Ets-1 Regulates fli-1 Expression in Endothelial Cells

IDENTIFICATION OF ETS BINDING SITES IN THE fli-1 GENE PROMOTER

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To understand the role of the Ets-1 transcription factor during angiogenesis, we have overexpressed it in endothelial cells and analyzed the levels of expression of several candidate target genes involved in angiogenesis. The transcripts levels of the ETS transcription factor fli-1 are specifically up-regulated in endothelial cells, which overexpress Ets-1, but not in fibroblasts. Analysis of the promoter of the mouse fli-1 gene reveals that the 1-kb region that comprises the transcription starts and part of exon 1 is responsible for the response of the promoter to Ets-1. The −270/-41 fragment contains two known Spi-1-responding Ets binding sites (EBS), which are also necessary for the activation by Ets-1. In contrast to Spi-1, a third EBS is necessary for the full response of this promoter fragment to Ets-1. The rest of the promoter activity has been located in the −986/-505 region, where three active EBSs have been identified. Furthermore, endogenous Fli-1 was found to be bound to its own gene promoter and to be able to promote the transactivation of its gene. These results suggest that Ets-1 activates an auto-regulatory loop of expression of fli-1 in endothelial cells, a mechanism that could have significant implications for the endothelial cell fate.

Ets-1 is the founding member of the ETS family of winged helix-turn-helix transcription factors, which is defined by a conserved DNA binding domain that recognizes the DNA core sequence GGAW (ETS binding site or EBS).1 Ets-1 is expressed in endothelial precursors during vasculogenesis and in endothelial cells during angiogenesis (reviewed in Ref. 1). Functional EBSs have been identified in numerous promoters of genes that are involved in angiogenesis, among which Tie-1 (2), Tie-2 (3), uPA (4), Flt-1 (5), or Flk-1 (6) are directly regulated by Ets-1. In a previous work, we reported that Ets-1 lowered endothelial cell density at confluence and regulated the expression of the endothelial specific VE-cadherin gene (7) by controlling two EBSs located in the proximal part of the promoter. Here, we investigated the changes in expression of several other angiogenesis-related genes in response to Ets-1 in endothelial cells and in fibroblasts. Among the analyzed genes, we found the novel Ets-1 target gene fli-1. The fli-1 gene was first identified as a common site for retroviral integration in Friend virus-induced erythroleukemias (8). During mouse embryonic development, fli-1 messengers are detected as soon as embryonic day 8.5 in the blood islands of the extra-embryonic visceral yolk sac, the structures that also express Ets-1 and give rise to the primary endothelial cells. Afterward, fli-1 is expressed in endothelial cells throughout the embryo and in the maternal decidua (9). fli-1 gene disruption leads to early embryonic mortality; fli-1−/− embryos display vascular defects evidenced by hemorrhages in brain tissues and changes in the expression of the endothelial specific receptor Tie-2 (10, 11). In other vertebrates such as birds, amphibians, and fishes (12–14), fli-1 gene homologs are also expressed by vascular endothelial cells, suggesting a conserved role for fli-1 in vascular development. As for ets-1, fli-1 expression is not strictly limited to endothelial cells as it is also associated with the hematopoietic system development. Megakaryocytes differentiation is impaired in fli-1−/− embryos (10, 11), and mice develop erythroleukemia upon infection by the F-MuLV retrovirus wherein provirus integration results in the activation of the fli-1 gene and in Fli-1 overexpression (8, 15). A similar Fli-1 overexpression is observed in mouse erythroleukemia resulting from SFFV retrovirus infection, which activates the Spi-1 gene, another member of the ETS family (16, 17).

We show here that Fli-1 is an endothelial specific target gene of Ets-1. Ets-1 activation of the fli-1 promoter is mediated by half through the region that includes the transcription start sites; the other half of the activity is a result of the interaction of Ets-1 with several EBSs located further up in the promoter. We also show that Fli-1 is a transactivator of its own promoter.

EXPERIMENTAL PROCEDURES

Materials—The pGL2basic plasmid and the mouse fli-1 gene promoter constructions corresponding to −270/-41Luc, EBS1mut−270/-41Luc, EBS2mut−270/-41Luc, and EBS1mut−270/-41Luc (17) were kindly provided by Dr. François Morle (Centre de Génétique Moléculaire et Cellulaire, CNRS UMR 5534, Villeurbanne, France). The pCMV-β-galactosidase was from Dr. Didier Monte (CNRS UMR 8526, Institut de Biologie de Lille, Lille, France), mouse brain capillary (MBE) endothelial cells (18) from Dr. Robert Auerbach (University of Madison, Madison, WI) and Dr. Marco Presta (University of Brescia, Brescia, Italy), and mouse heart endothelioma (H5V) and 3T3 fibroblasts from Dr. Philippe Huber (Commissariat à l’Energie Atomique, Grenoble, France). Paired sample t test statistical analysis was performed using Analyse-it software.

Cell Culture—MBE, H5V endothelial, and 3T3 fibroblasts were cultured in a humidified 5% CO2, 95% air atmosphere in Dulbecco’s modified Eagle’s culture medium (4.5 g/liter glucose) containing 10% heat-inactivated calf serum (HyClone), 8 μg/ml gentamycin, 50 units/ml penicillin, 50 μg/ml streptomycin. Sodium pyruvate (1 mM) was added...
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to the MBE culture medium. Endothelial cells were cultured on gelatin-coated dishes.

Plasmid Constructions—A 3.40-kb region of fli-1 promoter and intron-1 sequence was amplified by PCR from mouse genomic DNA (Atlas, Promega). The PCR product (pFli1-Luc) was prepared from total RNA using T4 DNA polymerase blunt-end-re-joining and ligation. Site-directed mutagenesis of EBS3 and EBS4 on the various -270/-41 Luc constructs were performed by the PCR overlap extension method (19) using the oligonucleotides

CTG AAG CGT GAC AGG G oligonucleotides. The fragment was cloned into the KpnI and XhoI sites of the pGL3basic reporter vector (Promega) and named pFli1-i-Luc. Successive 5’ and 3’ intron deletions of pFli1-i-Luc were performed by digestion with KpnI and PflJFI digestion of the products, which were then cloned at the corresponding sites in pGL3basic Reporter vector (Promega). Control vectors corresponding to the EcoRI- or KpnI-PflIFI-digested, end-filled, and -ligated pFli1-Luc vector. The Fli-1 cDNA was obtained by PCR amplification from H5V total RNA using Pfu polymerase and the primer pair CAG ACT TGC CAA TAT GGA GGC GAG CAT TAA G and TAG ATG CAG CAG ATA ATC TGC AAG TGA GAG C. The amplified fragment was cloned into the pcDNA3 expression vector (Stratagene). All vectors and mutated regions were sequenced to detect any spurious changes.

mRNA Expression Analysis—RT-PCR analysis was performed as previously described (7). 40 cycles of amplification of the fli-1 isoforms were performed using the following primer pairs and MgCl2 concentrations: for Fli-1, ACA ATA TGA TCT CGC AG and ATG GAT TCT GCT, and for PflIFI digestion of the products, which were then cloned at the corresponding sites in pGL3basic Reporter vector (Promega). Control vectors corresponding to the EcoRI- or KpnI-PflIFI-digested, end-filled, and -ligated pFli1-Luc vector. The Fli-1 cDNA was obtained by PCR amplification from H5V total RNA using Pfu polymerase and the primer pair CAG ACT TGC CAA TAT GGA GGC GAG CAT TAA G and TAG ATG CAG CAG ATA ATC TGC AAG TGA GAG C. The amplified fragment was cloned into the pcDNA3 expression vector (Stratagene). All vectors and mutated regions were sequenced to detect any spurious changes.

RESULTS

Overexpression of Ets-1 in Ets-1-overexpressing Endothelial Cells—Control and Ets-1-overexpressing cells were previously established following a retroviral strategy (7). Briefly, mouse brain capillary MBE endothelial cells and 3T3 fibroblasts were infected with a MFG-TagEts1 retrovirus that provides the cells a stable expression of Ets-1 together with resistance to neomycin (MBE-Ets-1 and 3T3-Ets-1). Control cells are noninfected (3T3 and MBE) or infected with the control MFG-Neo virus (MBE-Neo and 3T3-Neo). Control cells are noninfected (3T3 and MBE) or infected with the control MFG-Neo virus (MBE-Neo and 3T3-Neo). This expression strategy results in an homogenous population of infected cells, which express Ets-1 at relatively high levels (Fig. 1, top lane) (7).

When RT-PCR analysis of expression of several endothelial specific or angiogenesis-related genes was performed, the sole gene that was significantly up-regulated in response to Ets-1 among those tested was flt-1 (Fig. 1). Moreover, and in contrast to our previous study on VE-cadherin (7), flt-1 messenger levels increased in MBE-Ets-1 endothelial cells but not in 3T3-Ets-1 fibroblasts. The transcript levels of VEGF, VEGF-B, Flt-1, Flk-1, Tie-2, EphB4, ephrinB2, PECAM-1, and Tsp-1 and of the ETS factors Ets-2, Erg, and Tel did not vary in these cell lines (data not shown).

Identification of the flt-1 Transcript Expressed in MBE-Ets-1 Cells—flt-1 regulation is quite complex, as the gene gives rise to three transcripts through the use of alternative promoters and transcription start sites and to two proteins through the
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**Fig. 1.** RT-PCR analysis of the expression of fli-1 in infected cells. Analysis of expression was done by semiquantitative RT-PCR performed on total RNA extracted from noninfected 3T3 fibroblasts and MBE endothelial cells (Ctrl), or cells that were infected with the control MFG-Neo retrovirus (−Neo) or the MFG-TagEts1 virus (Ets-1). The top lanes show the control gene transcripts Etsa-1 (positive) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; unaffected). The fli-1 transcripts are barely detectable in MBE and MBE-Neo cells but strongly overexpressed in MBE-Ets-1 cells (arrowhead). fli-1 levels do not vary in non-endothelial 3T3 fibroblasts, which express detectable amounts of fli-1.

**Fig. 2.** Analysis of Fli-1 expression in Ets-1-overexpressing cells. A, representation of the transcription and translation starts of the fli-1 gene. fli-1 transcripts are initiated either at the −398 or the −204 site, in exon-1. fli-1b transcripts are initiated at the −1842 site, in exon-1. Identification of the fli-1 transcripts by RT-PCR analysis of MBE total RNA using primer pairs that specifically amplify fli-1 (top) or fli-1b (bottom). Only fli-1 transcripts are overexpressed in MBE-Ets-1 cells. +, HS-4 and mouse embryonic day 10.5 embryo total RNA are used as positive controls of fli-1 and fli-1b amplification, respectively. B, RT-PCR analysis of the fli-1 transcripts initiated either at the −398 or both −398 and −204 sites. The −398 form is the main transcript produced in response to Ets-1 in MBE cells. Nonspecifically amplified material is indicated by an asterisk. C, analysis of Fli-1 protein expression in MBE cells. 35S-Fli-1 was immunoprecipitated from 35S-labeled MBE cells and analyzed on 10% SDS-PAGE. 3T3 cells transfected with a mouse Fli-1 expression vector are used as positive control (+). The characteristic doublet of 51- and 48-kDa Fli-1 proteins is detected (arrow); nonspecifically immunoprecipitated material is indicated by asterisks. Ctrl, control; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

The use of alternative translation start codons. In F-MuLV-infected cells, fli-1 transcripts are initiated at the −398 and −204 sites relative to the ATG+1 start codon (Fig. 2A), whereas, in SFFV-infected cells and in normal spleen, fli-1 transcripts are initiated mainly at position −204 (17). In addition, in mice, fli-1 messengers give rise to a 51- and a 48-kDa Fli-1 protein by the use of ATG+1 and a second in-frame ATG codon located 100 bp downstream of ATG+1 (20). Another fli-1 mRNA has been identified and named fli-1b. It is produced from a second promoter on the same gene and gives rise to a transcript that starts by the noncoding exon-1b and leads to the production of the 48-kDa Fli-1 protein only (26). To identify which transcripts are up-regulated in response to Ets-1 in MBE-Ets-1 endothelial cells, RT-PCR analyses were performed using oligonucleotides that specifically recognize either exon-1, to detect fli-1, or exon-1b, to detect fli-1b. MBE-Ets-1 cells clearly over-express fli-1, as seen in Fig. 2A, whereas little to no fli-1 transcript is detected in control MBE or MBE-Neo cells. On the other hand, fli-1b is detected neither in control nor in Ets-1-expressing cells (Fig. 2A, bottom). The form of fli-1 transcripts initiated at the −398 site is overexpressed in MBE-Ets-1 cells as evidenced by RT-PCR (Fig. 2B, top). This −398 site appears to be the preferential transcription initiation site in MBE-Ets-1 cells, as the transcripts levels detected using oligonucleotides that allow the amplification of both the −398 and −204 transcripts are not much different from those observed when specifically amplifying the −398 transcripts (Fig. 2B). It remains possible that a minor amount of fli-1 messengers is initiated at the −204 site.

Accordingly to the observed overexpression of the fli-1 mRNA in MBE-Ets-1 cells, higher levels of the 51-kDa Fli-1 protein are seen in these cells after immunoprecipitation with an anti-Fli-1 antibody (Fig. 2C).

**Activation of the fli-1 Gene Promoter by Ets-1**—A 3.4-kb fragment of the fli-1 gene starting 660 bp into the fli-1b promoter and extending to intron-1 was isolated, cloned, and placed in front of a luciferase reporter gene. When used in transactivation experiments, this −2437/+954 fragment responds to Ets-1 activation by 2-fold (Fig. 3, pFli1+1-Luc). Successive 5′ deletions show that the region activated by Ets-1 is contained in the −986/+954 and that removal of the −986/−505 fragment results in the loss of approximately half of this activity, suggesting that both the −986/−505 and the −505/+954 regions contain Ets-1-responsive elements. On the other hand, activation of the promoter by Ets-1 is strongly increased when intron-1 is deleted from the construct (Fig. 3, pFli1-Luc), suggesting that intron-1 is involved in a negative regulation of the fli-1 promoter.

The −270/−41 Region of the fli-1 Promoter Contains Ets-1-responsive EBS—Analysis of the fli-1 promoter was performed in two parts. First, we studied the −505/−11 fragment because it contains the −270/−41 region that has been shown to contain two essential Spi-1-responding EBSs (17), named here EBS1 (EBS 5′ in Ref. 17) and EBS2 (EBS 3′ in Ref. 17). In EMSA, Ets-1 is able to specifically bind to EBS1, and, to a lower extent, to EBS2, but not to the mutated counterparts, binding to EBS1 is more efficient as unlabeled EBS2 is a less effective competitor of binding than unlabeled EBS1 (Fig. 4A). In transactivation experiments, Ets-1 induces a significant response of the corresponding −270/−41 promoter fragment (Fig. 4B) and
mutation of EBS1 reduces this response by 25%, whereas mutation of EBS2 has a minor effect, consistent with the observed differences in binding. In contrast to the response to Spi-1 (17), mutation of both EBS1 and EBS2 has a moderate effect. Mutation of EBS1 (2400 cpm/ng) and EBS2 (3100 cpm/ng). rETS-1 binds to the normal (wild type, wt) EBS1 and, to a much lower extent, to EBS2, but not to the respective 2P-labeled mutated versions (mt). The complexes formed by rETS-1 and EBS1 are specific because they can be competed for by the addition of an increasing molar excess of unlabeled probe (EBS1 × 10, × 100, × 1000), whereas EBS2 mt does not significantly inhibit rETS-1 binding to EBS1 (EBS1 mt). In accordance to the binding properties, the activation fold of the −270/−41 fragment of the fli-1 promoter by EBS1 (EBS1 mt) reduces the activation by 25%, whereas mutation of EBS2 (EBS2 mt) has a moderate effect. Mutation of both EBS1 and EBS2 (EBS1/2 mt) reduces the promoter fragment response to ETS-1 by 41.4%. Each histogram represents the average fold of induction of normalized values ± S.D. of triplicate points. * and **, p ≤ 0.05 and 0.01, respectively, versus −270/−41-Luc. The data are representative of a set of four experiments performed in similar conditions.
This construct and the -505/-11 Luc are still responding to Ets-1 because of the presence of the active 270/41 region studied above.

Fli-1 Binds to Its Own Promoter in Endothelial Cells—To verify that Ets-1 was present on the Fli-1 EBS in endothelial nuclear extracts, we performed EMSA analysis using DNA probes that corresponded to the identified EBS and nuclear extracts from H5V cells, which express fli-1 under normal culture conditions. H5V nuclear extracts contain several ETS factors, which bind to the various identified EBSs. When adding a specific anti-Ets-1 antibody, a very discrete supershift (Fig. 8A, lane 5, open arrow) of a complex formed by Ets-1 with EBS3 is observed. In addition, the antibody supershifts a faster migrating specific band (Fig. 8A, lanes 5 and 7, open arrowhead), which most probably corresponds to a degradation product of Ets-1. Such discrete Ets-1 supershifts are also observed with EBS1, EBS2, and EBS F (data not shown). On the other hand, when testing for the presence of Fli-1 in these complexes, a strong supershift was detected with EBS3 (lanes 6 and 8 in Fig. 8A, filled arrow), with EBS B (lane 7 in Fig. 8B, filled arrow), and with EBS1 and EBS2 (data not shown), together with EBS4 (lanes 9 and 10, filled arrow). Such discrete Ets-1 supershifts are also observed with EBS1, EBS2, and EBS F (data not shown). On the other hand, when testing for the presence of Fli-1 in these complexes, a strong supershift was detected with EBS3 (lanes 6 and 8 in Fig. 8A, filled arrow), with EBS B (lane 7 in Fig. 8B, filled arrow), and with EBS1 and EBS2 (data not shown), together with EBS4 (lanes 9 and 10, filled arrow).

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to induce a significant response of its own promoter (Fig. 9A). Deletion of the −986/−505 fragment leads to an approximately 40% decrease of the response, because of the loss of the EBS A−E region. Removal of intron-1 results in a marked increase in the promoter response. Furthermore, Fli-1 is also active on the −270/−41 promoter region. Mutation of EBS1 has almost no effect on the promoter response to Fli-1, whereas mutation of EBS2 results in the loss of approximately half of this activity (Fig. 9B), contrasting with the fact that the activation by Ets-1 was slightly more sensitive to EBS1 than to EBS2 mutation (Fig. 4). Mutation of both EBS1 and EBS2 does not result in the complete loss of the response to Fli-1, and the remaining activity is mediated by EBS3, as evidenced by the complete loss of activity of the EBS1/2/3 mutant (Fig. 9B). EBS4 has no relevant role because the EBS1/2/4 mutant displays an activity similar to that of the EBS1/2 mutant.

**DISCUSSION**

This work was originally undertaken to identify novel target genes of Ets-1 in endothelial cells. It was initiated on the basis of several reports, which suggested that Ets-1 could be important in endothelial cells during angiogenesis; in the embryo, Ets-1 transcripts are expressed in endothelial precursors and in endothelial cells (27–30) and also in various situations where endothelial cells undergo capillary formation, such as during wound healing (31) or vascularization of solid tumors in the adult (31, 32). Furthermore, Ets-1 expression in endothelial cells in vitro is increased by angiogenic factors (31, 33). We previously showed that Ets-1 increased the expression of the endothelial specific VE-cadherin gene together with a reduction of cell density at confluence of endothelial cells, suggesting that, although Ets-1 is not an endothelial specific transcription factor, it may participate to the definition or the maintenance of the quiescent endothelial phenotype through the regulation of this specific cadherin (7). Here, analysis of the expression of other angiogenesis-related genes in Ets-1-overexpressing cells was rather deceiving because most of them showed no significant changes of expression, although many of them had been shown to contain active ETS in their promoters (see Ref. 1 for review). Our results are, however, consistent with a recent report in which the authors used an adenoviral strategy to induce Ets-1 expression in human endothelial cells. DNA array analysis of the gene profile showed that the levels of expression of Tie-1, flt-1, the flk-1 human homolog KDR, EphB4, ephrinB2, or uPA genes did not vary either upon overexpression of Ets-1, whereas those of VE-cadherin, together with the VEGF receptor neuropilin-1, ephrin-A1, angiopoietin-2, the uPA receptor, or FGF-R1, increased slightly (Ref. 34; the status of fli-1 was rather deceiving because most of them showed no significant variations because of the use of an adenoviral strategy for Ets-1 induction). From this work and ours, it can be stated that Ets-1 is probably not a master transcription factor that controls the endothelial cell phenotype but rather participates in the regulation of few genes related to a particular cell metabolism such as cell quiescence, endothelium integrity (7), or possibly apoptosis (35–37).

Checking for the expression of ETS members in the same context was also motivated by several facts; erg and fli-1 are co-expressed with fli-1 in endothelial cells of the developing embryo (12, 27, 38, 39), inactivation of the fli-1 gene leads to hemorrhages in the neural tissue, possibly because of a disruption of the endothelial integrity (10, 11), and inactivation of tel in the mouse induces a lack of branching of the vitelline vessels (40). Among the ETS genes tested, fli-1 was the only one to show a significant variation of transcript levels following Ets-1 expression. This was very interesting because the regulation of the fli-1 gene in endothelial cells had not been studied before. In the embryonic context, this suggests that the expression of ets-1 in early endothelial progenitors of the blood islands can
control the subsequent expression of fli-1 in these cells. This is further strengthened by the fact that Ets-1 induces an increase of fli-1 expression only in MBE (and IBE; data not shown) capillary endothelial cells and not in 3T3 or L929 fibroblasts (data not shown), suggesting that the fli-1 promoter contains undocumented endothelial specific response elements placed under the control of ETS transcription factors. In an attempt to identify these elements, we have analyzed the activity of the 3.40-kb fragment of the fli-1 promoter in endothelial (MBE) versus fibroblasts (3T3) cells by transient transfection; however, no differences in activity could be detected (data not shown), either because the region involved in the endothelial specific regulation is not contained in the 3.40-kb fragment or because the surrounding chromatin structure plays a role in the specific response, a situation that is not reproduced in transient transfection. The search for these endothelial specific response elements needs to be pursued.

The levels of activation of the −2437/+954 fli-1 promoter region by Ets-1 (2.5-fold) correspond to the levels obtained on the VE-cadherin (7) or the GATA-1 (41) promoters. Still, this activation seems low when looking at the high levels of fli-1 transcripts detected by RT-PCR in Ets-1-expressing cells. This is probably because of the fact that fli-1 transcripts accumulate in response to the constitutive expression of Ets-1 in these cell lines. This also raises the question of whether the entire region of the promoter was analyzed; because the fli-1 promoter is located upstream of the fli-1 promoter and because these promoter regions are independent (Fig. 2 and Ref. 26), it is most probable that the regulatory regions of fli-1 are indeed located between exon-1b and exon-1. Nevertheless, we chose to analyze a large gene fragment (−2437/+954), which encompasses both fli-1 and fli-1b regulatory regions. This fragment includes the 1.5-kb region located upstream of exon-1 (the fli-1 promoter), plus exon-1b and 660 bp of fli-1b promoter, including the corresponding −410/−150 region of the fli-1b promoter, which was shown to control the transcription of exon-1b in human (26); this region as well as the upstream sequences are conserved in the mouse gene. Because fli-1b is not regulated in response to Ets-1 in endothelial cells (Fig. 2) and because the deletion of the fli-1b regulatory regions does not affect Ets-1 activity on the promoter fragment (Fig. 3, −1397/+954), it seems that the fli-1b regulatory regions are not involved in the regulation of the gene by Ets-1 or Fli-1 and that the main active regions controlling fli-1 expression are indeed those placed after exon-1b.

The regulation of the fli-1 gene by Ets-1 further illustrates the fact that ETS factors can specifically control the expression of other transcription factors of the same family, a situation that had been described in SFFV-infected cells where Fli-1, and not Ets-1, Ets-2, Elf-1, or GAPB/β, is specifically overexpressed in response to Spi-1 (17). This study and ours addressed the regulation of the fli-1 gene, making it probable that this particular gene is specifically regulated by other ETS factors depending on the cell context. On that matter, the auto-regulatory loop of Fli-1 supports that same idea. It had been suggested by two earlier reports where fli-1 transcripts detected by RT-PCR in Ets-1-expressing cells. This now clearly show that Fli-1 is a positive regulator of its own gene transcription. Further, our observations suggest that the transcriptional mechanisms involved in the regulation of the fli-1 gene by Spi-1, Ets-1, and Fli-1 itself are not similar; both EBS1 and EBS2 are clearly critical for the activity of Spi-1 in erythroleukemic cells (17) and for Ets-1 and Fli-1 here. However, a more dramatic effect of the mutation of EBS1 on the
response of the promoter to Ets-1 is noticed here (Fig. 4), whereas Spi-1 (17) and Fli-1 (Fig. 9B) are more sensitive to the mutation of EBS2. Furthermore, no remaining activity of the promoter in response to Spi-1 was reported when EBS1 and EBS2 were mutated (17), whereas we found that Ets-1 and Fli-1 are still active on this double mutant, leading to the identification of EBS3. Finally, Ets-1 and Fli-1 show other differences on the regulation of the fli-1 promoter as, when normalizing to the values of the empty vector, Fli-1 and Ets-1 activate the −2437/−11 promoter region almost as well (3.5-fold (Fig. 9) and 2.5-fold (Fig. 3), respectively), but Fli-1 shows a 4-fold stronger activity than Ets-1 on the −270/−41 region (Fig. 9B), probably in relation to its stronger activity on EBS2. These differences show that the regulation of the fli-1 gene by different ETS factors can be finely tuned through the use of the various EBSs by these factors. ETS factors are indeed quite different although they recognize a common DNA core and share a closely related DNA-binding domain; Spi-1, on the one hand, and Ets-1 and Fli-1, on the other, recognize different flanking DNA sequences around the GGAW EBS core (43). They also regulate different target genes as Spi-1 is mostly an activator of myeloid and lymphoid genes, an activity related to its restricted expression in these lineages (43), whereas Fli-1 possibly activates GPIX and Tie2 (11) but represses the expression of Rb (44) and Ets-1 has a much larger spectrum of known target genes (7, 34, 43, 45).

In relation to these noted differences in the transcriptional activators of the fli-1 gene, we also found that fli-1 mRNA transcription was mainly initiated at the −398 site in MBE-Endothelial cells, a situation similar to that observed in F-MuLV-transformed cells, whereas in SFFV-infected cells, which overexpress Spi-1, fli-1 transcripts are mainly initiated at the −204 site (17). Although the mechanisms responsible for the selection of the start sites are not known, these variations could be related to a different access of the transcription factors to exon-1 because this exon was shown to form an H-DNA structure (46) that may modulate transcription factor binding.

The role of the fli-1b RNA isoform is not clear. fli-1b transcripts have originally been identified in two human cDNA libraries and found to be expressed in pre-B cells (26), but other cell lines of different origin also express this transcript, although at lower levels (47). Fli-1b is absent from NIH-3T3 cells (47) as observed here (data not shown), and we found no evidence of its expression in MBE endothelial cells. The 48-kDa coded protein corresponds to an alternatively initiated protein of the original fli-1 transcript, which conserves the transcriptional activity of Fli-1 (26). This suggests the possible existence of a (tissue?) specific regulation of the fli-1 gene expression leading to the formation of the same protein. In that regard, the nature of the fli-1 transcripts detected in early endothelial cells during embryonic development is not known; it remains possible that fli-1b mRNA account for some of the detected signals. Regarding the fli-1b promoter region, several potential response elements have been localized, their analysis has not been done (26), and our results suggest that this promoter is not regulated by Ets-1 or Fli-1.

During the isolation of the 3.40-kb fragment of the fli-1 gene, restriction and sequence analysis revealed the presence of an additional 225 bp fragment (GenBank™ accession no. AF483910) starting 520 bp downstream of the intron-1 splice donor, which had not been seen earlier (46). This fragment was identified in mouse genomic DNA extracted from H5V cells, a cell line established from C57BL/6 strain, and also from Balb/c genomic DNA (CLONTECH), and from an NIH/3Swiss phage library, indicating that this fragment does not derive from polymorphic variations or PCR artifacts.

In this intron-1, one EBS had been shown to bind unidentified ETS-related factors (48). These factors may well be Fli-1 and Ets-1 and we found that both were able to bind to this isolated EBS in EMSA experiments. However, despite this binding, we found no enhancer activity of the isolated intron-1 in response to Ets-1 or Fli-1; rather, we found that intron-1 had a strong inhibitory activity on the activation of the fli-1 promoter by both factors. This inhibitory activity is reminiscent of the fli-1 gene, where the first intron strongly represses the activity of the promoter, possibly because of the presence of a transcription arrest site (49). The fli-1 intron-1 contains the GCC Ggc ACC caT T sequence, which is very close to the adenosine deaminase gene transcription arrest consensus (uppercase letters; Ref. 50), including the essential bases (bold). Whether this site is responsible for the dramatic effect of intron-1 on the response of the fli-1 promoter to Ets-1 and Fli-1 and whether the ETS factors detected on the intron participate in the negative regulation of the fli-1 gene remain to be determined.

By showing that Fli-1 is a target gene of Ets-1 in endothelial cells, our work raises the question of the role of Fli-1 in these cells and in angiogenesis. We found that VE-cadherin is overexpressed in Ets-1-cells and that Fli-1 is able to activate the VE-cadherin promoter in vitro (data not shown). We initially thought that Ets-1 and Fli-1 could therefore act together or in cascade in endothelial cells to promote the VE-cadherin gene in this cellular context. However, the VE-cadherin levels are not noticeably different in fli-1−/+ versus fli-1−/− embryos (11), making it less probable that Fli-1 participates to the regulation of this gene in vivo, safe for a rescue of the gene by other ETS members in fli-1−/− mice. A role of Fli-1 in the reduction of cell density at confluence seen in Ets-1-overexpressing endothelial cells (7) was also possible because Fli-1 had been associated with the regulation of cell proliferation and survival (44, 51, 52). The role of Fli-1 in these processes is unfortunately not clear yet; attributing a role to this transcription factor in the phenotype observed in endothelial cells that overexpress Ets-1 is, therefore, still risky.

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