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Activation of an Enhancer on the Syndecan-1 Gene Is Restricted to Fibroblast Growth Factor Family Members in Mesenchymal Cells

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Fibroblast growth factors (FGFs) induce a variety of biological effects on different cell types. They activate a number of genes, including immediate-early genes, such as the transcription factors Fos and Jun, which are also common targets for other tyrosine kinase receptor-activating growth factors. Here we describe a secondary far-upstream enhancer on the syndecan-1 gene that is activated only by members of the FGF family in NIH 3T3 cells, not by other receptor tyrosine kinase-activating growth factors (e.g., epidermal growth factor, platelet-derived growth factor, insulin-like growth factor, or serum). This FGF-inducible response element (FiRE) consists of a 170-bp array of five DNA motifs which bind two FGF-inducible Fos-Jun heterodimers, one inducible AP-2-related protein, a constitutively expressed upstream stimulatory factor, and one constitutive 46-kDa transcription factor. Mutational analysis showed that both AP-1 binding motifs are required, but not sufficient, for FiRE activation. Moreover, agents such as 12-*O*-tetradecanoylphorbol-13-acetate, okadaic acid, or forskolin, which are known to activate AP-1 complexes and AP-1-driven promoters, fail to activate FiRE. However, FiRE can be activated by the tyrosine kinase phosphatase inhibitor orthovanadate. Taken together, this data implies a differential activation of growth factor-initiated signaling on AP-1-driven regulatory elements.

Fibroblast growth factors (FGFs) are a family of heparin-binding peptides that currently include nine members. FGFs are known to induce the transcription of a number of genes, including transcription factors, components of the cytoskeleton, and ribosomal genes. Basic fibroblast growth factor (FGF-2), the best-characterized member of this family, is synthesized by and acts on various cell types and tissues. For example, in vitro it is a strong mitogen for cells of mesodermal origin, can modulate cell motility and differentiation, and is a potent angiogenic factor. In vivo, it potentiates neovascularization and stimulates proliferation of most of the cell types involved in wound healing, including keratinocytes, fibroblasts, and vascular and capillary endothelial cells (2, 3, 6, 29). It plays a crucial role in fetal development, in which it seems to possess various activities. Several studies have implicated FGFs as the prime candidates for the limb bud apical ectodermal ridge (AER) growth signal. FGF-2 is detectable in chick limb bud, and replacing the AER with FGF-2 restores limb development (13). FGF-2 can also induce additional limb formation in chick embryos, as placing FGF-2 beads in the embryos results in formation of complete ectopic limbs (7). Other members of the FGF family, FGF-4 and FGF-8, have also been implicated in the AER growth signal and have been shown to retain the outgrowth of the limb (10, 25, 36).

FGFs act through a family of transmembrane tyrosine kinase receptors (FGFRs) (23). Heparin or heparan sulfate proteoglycans facilitate the binding of FGF-2 to the FGFR (41, 62). Recent observations indicate that heparin is required for oligomerization of FGF-1 molecules leading to FGFR dimerization and further signaling (38, 50). It is still unclear how heparan sulfate proteoglycans participate in the regulation of FGF action. Several mechanisms, for both negative and positive regulation for FGF action by proteoglycans, have been postulated (48). An integral membrane heparan sulfate proteoglycan, syndecan-1 (46), can simultaneously bind FGF-2 and extracellular matrix molecules, and this complex is able to promote DNA synthesis in 3T3 cells (44). However, it is known that different heparin sequences can either activate or inhibit FGF-2 function (14) and that the composition and length of the syndecan side chains vary in a cell- and tissue-dependent manner (40, 43, 45). Negative regulation of FGF action by syndecan-1, which might be due to the glycosaminoglycan side chain modification or a different stoichiometric ratio of FGFR and coreceptor, has also been reported elsewhere (1, 28).

The expression of syndecan-1 follows morphogenetic rather than histological tissue boundaries (4). It is expressed at the four-cell stage (51), but during later development, it is expressed mainly by epithelia and only transiently by several condensing mesenchymes, including tooth (53), kidney (56), and developing limb mesenchyme (49). FGF-2 is also detected in limb bud mesenchyme (47), similar to FGF-4 (35) and FGF-8 (16). Furthermore, syndecan-1 is colocalized with FGF-3 in developing tooth mesenchyme (60) and with the heparin-binding growth factor-like molecule, midkine, in developing skin (33). Syndecan-1 expression is also induced up to 20-fold in keratinocytes during wound healing (11), suggesting that these growth factors might be involved in the regulation of syndecan-1 expression.

Several growth factors, including FGFs, can elicit immediate-early responses after their receptor tyrosine kinase (RTK) activation. Well-characterized examples are epidermal growth factor (EGF) and platelet-derived growth factor (PDGF), which induce, via the mitogen-associated protein (MAP) kinase pathway transcription factors Fos and Jun, the serum response factor and ternary complex factor (17). The cyclic AMP (cAMP) response element (CRE), bound by the CRE-

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binding protein homodimer or as heterodimers in association with members of the ATF family, is also under the influence of growth factors. EGF and PDGF, but not FGFs, are also able to induce activation of signal transducer and activator of transcription (STAT) transcription factors which act on Sis-inducible element (SIE) or interferon-stimulated response element (18, 24). While able to induce the same signaling molecules, like MAP kinases and subsequently several early genes such as AP-1, it remains less well understood how differential transcriptional activation elicited by different growth factors on AP-1-driven promoters is obtained.

Our previous data has indicated that simultaneous exposure of cultured 3T3 cells to FGF-2 and transforming growth factor β (TGF-β) enhances syndecan-1 expression (12), indicating that members of the FGF family are involved in the regulation of syndecan-1 expression. A search for the basis of the growth factor-induced upregulation resulted in the discovery of a novel enhancer that can be activated by FGF. In this paper, we describe this FGF-inducible response element (FiRE) and, furthermore, show that in 3T3 cells it appears to be activated selectively by members of the FGF family but not by other tyrosine kinase receptor-activating growth factors (EGF, PDGF, or insulin-like growth factor).

MATERIALS AND METHODS

Isolation of the 5′ regions of the gene and DNA sequencing. The mouse syndecan was previously cloned and sequenced to −9.4 kb upstream from the translation initiation site (58). To sequence further upstream, an *Xba*I fragment (Xb6) from the cosmid clone was subcloned into pBluescript KS M13(+/−) vectors (Stratagene). DNA sequencing was performed by the dideoxy chain termination method. Sequence database comparisons were made with the Wisconsin package (Genetics Computer Group, Inc.) and the Transcription Factor Database.

Construction of plasmids and transfections. For the gene expression analysis, different *Xba*I fragments were subcloned from mouse syndecan-1 genomic clones into the *Xba*I site of the pCATProm vector in which a mouse syndecan-1 promoter region (−1310 to +140) was fused to the chloramphenicol acetyltransferase (CAT) reporter gene (59). The pX-HIIICAT plasmid was prepared by cloning a *Hin*dIII-*Xho*I promoter fragment (−2400 to +140) into promoterless pCAT basic vector (Promega). For pXSp1 and -2 plasmids, *Sph*I fragments (−11.4 to −10.5 and −10.5 to −9.4 kb, respectively) were deleted from pXb6CAT (−11.6 to −9.4 kb). Constructs pXS2, pXB3, and pXX1 were generated by ligating *Sph*I-*Ear*I/blunt (−10.5 to −10.1 kb), *Ear*I/blunt (−10.1 to −9.8 kb), and *Ear*I/blunt-*Xba*I (−9.8 to −9.4 kb) fragments from pXSp1 into *Sph*I-*Acc*I/blunt, *Acc*I/blunt, or *Xba*I-*Sph*I/blunt sites, respectively, in the pCATProm vector. Blunt ending was done with T4 polymerase (Promega). For FiRE and FiRErev, a *Pst*I-*Sty*I/blunt fragment was subcloned into the *Pst*I-*Eco*RV or *Pst*I-*Sma*I sites of pBluescript vector and transferred to the *Xba*I-*Sph*I sites in pCAT Prom vector. p-271FiRE was constructed by deleting a 1-kb *Bgl*II-*Pst*I fragment of the syndecan proximal promoter from the pFiRE plasmid.

For transient transfections, 3T3 NIH cells were plated at equal density on six-well plates (Falcon) 2 days before transfection. Plasmid DNA was transfected into cells by the calcium phosphate method (8). A β-galactosidase-expressing plasmid (pSV-β-galactosidase; Promega) was cotransfected with CAT constructs to monitor transfection efficiencies. Three parallel transfections were used in every assay. Growth factors were added directly after transfection, the medium was changed the next day, and cells were harvested after 2 days. CAT activities were measured by liquid scintillation counting, and β-galactosidase activities were measured spectrophotometrically at 420 nm as described by Vihinen et al. (58). Stable transfections were made by transfecting simultaneously pMAMNeo plasmid and a 10-fold molar excess of p-271FiRE by the calcium phosphate method and selecting cells with 750 μg of G418 per ml.

Cell culture, hybridizations, and protein extracts. 3T3 NIH mouse fibroblasts were routinely cultured in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum (FCS) to approximately 70% confluence. For growth factor treatment, FCS was replaced with 2% carboxy methyl-Sephadex-eluted FCS 24 to 48 h before addition of growth factors. The human recombinant growth factors were purchased from PeproTech (FGF-1, -2, and -7; IGF-I; gamma interferon, and TGF-α), Boehringer (FGF-1 and FGF-2), Sigma (TGF-β), or Calbiochem (PDGF/BB and EGF). Growth factors were used at 10 ng/ml, except for TGF-β, which was used at 2 ng/ml.

For Northern blot analysis, cells were lysed in 4 M guanidine isothiocyanate and RNA was isolated by acid guanidium thiocyanate-phenol-chloroform extraction (9), run on a 1% agarose gel, and transferred to Hybond-N^+ (Amersham) nylon membrane. The membrane was prehybridized as recommended by the manufacturer and hybridized with a random-primed (Promega) labeled partial cDNA of the mouse syndecan-1 gene (PM-4). The membrane was washed as recommended by the manufacturer and rehybridized with a glyceraldehyde-3-phosphate dehydrogenase cDNA. Transcription run-on analysis was performed with an equal number of isolated nuclei in the presence of 100 μCi of [α-^{32}P]dUTP. Radiolabeled RNA was isolated and hybridized to nitrocellulose-immobilized plasmids specific for syndecan-1, β-actin, c-Jun, and Nur.

For nuclear extracts, 3T3 NIH cells were plated on 16-cm dishes and treated with or without FGF-2 for 2 to 4 h. Nuclear proteins were extracted by a modification described by Lee et al. (27). Protein concentrations were measured by the Bradford reaction, and approximately 3 μg of extract was used for each reaction. Whole-cell extracts were prepared by freezing the cells after harvesting and pelleting. They were subsequently resuspended in a 400 mM sodium salt buffer and ultracentrifuged (Sorvall RC-M120) for 5 min at 50,000 rpm, and the supernatant was used for gel shift analysis, with approximately 6 μg of protein extract for each reaction.

Cell proliferation assays were made by incubating cells for 4 to 6 h with 0.25 μCi of 5-[^{125}I]iodo-2′-deoxyuridine (5-[^{125}I]IdU; Amersham), washed several times with phosphate-buffered saline, and solubilized in 1 M NaOH. Radioactivity was measured by a gamma counter (Wallac).

DNase I footprinting, gel retardation analysis, and UV cross-linking. For footprinting, pBluescript carrying pFiRE was cut with *Hin*dIII, end labeled with [α-^{32}P]dCTP by using Klenow DNA polymerase (Promega), and digested with *Xba*I. The labeled and polyacrylamide gel electrophoresis (PAGE)-purified DNA was incubated for 10 min at room temperature with approximately 40 mg of crude nuclear extract, 2 μg of poly(dI-dC) (Boehringer Mannheim) in a reaction buffer (10 mM Tris-Cl [pH 8], 5 mM $MgCl_2$, 1 mM $CaCl_2$, 2 mM dithiothreitol, 50 mg of bovine serum albumin per ml, and 100 mM KCl), and 0.1 or 1 Units of DNase I (Boehringer Mannheim). The reaction was stopped after 2 min. A chemical G+A sequencing ladder (30) was run along with the digestion products in a 6% sequencing gel.

For gel mobility shift assays, double-stranded oligonucleotides were end labeled with [γ-^{32}P]dATP (ICN Biomedicals) by T4 polynucleotide kinase (Promega). Corresponding to footprint regions (see Fig. 3), oligonucleotides (top strand) were 5′-dGCTGGCACAC CCACCGTCAC GAGAGCTTCC-3′ (motif 1), 5′-TTGGCACACC TGGGAGGATG-3′ (motif 2), 5′-AGTGGTTCAG GGTGACTCT-3′ (motif 3), and 5′-AGGAGTGAGC CATGCCACC-3′ (motif 4), and 5′-CTGGGTCATT GATGACTGTT GTGTGGGATA CCTG-3′ (motif 5). In a 12-μl reaction mixture, 5 μg of nuclear extracts was incubated with labeled oligonucleotide, 2 μg of poly(dI-dC), and 2× reaction buffer (20 mM Tris [pH 7.5], 100 mM NaCl, 2 mM EDTA, and 10% glycerol) for 15 min at room temperature. Nonlabeled competitor oligonucleotides were used at 50× molar excess. The complexes were analyzed by electrophoresis in a 4.5% polyacrylamide gel. For supershifts, 1 μl of specific antibody (Santa Cruz) was added to the reaction 15 min before the labeled oligonucleotide.

For UV cross-linking experiments, gel mobility assays were run as described above. After the run, the gel was exposed to 245-nm UV light (3,600 J/cm^2) in a Stratagene UV cross-linker. The gel was exposed for several hours, and specific bands were cut from the gel, eluted overnight at +4°C, precipitated with ethanol, resuspended in Laemmli buffer, denatured at +95°C for 5 min, and run on a sodium dodecyl sulfate (SDS)–10% polyacrylamide gel together with a ^{14}C-labeled molecular weight marker. Calculated molecular masses of the oligonucleotides (330 Da/nucleotide) were subtracted from the masses revealed by the gel.

Nucleotide sequence accession number. The Xb6 sequence (sequence of the 5′ region of the syndecan-1 gene) has been deposited in the EMBL sequence data bank under accession no. Z22532.

RESULTS

Syndecan-1 gene is activated by FGF-2 in 3T3 cells. Syndecan-1 expression is usually very low in mesenchymal cells compared to that in epithelial cells. Yet, many mesenchymes can transiently induce syndecan-1 expression (56, 57). Our earlier work has indicated that syndecan-1 expression is upregulated in 3T3 cells after 24 h of simultaneous FGF-2 and TGF-β exposure (12). However, our previous data suggested that transiently FGF-2 alone could also activate the syndecan-1 gene. To demonstrate this, 3T3 cells were exposed to FGF-2 at 10 ng/ml in growth factor-depleted conditions and the syndecan mRNAs were quantified at various time points. As shown in Fig. 1A, the mRNA levels were increased severalfold already at 4 h after FGF-2 treatment. This induction reverted to low levels within the next 8 to 24 h, however. Furthermore, a nuclear run-on experiment revealed that this upregulation was transcriptional. In nuclei isolated 4 h after FGF exposure, the level of transcription of the syndecan-1 gene was elevated, as

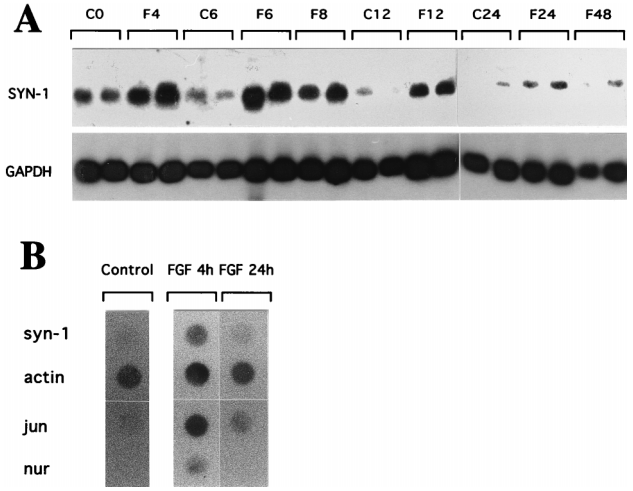

FIG. 1. The syndecan-1 gene is transcriptionally activated by FGF-2 in 3T3 NIH cells. (A) 3T3 NIH cells were exposed to 10 ng of FGF-2 per ml for 0, 4, 6, 8, 12, 24, and 48 h (C0 and F4 to F48) followed by RNA isolation and Northern analysis of mouse syndecan mRNA (SYN-1) and a loading control mRNA (glyceraldehyde-3-phosphate dehydrogenase [GAPDH]). Control time points with no FGF-2 treatment were 6, 12, and 24 h (C6 to C24). Two individual exposures for each time point are presented. (B) The same cells were exposed to 10 ng of FGF-2 per ml for 4 and 24 h or without exposure to FGF (Control) followed by isolation of nuclei for a run-on experiment. Transcription of c-Jun and that of Nur were used as positive controls, and β-actin was used as a loading control.

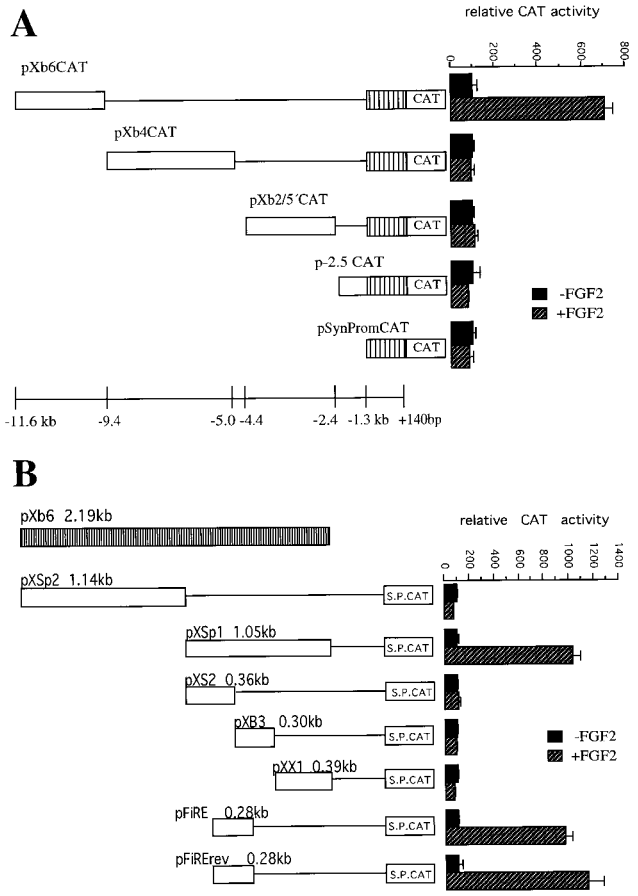

FIG. 2. The upregulation of syndecan-1 expression is caused by a far-upstream enhancer. (A) Transfection constructs consisting of different fragments of the 11.5-kb upstream regulatory region together with 1.1 kb of the proximal promoter of the syndecan-1 gene were assayed by transient transfection in 3T3 NIH cells with or without FGF-2 treatment. Only the most distal 2.2-kb fragment (pXb6) responded to FGF-2 treatment. (B) This FGF-2-inducible part of the gene was cleaved into further fragments and assayed similarly. A 280-bp element (FiRE) retained full FGF-2 response, regardless of its orientation within the plasmid (pFiRErev).

were the levels of c-Jun and Nur used as positive controls. The transcription of syndecan-1 was no longer detectable after 24 h (Fig. 1B), in agreement with the results of Northern hybridization.

In 3T3 cells, a novel FGF-2-specific, far-upstream element is responsible for enhanced syndecan-1 expression. In order to find transcriptional elements responsible for syndecan-1 expression following FGF-2 exposure, the 5′ region of the gene was cloned and sequenced 12 kb upstream from the translation initiation site. Fragments of the 5′ region ranging in size from 1.1 to 4.5 kb were fused with the proximal promoter (−1.1 kb upstream from the translation initiation site) of the syndecan-1 gene (19, 58) and inserted into a CAT reporter plasmid. These constructs (see Fig. 2A) were assessed by transiently transfecting them into 3T3 NIH cells, which were then treated with or without FGF-2. The most distal 2.2-kb part of the syndecan-1 gene produced a 7- to 10-fold enhancement when the reporter construct was expressed in FGF-2-treated cells (Fig. 2A). All other fragments, as well as the 1.1-kb proximal promoter clone alone (pCATProm), revealed no response to FGF-2 (Fig. 2A). The 2.2-kb FGF-responsive region was cut into halves (pXSp1 and pXSp2), and the derived pXSp1 was further cut into shorter fragments (Fig. 2B). These constructs were assessed for FGF-2-induced enhancer activity as described above. A 280-bp element, termed FiRE, was found to mediate the full FGF-2 response, regardless of its orientation (FiRErev) inside the reporter plasmid (Fig. 2B), thus demonstrating properties of a classical enhancer element. In addition, FiRE (studied with the larger Xb6 fragment) was functional in a reporter gene plasmid in which the syndecan-1 promoter was replaced with a heterologous simian virus 40 promoter (data not shown).

FiRE binds FGF-2-inducible and noninducible nuclear factors. DNase I footprinting was performed with the end-labeled FiRE fragment and nuclear extracts derived from FGF-2-treated or nontreated 3T3 NIH cells in order to find DNA-protein interactions along the enhancer sequence. Five protein binding sites, ranging from 14 to 38 bp in length, close to each other and covering a total of 170 bp, were revealed (Fig. 3A). Binding of nuclear proteins by motifs 1 and 2 was evident both in FGF-2-stimulated and in nonstimulated 3T3 cells, but motifs 3, 4, and 5 clearly indicated binding of FGF-2-dependent nuclear factors (Fig. 3A).

Gel retardation assays were performed with double-stranded oligonucleotides corresponding to all five motifs. An SP1 consensus oligonucleotide was routinely used as a control to check the functionality of the nuclear extracts. Binding of one or more protein complexes to each motif was observed. To reveal specific binding, each oligonucleotide was competed with a 50× molar excess of specific and nonspecific oligonucleotides. The results from these experiments indicated that all motifs shifted at least one specific band in the gel retardation assay. The motifs 1 (Fig. 4A) and 2 (Fig. 4B) revealed no FGF-2-inducible nuclear factors, as observed earlier in the footprint experiment. Instead, motifs 3 (Fig. 4C), 4 (Fig. 4D), and 5 (Fig. 4E) all showed binding of at least one specific nuclear protein induced by FGF-2. To further support the finding that motifs 1 and 2 are occupied by constitutively expressed nuclear proteins but motifs 3, 4, and 5 are occupied

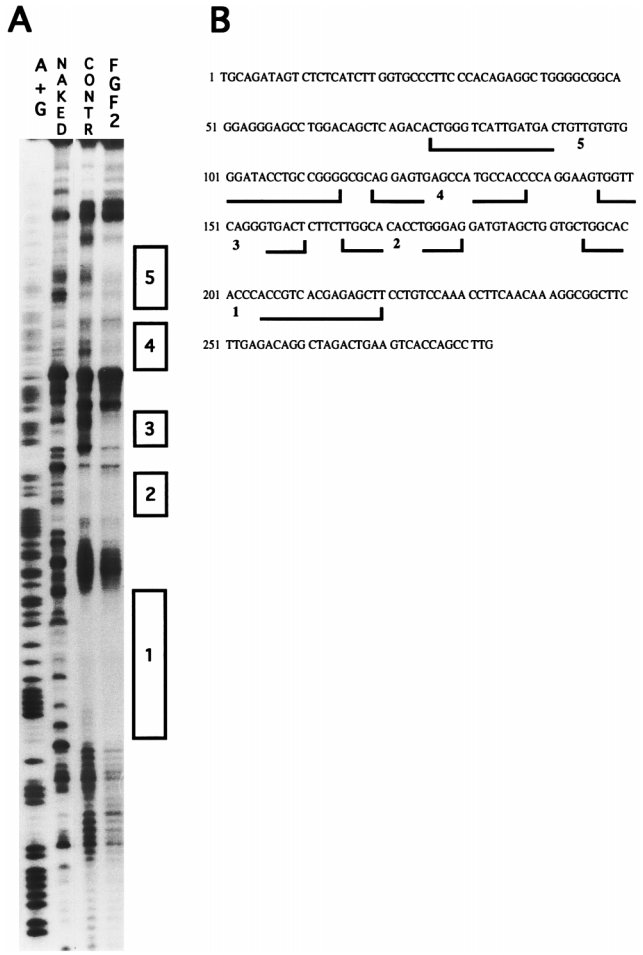

FIG. 3. The enhancer consists of five DNA binding motifs. (A) DNase I footprinting was performed with end-labeled pFiRE vector alone (NAKED) or together with FGF-2-induced (FGF2) and noninduced (CONTR) nuclear extracts. The A+G sequence of pFiRE was run alongside. Five footprinted motifs are marked with numbered boxes. (B) Sequence of FiRE. Underlined motifs indicate each footprinted region. Motifs 5 and 4 host AP-1-like binding sites, motif 2 has an E box, and motifs 3 and 1 have unknown transcription factor recognition sites.

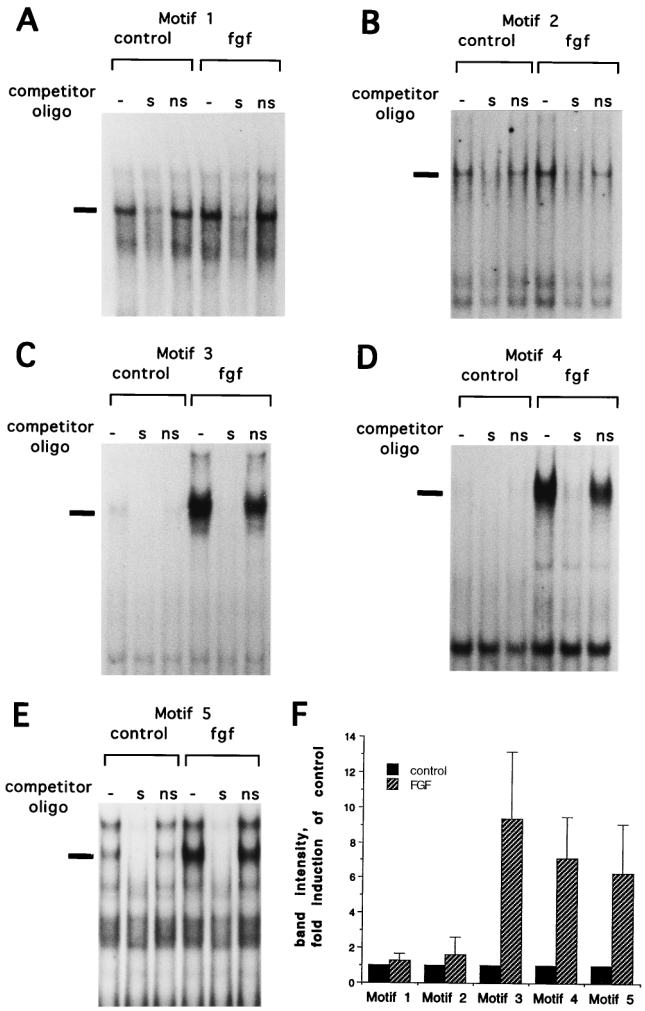

FIG. 4. The enhancer binds both FGF-2-inducible and noninducible nuclear factors. Gel retardation analysis for each motif was performed to reveal protein complex formation with nuclear extracts derived from 2-h FGF-2-treated (fgf) or untreated (control) 3T3 cells. Motifs 1 (A), 2 (B), 3 (C), 4 (D), and 5 (E) were also competed with a 50× molar excess of specific (s) or nonspecific (ns) oligonucleotides (oligo). SP-1 consensus oligonucleotide was used as a nonspecific competitor. Specific binding is indicated by a horizontal bar. To further illustrate the inductivity of motifs 3 to 5, five independent gel shift assays were run and quantified by gel scanning. The means of these five measurements are presented as fold induction between FGF-treated and nontreated 3T3 NIH cells (F).

only after FGF exposure of 3T3 cells, five independent gel shift assays were performed and the specific bands (indicated by a line in Fig. 4) were scanned. These results are shown in Fig. 4F and clearly indicate a 6- to 10-fold induction for the nuclear factors binding to motifs 3 to 5 but only a 1.2- and a 1.6-fold induction for motifs 1 and 2, respectively.

Characterization of FiRE-binding nuclear proteins. The nucleotide sequence of FiRE (Fig. 3B) was compared to the known sequences of different transcription factor binding elements stored in the Transcription Factor Database. Only motif 2 revealed a known transcription factor binding consensus site, an E box. Motif 4 contained an AP-1-like consensus binding site with one mismatch, and motif 5 contained two AP-1-like consensus sites. Motifs 1 and 3 were not found to contain any known consensus sequences for transcription factors. Therefore, a large array (including AP-1, AP-2, AP-3, Ets, GATA, SIE, CRE, MEF-1, MEF-2, Max-Myc, SP-1, NF-κB, and C/EBP) of commercial consensus oligonucleotides were competed with each binding site in order to reveal possible protein binding outside the established consensus sites. As expected, a Max-Myc consensus oligonucleotide was able to abolish the binding to motif 2 (Fig. 5D). For motifs 4 and 5, AP-1 consensus oligonucleotides also competed the binding (Fig. 5A and B). Surprisingly, binding on motif 3 was abolished by AP-2 oligonucleotide although there is no AP-2 consensus site present in motif 3 (Fig. 5C). Motif 1 could not be competed by any of the consensus oligonucleotides tested (data not shown). These competition assays suggested that motifs 4 and 5 may bind an AP-1 complex, motif 3 may bind an AP-2 complex, motif 2 may bind a helix-loop-helix factor, and motif 1 may bind an unknown nuclear factor.

Based on the competition experiment results above, specific antibodies were tested by adding them to the gel retardation reaction. As indicated in Fig. 5, Jun and Fos antibodies were able to remove the specific binding on motifs 5 (Fig. 5A) and 4 (Fig. 5B) and also to produce supershifts. Anti-USF or anti-ATF-3 antibodies analyzed at the same time had no effect. As an AP-2 consensus oligonucleotide was able to abolish the

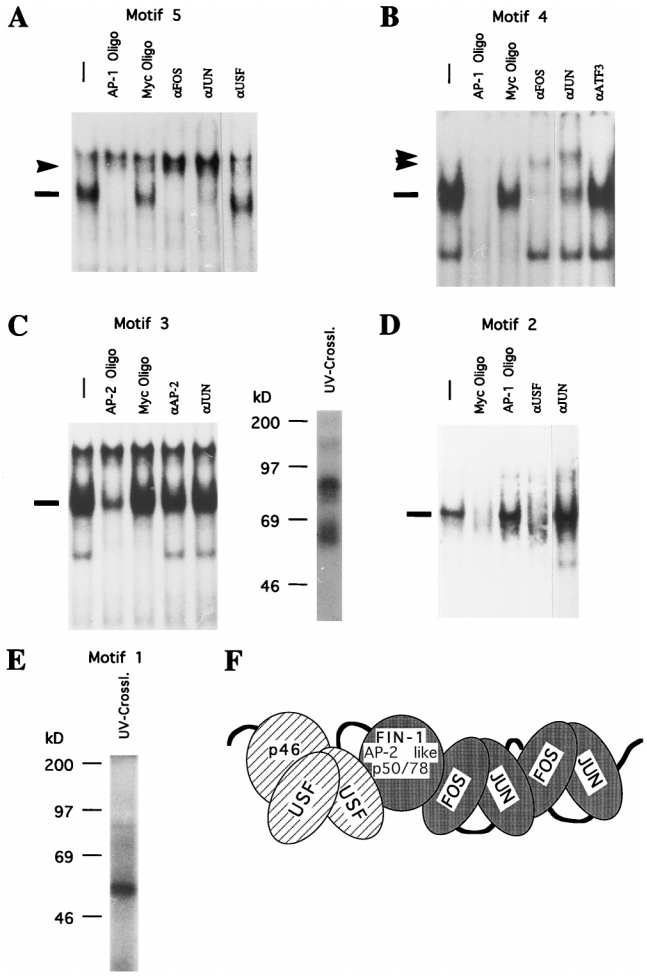

FIG. 5. The FiRE involves FGF-inducible AP-1s and constitutively expressed USF. (A) Specific binding (indicated by a line) on motif 5 was competed by a molar excess of AP-1 consensus oligonucleotide but not by E box consensus oligonucleotide. Antibodies against Fos (c-Fos [K-25; Santa Cruz]) and Jun (c-Jun [D; Santa Cruz]) removed the specific band and produced a supershift (arrowhead), which comigrates with the top band. Antibody against USF, used as a negative control, had no effect. (B) For motif 4, effects of AP-1 and E box consensus oligonucleotides as well as of Fos and Jun antibodies were the same as those for motif 5. Antibody against ATF-3 had no effect. (C) Specific binding on motif 3 was competed by a molar excess of AP-2 consensus oligonucleotide but not recognized by AP-2 or AP-1 antibodies. AP-2 antibody was found to be functional, as it produced a supershift in a reaction of labeled AP-2 oligonucleotide and 3T3 cell nuclear extract (data not shown). For UV cross-linking, a retardation gel equal to that shown in panel A was run and exposed to UV light. Areas with specific bands were cut out, eluted overnight, and loaded onto SDS-PAGE gels to analyze their molecular masses, with molecular weight markers. The two reproducible bands for motif 3 are shown. Molecular weights of nuclear factors were estimated after subtracting the mass of each oligonucleotide from the complex mass. (D) Specific binding (indicated by a line) on motif 2 was competed by a molar excess of E box consensus oligonucleotide, but not by AP-1 consensus oligonucleotide. Antibody against USF abolished the specific band, but the c-Jun antibody had no effect. (E) UV cross-linking was performed for motif 1, revealing one reproducible band. Nuclear extracts induced with FGF-2 for 4 h were used in all experiments. (F) Schematic presentation of FiRE in activated form.

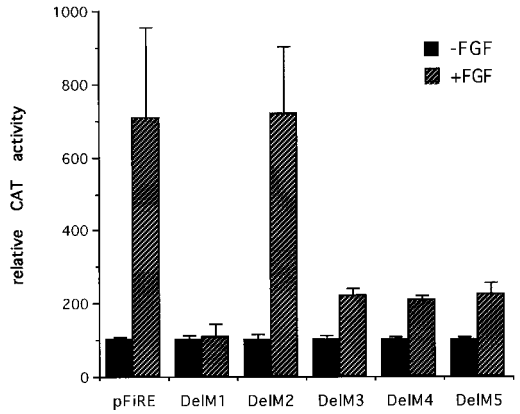

FIG. 6. AP-1s are required but not sufficient for FiRE activation. To estimate the importance of each motif, five deletion mutants for all the protein binding DNA regions were generated by PCR (see Materials and Methods). For DelM1 and DelM5, motifs 1 and 5 were totally deleted. For DelM2, the E box (CACC TG) on motif 2 was changed to a *Kpn*I recognition site (GGTACC). For DelM3, a central part of the motif (TCAGGGT) was replaced by a *Spe*I site (AATCACTAGTGA). For DelM4, the AP-1 site (GGAGTGAGCCATGCC) was replaced by a *Spe*I site (AATCACTAGTGATT). Transfections and CAT assays were performed as for Fig. 2. Except for the binding domain for USF (DelM2), deletion of each motif (DelM1 and DelM3 to M5) dramatically decreased the activation of FiRE by FGF-2.

binding by motif 3, an AP-2 antibody was tested with it. This, however, had no effect (Fig. 5C). The AP-2 antibody was also tested with labeled AP-2 oligonucleotide by using FGF-2-induced 3T3 cell nuclear extracts, and it was shown to be functional, as it produced a supershift (data not shown). This data suggests that the protein bound by motif 3, which is able to bind an AP-2 consensus site but is not AP-2, is perhaps an AP-2-related transcription factor and may bear homology within the DNA binding domain. This protein was named FIN-1 for FGF-inducible nuclear factor. For motif 2, neither Max nor Myc antibodies had any effect. However, another basic helix-loop-helix protein, USF, which is known to be constitutively expressed in 3T3 cells (32), was shown to occupy motif 2, as USF antibody removed the specific band (Fig. 5D) while not influencing the binding of other motifs.

As the proteins binding to motifs 1 and 3 remained unknown, we performed a UV cross-linking experiment to reveal their approximate molecular weights. Gel retardation was run as for Fig. 4, and the specific bands were cut, eluted, and run on SDS-PAGE gels. The oligonucleotide mass was subtracted from the estimated molecular weights revealed by the gel analysis. This method indicated one reproducible 46-kDa band for motif 1 and two bands of 78 and 50 kDa for motif 3 (Fig. 5C and E). Interestingly, AP-2 is known to be a 50-kDa protein. Figure 5F summarizes the structural model of FiRE, which includes constitutive and FGF-inducible transcription factors, in an active form.

Newly synthesized AP-1s are required but not sufficient for FiRE activation. To determine whether all the binding sites are required for enhancer activation, deletion mutants for each motif were generated by PCR. The E box on motif 2 was replaced by a *Kpn*I recognition site. For motifs 4 and 3, a central 10-bp sequence was replaced with a 10-bp sequence including an *Spe*I recognition site. For motif 1, the 3′ end of FiRE was deleted, and for motif 5, a PCR product ranging from motif 4 to motif 1 was generated. The mutant products were ligated into the CAT reporter plasmid with the 1.1-kb syndecan proximal promoter and transfected as described above. Cells were treated with FGF-2, and CAT assays were performed as described above. Modifications for motifs 5, 4, 3, and 1 resulted in a remarkable loss of FiRE activation. Modification of motif 2 had only slight or no effect (Fig. 6). This suggested that at least four of the five motifs are required for full activation of FiRE by FGF. Furthermore, it shows that at

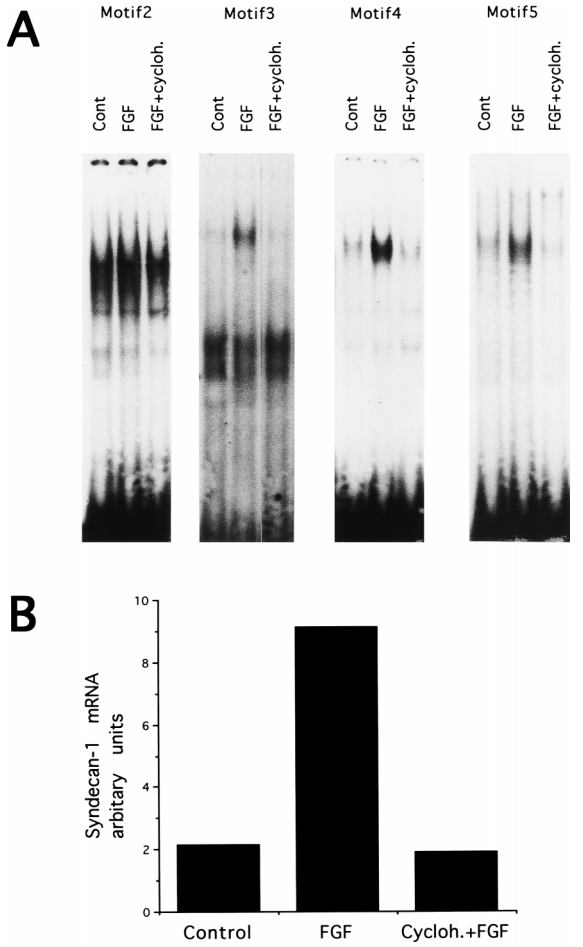

FIG. 7. Induction of syndecan-1 mRNA and FGF-inducible transcription factors requires de novo protein synthesis. To study whether protein synthesis is needed for the induction of FGF-inducible transcription factors, cells were treated with FGF-2 for 4 h and with or without simultaneous translation inhibitor cycloheximide (10-μg/ml final concentration). Whole-cell extracts were prepared, and gel mobility shifts were run as described in Materials and Methods. Each motif (2 to 5) is shown with a separately run control (Cont), FGF-2 (FGF), and simultaneous FGF-2 and cycloheximide treatment (FGF+cycloh.). The cycloheximide treatment abolished the specific binding on motifs 3, 4, and 5 but not on motif 2 (A). To demonstrate that cycloheximide also blocks the effects of FGF on syndecan-1 mRNA, a Northern analysis was performed, and the blot was probed with syndecan-1 and subsequently with a probe recognizing the ribosome 28S as a loading control (B).

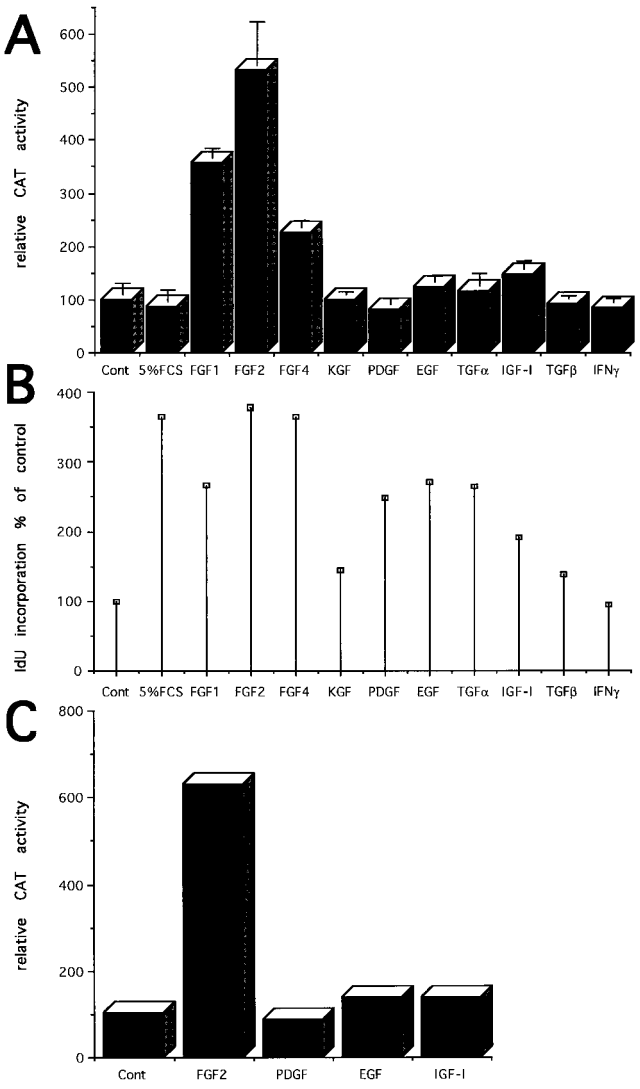

FIG. 8. The enhancer activation is specific for FGFs in NIH 3T3 cells. (A and B) pFiRE plasmid was transfected into 3T3 NIH cells as for Fig. 2. The cells were starved in medium supplemented with carboxymethyl-Sephadex serum (Cont) or exposed to various growth factors in the same medium or to 5% FCS-supplemented medium, and the reporter gene activity was tested by CAT assays. Only FGFs revealed clear enhancer activation not detectable with other growth factors or 5% FCS (A), independent of the DNA synthesis induced by these growth factors as tested by 5-[^{125}]IdU incorporation assay (B) after 4-h growth factor treatment. (C) To rule out the possible suppressive action of the syndecan proximal promoter on the growth factor action, only a 98-bp minimal promoter fragment (p-271FiRE), including the putative TATA box but without any upstream activator binding sites, was used. This was stably transfected into 3T3 NIH cells following FGF-2, PDGF, EGF, and IGF-I treatments and CAT assays.

least two Fos-Jun complexes are required and that alone they are not sufficient to activate FGF-induced transcription.

To study whether the activation of the different inducible components of FiRE is due to direct posttranslational modifications or whether they are newly synthesized, simultaneous cycloheximide and FGF treatment and subsequent protein extraction were performed. As shown in Fig. 7A, the FGF-induced binding of AP-1s to motifs 4 and 5, as well as the binding of FIN-1 to motif 3, was abolished when translation was blocked by cycloheximide. This suggests that all the inducible proteins involved in FiRE require de novo protein synthesis and, furthermore, that FiRE represents a secondary response element in FGF-initiated signaling. This is further demonstrated by the fact that cycloheximide also blocks the FGF-induced activation of syndecan-1 mRNA (Fig. 7B).

FiRE shows selectivity for FGFs in 3T3 cells. To examine the responsiveness of FiRE to other growth factors and serum, the FiRE-CAT construct was transfected into 3T3 NIH cells treated with FGF-1, FGF-2, FGF-4, FGF-7 (KGF), PDGF/BB, EGF, IGF-I, TGF-α, TGF-β, gamma interferon, and 5% FCS. Although all of the growth factors known to act on 3T3 cells stimulated 3T3 proliferation, as assayed by 5-[^{125}I]IdU incorporation after a 24-h growth factor treatment (Fig. 8B), FGFs were the only growth factors to clearly increase the reporter gene activity (Fig. 8A). Interestingly, FGF-1 and FGF-4 had less effect than FGF-2 (Fig. 8A). FGF-7 is known to act only on epithelial cells and had no effect. Although serum is known to contain several growth factors, 5% FCS also gave no response. PDGF, IGF, and EGF clearly enhanced cell proliferation but

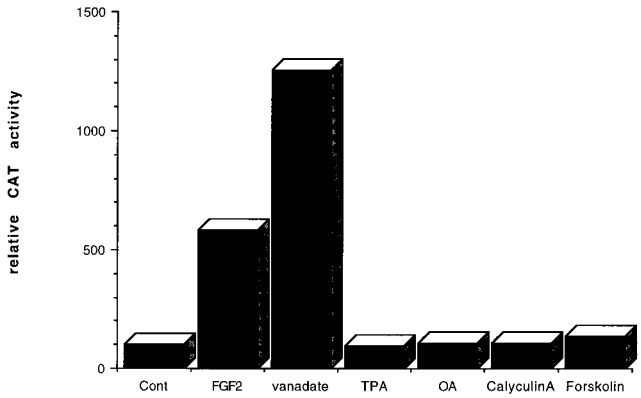

FIG. 9. TPA or other activators of AP-1 do not activate FiRE. Different agents, known to induce Fos-Jun complexes and activate AP-1-driven promoters, were tested for FiRE activation in a CAT assay with the minimal promoter FiRE (p271FiRE) stably transfected 3T3 NIH cells. The PKC activator TPA (10-nmol final concentration), the protein phosphatase-1 and -2 inhibitors okadaic acid (OA) (10 nmol) and calyculin A (10 nmol), the cAMP activator forskolin (5 μmol), and the tyrosine kinase phosphatase inhibitor orthovanadate (100 μmol) were used.

had no effect on the enhancer activity. Thus the stimulation of different tyrosine kinase receptors does not lead to activation of FiRE, nor does the proliferation alone correlate with the activation of FiRE.

As the original construct contained over 1 kb of syndecan-1 proximal promoter, we wanted to rule out the possible suppressive action of this basal promoter on the function of the other growth factors. Therefore, nearly all of the promoter was deleted, leaving only 98 bp of the proximal promoter (p-271CAT) which included only the putative TATA box without any upstream regulatory elements (58). As shown in Fig. 8C, the removal of the syndecan promoter had no effect on the pattern of growth factor-induced FiRE activation, indicating that the growth factor specificity is not regulated by the proximal promoter.

Agents activating AP-1 are not sufficient for FiRE activation. Since FiRE binds AP-1 transcription factors, whose activation is well characterized, we tested chemicals known to cause AP-1-dependent gene activation. These included the protein kinase C (PKC) activator 12-*O*-tetradecanoylphorbol-13-acetate (TPA), the protein phosphatase-1 and -2 inhibitors okadaic acid and calyculin A, the cAMP activator forskolin, and the tyrosine kinase phosphatase inhibitor orthovanadate (Fig. 9). As expected, inhibition of the tyrosine kinase phosphatase, causing continuous activation of tyrosine kinases, resulted in activation of FiRE. This suggests that all the nuclear factors bound to FiRE could be activated by tyrosine kinase activation. Surprisingly, however, none of the other compounds known to induce the immediate-early genes *fos* and *jun* and AP-1-driven promoters were able to activate FiRE. This suggests that more than simple activation of the pathways elicited by these agents (e.g., PKC- or cAMP-dependent pathways) is required for the response of FiRE induced by FGF.

DISCUSSION

In this paper, we have characterized a response element for members of the FGF family, factors which are known to influence a variety of biological systems. This far-upstream FiRE employs a rather complex composition of different nuclear factors. It consists of an array of five binding motifs, bound by several different transcription factors, and presents a novel possibility for FGFs to induce differential gene activation. Combining growth factor specificity and ability to upregulate an FGF-binding cell surface molecule, syndecan-1, the model also presents a mechanism by which members of the FGF family could autoregulate their own function.

A novel FiRE. The 280-bp FiRE described in this paper is located 11.6 kb upstream from the translation start site of the syndecan-1 gene and shows at least a 10-fold activation in FGF-2-treated 3T3 cells. In mesenchymal cells, this activation seems to be restricted to FGFs and shows no response to serum or other growth factors tested.

FiRE consists of several FGF-inducible and noninducible nuclear factors in an organized array (Fig. 5F). Components of AP-1, Fos and Jun, are rapidly activated by numerous extracellular stimuli. Their activation is brought about by either direct gene activation or posttranslational modifications (24). In several studies, FGFs have been shown to activate c-Fos. FGF-2 is also known to induce the expression of c-Fos and c-Jun in 3T3 cells (22). Our results show that FGF treatment results in transcriptionally active Fos-Jun heterodimers and that FiRE is bound by at least two AP-1 complexes, both of which seem to be required for FGF induction. Removal of the binding site of one AP-1 (motif 4 or motif 5) dramatically reduces the level of the FGF effect, as does the removal of the binding site for FIN-1. This indicates that FiRE requires concatenation of at least three FGF-inducible transcription factors on the same array of DNA. However, this seems not to be sufficient, since removing motif 1 also nearly abolishes the FGF response. This suggests that, besides gathering inducible factors together, the element demands interactions between inducible and constituent DNA-binding proteins. USF is a ubiquitously expressed homodimeric transcription factor (15) constantly expressed in 3T3 cells (32). It is not known to be under the influence of any growth factor. In FiRE, USF is not activated by FGF, but it is involved in the complex with inducible components. Replacement of the E box does not, however, reduce the FGF effect on FiRE. This might be due to replacement of USF with another constantly active transcription factor which would be able to interact with the inducible components. Alternatively, protein-protein interactions might hold USF in the FiRE complex despite the removal of its optional DNA binding target.

Besides AP-1 and USF, FiRE involves another noninducible component as well as a putatively novel FGF-inducible AP-2-like transcription factor. AP-2 is a cell-specific 50-kDa transcription factor expressed by several tissues at high levels (26). Multiple forms of AP-2 are generated by alternative splicing (31), and also one AP-2-related transcription factor, AP-2b, has been cloned (34). FIN-1, the FGF-inducible nuclear factor that binds motif 3 and could be competed with an AP-2 consensus oligonucleotide but was not recognized by AP-2 antibody, might also be a member of a larger AP-2 family. It is known that AP-2 can form heterodimers without DNA binding and that AP-2 dimerization is required for binding (61). This implies that the larger of the two bands seen in our cross-linking analysis might represent a dimer form of FIN-1 (Fig. 5). Our current effort is targeted to the cloning of this factor.

The interactions between far-upstream regulatory gene elements and basal transcriptional complexes are currently not fully understood. However, looping of DNA and physical interactions between these two separate elements are supposed to occur. The exchange of the syndecan-1 promoter with the simian virus 40 promoter did not inactivate the enhancer, and neither did removal of most of the proximal promoter, suggesting that the FiRE-type element does not require any specific upstream activators on proximal promoter and, further-

more, that a similar element might be able to activate other genes besides syndecan-1.

FGFs, EGF, PDGF, and IGF signal by binding to their own cell surface tyrosine kinase receptors (RTKs). FGFs, as well as the other growth factors, activate the MEK-MAP kinase pathway downstream from the receptor activation via *ras* and *raf* (5, 18, 29, 37, 55), but other signal transduction pathways also might contribute to the FGF signaling (29). Several endpoints for growth factor signaling on gene elements have been described elsewhere. These include (i) EGF and PDGF activation of the serum response element (SRE), which is bound by a ternary complex factor-serum response factor complex; (ii) activation of the CRE, which besides CRE-binding protein is also bound by AP-1 and ATF family members; (iii) response elements for signal transducer and activator of transcription factors (interferon-stimulated response element and SIE), which are activated through Janus kinases by EGF and PDGF as well as by various cytokines like interferons; and (iv) activation of NF-κB by TGF-α (18, 24). FGFs are also reported to activate the SRE (39) and, together with forskolin, also the CRE (52) and subsequent transcription of cellular genes. Together with these examples, FiRE, which does not contain either SRE or CRE, illustrates the multiple ways in which FGFs can induce transcription.

All the RTK-activating growth factors can induce cell proliferation and activate Fos and Jun transcription factors in 3T3 cells. They still have different biological effects and can induce partly different subsets of secondary target genes. As the cycloheximide inhibition of the FGF response reveals, FiRE is a secondary response element in FGF-induced signaling. Furthermore, based on the mutational analysis and the treatment with several AP-1-activating agents, it is clear that, while AP-1 activation is mandatory for FiRE activation, it is not sufficient for it. This element can also distinguish the action of different RTK-stimulating growth factors upon an AP-1-driven DNA element, since only FGFs, not other AP-1-inducing growth factors or chemicals, can activate it. The mechanisms underlying this specificity still remain to be studied. Several possibilities exist as to why FGFs are able, but other growth factors and serum fail, to activate FiRE. The unresponsiveness of FiRE to serum could be explained by the low concentration of FGFs in it, as the most abundant growth factor in serum is thought to be PDGF. The activation of RTKs other than FGFRs can result in differential activation of a diverse subset of cytoplasmic kinases and, subsequently, also different transcription factors such as members of the Fos and Jun families. This again could result in a switch of one family member to another that binds to FiRE while lacking the inductive capacity. Equally, it is also possible that the other growth factors cannot induce the FIN-1 transcription factor while they are still able to activate AP-1s. One possible mechanism would be different posttranslational modification, such as phosphorylation or dephosphorylation on the inducible (e.g., Fos or FIN-1) or constituent (e.g., USF) proteins. For example, c-Jun is known first to require dephosphorylation to bind on DNA and then to require further phosphorylation in order to activate transcription (for a review, see the work of Karin [24]). Finally, signaling through RTKs other than FGFR could induce inhibitory transcription factors that bind to FiRE but lack the capacity to activate it. For example, the high PDGF content in serum might activate signaling pathways downregulating FiRE. Therefore, FiRE, as an end point for signaling, can distinguish various RTK-activated cascades and may be a very useful tool for future studies elucidating biological differences such as the differential activation of these cascades.

Physiology of FiRE. FGF-2 is produced by fibroblasts and is also found in association with extracellular matrix and basement membranes, where it can be released by proteolytic activity. FGF-2 enhances the accumulation and proliferation of fibroblasts, keratinocytes, endothelial cells, and macrophages. In animal models, it induces neovascularization, cell migration, and granulation tissue formation, and during development, it seems to possess a vast number of different functions, including induction of mesenchyme. In adult tissues, syndecan-1 is expressed mainly in epithelia, but during embryogenesis also transiently in mesenchymes, where it correlates with FGF expression. Syndecan-1 expression is strictly regulated following morphological boundaries, e.g., in limb bud, tooth, and kidney. For example, in tooth organogenesis syndecan is first detected in epithelium, but during induction of mesenchyme, syndecan appears in condensing and proliferating mesenchyme (53, 56). Syndecan can be colocalized to the target tissues of many members of the FGF family, for example, the mesenchyme underlying ectoderm in limb bud (49), which is a target for FGF-2 (13), FGF-4 (36), and FGF-8 (10). FGF-8 can also be colocalized with syndecan-1 in limb bud (16). In tooth development, syndecan is colocalized with FGF-3 and is also detected in the mesenchyme and epithelium, which are putative target tissues for FGF-4 (54). This raises the possibility that FiRE is the regulatory element and the end point for FGF-initiated signaling of syndecan upregulation in mesenchyme.

Heparin is shown to be required for FGF oligomerization and subsequent FGFR dimerization and signal transduction (50). Heparin is in the form of heparan sulfate proteoglycans at the cell surface, and it is not yet fully understood how proteoglycans can participate in the regulation of FGF. Syndecan-1 can bind simultaneously FGF-2 and an extracellular matrix molecule, and this complex can promote FGF-induced cell proliferation (44). However, there is also evidence that syndecan-1 expression could be inhibitory for FGF action. Syndecan-1 isolated from lung fibroblasts has been shown to inhibit FGF binding to FGFR (1). Furthermore, the overexpression of syndecan-1 on the cell surface of 3T3 NIH cells by transfection abolishes the proliferative response of FGF-2 (28). Several possibilities can explain this type of inhibition, including an unfavorable stoichiometric ratio of FGFR and syndecan-1 that does not support ternary complex formation. Alternatively, an altered heparan sulfate structure may generate antagonistic activity and subsequent release of growth factor from FGFR. Whatever the mechanism, this inhibition provides an interesting inhibitory loop for FGF action. Cells with a low cell surface proteoglycan content can activate the FGF signaling pathway resulting in the activation of FiRE. This results in the enhancement of syndecan-1 expression, which subsequently could block further FGF action. This type of restriction of FGF action would be extremely useful during development, for example, as FGFs could otherwise cause inappropriate proliferation of mesenchymal cells in time and space. This principle could be applied also to other physiological and pathophysiological conditions in which proliferation of mesenchyme or fibroblasts by growth factors occurs.

Cancerous cells are also known to be able to activate AP-1 complex, and it is known that c-Fos is required for malignant tumor progression (42). However, in many cases, syndecan-1 is shown to disappear when cells transform and become invasive (20, 21). It is therefore tempting to speculate that, if FiRE is needed for high-level expression of syndecan-1, then perhaps the activation of the non-AP-1 transcription factors, such as FIN-1 and USF, of FiRE may be disturbed in malignant cells while Fos and Jun dimers remain active, resulting in decreased syndecan-1 levels.

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