Sp1- and Sp3-mediated Transcriptional Regulation of the Fibroblast Growth Factor Receptor 1 Gene in Chicken Skeletal Muscle Cells*

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Expression of the fibroblast growth factor receptor 1 (FGFR1) gene in skeletal muscle is positively regulated in proliferating myoblasts and declines during differentiation. We have characterized the cis-regulatory elements in the proximal region of the FGFR1 promoter which render positive transcriptional activity. Multiple elements between −69 and −14 activate the FGFR1 promoter. Myoblast transfections revealed that potential Sp transcription factor binding sites are required for promoter activity. Electromobility shift assays indicated that myoblast nuclear proteins specifically bind to these cis-elements and that differentiated myotube nuclear extracts do not form these same complexes. In addition, Southwestern blot analysis detected binding of the most proximal Sp motif to a Sp1-like protein present in myoblast nuclear extracts but not in myotubes. In corroboration, Sp1 and Sp3 proteins were detected only in myoblasts and not in differentiated myotubes. Finally, transfection of Drosophila SL2 cells showed that Sp1 is a positive regulator of FGFR1 promoter activity and that Sp3 is a coactivator via the proximal Sp binding sites. These studies demonstrate that the FGFR1 promoter is activated by Sp transcription factors in proliferating myoblasts and demonstrate at least part of the mechanism by which FGFR1 gene expression is down-regulated in differentiated muscle fibers.

During vertebrate myogenesis, mesodermally derived cells within myogenic lineages proliferate as mononucleated myoblasts before differentiation into postmitotic, multinucleated muscle fibers. Both the sustained proliferation of myoblasts and subsequent withdrawal from the cell cycle as a part of differentiation are regulated by signal transduction cascades initiated by environmental signals including growth factors. Members of the fibroblast growth factor (FGF) family of signaling molecules are capable of sustaining myoblast proliferation and delaying differentiation. The cellular effects of FGF signaling are mediated through a small family of fibroblast growth factor receptors (FGFRs). FGFR1 and FGFR2 possess well-documented mitogenic activity for skeletal myoblasts, and these factors bind to FGFR1 in the cell surface of proliferating myoblasts. In addition to skeletal muscle myoblasts, FGFR1 is also expressed during development of the brain, skin, bones, and cardiac muscle (1). It has recently been shown that FGFR1 can be translocated to the nucleus via importin B and that it plays an important role in the regulation of the cell cycle by inducing nuclear target genes (2). However, FGF signaling declines during myoblast differentiation. This decline is the result of loss of cell surface receptor and a coordinate decrease in FGFR1 mRNA (3–5).

The significance of the developmentally regulated expression of FGFR1 in relation to muscle growth and patterning in vivo has been partially examined. Myoblast differentiation and muscle fiber formation were delayed in chick limb musculature overexpressing wild type FGFR1. Conversely, premature differentiation with decreased muscle mass occurred in chicken embryos expressing a dominant negative mutation of FGFR1 (6). Muscle patterning within these embryos was disorganized, suggesting a role for FGFR1 function at multiple levels of developmental regulation (7). Coupled with studies in vitro correlating FGFR1 availability and myoblast proliferation versus differentiation (8), these studies demonstrate the requirement of developmentally regulated FGFR1 gene expression for normal skeletal muscle development.

Although regulation of FGFR1 gene expression plays an important role in the development and growth of skeletal muscles, the mechanisms involved in its regulation are not well understood. Nonetheless, studies focusing on other growth factor receptor gene promoters have provided some insight into the possible mechanisms of FGFR1 promoter regulation. Structural analysis of the promoters of the human and rat insulin-like growth factor I and human transforming growth factor β receptor genes revealed a lack of consensus TATA and CCAAT motifs in the proximal promoter regions (9–12). Similarly, the human epidermal growth factor receptor and FGFR3 gene promoters lack TATA elements (13, 14). Our previous work on the FGFR1 promoter demonstrated that the promoter was structurally and functionally divided into proximal and distal regions. The distal region is located more than 1 kb upstream from the start of transcription and positively regulates FGFR1 gene expression in myoblasts (5). The distal region contains two Sp transcription factor binding sites, both of which are required for FGFR1 promoter activity in proliferating myoblasts. Recently, it has been shown that the two functional Sp binding sites within the distal promoter region bind Sp1 in myoblast nuclear extracts (15). Although the distal Sp1 binding sites were required for full FGFR1 promoter activity and conferred increased transcriptional activity to a minimal thymidine kinase promoter, the distal region alone was not sufficient for promoter activity. The proximal region was also required. Therefore, additional sites in the FGFR1 proximal promoter, possibly other than CCAAT and TATA elements, regulate gene transcription.

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The Sp family of transcription factors plays a concerted role in regulating many different promoters. Four zinc finger Sp transcription factors have been identified. Like Sp1, both Sp3 and Sp4 can bind to the GC box or GT motifs, whereas Sp2 has a much weaker binding affinity to GT motifs. Sp1 and Sp3 are generally regarded as ubiquitously expressed, and Sp4 expression is restricted to the brain (16). In addition, Sp1 and Sp4 are considered to be transcriptional activators, whereas Sp3 often functions as a transcriptional repressor (17–20).

We report here the structural and functional characterization of the proximal region of the FGFR1 gene promoter. The proximal region contains multiple cis-elements that differentially regulate FGFR1 gene expression in proliferating myoblasts. These sites interact with specific transcription factors differentially expressed in proliferating myoblasts versus differentiated myotubes to confer myoblast-specific expression of the FGFR1 gene. Southwestern blot analysis and functional transient transfection assays indicate that the transcription factors Sp1 and Sp3 coactivate the FGFR1 promoter. In addition, developmentally regulated expression of the FGFR1 gene in proliferating myoblasts versus differentiated muscle fibers may be explained by differential expression of Sp transcription factors during differentiation.

MATERIALS AND METHODS

Construction of Mutant Plasmids—The plasmid 3284FGFRICAT containing the full-length promoter was described previously (5). A series of mutations was made in the proximal promoter region by utilizing QuikChange site-directed mutagenesis kit (Stratagene) with each of the following forward primers and their antisense oligonucleotides as reverse primers:

- (m23) CTGCCCCGACTTTCTTCTCATTCCACAGCTCACGGCC
- (m24) CAGGGGCTGCGACTTCTATCCTGACTCTTCTTTCCTCCCTC
- (m54) CAGGACGAGCAGACGGGTTAACATGCAGTCCTCGGC

Briefly, 250 ng of 3284FGFRICAT promoter plasmid was used in a PCR containing 5 μl of Pfu polymerase buffer, 1 μl of Pfu turbo DNA polymerase, 10 μM dNTPs, 125 ng of each respective oligonucleotide and H2O to 50 μl. After temperature cycling (94°C for 1 min, 25°C for 30 sec, 72°C for 1 min) treatment was performed to cleave parental DNA and to improve the efficiency of screening mutant plasmids. The reaction mixture was then transferred into JM109 cells on LB ampicillin plates, and the mutant plasmids were confirmed by DNA sequencing.

Cell Culture, DNA Transfections, and Reporter Assay—Fetal (embryonic days 12–13) chick myoblasts were isolated from leg muscles and HeLa cells (Geneka) were denatured in 2× SDS sample buffer, boiled for 5 min, and electrophoresed in a 7.5% SDS-polyacrylamide gel. Fractionated proteins were then electroblotted onto a nitrocellulose membrane (Schleicher & Schuell, BA-85). The membrane was prehybridized with 100 ml of hybridization buffer containing 10× Tris-HCl, pH 7.5, 1 mM MgCl2, 0.5 mM EDTA, 0.5 mM dithiothreitol, 50 mM NaCl, and 5% nonfat dry milk and were fixed with 100% methanol for 5 min. Cultures were washed as above and blocked for 1 h at room temperature or overnight at 4°C. The membrane was then rinsed twice with the hybridization buffer containing 0.25% nonfat dry milk. Subsequently, the membrane was hybridized with the end-labeled probe (107 cpm) in 25 ml of hybridization buffer containing 0.25% nonfat dry milk and 250 μg of poly(dIdc) at room temperature for 3 h. The hybridized membrane was washed four or five times with hybridization solution containing 0.25% nonfat dry milk, dried, and exposed overnight to an x-ray film.

Antibodies—Rabbit anti-Sp1, Sp2, Sp3, and Sp4 sera and horseradish peroxidase-conjugated anti-rabbit IgG were obtained from Santa Cruz Biotechnology, Inc. DAPI was from Molecular Probes, Inc. Texas Red streptavidin, biotinylated anti-rabbit IgG (H+L), and fluorescein-conjugated anti-mouse IgG were from Vector Laboratories, Inc.

Immunohistochemistry—Myogenic cultures were immunostained 24 h or 10 days after plating. Cells were washed three times with PBS and were fixed with 10% methanol for 5 min. Cultures were washed as above and blocked for 1 h with BS containing 0.3% bovine serum albumin and 0.1% Tween in PBS. Polyclonal anti-rabbit Sp family antibodies (1:1,000) and F59, an anti-fast myosin heavy chain monoclonal antibody, were used to detect the total DNA up to 7 μg/plate. After transfection, cells were maintained in growth medium. Cells were harvested 48 h after transfection, and CAT assays were performed (5). For all transfection assays, four or five independent experiments were performed.

Nuclear Extracts and Electromobility Shift Assay (EMSA)—Nuclear extracts from proliferating chicken myoblasts and differentiated myotubes were prepared after 24 h of culture for myoblasts and 10 days for myotubes and stored at −80°C as described previously (23). Protein content was determined by BCA protein assay (Pierce).

Complementary oligonucleotides were commercially synthesized (Operon). The oligonucleotides (10 μg) each were boiled in 10× kinase buffer and allowed to cool gradually to room temperature. Approximately 200 ng of double stranded oligonucleotide was 5’-end labeled using T4 kinase (Promega) and [γ-32P]ATP (PerkinElmer Life Sciences). Each binding reaction, 2 μg of double stranded poly(dIdc), 5× binding buffer (40 mM KCl, 15 mM HEPES, pH 7.9, 1 mM EDTA, 0.5 mM MgCl2, and 5% glycerol), and 20 μg of nuclear extracts in a 20-μl reaction volume were incubated at room temperature for 30 min. Competitive EMSAs were carried out under identical conditions except that a 100 or 250 molar excess of nonlabeled double stranded oligonucleotides was added to the binding reactions. Labeled probe (70,000 cpm in 1 μl) was added and incubated further for 15 min on ice. DNA-protein complexes were resolved by 5% nondenaturing polyacrylamide gel electrophoresis with 1× Tris borate EDTA buffer. The gel was dried and exposed to x-ray film overnight.

Southern Analysis—Southwestern blot analysis was performed as described previously (24) with few modifications. Briefly, 75 μg of nuclear extracts from myoblasts, myotubes, and HeLa cells (Geneka) were denatured in 2× SDS sample buffer, boiled for 5 min, and electrophoresed in a 7.5% SDS-polyacrylamide gel. Fractionated proteins were then electroblotted onto a nitrocellulose membrane (Schleicher & Schuell, BA-85). The membrane was prehybridized with 100 ml of hybridization buffer containing 10× Tris-HCl, pH 7.5, 1 mM MgCl2, 0.5 mM EDTA, 0.5 mM dithiothreitol, 50 mM NaCl, and 5% nonfat dry milk and 250 μg of poly(dIdc) at room temperature for 3 h. The hybridized membrane was washed four or five times with hybridization solution containing 0.25% nonfat dry milk, dried, and exposed overnight to an x-ray film.
clonal antibodies (1:10) were diluted in BS. Cultures were incubated in primary antibodies for 1 h at room temperature and washed as above. Fluorescein-conjugated anti-mouse IgG (1:100) and biotinylated anti-rabbit IgG (1:100) were diluted in BS and added to the cultures. After a 1-h incubation at room temperature the cells were washed as above. Two drops of 2.5% diazabicyclooctane in glycerol:PBS (9:1) and coverslips were applied, room temperature and washed five times with PBS. Two drops of 2.5% 32P-orthophosphate (32P-OP) at 300 mM concentration in PBS was added to the cultures for 10 min at room temperature, and the cultures were washed three times. DAPI Texas Red streptavidin (1:1,000 in BS) was added to the cultures for 1 h at 37°C. The cultures were washed three times. Finally, the cultures were incubated in anti-mouse IgG (1:100) for 1 h at room temperature the cultures were washed as above. Rabbit IgG (1:100) were diluted in BS and added to the cultures. After 1-h incubation at room temperature the cells were washed as above. The cultures were incubated in Fluorescein-conjugated anti-mouse IgG (1:100) and biotinylated anti-rabbit IgG (1:100) were diluted in BS. Cultures were incubated in primary antibodies for 1 h at room temperature and washed as above. Fluorescein-conjugated anti-mouse IgG (1:100) and biotinylated anti-rabbit IgG (1:100) were diluted in BS and added to the cultures. After a 1-h incubation at room temperature the cells were washed as above. Texas Red streptavidin (1:1,000 in BS) was added to the cultures for 1 h at room temperature, and the cultures were washed three times. DAPI at 300 mM concentration in PBS was added to the cultures for 10 min at room temperature and washed five times with PBS. Two drops of 2.5% diazabicyclooctane in glycerol:PBS (9:1) and coverslips were applied, and the cultures were viewed with fluorescence microscopy.

RESULTS

Identification of cis-Elements in the Proximal Promoter Region of the FGFR1 Gene Promoter—A SacI DNA fragment of a chicken genomic clone containing exon 1 and the 5′-end of the first intron as well as more than 1 kb of DNA upstream from the start of published mRNA sequence encoding FGFR1 (27) was isolated and sequenced (Fig. 1). Sequence analysis of the proximal upstream DNA revealed four potential Sp binding sites located within a 2.2-kb promoter segment of the wild type 3,284-bp promoter. The promoter activity of each mutant plasmid was then evaluated by CAT assay of extracts from transfected, proliferating myoblasts and differentiated myotubes (Fig. 2B). Cells transfected with promoterless pCAT3Basic vector alone showed basal levels of CAT activity. Myoblasts transfected with the wild type promoter (3284FGFR1) had more than a 50-fold increase in CAT activity, whereas its activity in myotubes was reduced substantially to basal levels. This result was similar to the previous results of experiments performed with myoblasts and myotubes (5). With 3284FGFR1 promoter activity set at 100% activity, CAT activities were reduced significantly in extracts from myoblasts transfected with the mutant promoters. Mutation of the −23 Sp site significantly reduced (9%) CAT activity in myoblasts. Mutations of the Sp sites at −42 and −54 resulted in 50 and 21% CAT activity, respectively. Combinatorial mutations further reduced CAT activities in myoblast extracts. CAT activities in extracts of myoblasts transfected with the wild type promoter (3284FGFR1) had more than a 50-fold increase in CAT activity, whereas its activity in myotubes was reduced substantially to basal levels. This result was similar to the previous results of experiments performed with myoblasts and myotubes (5). With 3284FGFR1 promoter activity set at 100% activity, CAT activities were reduced significantly in extracts from myoblasts transfected with the mutant promoters. Mutation of the −23 Sp site significantly reduced (9%) CAT activity in myoblasts. Mutations of the Sp sites at −42 and −54 resulted in 50 and 21% CAT activity, respectively. Combinatorial mutations further reduced CAT activities in myoblast extracts. CAT activities in extracts of myotubes transfected with all constructs were reduced significantly compared with 3284FGFR1 promoter activity in myoblasts. These results indicate that the Sp site at −23 bp is a functional cis-element responsible for transcriptional activation of the FGFR1 promoter in myoblasts. Furthermore, the two other Sp sites in the proximal region confer additional activation.

Different Nuclear Proteins from Myoblasts and Myotubes Bind to the Sp Binding Sites of the Proximal Promoter Region—To determine whether each of the Sp consensus binding sites interacts with a nuclear protein component(s), EMSAs were performed with three oligonucleotides spanning the region −69 to −14 (Fig. 3A). These probes were incubated with myoblast and myotube nuclear extracts. Each oligonucleotide

![Fig. 1. Nucleotide sequence of the proximal region of the FGFR1 gene promoter. DNA sequence extending 1,052 bp upstream from exon 1 is shown.](image-url)
formed a major DNA-protein complex and several additional complexes (Fig. 3B). A 100-fold molar excess of unlabeled double stranded oligonucleotide (−23) inhibited complex formation between the labeled −23 oligonucleotide and nuclear proteins, whereas a 100-fold molar excess of the mutant oligonucleotide (m23) did not compete in binding, demonstrating specificity of the DNA-protein interactions. The major DNA-protein complex (indicated by †) was present in both myoblast and myotube nuclear extracts. However, the slower migrating complexes (indicated by ‡) formed only from myoblast nuclear extracts when shifted with the −23 probe and were absent in DNA-protein complexes formed from myotube nuclear extracts. Competitive binding reactions with wild type competitors of labeled −42 and −54 double stranded oligonucleotides also reduced formation of detectable DNA-protein complexes formed by both myoblast and myotube nuclear extracts with the corresponding Sp binding site (data not shown).

To begin to define the nature of the DNA-protein complexes formed by the −23 Sp site and myoblast nuclear extract, competitive binding reactions using unlabeled −23 oligonucleotide and a Sp1 consensus oligonucleotide were performed (Fig. 3C). The formation of all DNA-protein complexes was significantly reduced by the addition of a 100-fold molar excess of −23 oligonucleotide. However, only formation of the slower migrating DNA-protein complexes was inhibited competitively by the Sp consensus oligonucleotide. These results indicate that the slower migrating DNA-protein complexes contain a Sp site binding factor.

The Most Proximal Site (−23) Binds the Sp1 Transcription Factor—To characterize further the most proximal (−23) Sp binding site, Southwestern blot analysis of myoblast and myotube nuclear extracts was carried out using the −23 oligonucleotide as a probe. In addition, a double stranded 22-bp Sp1 consensus oligonucleotide (5′-ATTCCATCGGCGGCGGCGG-3′) was also used as a probe in a comparative analysis. This Sp1 consensus oligonucleotide bound to a DNA-binding protein with molecular mass of 105 kDa in myoblast nuclear extracts (Fig. 4A). This corresponds to the size of reported Sp1 protein (28). Similarly, a 105-kDa protein was detected in myoblast nuclear extracts by the −23 Sp probe. This protein was not detected by the −23 probe bearing a mutation at the Sp binding site. The mutated site did bind to a lower molecular mass component in the myoblast nuclear extract, the nature of which is undefined. Interestingly, the 105-kDa protein was present only in myoblast nuclear extracts and was not detected in extracts from differentiated myotubes. As a control, a Southwestern blot with HeLa cell nuclear extract was performed using the Sp1 consensus oligonucleotide and the −23 oligonucleotide as labeled probes. Both probes detected a similar band of 105 kDa in the HeLa cell nuclear extracts (Fig. 4B). These results indicate that the −23 proximal Sp site of the FGFR1 promoter binds the Sp1 transcription factor.

Sp1 Transactivates the FGFR1 Gene Promoter, and Sp3 Coactivates through the Proximal Sp Binding Motifs—Although Southwestern analysis indicated that the most proximal Sp site (−23) bound the Sp1 transcription factor (Fig. 4B), it was not determined whether Sp transcription factors can functionally transactivate the FGFR1 promoter. To determine whether members of the Sp transcription factor family of proteins can transactivate the FGFR1 promoter, expression plasmids pPacSp1 and pPacSp3, encoding Sp1 and Sp3, respectively, were cotransfected with the wild type 3284FGFR1CAT promoter construct or its mutant derivatives into Drosophila SL2 cells. Drosophila SL2 cells lack the endogenous Sp transcription factors and have been useful in studying the function of the Sp family of proteins (16, 17, 29). As shown in Fig. 5A, with CAT activity derived from cells transfected with 3284FGFR1CAT and 3 μg of pPacSp1 as 100% activation, Sp1 specifically transactivated the FGFR1 promoter in a dose-dependent manner.

CAT activities resulting from cotransfection of mutated FGFR1CAT promoter and pPacSp1 constructs were measured. Mutations in the Sp motifs of the proximal region of the promoter reduced transactivation by Sp1 significantly (Fig. 5B). Mutation of the Sp1 site at −23 reduced (20%) Sp1 transactivation significantly. In addition, mutations of the Sp sites at −42 also reduced FGFR1 promoter transactivation significantly to 60%, although this mutation did not reduce transactivation to the same extent (20%) as the mutation of the −23 Sp1 site. Combinatorial mutations of the Sp sites further abrogated Sp1 transactivation.

Sp3 is a bifunctional transcription factor that can either activate or repress transcription (19, 30). Therefore, we exam-
Transcriptional Regulation of the FGFR1 Gene by Sp1 and Sp3

**Panel A**, sequence of the proximal promoter of the FGFR1 gene containing the three identified Sp binding sites. Three double stranded oligonucleotides used as probes and wild type competitors are indicated by the brackets above and below the sequence. The underlined sequences indicate the potential Sp binding sequences and the sites mutated for competitive binding experiments. Panel B, EMSAs were performed with the above oligonucleotides as probes in the absence or presence of a 100-fold molar excess of unlabelled competitors. Lanes 1, 6, and 9 have probe alone; lanes 2, 4, 5, 7, and 10 contain 20 μg of myoblast nuclear extract; lanes 3, 8, and 11 contain 20 μg of myotube nuclear extract; lane 4 contains −23 oligonucleotide as a wild type competitor, and lane 5 contains the mutated −23 oligonucleotide competitor (m23). Major complexes are indicated (− and <), and the slow migrating bands are indicated by an asterisk. The slow migrating DNA-protein complexes were abolished, and the faster, major DNA-protein complex was reduced significantly by competition with the −23 oligonucleotide. Competitive binding of the −23 oligonucleotide with the myoblast nuclear extracts components in the slower migrating complexes was reduced effectively in the presence of the consensus Sp1 oligonucleotide competitor.

**Panel C**, EMSAs were performed with 20 μg of myoblast nuclear extract using −23 oligonucleotide as a labeled probe. The −23 and consensus Sp1 oligonucleotides were used as wild type competitors in the indicated molar excess to test the specificity and nature of the DNA-protein interaction. Lane 1 contains probe alone, and lane 2 contains probe and nuclear extract. The major complexes are indicated by >, and the slow migrating bands are indicated by an asterisk. The slow migrating DNA-protein complexes were abolished, and the faster, major DNA-protein complex was reduced significantly by competition with the −23 oligonucleotide. Competitive binding of the −23 oligonucleotide with the myoblast nuclear extracts components in the slower migrating complexes was reduced effectively in the presence of the consensus Sp1 oligonucleotide competitor.

**Panel D**, EMSAs were performed with 20 μg of myoblast nuclear extract using −23 oligonucleotide as a labeled probe. The −23 and consensus Sp1 oligonucleotides were used as wild type competitors in the indicated molar excess to test the specificity and nature of the DNA-protein interaction. Lane 1 contains probe alone, and lane 2 contains probe and nuclear extract. The major complexes are indicated by >, and the slow migrating bands are indicated by an asterisk. The slow migrating DNA-protein complexes were abolished, and the faster, major DNA-protein complex was reduced significantly by competition with the −23 oligonucleotide. Competitive binding of the −23 oligonucleotide with the myoblast nuclear extracts components in the slower migrating complexes was reduced effectively in the presence of the consensus Sp1 oligonucleotide competitor.

**Fig. 3.** Gel retardation experiments with the proximal Sp binding motifs of the FGFR1 promoter. Panel A, sequence of the proximal promoter of the FGFR1 gene containing the three identified Sp binding sites. Three double stranded oligonucleotides used as probes and wild type competitors are indicated by the brackets above and below the sequence. The underlined sequences indicate the potential Sp binding sequences and the sites mutated for competitive binding experiments. Panel B, EMSAs were performed with the above oligonucleotides as probes in the absence or presence of a 100-fold molar excess of unlabelled competitors. Lanes 1, 6, and 9 have probe alone; lanes 2, 4, 5, 7, and 10 contain 20 μg of myoblast nuclear extract; lanes 3, 8, and 11 contain 20 μg of myotube nuclear extract; lane 4 contains −23 oligonucleotide as a wild type competitor, and lane 5 contains the mutated −23 oligonucleotide competitor (m23). Major complexes are indicated (− and <), and the slow migrating bands are indicated by an asterisk. The slow migrating DNA-protein complexes were abolished, and the faster, major DNA-protein complex was reduced significantly by competition with the −23 oligonucleotide. Competitive binding of the −23 oligonucleotide with the myoblast nuclear extracts components in the slower migrating complexes was reduced effectively in the presence of the consensus Sp1 oligonucleotide competitor.

**Fig. 6A**, cotransfection of a constant amount of pPacSp1 (750 ng) and increasing amounts of pPacSp3 together with the 3284FGFR1CAT construct, yielded dose-dependent increases in CAT activities. These results indicate that Sp3 functions as a coactivator in the presence of Sp1. In another set of SL2 cell transfection experiments, wild type or mutated FGFR1CAT constructs were cotransfected with pPacSp1 and pPacSp3 (Fig. 6B). As before, cotransfection of both pPacSp1 and pPacSp3 increased FGFR1 promoter activity significantly relative to cotransfection of only pPacSp1. Mutation of each Sp site alone and in combination caused a significant decline in FGFR1 promoter activity in pPacSp1 and pPacSp3 cotransfections. These results indicate that Sp1 can transactivate the FGFR1 promoter and Sp3 functions as a coactivator in the presence of Sp1.

**Sp1 and Sp3 Are Expressed Only in Proliferating Myoblasts**—Southwestern blot analysis revealed the presence of a 105 kDa band corresponding to Sp1 only in the nuclear extracts of myoblasts. To determine whether Sp transcription factors are expressed in differentiated myotubes, Western blot analysis was carried out using the nuclear extracts from myoblasts and myotubes. Protein bands of −105, 65, and 60 kDa were detected using a Sp1-specific antibody. An Sp3-specific antibody detected 135, 110, and 60 kDa protein bands in the nuclear extracts of myoblasts (Fig. 7). Very faint bands of Sp3 (135 and 60 kDa) were detected in the myotube nuclear extracts. Sp2 and Sp4 proteins were not detected in either myoblast or myotube nuclear extracts (data not shown). These experiments were repeated four times with fresh nuclear extracts. The molecular masses of Sp1 and Sp3 proteins were consistent with results published previously (32).

To confirm further the results of Southwestern and Western blot analyses, proliferating myoblasts and differentiated myotubes were immunostained with Sp1, Sp2, Sp3, and Sp4-specific antibodies. As observed earlier in Western blots, Sp2 and Sp4 proteins were not detected in myoblasts or myotubes (data not shown). Nuclear immunolocalization was observed in myoblasts immunostained with the anti-Sp1 antibody (Fig. 8). Similar nuclear immunolocalization of Sp3 was also observed in myoblasts (not shown). However, nuclear immunostaining of Sp1 or Sp3 was not detected in myotubes, confirming the results of Western and Southwestern blots.

In total, these results demonstrate that the proximal Sp binding site motifs of the FGFR1 promoter are necessary for maximal transcriptional activity in myoblasts. The most proximal Sp1 site (−23) binds the Sp1 transcription factor and, in
FIG. 4. Identification of the proteins that interact with the −23 Sp site by Southwestern blot analysis. Panel A, detection of a 105-kDa protein that interacts with the −23 Sp site. Myoblast (MB) and myotube (MT) nuclear extracts (75 μg) were resolved by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was cut and probed with a consensus Sp1 oligonucleotide (lane 1), −23 oligonucleotide (lanes 2 and 4), and a mutant (m23) oligonucleotide (lane 3). The positions of the Sp1 protein and molecular size markers (Invitrogen) are indicated. Myoblast nuclear extract contained a 105-kDa protein that bound both the consensus control Sp binding site as well as the −23 oligonucleotide. This protein was not detected in myotube nuclear extract with the mutated −23 oligonucleotide or in myotube nuclear extract with the wild type −23 oligonucleotide. Panel B, binding of the 105-kDa protein in HeLa cell nuclear extracts (75 μg) with the −23 (lane 1) and the Sp1 control (lane 2) oligonucleotides as probes. The position of Sp1 protein is marked. The experiment was repeated three times.

DISCUSSION

The proximal region of the chicken FGFR1 promoter has three potential Sp transcription factor binding sites necessary for its activation. In this study we sought to identify a cis-regulatory element responsible for the transcriptional activation of the FGFR1 promoter in proliferating myoblasts. Using transient transfection experiments, we assayed the function of individual Sp sites in the proximal region of the promoter with single or multiple mutations. In primary cultures of chicken myoblasts, mutation at individual Sp sites reduced promoter activity significantly. Mutation of the −23 Sp site yielded a 10-fold reduction in CAT activity, indicating that it is a positive cis-regulatory element. Mutation of the −42 and −54 Sp binding sites caused a different pattern of reduction in promoter activity (50 and 21%), indicating that these sites are weaker positive elements. Although a fourth potential Sp binding site was identified by sequence analysis at the start of transcription, this site was not mutated in these studies to ensure proper transcription initiation as regulated by the upstream Sp elements.

In Drosophila SL2 cells, mutation of the most proximal Sp site reduced Sp1 transactivation to 20%, demonstrating that this site mediates transactivation by Sp1. Mutation of the other two sites (−42 and −54) also reduced Sp1 transactivation, but to lesser extents (60 and 82%, respectively). As found in other promoters, Sp3 functioned as a coactivator of the FGFR1 promoter when cotransfected with a Sp1 expression construct into Drosophila SL2 cells (30, 32, 33).

EMSAAs detected several DNA-protein complexes formed by myoblast and myotube nuclear extracts with the three individual Sp sites. A prominent complex was detected at each site with each extract. However, formation of this DNA-protein complex was not competed against by consensus binding sites for Sp transcription factors. Therefore, the proximal Sp binding sites may also interact with novel DNA-binding proteins or may bind differentiation response elements (34) or G-C homopolymer binding factor (35, 36).

Multiple DNA-protein complexes formed with the −23 probe and nuclear extracts derived from myoblasts. Although the
prominent DNA-protein complex did not consist of Sp1, several lines of evidence indicate that Sp1 bound to the most proximal (−23) Sp site of the FGFR1 promoter in a slower migrating complex. Southwestern blot analysis showed that a 105 kDa Sp1-like band detected by the −23 probe from the nuclear extracts of myoblasts and HeLa cells co-migrated with the band detected by the consensus Sp1 binding site in nuclear extracts of HeLa cells. Additionally, the mutated −23 probe (m23) did not detect a Sp1 band in myoblast nuclear extracts. Supershifts with the Sp1 monoclonal antibody were unsuccessful presumably because of masking of the epitope in the DNA-protein complex. Detection of Sp1 binding to the −23 probe in the Southwestern blot as a major band versus Sp1-DNA complex formation as a minor band in the EMSA may reflect differences in binding affinities under the differing conditions of the two assays. Therefore, although the −23 Sp site and Sp1 do not form the major DNA-protein complex in the EMSAs, formation of this complex is required for FGFR1 promoter activity.

Western blot and immunostaining demonstrated that Sp1 and Sp3 are present in proliferating myoblasts and not in differentiated myotubes. This pattern of Sp transcription factor localization is coincident with FGFR1 gene expression in proliferating myoblasts and down-regulation in differentiated myotubes (5). This specific correlation is extended to more general findings of elevated Sp1 levels in early neonatal mice and subsequent reduced levels in adults (38). Sp1- and Sp3-deficient embryos are growth-retarded and either die after 10 days of embryonic development (39) or after birth because of respiratory failure (40). Some mechanisms of inhibition of Sp1 function have been reported. The cell cycle regulatory protein p107 specifically represses Sp1-dependent transcription (41). Furthermore, G10BP protein and Sp3 can compete with Sp1 for
FGFR1 gene expression. Actions occur at a distance along the promoter may require This type of mechanism in which direct Sp1-Sp1 protein interactions interact to regulate FGFR1 gene transcription, and it is likely that the interaction occurs via the proteins associated with TATA elements (14). It has been proposed that such multiple Sp binding sites and associated protein(s) may stabilize the transcriptional machinery and establish a site of transcription initiation in promoters without TATA elements (14).

Although the proximal Sp binding sites of the FGFR1 promoter are required for transcriptional activation, these sites are not sufficient. We have previously located two additional Sp1 binding sites in a distal region of the promoter located more than 1 kb upstream from the proximal Sp binding sites (15). These two distal sites are also required for FGFR1 promoter activity. Therefore, it appears likely that these two regions interact to regulate FGFR1 gene transcription, and it is likely that the interaction occurs via the proteins associated with the Sp sites. A similar regulatory mechanism has been suggested by others for Sp1-dependent gene regulation (29, 31).

This type of mechanism in which direct Sp1-Sp1 protein interactions occur at a distance along the promoter may require looping of the intervening promoter region bringing the distal Sp1 sites near the start of transcription (14). It is currently not known whether such a looping mechanism exists to regulate FGFR1 gene expression.

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