with pRsv-βgal, which express β-galactosidase under control of the Rous sarcoma virus long terminal repeat, as an internal control. CAT activities were normalized to the β-galactosidase activity assay as described [Sambrook et al., 1989].

Plasmids and Electrophoretic Mobility Shift Assay

The control plasmid pRc/CMV (Invitrogen, CA) and pRc/CMV-sHHO-1, containing the entire protein-coding region of HHO-1 [Abraham et al., 1995] were used for stable transfection. Two plasmids were used for 5′ deletion analysis of the HHO-1 promoter. A was constructed by inserting the *Eco*RI-*Xho*I fragment (nucleotide residues −1500 to +19) of the HHO-1 promoter [Shibahara et al., 1989] in the skII plasmid (Stratagene, San Diego, CA) in the *Eco*RI-*Xho*I sites. Then, the *Bam*HI-*Xho*I fragment containing the HHO-1 promoter (from the construct described above) was inserted into the *Bam*HI-*Xho*I site of the tk-CAT plasmid instead of the tk promoter. B was constructed from A by digestion with *Hind*III; the ends were filled in using Klenow enzyme and dNTPs; the product was digested with *Bam*HI and *Xho*I to obtain (−60 bp to +20 bp). The CMV-gal plasmid (cytomegalovirus [CMV] promoter region fused to the gal gene) was purchased from Stratagene (San Diego, CA). Electrophoretic mobility shift assay (EMSA) was performed as described elsewhere [Lavrovsky et al., 1994b].

For the competitive EMSA, a 21-nucleotide sequence from HHO-1 promoter overlapping the putative Ets binding site (EBS) located between nucleotides 1023 and 1002, was synthesized and labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase. Then 0.2 pmol of labeled double-stranded DNA was incubated during 20 min at room temperature in gel binding buffer (4% glycerol, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM Tris HCl pH 7.5, 0.05 mg/ml poly(dI-dC) with 10 µg of crude EC nuclear extract with or without 2 pmol of specific competitor (consensus ets-1 motif 5′-AAACAGGAAGGACTTCCTCAGG-3′) or nonspecific competitor (consensus TFIIA motif from Promega, Madison, WI: 5′-GCAGAGCATATAAGGTAGG-3′). The mix was then loaded onto a 6% acrylamide-bisacrylamide gel 29:1 buffered with Tris-glycine-EDTA and the electrophoresis was performed at 4°C, 12V/cm, for 3 h. For the combined AP-1/ETS protein EMSA a 131-bp-long oligonucleotide containing the putative Ets binding site (EBS) and AP1 motif located between the nucleotides −1076 and −946, was amplified using [α^{32}P]dCTP by polymerase chain reaction (PCR). Then 0.2 pmol of labeled double-stranded DNA was incubated for 20 min at room temperature in gel binding buffer consisting of 4% glycerol, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM Tris-HCl pH 7.5, 0.05 mg/ml poly(dI-dC), with 0.5 fpU of purified AP-1 protein (cFos homodimer) and/or with 3 µl of erg/ets/fli-1-translation/transcription mix (TnT-coupled transcription-translation kit; Promega). The mix was then loaded onto a 6% polyacrylamide gel 29:1 buffered with Tris-glycine-EDTA and the electrophoresis was performed at 4°C, 12 V/cm, for 3 h.

RESULTS

Sequence Analysis

Computer analysis using the sequence of the human HO-1 promoter region (Accession No. X14782) and the National Institutes of Health (NIH) database were used to examine the presence of Ets putative response elements GGA(A/T) on HO-1. Our goal is to evaluate the effect of modulation of HO-1 gene activation on angiogenesis and to examine the presence of Ets putative regulatory elements on HO-1. The role of Ets family proteins on upregulation of HO-1 gene expression was assessed in *Xenopus* oocytes. The results have shown that HO-1–1500 to +1 contain 26 putative Ets core motives (Fig. 2). It was of interest to check whether some of these putative EBS (Ets-binding sequence) would be involved in the control of HO-1 transcription by proteins of the Ets-family.

Synthetic mRNAs Are Efficiently Translated in *Xenopus* Oocytes

An experiment was conducted to check how long the FLI protein would be present at high

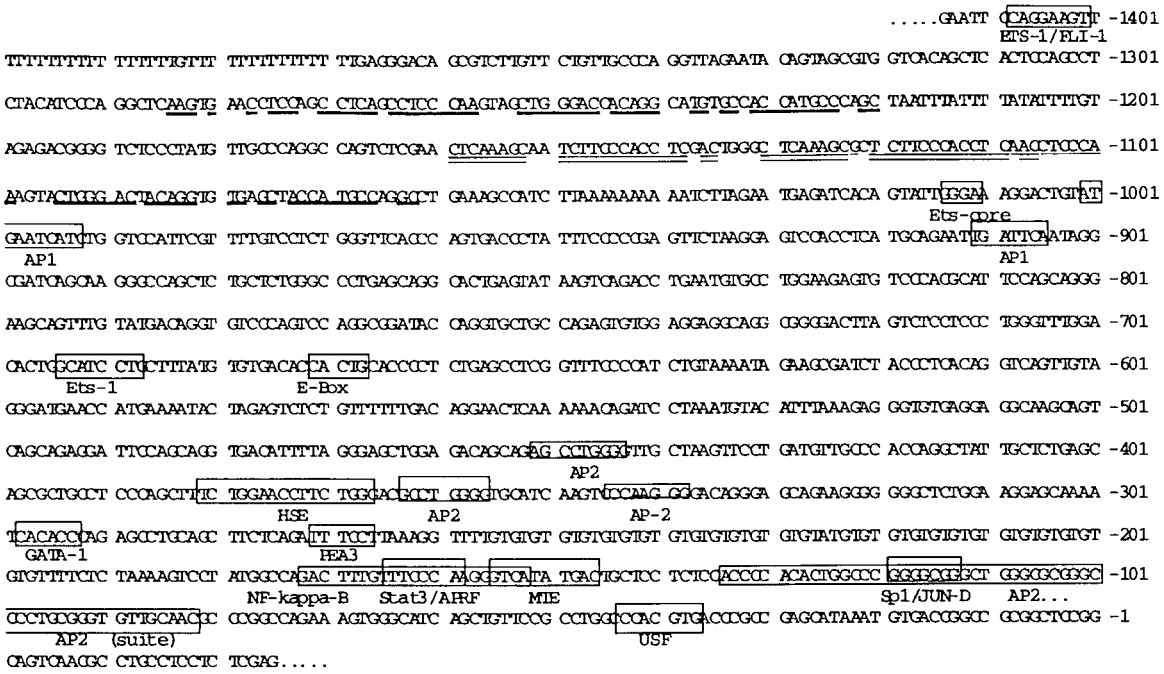

Fig. 2. Schematic representation of the human heme oxygenase-1 promoter region. Consensus binding sites and motifs ETS-1/FLI-1, ETS-core, AP1, ETS-1, E-Box, AP-2, HSE, AP-2, GATA-1, PEA3, poly[d(G-T)], NFκB, STAT3, MTE, Sp1, AP2, and USF.

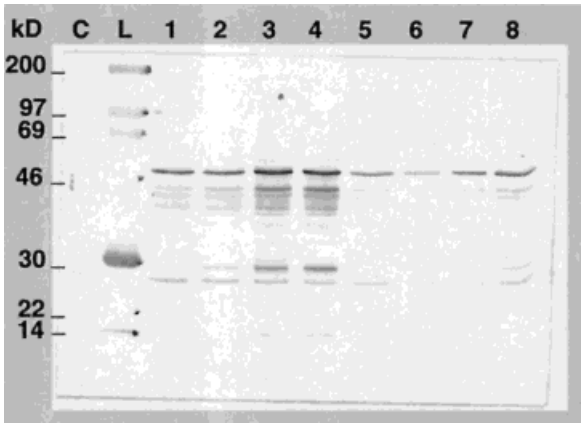

Fig. 3. Control of the efficiency of translation of the synthetic mRNAs. Western blot showing the presence of the FLI protein from 4 h (**lanes 1–4**) to 15 h (**lanes 5–8**) after injection of, respectively, 2 ng (lanes 1,2,5,6) and 10 ng (lanes 3,4,7,8) of synthetic transcript in the oocytes. No endogenous maternal FLI protein can be detected in control oocytes (**lane C**). The molecular weights were assessed by comparison with a protein standard mix (**lane L**).

levels in oocytes after injection of a synthetic *fli* mRNA. Figure 3 shows a Western blot analysis of the FLI protein, after injection of 2 ng (lanes 1, 2, 5, 6) or 10 ng (lanes 3, 4, 7, 8) of synthetic mRNA and, respectively, 4 h (lanes 1–4) and 15–h (lanes 5–8) incubations at 19°C. A control with noninjected oocytes is shown in lane C. Whereas no endogenous FLI (51-kDa) protein can be detected in the oocyte (lane C), large amounts of FLI can be observed as soon as 4 h postinjection. Significant levels are still observed 15 h after injection, despite an active degradation, clearly visualized by the low-molecular-weight bands in lanes 1–4.

HO-1 Promoter Regulation by ETS-1, FLI, and ERG Proteins in *Xenopus* Oocytes

Two different promoters of the human hemoxygenase were tested. Construct A containing a CAT reporter-gene driven by a full-length promoter and construct B containing a minimal promoter (see under Materials and Methods, for a more accurate description of the control sequences). Oocytes were injected in the cytoplasm with 2 ng of, respectively, *ets*-1, *fli*, and *erg* synthetic transcripts. At 4 h later, they were co-injected in the nucleus with DNA of the above reporter constructs and a control β-galactosidase expression vector (see under Materials and Methods). At 18 h later, the levels of β-galactosidase and CAT were mesasured (see Fig. 1).

Figure 4 shows the transcriptional levels of the reporter gene either in the absence of added

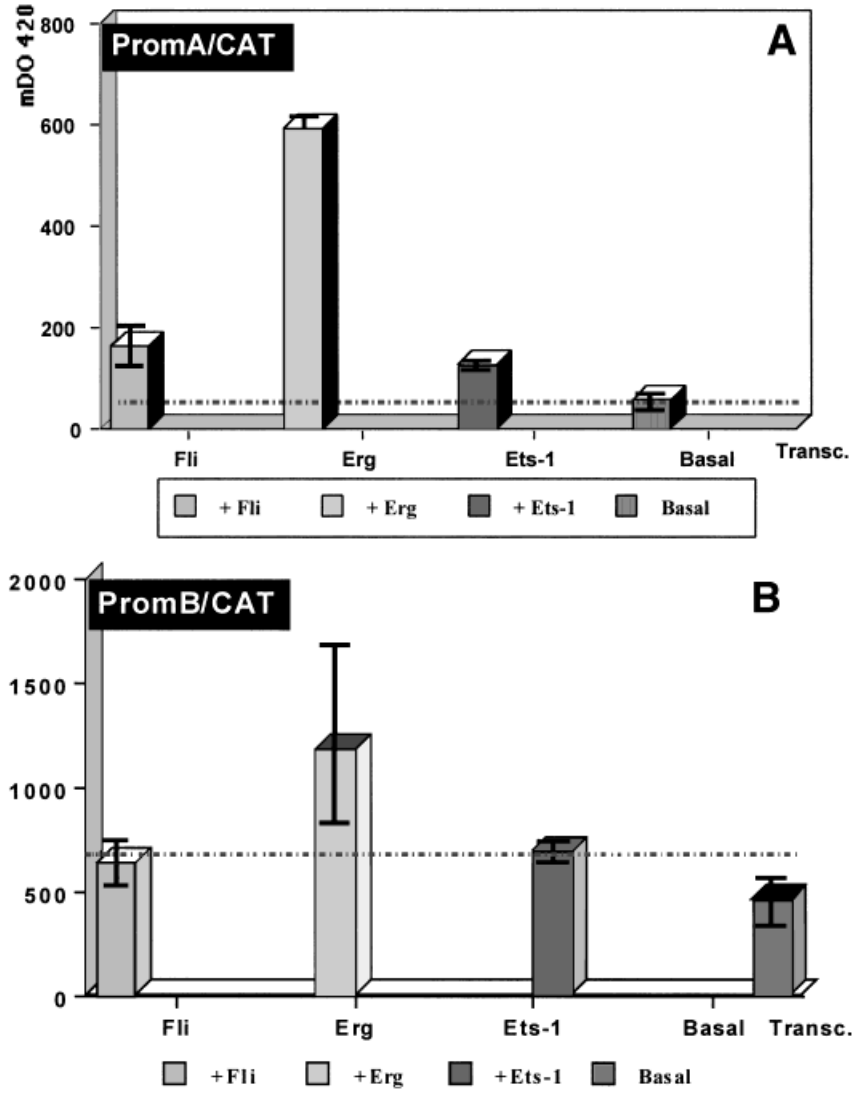

Fig. 4. **A:** Transactivation of the full-length HO-1 promoter by FLI, ERG, and ETS-1. Dotted line, mean basal transactivation. **B:** Transactivation of the reduced HO-1 promoter by FLI, ERG, and ETS-1. Dotted line, mean basal transactivation.

synthetic transcript (basal transcription) or after injection of 2 ng of synthetic mRNAs encoding the FLI, ERG, and ETS-1 proteins. All three proteins appear to have a positive effect on the "full-length" promoter (construct A). However, only ERG exhibits a significant stimulation (×10.6). The transcription stimulation observed after injection of a *fli* synthetic mRNA (×3) is underestimated, however. Indeed, we observed that increasing amounts of *fli* synthetic mRNA yielded a decreasing expression of the β-galactosidase internal standard (data not shown). A correction for this inhibition together with dose-response experiments yielded a 8.5-fold stimulation of the basal transcriptional activity for an injection of 1 ng of synthetic *fli* mRNA. The shortened "minimal promoter" does not appear to respond significantly to ETS-1, FLI, or ERG proteins, the variations observed being close to experimental errors (Fig. 4B, Table I). However, it should be noted that the basal activity of this promoter is much higher (×8.3) than that of "full-length" promoter. This results suggest the existence of negative regulation elements in the full-length HHO-1 promoter.

Chloramphenicol Acetyltransferase Assays in Endothelial Cells

We carried out a pilot experiment to assess whether constructs A and B contains a negative

TABLE I. Basal Transcriptional Activities of the "Full-Length" and Minimum HO-I Promoters and Their Stimulations by ETS-1, FLI, and ERG Proteins

pg CAT promoter	Basal	+ETS-1	+FLI	+ERG
A stimulation	56 ± 15	128 ± 8	164 ± 40	593 ± 25
	/	2.3	3[a]	10.6
B stimulation	464 ± 100	685 ± 50	632 ± 110	1,189 ± 500
	/	1.5	1.4	1.7

CAT, chloramphenicol acetyltransferase.
[a]This figure is most probably underestimated, due to marked inhibition of the general transcriptional/translational activity, as shown by the inhibition of the internal standard β-galactosidase (see text). Dose-dependent measurement of the transactivation by FLI, corrected for this inhibitory effect, shows a 8-fold stimulation of transcription for an injection of 1 ng of synthetic transcript.

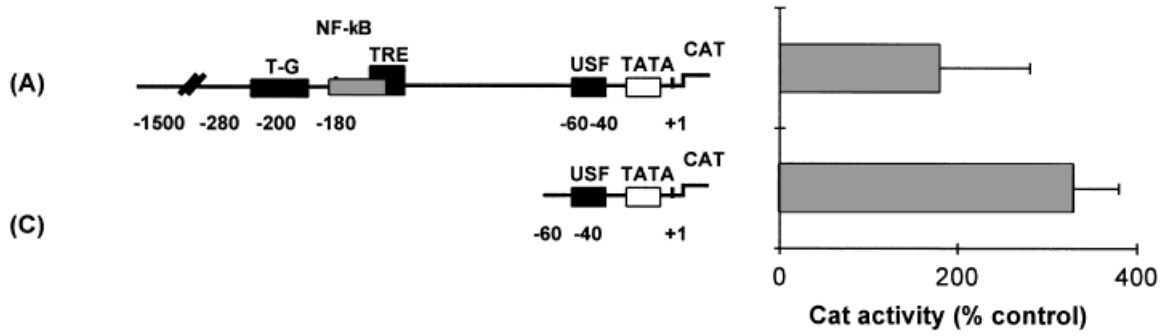

Fig. 5. Transient transfection of the human heme oxygenase promoter. Left: Schematic representation of the HHO-1 promoter (construct A) and the deleted one (construct B). Right: Corresponding corrected chloramphenicol acetyltransferase (CAT) transactivation.

regulator. We performed CAT assay in EC and results are presented in Figure 5. As seen there is a substantial increase in CAT activity of construct B of HHO-1 promoter, suggesting a presence of a negative regulator for HO-1 activity in HHO-1 promoter region between −1500 to −60.

Electrophoretic Mobility Shift Assays

We examined the binding of Ets-family proteins and nuclear proteins from endothelial cells to human HO-1 promoter. We used double-stranded oligonucleotides corresponding to the putative Ets binding sites (EBS) that might be involved in the HO-1 promoter. The binding specificity to ETS-1 was confirmed by EMSA using both specific and nonspecific competitor oligonucleotides. Nuclear extracts from control EC and from EC where HHO-1 was overexpressed were incubated with the labeled putative sequences from HHO-1 promoter. Incubation of nuclear extract from transfected cells with EBS resulted in a marked increase in binding activity (Fig. 6, lane 3). Addition of 10-fold molar excess of unlabeled nonspecific competitor did not change ets binding activity (Fig. 6, lane 5). By contrast, addition of 10-fold unlabeled specific competitor (consensus EBS) decreased the binding of nuclear extract proteins to HHO-1 oligonucleotide. Similarly, control EC nuclear extract did not result in a marked binding to ets-1 sequence on HO-1 promoter. This result suggests that overexpression of HO-1 gene may result from cross-talk between several transcription factors on binding site of HHO-1 promoter.

The HHO-1 promoter contains several regulatory motives, such as NF-κB, AP-1, and AP-2. Since AP-1 has been shown to interact with several members of *ets* gene family [Thomas et al., 1997; Westermark et al., 1997; Defossez et al., 1997], we examined whether a similar interaction, and possibly synergistic action, for binding HHO-1 promoter might be. To determine this, EMSA was performed using AP-1, ETS-1, ERG, and FLI-1 proteins. The results are presented in Figure 7. Incubation of AP-1 with ETS-1, ERG, or FLI-1 protein with a 130-bp probe containing AP-1- and Ets-binding sites showed that AP-1 synergistically enhances the

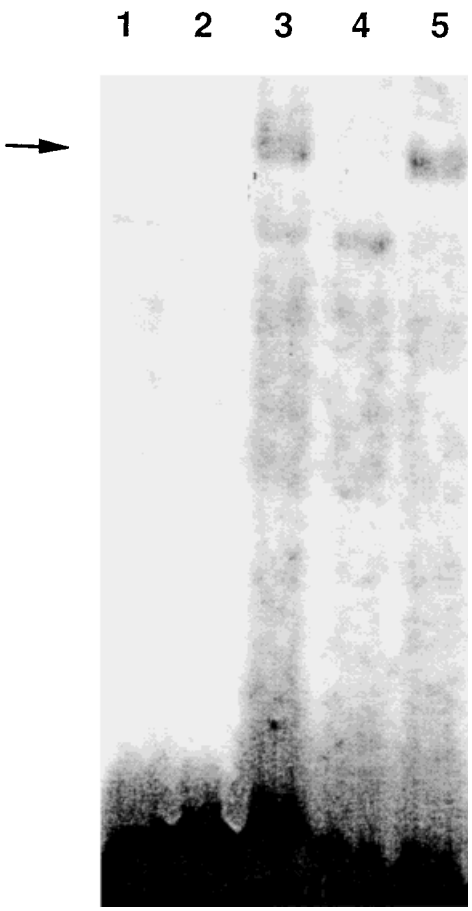

Fig. 6. Competitive electrophoretic mobility shift assay of the ETS binding site. EMSA using oligonucleotides corresponding to the ETS binding site at the position −1015 on the HHO-1 promoter. Arrow, the retarded band composed of the probe and the nuclear protein. **Lane 1**, oligonucleotide + control RCME nuclear extract; **lane 2**, the oligonucleotide alone; **lane 3**, oligonucleotide and RCME-HHO nuclear extract; **lane 4**, same as lane 3 with unlabeled ETS consensus motif as specific competitor; **lane 5**, same as lane 3 with unlabeled TFIIA consensus motif as nonspecific competitor.

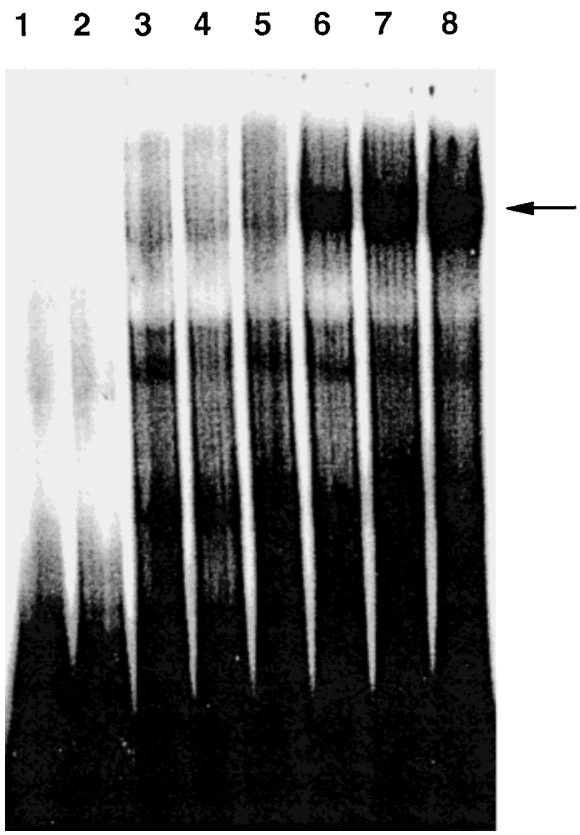

Fig. 7. Electrophoretic mobility shift assay of the AP-1 and ETS biding sites. EMSA using the fragment located between nucleotides −1076 and −946 of the HHO-1 promoter and AP-1, ETS, ERG, and FLI-1 proteins. The motives are represented in Figure 2. Arrow, the retarded band composed of the probe and both AP1 and ets family members. **Lane 1**, DNA fragment alone; **lane 2**, DNA fragment and purified AP-1 protein; **lane 3**, DNA fragment and neosynthesized ETS-1 protein; **lane 4**, DNA fragment and neosynthesized ERG protein; **lane 5**, DNA fragment and neosynthesized FLI-1 protein; **lane 6**, DNA fragment with purified AP-1 and neosynthesized ETS-1 protein; **lane 7**, DNA fragment with purified AP-1 and neosynthesized ERG protein; **lane 8**, DNA fragment and with purified AP-1 and neosynthesized FLI-1 protein.

binding of all three Ets-family proteins to HHO-1 promoter. As shown in Figure 6, lanes 6–8, a strong band was seen as compared with the absence of AP-1 protein (lanes 3–5). These results suggest a cross talk between AP-1 and *ets* gene family members on binding to HHO-1 promoter.

DISCUSSION

Members of the Ets-family of transcription factors were reported to play an important role in the control of vasculogenesis and angiogenesis, under both normal and pathological conditions [Vandenbunder et al., 1989; Wernert et al., 1992]. Several of these genes are actively expressed in the endothelial cells of forming heart and blood vessels [Mager et al., 1998; Meyer et al., 1995, 1997].

Recently, we showed that overexpression of HO-1 activity by gene transfer into EC potentially promotes angiogenesis [Deramaudt et al., 1998]. Strikingly, the human HO-1 promoter contains 26 GGAA/T core motives, potential targets for transcription factors belonging to the Ets-family. The latter usually exhibit low selectivity in binding site preference. This finding suggests that in addition to protein–DNA interactions, the selectivity of promoter elements recognition also relies on cooperation

with other transcriptional effectors or cofactors. Such interactions are also likely to allow coupling to signal transduction pathways [Hipskind et al., 1994]. Therefore, it was worth investigating a possible regulation of HO-1 promoter by Ets-family transcription factors. Among the different putative target sequences, two deserve more consideration: (1) the CAGGAAGT motif at position −1409, which is a very good consensus sequence for ETS-proteins [see the review by Sharrocks et al., 1997]; and (2) the core motif at position −1013, despite the lower homology with identified EBS, because of the vicinity of an AP1 binding sequence (−1002/−993). Indeed, Ets-family transcription factors often cooperate with AP1 complexes. Such synergistic effects have already been reported in the control of granulocyte-macrophage colony-stimulating factor (GM-CSF) transcription upon lymphocyte activation [Thomas et al., 1997; Wang et al., 1994], in the regulation of urokinase-type proteases and metalloproteinases and their inhibitors [Aho et al., 1997; D'Orazio et al., 1997; White et al., 1997; for review, see Borden and Heller, 1997]. These interactions may involve direct protein–protein contacts, as shown in several instances [Bassuk and Leiden, 1995; Logan et al., 1996; Sieweke et al., 1996; Bradford et al., 1995; Dalton and Treisman, 1992; Nerlov et al., 1992]. We therefore studied the transactivation of the human HO-1 promoter in the *Xenopus* oocyte system, upon forced expression of the ETS-1, FLI, and ERG proteins.

In this paper, we show that these proteins exhibit a transactivation potential toward the full-length promoter. The most efficient transactivation was achieved by ERG (10.6-fold), followed by FLI (3- to 8.5-fold). ETS-1 only afforded a very moderate (2.3-fold) gain of expression of the reporter gene. In the specific case of FLI, increasing amounts of synthetic transcripts yielded a nonspecific inhibition of reporter genes expressions (both for the HO-1 promoter-driven CAT activity and for the control β-galactosidase activity). Such a situation could be thought to be artifactual, simply reflecting a competition between the injected *fli* and the newly synthesized CAT or β-gal mRNA, at the level of the translation machinery. Indeed, the synthetic *fli* mRNA contains a highly efficient ribosome entry point, in the fused 5′-UTR of the globin mRNA. In agreement with this idea, the *erg* mRNA, which does not contain this 5′-UTR, although moderately activating the HO-1 promoter, does not show inhibition for increasing doses. An alternative explanation could involve lower-affinity EBS sites of the promoter leading to a negative regulation, or a squelching of other components of the transcription machinery, upon formation of specific complexes. Negative regulatory elements indeed appear to be present in the full-length promoter (between positions −1500 and −160), as the basal activity of the restricted minimal HO-1 promoter was found significantly higher (8-fold in the oocyte assay) than that of the full-length promoter. This restricted promoter (construct B) does not seem to be significantly transactivated by ERG, FLI or ETS-1 proteins, as the faint effects observed are in the same range as experimental variations. This result is in favor of specific interactions with EBS motives between positions −1500 and −160 of the full-length promoter.

Using EMSA, we have been assessing the potential binding of AP1, ETS-1, ERG, and FLI to a 131-bp probe (spanning positions −1076 to −946) and containing both the EBS core motif at position −1013 and the AP1 site at position −1002. AP1 alone does not bandshift the DNA probe and isolated ETS-1, FLI, or ERG proteins only give low to moderate amounts of complexes. Strikingly, the co-incubation of AP-1 with any of the three Ets-family proteins investigated leads to an efficient complex formation, demonstrating a synergistic binding of both transcription factors to their neighbor target sequences, suggesting a direct interaction between the proteins belonging to the two different families. Earlier studies have indeed revealed direct interactions between AP1-family and Ets-family transcription factors. These contacts appear to involve the basic domain of Jun and the DNA-binding domain (DBD) of Ets-family proteins [Bassuk and Leiden, 1995]. They could be responsible for a conformational change increasing the affinity of ETS-family proteins for the DNA sequence. One of the best studied examples is ETS-1, the DNA-binding activity of which is inhibited by tertiary interactions between an α-helix located C-terminal with respect to the ETS domain and two α-helices located N-terminally to the DBD. A conformational change involving unfolding of one of the N-terminal α-helices has been associated with relief of this intramolecular inhibition [Jonsen et al., 1996; Petersen et al., 1995].

The specificity of the binding to the EBS at position −1013 is assessed by the specific displacement of the complex upon incubation with a 10× excess of a specific competitor (consensus ETS-1 motif), whereas no displacement is observed with a nonspecific competitor (consensus TFIIA motif). Whereas no bandshift is observed when the double-stranded oligonucleotide is incubated in the presence of a nuclear extract of control EC cells, efficient complex formation occurs with a nuclear extract of EC cells overexpressing HO-1 in a stable manner.

As we showed earlier that overexpression of HO-1 by gene transfer into EC cells promotes angiogenesis [Deramaudt et al., 1998], the above results open the possibility that HO-1 overexpression leads to an "activated" state for EC, resembling the one of endothelial cells in forming blood vessels. These results suggest the possible use of HO-1 gene transfer to enhance the blood supply in pathological situation is needed for development of coronary collateral circulation to the myocardium at risk. Indeed, ETS-1 expression is high in the endothelial cells of forming blood vessels and low when those cells are in a resting state. This is true both for normal conditions, such as vasculogenesis, or for wound healing [e.g., Ito et al., 1998; Meyer et al., 1995], as well as for pathological conditions, such as the neoangiogenesis of tumors [Vandenbunder et al., 1994]. Site-specific manipulation of HO-1 may provide the means by which HO-1 can be inhibited in tumors, as well as its overexpression in myocardium. Clearly, the above results require further investigation of the regulatory sequences present in HO-1 promoter.

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