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Fli-1 Inhibits Collagen Type I Production in Dermal Fibroblasts via an Sp1-dependent Pathway*

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Fibrosis is characterized by the excessive deposition of extracellular matrix (ECM), especially collagen. Because Ets factors are implicated in physiological and pathological ECM remodeling, the aim of this study was to investigate the role of Ets factors in collagen production. We demonstrate that the expression of collagenous proteins and collagen α2(I) (COL1A2) mRNA was inhibited following stable transfection of Fli-1 in dermal fibroblasts. Subsequent analysis of the *COL1A2* promoter identified a critical Ets binding site that mediates Fli-1 inhibition. In contrast, Ets-1 stimulates *COL1A2* promoter activity. *In vitro* binding assays demonstrate that both Fli-1 and Ets-1 form DNA-protein complexes with sequences present in COL1A2 promoter. Furthermore, Fli-1 binding to the *COL1A2* is enhanced via Sp1-dependent interaction. Studies using Fli-1 dominant interference and DNA binding mutants indicate that Fli-1 inhibition is mediated by both direct (DNA binding) and indirect (via protein-protein interaction) mechanisms and that Sp1 is an important mediator of the Fli-1 function. Furthermore, experiments using the Gal4 system in the context of different cell types as well as experiments with the *COL1A2* promoter in different cell lines demonstrate that the direction and magnitude of the effect of Fli-1 is promoter- and cell context-specific. We propose that Fli-1 inhibits *COL1A2* promoter activity by competition with Ets-1. In addition, we postulate that another factor (co-repressor) may be required for maximal inhibition after recruitment to the Fli-1-Sp1 complex. We conclude that the ratio of Fli-1 to Ets-1 and the presence of co-regulatory proteins ultimately control ECM production in fibroblasts.

ECM[1] remodeling is a complex and tightly regulated process that occurs during embryogenesis, the female reproductive cycle, angiogenesis, and wound repair. However, in the majority of normal adult tissues only limited turnover of the ECM takes place. In contrast, in many pathological conditions the balance between ECM synthesis and degradation is disrupted, leading to abnormal ECM remodeling. Excessive ECM breakdown is associated with rheumatoid arthritis, osteoarthritis and periodontitis as well as tumor invasion and metastasis (1–4), whereas excessive ECM deposition occurs in fibrotic diseases such as scleroderma, liver cirrhosis, and glomerulosclerosis (5–7). The type I collagen, a product of two coordinately regulated genes, *α1(I)* and *α2(I)* (*COL1A2*), is a predominant ECM component of the fibrotic lesion. Studies using diverse experimental systems have indicated that transcriptional regulation of the type I collagen genes is species- and tissue-specific (5). The *COL1A2* promoter has been extensively used as an experimental model system to delineate transcriptional regulation of the collagen gene in human tissues. This promoter constitutively exhibits high levels of expression in activated dermal fibroblasts, which are "turned on" by routine *in vitro* tissue culture conditions such as growth on plastic in the presence of serum. Previous studies have identified the ubiquitous factors, Sp1 and Sp3, and CAAT binding factor as primary activators of this promoter (9, 10). In contrast, very little is currently known about the nature of the transcriptional repressors that may counteract or modulate the potent stimulatory effects of Sp1 and Sp3 on this promoter. The activity of the *COL1A2* promoter can be modulated further by transcriptional factors mediating cytokine responses. Thus, Smad3 in cooperation with p300/CREB-binding protein mediates activation of this promoter by TGF-β (8, 11–13), whereas CAAT/enhancer-binding protein as well as nuclear factor-κB seem to be involved in tumor necrosis factor-α inhibition of the *COL1A2* activity (14, 15). Moreover, recent studies provide evidence for Sp1 as a critical mediator of TGF-β induction of this promoter via interaction with Smad3 (8, 16).

Ets proteins comprise a family of transcription factors with over 30 members identified to date that are characterized by the presence of a highly conserved DNA binding domain termed the Ets domain (17). Ets proteins are grouped into subfamilies based on the sequence similarity of the Ets domain and its location within the protein as well as additional sequence similarities (17). Fli-1 and the closely related Erg are the members of the ERG subfamily. Extensive *in vitro* studies as well as the data obtained from various Fli-1 null mice support a crucial role for Fli-1 in hematopoiesis (18). Fli-1 is also expressed in embryonic tissues at the sites of cell migration and epithelial-mesenchymal transition, suggesting additional functions during development (19). It may be relevant that Fli-1 is an inducer of tenascin-C, an ECM molecule, which is also expressed at the sites of cell migration (20). However, the specific role of Fli-1 in these processes is presently not well

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[1] The abbreviations used are: ECM, extracellular matrix; *COL1A2*, type I collagen α2(I); TGF, transforming growth factor; MMP, matrix metalloproteinase; EBS, Ets binding site; EMSA, electrophoretic mobility-shift assay; DBD, DNA binding domain; bp, base pair; GABP, GA-binding protein.

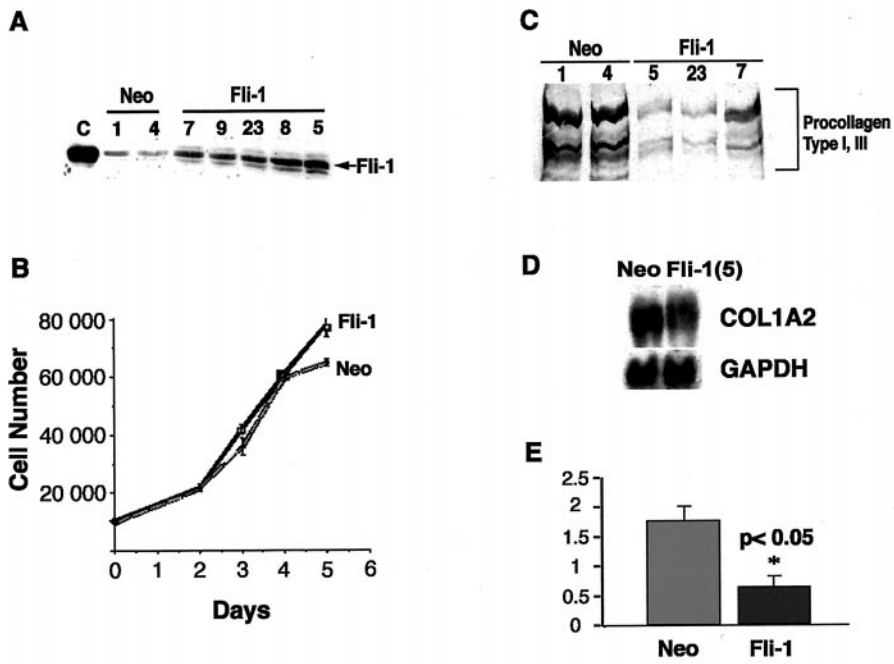

FIG. 1. **Characterization of stable transfectants overexpressing Fli-1.** *A*, Fli-1 protein levels in independent dermal fibroblast clones carrying empty vector (*Neo*) or Fli-1 cDNA. In the first line (*c*) an *in vitro* translated (TNT) Fli-1 was used. The expression level of Fli-1 was examined by Western blot with a polyclonal anti-human Fli-1 antibody. *B*, growth curve of Fli-1 (clone 5) and control transfectants. *C*, Fli-1 overexpression decreases collagen protein level. Newly synthesized collagenous proteins in control (*Neo*) and Fli-1 stable transfectants were measured in a [^{3}H]proline incorporation assay. Conditioned medium normalized for cell number was analyzed for collagenous protein content via SDS-polyacrylamide gel electrophoresis and fluorography. *D*, Fli-1 overexpression inhibits *COL1A2* mRNA expression. The RNA isolated from the control (empty vector) and Fli-1 stable transfectants was analyzed simultaneously by Northern blot. Blots were hybridized with probes for *COL1A2* and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as the loading control. *E*, a summary of quantitative analysis of the Northern blots from three different clones. All values were corrected for the loading differences by normalizing to the glyceraldehyde-3-phosphate dehydrogenase mRNA intensity.

understood. Targeted disruption of the Fli-1 gene resulted in hemorrhage into the neural tube and the ventricles of the brain at E11, resulting in embryonic death shortly thereafter. In addition to hematopoietic defects, these mutant embryos demonstrated a disruption of basement membrane tissues (18).

There is increasing evidence that some Ets proteins, particularly the members of Ets-1 and E1AF/PEA3 subfamilies, play an important role in regulating transcriptional activation of ECM-degrading enzymes including serine proteases and matrix metalloproteinases (MMPs) (21, 22). Extensive previous studies of the polyoma virus enhancer as well as urokinase plasminogen activator and several MMP promoters have established a paradigm for the role of Ets in the regulation of this group of genes (23). These promoters contain functional Ets binding site(s) (EBS(s)) and AP-1 site(s). The members of the Ets and Jun families that bind to the Ets-AP-1 composite element are also targets of the Ras-mitogen-activated protein kinase signaling pathway (24). Thus, the transcriptional activation of this group of genes depends on the nature of interacting proteins (both Ets and AP-1 are multiprotein families) as well as the exogenous stimuli activating the Ras cascade (25).

Based on our recent observation that a matrix protein, tenascin-C, is also regulated by Ets factors (20), we postulated that the role of Ets in ECM remodeling may not be limited to regulation of the degradative pathways but may also include regulation of synthesis of the ECM proteins (26). To test this hypothesis we selected a well characterized system with physiological and pathological relevance, the human *COL1A2* gene. In the present study we demonstrate that the collagen gene is indeed a target for Ets factors. Significantly, we provide evidence that Fli-1 acts as a transcriptional repressor of this gene, whereas Ets-1 acts as an activator. Furthermore, we show that cellular context plays an important role in defining both the magnitude and direction of response to Fli-1.

MATERIALS AND METHODS

Cell Culture and Generation of Stable Transfectants—Human dermal fibroblast cultures were established from newborn foreskins obtained from the delivery suits of local hospitals. Foreskin tissue was dissociated enzymatically by 0.25% collagenase type I (Sigma) and 0.05% DNase (Sigma) in Dulbecco's modified Eagle's medium with 20% fetal calf serum (Life Technologies, Inc.). Human mesangial cells were purchased from Clonetics (Walkersville, MD). HepG2 and HeLa cells were purchased from the American Type Culture Collection (Manassas, VA). Fli-1 and control stable transfectants were generated in human foreskin fibroblasts transfected by the calcium phosphate technique with either pSG5 Fli-1 or pSG5 control vector and were grown in the presence of 100–200 μg/ml of geneticin (Life Technologies, Inc.). Individual antibiotic-resistant colonies were expanded and analyzed for the expression of Fli-1 protein.

Procollagen Analysis by [^{3}H]Proline Incorporation, SDS-Polyacrylamide Gel Electrophoresis, and Autoradiography—The analysis of labeled proline incorporation into secreted protein was performed as described previously (27). Fibroblasts were plated in 12-well plates and grown to visual confluency. The medium was changed to serum-free medium (Dulbecco's modified Eagle's medium) supplemented with 50 mg/ml ascorbic acid and 64 mg/ml β-amino propiontrile for the duration of the experiment. After a 24-h incubation in serum-free medium, 10 μCi/ml L-[2,3,4,5-^{3}H]proline (specific activity 3.6 TBq/mmol) (Amersham Pharmacia Biotech) was added for 24 h. The medium was harvested from each well, and the cells were trypsinized and counted. The medium was dehydrated in a SpeedVac (Savant) and resuspended in SDS/dithiothreitol sample buffer and boiled to denature. The volume of sample buffer added for resuspension was normalized according to the measured cell count of each well. After electrophoresis, gels were enhanced by immersion in 2,5-diphenyloxazole and visualized by autoradiography.

Western Blot—To determine the levels of expression of Ets-1 and Fli-1 proteins, total cell lysates (100 μg/lane) were used. To detect Ets-2 protein, nuclear extracts (35 μg/lane) were used. Samples were electrophoresed in 12% SDS-polyacrylamide gel and transblotted onto polyvinylidene difluoride membrane (Millipore). After blocking with 5% milk, the membranes were incubated with primary antibodies, followed by incubation with horseradish peroxidase-conjugated secondary antibod-

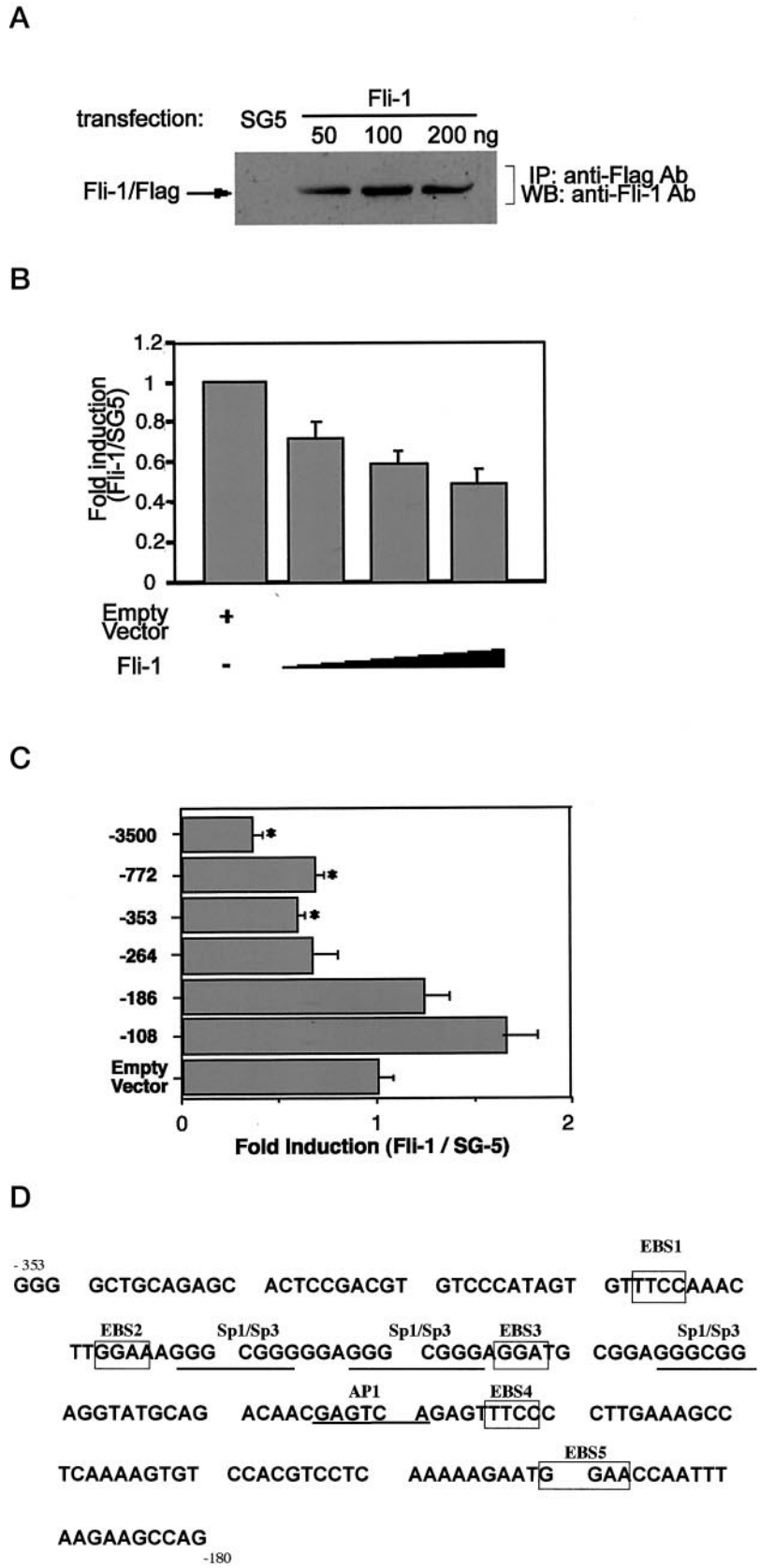

FIG. 2. **The effect of Fli-1 overexpression on the *COL1A2* promoter activity and identification of the *COL1A2* promoter region mediating Fli-1 inhibition.** *A*, expression of exogenous Fli-1 protein. Dermal fibroblasts were transfected with indicated amounts of Fli-1/Flag expression vector or empty vector (pSG5) (line 1). Protein levels of Fli-1/Flag in transfected cells were analyzed by immunoprecipitation with anti-Flag M2 monoclonal antibody (Sigma), and Western blot of the precipitates was analyzed with anti-Fli-1 polyclonal antibody. Equivalent amounts of protein were used for the immunoprecipitations. *B*, dose-dependent inhibition of the −353*COL1A2/CAT* promoter by Fli-1. The *COL1A2* promoter construct (0.1 μg) was co-transfected with increasing concentrations of Fli-1 expression vector. To ensure an equal amount of co-transfected expression vectors under each condition, appropriate amounts of pSG5 were added to individual co-transfections. *C*, transient transfections of human dermal fibroblasts with the indicated deletion constructs of the *COL1A2/CAT* promoter and either empty vector (pSG5) or Fli-1 expression vector. The *bar graph* represents the -fold induction of the promoter activity of each construct co-transfected with Fli-1 vector relative to the activity of the promoter co-transfected with empty vector (pSG5), which was arbitrarily set at 1. The average of at least four independent experiments is shown. The Fli-1-responsive element was mapped between −353 and −186. (*, $p \leq 0.05$). *D*, the nucleotide sequence of the *COL1A2* promoter region between bps −353 and −186. The five potential Ets binding sites are *boxed*. Sp1/Sp3 binding sites are *underlined*.

ies and washing with Tris-buffered saline buffer. Immune complexes were detected using enhanced chemiluminescence (Amersham Pharmacia Biotech). The monoclonal anti-Ets-1 antibody was obtained from Transduction Laboratories, the polyclonal anti-Ets-2 antibody was obtained from Santa Cruz Biotechnology, and the polyclonal anti-Fli-1 antibody was described previously (18).

Northern Blot—Total RNA was extracted from confluent fibroblasts and analyzed by Northern blotting as described previously (27). Filters were sequentially hybridized with radioactive probes for α2(I) procollagen and glyceraldehyde-3-phosphate dehydrogenase. The filters were scanned with a PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA).

TABLE I
List of the EBS mutants in the COLIA2 promoter

EBS mutants	Wild-type sequence	Mutation
1,2	GTTTCCAAACTTGGAAAGGG (−320) (−300) CGGGAGGATGC (−290) (−279)	GTTTAAAAACTTTTAAAGGG (−320) (−300) CGGGAGTATGC (−290) (−279)
3	AGAGTTTCCCCTT (−250) (−238)	AGAGTTTAACCTT (−250) (−238)
4	AAGAATGGAACCAA (−207) (−194)	AAGAATTTAACCAA (−207) (−194)
5		

Plasmid Constructs—The −353 *COL1A2* construct containing sequences between −353 and +58 linked to the CAT reporter gene was described previously (10). The −353 *COL1A2/LUC* was generated by recloning the −353 to +58 promoter fragment into pGL2 basic (Promega). The EBS promoter mutants were constructed by replacing guanosine in the EBS core sequence with thymidine using the −353 *COL1A2* promoter construct as a template with the use of the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's specifications (Table I). EBS mutations used in electrophoretic mobility-shift assays (EMSAs) contain EBS 1–4 mutations. The *COL1A2* promoter AP-1 binding site mutation was generated as described previously (28). Fli/W321R contains a single amino acid mutation that abolishes DNA binding. Fli/DBD contains DNA binding domain 267–384. pSG5Fli-1, pSG5Ets-1, and pSG5Ets-2 were described previously (20). Gal4-Sp1 and Gal4-Sp3 were a generous gift of G. Suske (Marburg University, Germany). The PG5luc vector was purchased from Promega.

Cell Culture Transfection and Reporter Gene Assays—Transient transfections with the indicated reporter, expression, and control constructs were performed in duplicate in 6-well plates using FuGene 6 reagent (Roche Molecular Biochemicals) according to the manufacturer's specifications. Transfections were repeated at least four times using two different plasmid preparations. CAT and luciferase activities in aliquots containing equal amounts of protein were determined 48 h post-transfection.

Preparation of Nuclear Extracts—Nuclear extracts were prepared according to Andrews and Faller (29) with minor modifications. *In vitro* transcribed and translated human Fli-1, Ets-1, and Ets-2 proteins were prepared using the TNT coupled reticulocyte lysate system (Promega).

DNA Binding Assays—EMSAs were performed with ^{32}P-labeled probes as described previously (30). Briefly, 7 μg of nuclear extracts were incubated for 30 min on ice in 24 μl of the binding buffer (20 mM HEPES-KOH, pH 7.9, 50 mM KCl, 1 mM $MgCl_2$, 0.2 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 10% glycerol, 10 μg/ml aprotinin, 2 μg/ml leupeptin, and 2 μg/ml pepstatin) containing 50,000-cpm labeled probe and 2 μg of poly(dI-dC)-poly(dI-dC) (Amersham Pharmacia Biotech). In some assays, double-stranded competitors (50-fold molar excess) or antibodies were preincubated with nuclear extracts 30 min prior to the addition of a radioactive nucleotide probe. Polyclonal anti-Sp1 and anti-Sp3 antibodies were purchased from Santa Cruz Biotechnology, and monoclonal anti-Ets-1 was purchased from Transduction Laboratories. Polyclonal anti-Fli-1 antibody was characterized previously (18). The separation of free radiolabeled DNA from DNA-protein complexes was carried out on a 5% nondenaturing polyacrylamide gel. Electrophoresis was performed in 0.5× Tris borate electrophoresis buffer at 250 V at 4 °C. The gels were dried and exposed to x-ray film at −80 °C.

Statistical Analysis—The Mann-Whitney *U* test or Wilcoxon test was used to determine statistical significance.

RESULTS

Fli-1 Inhibits Production of Collagenous Proteins and mRNA in Human Dermal Fibroblasts—In the first set of experiments we asked whether Fli-1 regulates ECM production by generating stable Fli-1 transfectants in human dermal fibroblasts. Several clones that exhibited elevated levels of Fli-1 protein were selected for further investigation (Fig. 1*A*). Overexpression of Fli-1 in dermal fibroblasts did not affect their proliferative rate (Fig. 1*B*). To assess the effect of Fli-1 on the production of collagenous proteins, conditioned medium from cells metabolically labeled with [^{3}H]proline was analyzed by SDS-polyacrylamide gel electrophoresis. As shown in Fig. 1*C*, increased expression of Fli-1 protein inversely correlated with the production of collagenous proteins. These data indicate that elevated expression of Fli-1 has a potent inhibitory effect on the newly synthesized collagenous proteins. Decreased levels of the collagenous proteins in conditioned medium may result either from the activation of the degradative pathways (*e.g.* MMPs), decreased collagen synthesis, or both. To determine whether Fli-1 directly affects collagen gene expression, COL1A2 mRNA levels were compared in Fli-1 stable transfectants and control clones. As shown in Fig. 1, *D* and *E*, Fli-1 decreased COL1A2 mRNA levels by at least 1.5-fold, suggesting that its inhibitory effects occurs at least partially via regulating collagen mRNA expression levels.

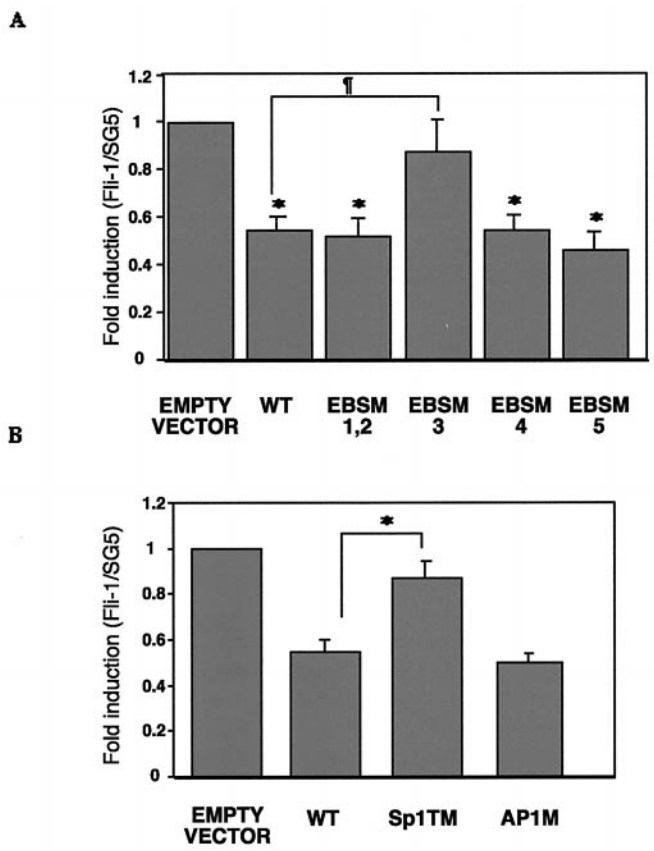

FIG. 3. *A*, identification of a functional Fli-1 response element in the human *COL1A2* promoter. The −353 *COL1A2/CAT* constructs harboring mutated EBSs (*EBSM*) (see Fig. 2*D*, Table I, and "Materials and Methods") were co-transfected with either Fli-1 expression vector or empty vector (pSG5). The *bar graph* represents the -fold induction of the promoter activity of each construct co-transfected with Fli-1 relative to the activity of the promoter co-transfected with pSG5, which was arbitrarily set at 1. Means ± S.E. of five independent experiments are shown. Comparisons of *COL1A2* promoter activity were made between cells transfected with empty vector and with Fli-1 expression vector (*, < 0.01), and between cells transfected with wild-type *COL1A2* and EBS mutated *COL1A2* constructs (¶, < 0.01). *B*, Sp1/Sp3 binding is required for the inhibitory effect of Fli-1. The *COL1A2/CAT* promoter constructs harboring either Sp1/Sp3 triple mutations (*Sp1TM*) (30) or the AP-1 mutation (*AP1M*) (28) were co-transfected with Fli-1 expression vector or empty vector pSG5. Comparisons of *COL1A2* promoter activity were made between cells transfected with wild-type and mutated promoter constructs (*, $p < 0.01$). The Fli-1 inhibitory effect on *COL1A2* was diminished in Sp1/Sp3 triple mutations.

Fli-1 Inhibits COL1A2 Promoter Activity—The effect of Fli-1 on the activity of the *COL1A2* promoter was examined next. Consistent with the effect of Fli-1 on the COL1A2 mRNA levels, Fli-1 inhibited *COL1A2* promoter activity in a dose-dependent manner (Fig. 2, *A* and *B*). To map the Fli-1 response element in the *COL1A2* promoter, we utilized previously generated pro-

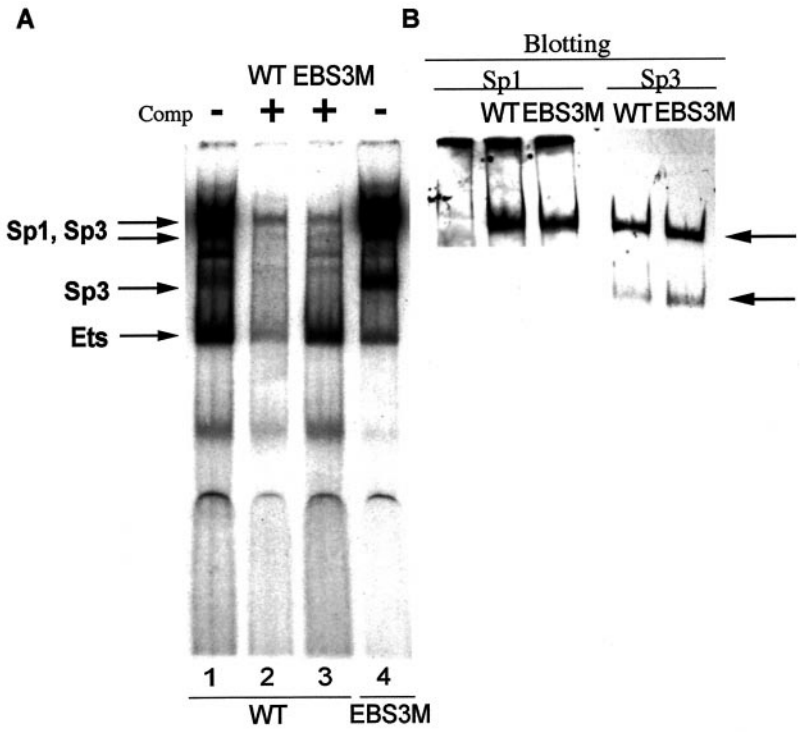

FIG. 4. **Sp1/Sp3 binding to the *COL1A2* promoter is independent of EBS 3.** *A*, EMSA with competitor oligonucleotides. The wild-type probe (*WT*) (*lanes 1* to *3*) or the EBS 3-mutated probe (*EBS3M*) (*lane 4*) was incubated with human fibroblast nuclear extracts (5 μg/lane) in the presence of wild-type unlabeled competitor (50-fold molar excess) (*lane 2*) or EBS 3-mutated unlabeled competitor (*lane 3*). *B*, detection of Sp1 and Sp3 in DNA-protein complexes. EMSAs were performed with equal amounts of unlabeled wild-type and EBS 3-mutated probes, followed by Western blot detection with anti-Sp1 and anti-Sp3 antibodies.

moter deletion constructs (10). The strongest inhibition (2.8-fold) was observed with the −3.5-kilobase *COL1A2* construct, whereas the −353 deletion was inhibited about 1.7-fold. The −264 deletion produced variable results, although the −186 and −108 deletions were modestly stimulated by Fli-1 (Fig. 2*C*). Thus, the inhibitory effect of Fli-1 was mediated by at least two separate *COL1A2* promoter regions: one region located between bp −3500 and bp −772 and the second region located between bp −353 and bp −185. Because the proximal 353-bp region of the *COL1A2* promoter is well characterized, we focused on this region to further delineate the molecular mechanism of the *COL1A2* repression by Fli-1. Inspection of the nucleotide sequence of this promoter region revealed the presence of five putative Fli-1 response elements (termed EBSs 1–5) located at bp −317, −307, −284, −244, and −200 (Fig. 2*D*). Significantly, EBSs 1–3 are located within the previously identified 60-bp DNase-1-protected region (−322 to −262) that included three Sp1 binding sites (30).

EBS 3 and Sp1/Sp3 Binding Sites Function as an Fli-1 Response Element—To determine which of the EBSs were responsible for mediating the Fli-1 inhibition, the putative EBSs were mutated individually and in combination (as described under "Materials and Methods"; see Table I). The −353 *COL1A2/CAT* constructs carrying mutated GGAA motifs were tested in transient transfection assays. Substitution mutation in the EBS 3 significantly reduced Fli-1 inhibition of the *COL1A2* promoter, whereas the mutations in other EBSs had no effect. (Fig. 3*A*). Because Ets factors are known to cooperate with AP-1 in the regulation of urokinase plasminogen activator and several MMP promoters (31), we asked whether the previously characterized AP-1 site (28) in the *COL1A2* promoter could be involved in the Fli-1 inhibition. The inhibitory effect of Fli-1 on the *COL1A2* promoter was not affected by mutation of the previously characterized AP-1 response element. However, the *COL1A2* promoter containing mutations in the three Sp1/Sp3 binding sites surrounding EBS 3 (see Fig. 2*D*) become significantly less responsive to the inhibitory effects of Fli-1 (Fig. 3*B*). This is unexpected because Sp1 and Sp3 have been shown to be potent activators of this promoter and their absence should have resulted in a further decrease of the promoter activity. In fact, promoter mutations in either Sp1/Sp3 or AP-1 binding sites result in a decrease of the basal promoter activity; however, only mutations in the Sp1/Sp3 binding sites affect the inhibitory response to Fli-1. This unexpected result suggests that Sp1 and Sp3, in this promoter context, may be considered co-repressors for Fli-1.

Fli-1 Does Not Compete with Sp1 for DNA Binding—To further examine the possible functional interactions between Fli-1 and Sp1/Sp3, we first evaluated whether Fli-1 could directly interfere with Sp1/Sp3 DNA binding. To test this potential mechanism, we compared binding of the Sp1 and Sp3 to the −307 to −269-bp *COL1A2* promoter fragment (39-bp oligomer) carrying all three Sp1 binding sites and either wild-type or mutated EBS 3. EMSAs were performed using nuclear extracts from human fibroblasts and a radiolabeled 39-mer probe (Fig. 4*A*). In agreement with previously published data (9, 30), several specific DNA-protein complexes were formed that could be competed by an excess of unlabeled wild-type probe (*lane 2*). One of the complexes was not competed by an excess of probe harboring EBS 3 mutation (*lane 3*), suggesting that the binding protein(s) in this complex is related to Ets factor(s). Furthermore, formation of the same complex was diminished when 39-mer-containing mutated EBS 3 was used as a probe (*lane 4*). The presence of Sp1 and Sp3 in the complexes formed with the wild-type and mutated probes were directly analyzed by Western blotting with Sp1 and Sp3 antibodies. As shown in Fig. 4*B*, the EBS 3 mutation did not affect either Sp1 or Sp3 binding, suggesting that competition for DNA binding does not contribute to the inhibitory effect of Fli-1.

Fli-1 Interacts Directly and Indirectly with the COL1A2 Promoter—To examine interaction of Fli-1 with the collagen promoter, we used the −348 to −234-bp fragment of the *COL1A2* promoter and nuclear extracts from human dermal fibroblasts. This promoter fragment was shown in our previous footprinting analyses to contain a large (over 60-bp) protected area, indicative of a multiprotein complex interacting with this promoter region. Sp1 and Sp3 have been identified as the components of this complex (9). Using EMSA we confirmed interac-

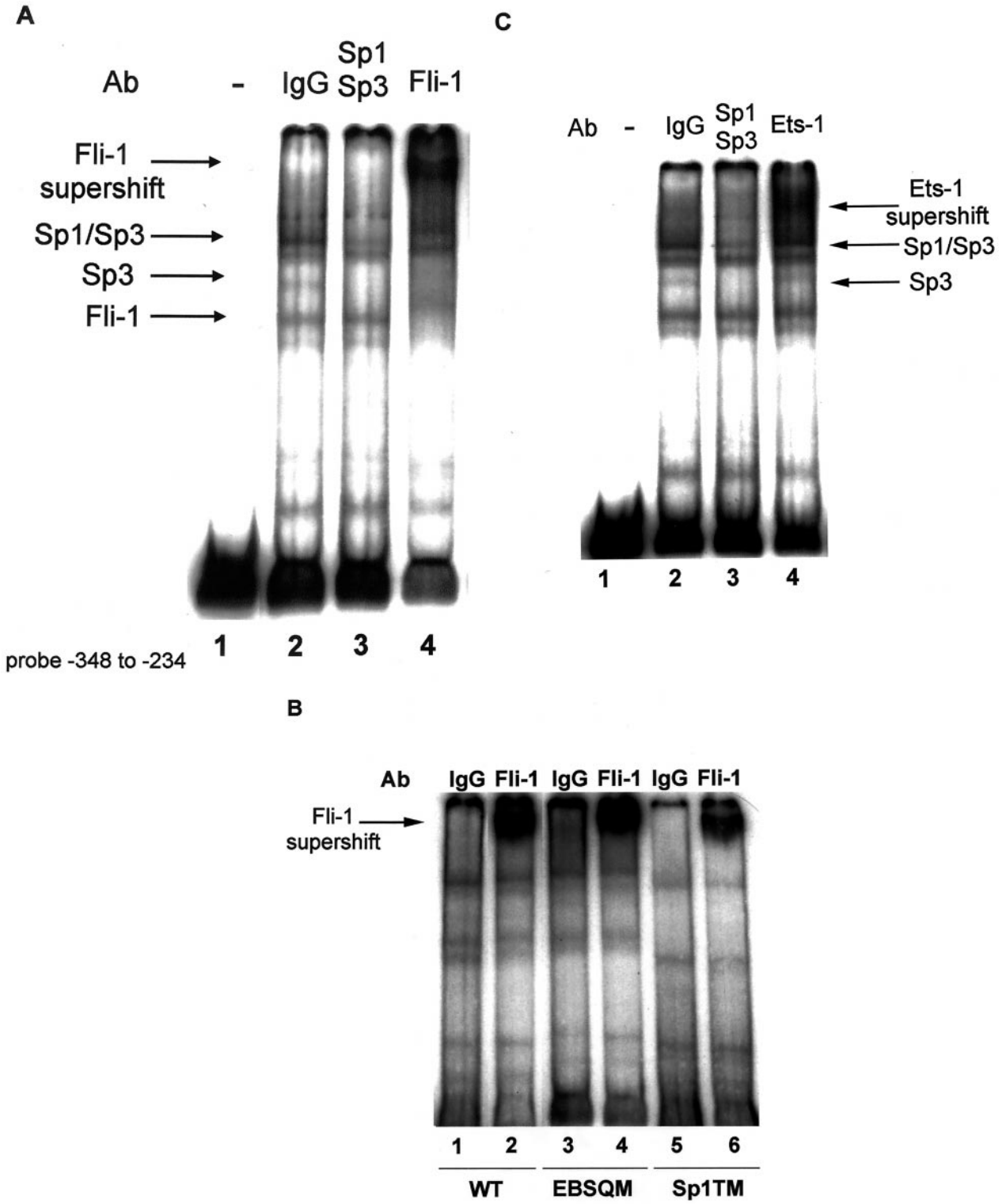

FIG. 5. **Fli-1 interacts with the human *COL1A2* promoter.** *A*, an EMSA with antibodies against Sp1, Sp3, and Fli-1 was performed. The wild-type fragment of the *COL1A2* promoter (−348 to −234) (*lanes 1-4*) was incubated with nuclear extracts (7 μg/lane) from human dermal fibroblasts in the presence of 1 μg of specific antibody as indicated on the *top*. In *lane 2*, anti-human polyvalent IgG was used as a control. *Lane 1* contains probe alone. Specific DNA-protein complexes and supershifted complexes are indicated by *arrows*. *B*, Sp1/Sp3 binding facilitates Fli-1 binding to the human *COL1A2* promoter. An EMSA was performed with the wild-type probe (*WT*) (*lanes 1-2*), the probe containing mutated EBS 1–4 (*EBSQM*) (*lanes 3-4*), and the probe containing mutated Sp1/Sp3 binding sites (*Sp1TM*) (*lanes 5-6*). The probes were incubated with 7 μg of nuclear extract from dermal fibroblasts and 1 μg of specific antibodies. Fli-1 binding was significantly decreased with the probe containing mutated Sp1/Sp3 binding sites as indicated by diminished supershift with anti-Fli-1 antibody. *C*, Ets-1 interacts with the human *COL1A2* promoter. The wild-type probe of the *COL1A2* promoter (−348 to −234) (*lanes 1-4*) was incubated with nuclear extracts (7 μg/lane) from human fibroblasts in the presence of 1 μg of specific antibody as indicated on the *top*. The *arrow* indicates the Ets-1 supershift. *Ab*, antibody.

tion of Sp1 and Sp3 with the −348 to −234-bp promoter region (Fig. 5*A*, *lane 3*). Fli-1 also binds to this promoter region as indicated by a supershift with the anti-Fli-1 antibody (*lane 4*). The ability of the anti-Fli-1 antibody to shift Sp1 and Sp3 complexes in addition to the Fli-1 complex suggests the occurrence of protein-protein interaction between these proteins. To

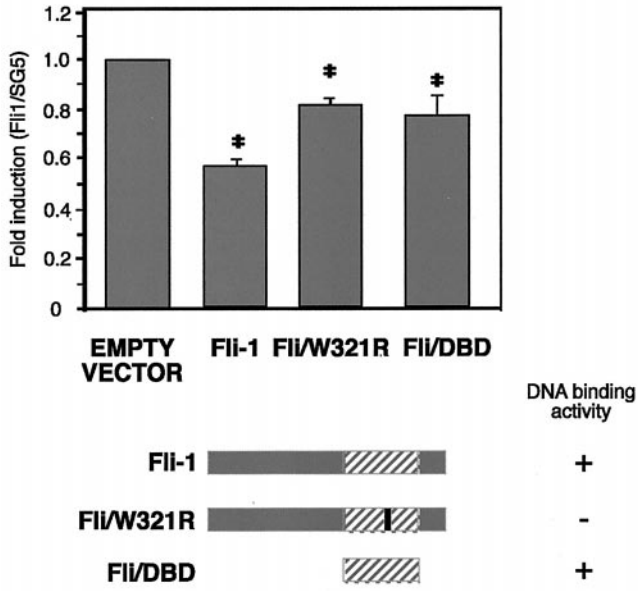

FIG. 6. **Fli-1 mutants inhibit the *COL1A2* promoter activity.** Transient transfections were performed with Fli-1 DNA binding mutant (Fli/W321R), Fli-1 dominant interference mutant (Fli/DBD), and the −353 *COL1A2* promoter. The Fli-1 DNA binding mutant contains a single amino acid mutation that abolishes DNA binding and the Fli-1 dominant interference mutant contains the Ets domain. Both Fli-1 mutants significantly decreased the *COL1A2* promoter activity (*, $p \leq 0.05$) but were less efficient than wild-type Fli-1.

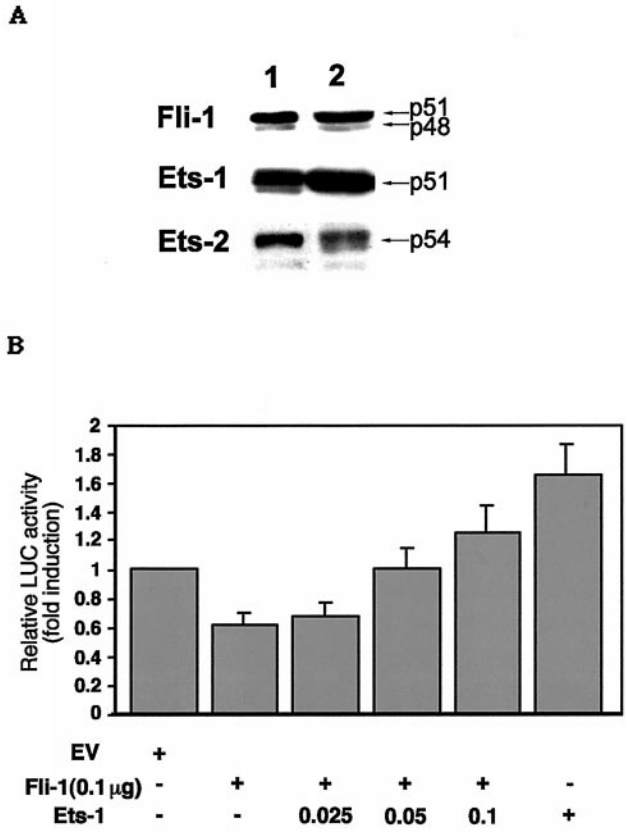

FIG. 7. **Ets family members expressed in human fibroblasts have distinct effects on the transcriptional activation of the *COL1A2* promoter.** *A*, dermal fibroblasts express Fli-1, Ets-1, and Ets-2. *Lane 1*, corresponding control Ets TNT product; *lane 2*, endogenous Ets protein. *B*, overexpression of Ets-1 stimulates the *COL1A2* promoter activity and abolishes Fli-1 inhibition of the *COL1A2* promoter in dermal fibroblast. Human dermal fibroblasts were transiently co-transfected with the −353 *COL1A2/LUC* promoter construct (0.9 μg) and either Fli-1 (0.1 μg) or Ets-1 (0.1 μg) was added individually or with a constant amount of Fli-1 expression vector (0.1 μg) added together with increasing amounts of Ets-1 (0.025, 0.05, and 0.1 μg) expression vector. To ensure an equal amount of co-transfected expression vectors under each condition, appropriate amounts of pSG5 were added to individual co-transfections. The *bar graph* represents the -fold induction of the −353 *COL1A2* promoter activity co-transfected with Fli-1 or Ets-1 individually or together relative to the activity of the promoter co-transfected with pSG5, which was arbitrarily set at 1. The average ± S.E. from four independent experiments is shown.

assess the effects of mutations in EBS on binding of Fli-1, we used the −348 to −234 fragment containing mutations in EBSs 1–4. Although functional assays indicated that EBS 3 mediates the Fli-1 response (Fig. 3), we chose to mutate other putative EBSs to test the possibility of binding to weaker potential sites. Neither Fli-1 nor Sp1 binding to this promoter region was affected by EBS 1–4 mutations (Fig. 5*B*, compare *lanes 1* and *3*). Quantitation of the Fli-1 supershift band from nine independent experiments shows no significant difference between Fli-1 binding to the wild-type probe and the probe containing EBS mutations.

To determine the role of Sp1/Sp3 in Fli-1 interactions with the *COL1A2* promoter, we used as a probe the promoter fragment in which all three Sp1 binding sites were mutated. As previously shown, these mutations abolish Sp1/Sp3 binding (Fig. 5*B*, *lane 5*). Significantly, Fli-1 binding was also substantially decreased with this probe as indicated by diminished supershift with the anti-Fli-1 antibody (Fig. 5*B*, *lane 6*). Quantitative analysis of six independent experiments shows a statistically significant 25% ($p < 0.05$) decrease in Fli-1 binding between the wild-type probe and the probe containing mutations in Sp1/Sp3 sites. Taken together, these *in vitro* binding data indicate that Fli-1 interacts with the *COL1A2* promoter even in the absence of EBSs. On the other hand, the binding of Sp1/Sp3 to this promoter region seems to facilitate Fli-1 binding.

To explore this dual mechanism further, we utilized two Fli-1 mutants: an Fli/W321R mutant harboring a single amino acid mutation in the Ets domain that abolishes its ability to bind DNA[2] (32) and a deletion mutant containing only the Ets domain (Fli/DBD). The effects of overexpression of these two Fli-1 mutants on the *COL1A2* promoter were investigated (Fig. 6). In comparison to the wild-type Fli-1, both mutants caused more modest but consistently reproducible decreases in the *COL1A2* promoter activity, suggesting that direct (via DNA binding) and indirect (via protein-protein interaction) mechanisms contribute to the effects of Fli-1 on the *COL1A2* promoter.

Ets-1and Fli-1 Have Antagonistic Effects on the COL1A2 Promoter and Compete with Each Other in Regulation of the COL1A2 Promoter Activity—What is the molecular mechanism for the Fli-1-mediated repression? Human dermal fibroblasts express several Ets factors including Fli-1, Ets-1, and Ets-2 (Fig. 7*A*). In contrast to Fli-1, Ets-1 and Ets-2 stimulated the *COL1A2* promoter in dermal fibroblasts. Ets-1 stimulated the *COL1A2* promoter with slightly higher potency than Ets-2 (data not shown) and was selected for further investigation. Although we have previously shown that GABPα and -β are expressed in dermal fibroblasts and that GABPα and -β are activators of the tenascin-C promoter in fibroblasts (20), it is unlikely that GABP is involved in the *COL1A2* regulation. GABPα and -β, which binds to DNA as a heterotetramer, requires two tandem repeats of the GGA motif, which are not present in the proximal region of the *COL1A2* gene.

To assess the possibility of competition between Ets-1 and Fli-1 for the *COL1A2* promoter, a constant amount of Fli-1 and

[2] D. K. Watson, unpublished observations.

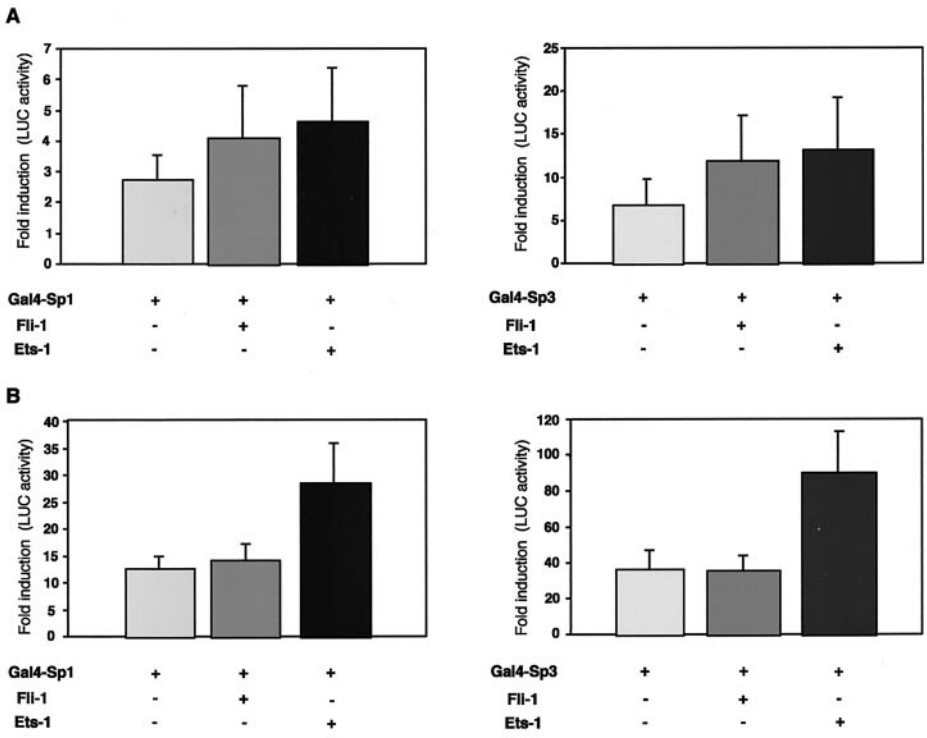

FIG. 8. *A*, Fli-1 and Ets-1 cooperate with Sp1 and Sp3 in activation of the Gal4 promoter in dermal fibroblasts. Human dermal fibroblasts were co-transfected with 0.4 μg of a Gal4-dependent luciferase (Gal4/LUC) reporter construct and with 0.4 μg of expression constructs of fusion proteins containing the DNA binding domain of Gal4 (Gal4DBD) and Sp1 and Sp3, Gal4-Sp1, and Gal4-Sp3, respectively. In some experiments 0.1 μg of Fli-1 or Ets-1 expression vectors were also co-transfected. The *bar graphs* represent means ± S.E. of the -fold induction of Gal4 promoter activity from experimental conditions relative to the activity of the Gal4 promoter co-transfected with Gal4DBD only, which was arbitrarily set at 1. *B*, Ets-1 but not Fli-1 cooperates with Sp1 and Sp3 in activation of the Gal4 promoter in HepG2 cells. The same experimental conditions were used in transfections with HepG2 cells.

the *COL1A2/LUC* was co-transfected with increasing concentrations of Ets-1 into dermal fibroblasts (Fig. 7*B*). Ets-1 was able to reverse the inhibitory effects of Fli-1 in a dose-dependent manner, suggesting that these two factors may compete for binding to the *COL1A2* promoter. Consistent with this model, Ets-1 binding to the *COL1A2* promoter was observed by EMSA; however, Ets-1 binding seemed to be less prominent than that observed with Fli-1 (Fig. 5*C*).

To compare the binding affinity of Fli-1 and Ets-1 to the *COL1A2* promoter, the *in vitro* translated Ets-1 and Fli-1 were reacted with the short promoter fragments containing individual EBSs in the presence or absence of the corresponding antibodies. The Fli-1 protein did not react with any of the promoter fragments (data not shown), whereas the Ets-1 bound weakly only to the promoter fragment containing EBS 3 (data not shown). These data suggest that either Ets-1 or Fli-1 alone have low binding affinity for the *COL1A2* promoter. Thus, their presence in the DNA-protein complexes most likely depends on the presence of other transcription factors interacting with this promoter. As suggested previously, the candidate cofactors may be Sp1 and Sp3.

Ets-1 and Fli-1 Differ in Their Ability to Functionally Interact with Sp1/Sp3 in the Gal4 System—To examine the interaction between Sp1/Sp3 and Ets factors further, we utilized the Gal4 system. Either Sp1 or Sp3 fused to the Gal4 DBD domain-transactivated Gal4-responsive promoter in human fibroblasts (Fig. 8*A*). Interestingly, Sp3 was a more potent activator than Sp1 in the context of the Gal4 system. Co-transfection of Ets-1 with either Gal4-Sp1 or Gal4-Sp3 further enhanced Gal4-responsive promoter activity (Fig. 8*A*). This supports a model for functional interaction between Sp1/Sp3 and Ets-1 in dermal fibroblasts. Surprisingly, Fli-1 was also able to synergize with the Gal4-Sp1 and Gal4-Sp3 chimeric proteins under these experimental conditions. These observations, together with our previous data using the tenascin-C promoter (20), suggest that Fli-1 can behave either as a repressor or an activator depending on the promoter context. In contrast to fibroblasts, in HepG2 cells, Fli-1 did not have a significant effect on either Gal4-Sp1 or Gal4-Sp3 activation potential, whereas Ets-1 synergized with both Sp1 and Sp3 in activating the Gal4 promoter (Fig. 8*B*). These data suggest that cell type-specific cofactors may be required to facilitate Fli-1 interactions with Sp1/Sp3. Consistent with this possibility, previous studies have demonstrated that the formation of a ternary complex and the functional interaction between Sp1 and Ets-1 required presence of the Tax protein (33). The difference between Fli-1-Sp1 interaction observed here further suggests that distinct cofactors facilitate Ets-1 and Fli-1 interaction with Sp1/Sp3. Together, these data suggest that the repressor function of Fli-1 depends on cell and promoter context.

Effects of Fli-1 and Ets-1 on the COL1A2 Promoter Are Cell Type-specific—To further examine the variable responses of different cell types to Fli-1, we utilized several cell lines of different origins in the analyses of Ets-1 and Fli-1 regulation of the *COL1A2* promoter (Fig. 9*A*). Ets-1 stimulated the *COL1A2* promoter activity in all cell lines tested. In contrast, Fli-1 showed cell line-specific effects. In human lung fibroblasts, Fli-1 significantly inhibited the *COL1A2* promoter. In human mesangial cells and in HepG2 cells, Fli-1 had a slight but consistent inhibitory effect, whereas in HeLa cells, Fli-1 was stimulatory for the *COL1A2* promoter. We have also determined the endogenous expression of Ets-1 and Fli-1 proteins in the cells used in this experiment. Ets-1 and Fli-1 were expressed in all cell types and although there were some differences in expression levels, the ratio of Ets-1 to Fli-1 protein seemed to be similar in all cells (Fig. 9*B*).

DISCUSSION

The present study establishes for the first time that Ets factors play a critical role in regulating the expression of the collagen type I gene in dermal fibroblasts. The following observations support this conclusion. First, the exogenous expression of Fli-1 in dermal fibroblasts led to a dramatic decrease of the newly synthesized collagenous proteins and the *COL1A2* mRNA. Second, transient transfection experiments have defined a functional EBS in the *COL1A2* promoter. Third, binding assays have demonstrated that Fli-1 and Ets-1 form DNA-protein complexes with sequences present in the *COL1A2*

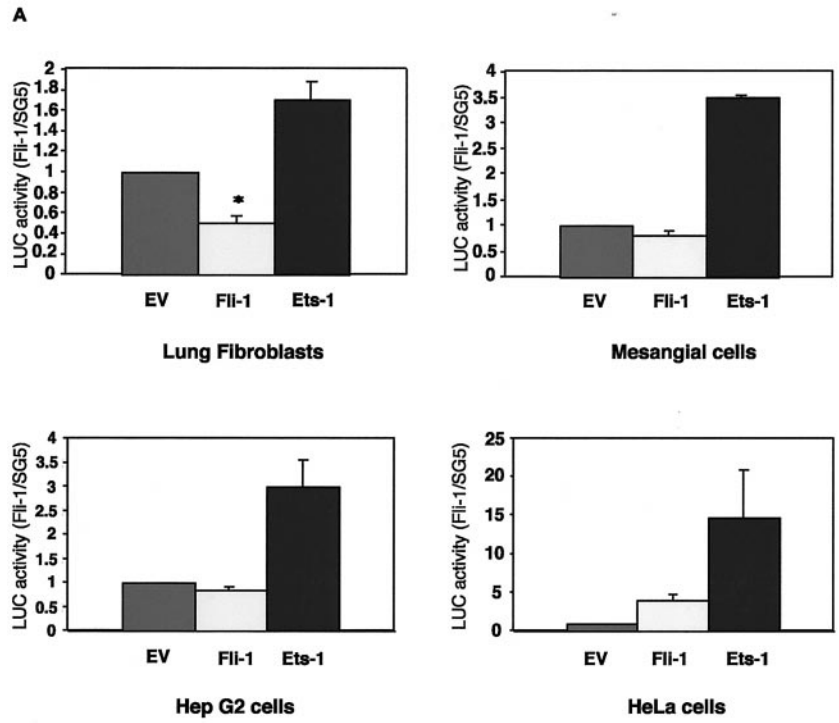

FIG. 9. **Transactivation of the *COL1A2* promoter by Ets factors is cell context-dependent.** *A*, human lung fibroblasts and mesangial , HepG2, and HeLa cells were transiently co-transfected with the −353 *COL1A2/LUC* promoter construct and Fli-1 or Ets-1 expression vectors. The bar graph represents the -fold induction of the *COL1A2* promoter activity relative to the activity of the promoter co-transfected with pSG5, which was arbitrarily set at 1. The average ± S.E. of at least three independent experiments is shown (*, $p \leq 0.05$). *B*, expression of endogenous Fli-1 and Ets-1 proteins in different cell types. *C*, corresponding Ets TNT product; *FB*, foreskin fibroblasts; *LF*, lung fibroblasts; *HMC*, human mesangial cells.

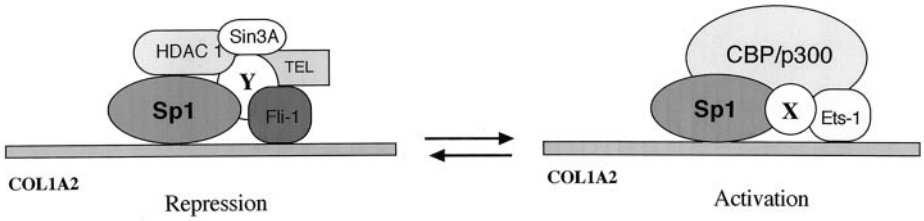

FIG. 10. **A hypothetical model for Sp1-mediated transcriptional repression or activation.** Details described under "Discussion."

promoter. Furthermore, this study has also begun to unravel specific regulatory mechanisms involved in the transcriptional control of the *COL1A2* gene by Ets factors. Experiments using Fli-1 dominant interference and DNA binding mutants indicate that Fli-1 inhibition is mediated by both direct (DNA binding) and indirect (protein-protein interaction) mechanisms. Based on the functional studies with the Sp1/Sp3 promoter substitution mutants as well as the EMSA results, we postulate that functional interaction of Fli-1 with Sp1 and Sp3 is essential for the inhibitory function of Fli-1. It also seems that additional tissue-specific bridging factors are involved in these interactions. The nature of these cofactors is presently unknown. Furthermore, the competition experiments suggest that Fli-1 inhibits the *COL1A2* promoter by displacing Ets-1. Together, these findings imply that the ratio of Fli-1 to Ets-1 and the presence of co-regulatory proteins may contribute to the control of collagen production in fibroblasts.

Our findings suggest that in the context of the *COL1A2* promoter, Sp1 may play a dual role of an activator and a repressor of this promoter (Fig. 10). Moreover, Fli-1 and a tissue-specific co-repressor(s) may contribute to formation of this repressor complex. Recent observations indicating direct interaction between Sp1 and histone deacetylase-1 further support this possibility (34). A possible mechanism explaining such a dual role has been proposed for a tumor suppressor protein, p53 (35, 36). It has been demonstrated that p53-mediated repression of specific target genes depends on association of p53 with histone deacetylase-1 and Sin 3A (35), whereas the activator status of p53 can be enhanced through acetylation by p300 (36). It is intriguing that in our system, Sp1 can behave as an activator and a co-repressor in the context of the same promoter. What determines the switch between these two states remains to be elucidated. However, it has been shown recently that Sp1 is involved in mediating TGF-β activation of this promoter (8), suggesting a possible role of TGF-β-induced signaling in this process.

Studies using dermal fibroblasts indicate that Fli-1 can act as a repressor in the context of the collagen promoter, and it is a potent activator for the tenascin-C promoter (20). What determines this dual function of Fli-1 is currently not known. Only a subset of Ets factors has repressor activity (*e.g.* ERF, YAN, TEL, and NET), and most are generally felt to be activators of transcription. However, previous studies have demonstrated that other proteins can block their ability to activate

transcription via interaction with Ets. For example, interaction of EAP1/Daxx with Ets-1 has been shown to down-regulate Ets-1-mediated activation of MMP1 and BCL2 genes (37). In another system, MafB, an AP-1 like protein, has been shown to interact with the Ets domain of Ets-1 and repress Ets-1 transactivation of Ets-responsive promoters (38). More importantly, Fli-1 has been shown to be an interacting partner of TEL, a member of the Ets family with repressor function. Furthermore, co-transfection of TEL with Fli-1 has been shown to inhibit Fli-1-mediated transcriptional activation (39). TEL contains two autonomous repression domains, suggesting two distinct repression mechanisms (40). One of the potential mechanisms involves the recruitment of a repression complex including silencing mediator for retinoid and thyroid hormone receptors and mSin3A (41). Whether TEL is involved in Fli-1-mediated repression of the *COL1A2* remains to be elucidated. Significantly, however, stable overexpression of TEL in NIH3T3 fibroblasts led to the down-regulation of collagen type I (42).

In support of the conclusions from our studies, it was observed that the exogenous expression of Ewing's sarcoma protein/FLI1 and Ewing's sarcoma protein/ETV1 inhibited deposition of collagen in NIH3T3 cells (43). Significantly, we have found that a subset of lesional scleroderma fibroblast cell lines expresses reduced levels of Fli-1 protein as compared with healthy control fibroblasts, whereas the expression of Ets-1 remains constant. The reduced levels of Fli-1 correlate with increased collagen production by these cells.[3] Thus, Fli-1 and other Ets factors may play a role in the pathology of fibrotic diseases. It will be important to examine *in vivo* expression of Ets factors in fibrotic lesions and in animal models of fibrosis.

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