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Regulation of inhibitor of differentiation gene 3 (Id3) expression by Sp2-motif binding factor in myogenic C2C12 cells: Downregulation of DNA binding activity following skeletal muscle differentiation

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Abstract

Id3 is a member of the Id family of transcriptional regulators that have been implicated in the development of multiple tissues. Altered expression of the Id genes and proteins contribute to carcinogenesis and atherosclerosis. Id3 is highly expressed in proliferating skeletal muscle cells but becomes downregulated upon terminal differentiation. We have identified several DNase I protected footprints within a proximal region of the mouse Id3 promoter that has been shown previously to support high levels of transcriptional activity in proliferating skeletal muscle cells. Two of these sites interacted, respectively, in vitro with Sp2 and Egr-1 proteins present in muscle cell nuclear extracts. Mutation analysis revealed that the Sp2 site accounted for a major part of the Id3 promoter activity in proliferating muscle cells whereas the Egr-1 site was dispensable. Consistent with the previously observed downregulation of the endogenous Id3 gene, protein binding to the Sp2 site was substantially reduced with extracts from differentiated muscle cells. Our results reveal Id3 as a potential target for Sp2 and raise the possibility that acute activation and the chronic and maintained expression of Id3 gene might be regulated by different mechanisms.

Keywords: Myogenesis; Basic helix–loop–helix; Id gene; Id3 promoter; Egr-1; Transcriptional regulation

1. Introduction

Id proteins are transcriptional regulators that profoundly influence the proliferation and differentiation of many cell types (reviewed in [1,2]). Four distinct genes, Id1–Id4 have been identified in human and mouse. Id1 was originally isolated based on its ability to inhibit skeletal muscle differentiation [3]. All Id proteins contain a helix–loop–helix (HLH) structural motif and function primarily (although perhaps not exclusively) by forming heterodimers with the ubiquitous basic helix–loop–helix (bHLH) transcription factors known as E-proteins [4,5]. Sequestration of E-proteins prevents them from forming transcriptionally active dimers with tissue-specific bHLH proteins.

Id genes are expressed primarily in proliferating tissues and downregulated in terminally differentiated cells [6]. Their expression can be induced by serum and growth factors [7–9]. Mouse Id3 was initially identified as an immediate early gene induced in cultured fibroblast following serum stimulation [7]. Id3 transcripts are expressed in developing somites [10,11] and are upregulated in myogenic satellite cells activated to proliferate [12]. Expression of Id3 could play an important role in the muscle regenerative process following tissue injury.

It is becoming clear though that the involvement of the Id gene family extends far beyond the skeletal muscle system. Experimental manipulations of individual Id genes have been linked to defects in immune system development [13], in spermatogenesis [14] and in mammary epithelial development during lactation [15]. Simultaneous inactivation of Id1 and Id3 results in embryonic lethality with defects in angiogenesis and

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neurogenesis, while mutant mice hemizygous for Id1 and lacking both copies of Id3 genes are resistant to tumor xenografts because of an inability to support tumor-induced angiogenesis [16]. Conversely, elevated expression of one or more of the Id genes occurs in colon, breast and prostate cancers [17] and an alternatively spliced Id3 transcript has been implicated in the formation of atherosclerotic plaques [18]. Recently, expression Id1 and Id3 has also been implicated in mediating the bone morphogenetic protein-induced self-renewal of embryonic stem cells [19]. Thus, studying the mechanism regulating the expression of Id3 and other Id genes could shed light on the mechanism of stem cell renewal and might provide novel targets for therapeutic interventions in a variety of diseases.

Id proteins could partially compensate for each other, but they are not functionally equivalent or redundant. Individual Id proteins show unique patterns of protein interaction [20] and contain structural domains that are not completely interchangeable [21–23]. Individual Id genes also exhibit overlapping yet distinct patterns of expression [24–28]. While the molecular mechanisms regulating the transcription of the other three Id genes have been reported, relatively little is known about the regulation of the Id3 promoter activity.

We previously found that a 180-bp region 5′ of the transcription start site of the mouse Id3 promoter could confer high level of transcriptional activity to a reporter gene in proliferating myogenic cells [29]. In this report, we have used DNase protection footprint analysis to identify putative transcription factor binding sites within this region. Electrophoretic mobility shift assays (EMSA) were used to identify nuclear proteins from proliferating muscle cells binding to these footprints. Mutation analyses were used to define the DNA sequence requirements for protein binding and transcriptional activity. Our data suggest that interaction of transcription factors at one or more sites within this region work together to drive the expression of the Id3 gene in proliferating skeletal muscle cells. Consistent with this idea, protein binding to one of the sites in vitro was substantially reduced following muscle differentiation.

2. Materials and methods

2.1. Cell culture

Mouse myogenic C2C12 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 0.1% gentamycin at 37 °C in humidified air with 5% CO_2. Myogenic differentiation was initiated by exposing cells at 70–80% confluence to DMEM supplemented with 5% horse serum (HS) and 0.1% gentamycin. To obtain a highly homogeneous population of differentiated myotubes, C2C12 cells were exposed to HS for 4–5 days and enriched for myotubes using a pre-plating protocol. Briefly, differentiated cultures were rinsed with Hanks balanced saline and dissociated from the dish by the addition of trypsin. The cell suspension was then diluted with the addition of 5 volumes of 5% HS. Undifferentiated mononucleated cells that had remained in culture were allowed to reattach to the dish for 10 min at 37 °C. The medium which contained the floating myotubes was carefully transferred to a new dish and incubated for another hour. Cells that still remained unattached were then collected by centrifugation. Microscopic examination of the final cell suspension confirmed the presence of predominantly multinucleated myotubes.

2.2. Plasmid constructs

The Id3 reporter construct, p-180Id3CAT, was made by deleting the −389/−180 fragment from p-389Id3CAT [29] using the *Sac*II restriction enzyme. Mutant reporter constructs were generated as described below.

2.3. Transient transfection and chloramphenical acetyltransferase (CAT) reporter gene assay

C2C12 cells were plated onto a 6-well plate (1×10^5 cells/well) and grown for 24 h before transfection. One and half μg of the various Id3 promoter CAT reporter constructs and 0.5 μg pSV-β-Gal expression vector were transfected into cells using lipofectamine (Life Technologies, Inc.) according to manufacturer's suggested protocol. The β-galactosidase activity expressed from the pSV-β-Gal vector served as internal control to normalize for variations in transfection efficiency. Typically, cells were exposed to the lipofectamine–DNA mixture for 6 h in 1 ml of serum-free DMEM before being fed 1 ml of fresh 20% FBS-DMEM. Eighteen hours later, the culture medium was removed and the cells were fed 2 ml DMEM+10% FBS and cultured for an additional 24 h before harvesting. The β-galactosidase and CAT assays were performed as described [23] and quantified, respectively, by spectrophotometry and by liquid scintillation counting.

2.4. Nuclear extract preparation

To prepare nuclear extracts from proliferating cells, logarithmically growing C2C12 cells in 100 mm diameter tissue culture dishes (at about 75% confluence) were rinsed with ice-cold phosphate-buffered saline (PBS). The cells were scraped into 1 × PBS and centrifuged. The cell pellets were resuspended in hypotonic lysis buffer (10 mM HEPES pH 7.9, 10 mM KCl, 1.5 mM MgCl2, 0.1 mM EDTA/EGTA, 1 mM DTT) with proteinase inhibitor cocktail (Sigma). The cell suspension was then centrifuged at 4 °C for 20 min at 3,000 rpm (Hermle, Z360K). The nuclei pellets were resuspended in hypertonic buffer (420 mM NaCl, 20 mM HEPES, pH 7.9, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM DTT, 25% glycerol) supplemented with proteinase inhibitor cocktail (Sigma). The resuspended nuclei were then extracted by rocking in a cold room for 1 h, then centrifuging for 20 min at 10,000 rpm. The supernatant containing nuclear proteins was aliquoted, frozen immediately in liquid nitrogen and stored at −80 °C. Protein concentration of the extract was determined using the Bio-Rad protein assay, according to manufacturer's protocol using bovine serum album as the concentration standard. Nuclear extracts from differentiated C2C12 cultures were prepared in a similar manner except that the population was first enriched for the presence of multinucleated myotubes as described earlier.

2.5. DNA probes for footprinting analysis

Footprinting probes corresponding to the −180 bp Id3 promoter region upstream of the transcription initiation site labeled on a single strand were generated by separate 3′ fill-in reaction of either the + or the − strand using the Klenow fragment of DNase Polymerase I (Promega). Briefly, the −180Id3CAT plasmid containing 180 bp of the Id3 promoter was digested separately with *Hin*dIII (5′ end of the promoter) or *Xba*I (3′ end of the promoter). After phenol/chloroform extraction and ethanol precipitation, the digested plasmids were labeled with α-^{32}P-dCTP with Klenow enzyme (Promega) using standard method. After Sephadex G-25 column chromatography to remove the unincorporated nucleotides, the labeled plasmids were subject to digestion with the second enzyme, *Xba*I (to digest *Hin*dIII-cut labeled plasmids) or *Hin*dIII (to digest *Xba*I-cut labeled plasmids) to release the end-labeled probes. The digestion products were separated by electrophoresis in 5% non-denaturing polyacrylamide gel. Individual 180 bp promoter fragments labeled at the 3′ end of either the + or the − strand were purified from the gels [30] and used as probes in the footprinting reaction.

2.6. DNase I footprinting assay

Five to fifteen μg nuclear extract proteins were incubated with p^{32} labeled probes (1–10 μg) and 2 μg of salmon sperm DNA for 30 min at 25 °C in a buffer containing 20 mM HEPES pH 7.9, 50 mM KCl, 20% glycerol and 2 mM DTT in a 20-μl reaction volume. The binding reactions were adjusted to 2 mM $MgCl_2$ and subjected to DNase I (RQ1 DNase, Promega) digestion for 30 s at 25 °C using varying concentrations of DNase I. The resulting DNA fragments were separated by electrophoresis on a 6% polyacrylamide sequencing gels. The locations of individual footprints were determined by comparison to the product of a G-sequencing reaction using probes cleaved at guanine nucleotides with dimethyl sulfate and piperidine (Sigma) [31].

2.7. Electrophoretic mobility shift assay (EMSA)

EMSAs were performed with double-stranded synthetic oligonucleotides radioactively labeled by 3′ fill-in with Klenow enzyme (Promega) using standard methods [30]. Two to three μg of nuclear extract proteins were incubated with different probes (0.05–0.3 ng) for 20 min at 25 °C with 2 μg of salmon sperm DNA in a buffer containing 125 mM HEPES pH 7.9, 5 mM EDTA, 5 mM DTT, 250 mM NaCl and 50% glycerol. The reaction mixture was then immediately loaded on a 5% non-denaturing polyacrylamide gel (29:1 acrylamide-bis-acrylamide) and electrophoresed in 0.5× TBE (50 mM Tris-borate and 1 mM EDTA) at 8–10 V/cm. The gels were fixed in 10% methanol, 10% glacial acetic acid, dried and exposed to X-ray films. Where indicated, the nuclear extracts were pre-incubated with unlabeled competitor DNA for 5–10 min prior to the adding the labeled probe. For antibody supershift assays, 1–2 μl of individual specific antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was added to each gel shift reaction for 10 min prior to the addition of probe.

The oligonucleotides used as EMSA probes include S1 (5′-GTCTGTTT-TGAATAAGGGGGTGTGTCCTAG-3′), S2 (5′-GTGCCCAGGGCGGGCGG-GGGTGGACCC-3′), and the E-box oligo EB (5′-GATCCCCCCAACACCT-GCTGCCTGAGATC-3′). Sequences for the competitor oligos are indicated in the corresponding figures.

2.8. Site-specific mutagenesis

Point mutations in the Id3 promoter reporter constructs were generated by overlapping polymerase chain reactions (PCRs) using synthetic oligonucleotides containing the mutated bases. To generate −180 mut-Sp2CAT, one reaction mixture contains the primer pairs HLH-Pro-1 (5′-TTCAAGGGATT-TATGACCTC-3′), and mutated oligonucleotide M6R (5′-CTAGGACACAC-CACCTTATTCAAAACAGAC-3′), and the second reaction mixture contains the primer pair HLH Pro-R1 (5′-CACTGTTTGCTGCTCGAGGTGTCT-3′) and mutated oligonucleotide M6 (5′-GTCTGTTTTGAATAAGGTGGTG-TGTCCTAG-3′). A *Hin*dIII digested -389Id3CAT (linearized) plasmid was used as template for both reactions. The PCR products were gel purified, combined in approximately equimolar ratio and amplified again using Tag DNA polymerase and 5′ HLH Pro-1 and 3′ HLH Pro-R1 as primers. The final product was purified and cloned into the pGEM-T-easy vector (Promega). The mutated Id3 promoter fragment was then removed from pGEM-T-easy with restriction endonucleases *Sal*I and *Xho*I and transferred into the *Sal*I site upstream of the CAT gene in pCAT-Basic (Promega). The-180 mut-Sp2CAT construct was generated by deleting the −389/−180 fragment from the Id3 promoter using the *Sac*II restriction enzyme. The −180 mut-Egr1CAT plasmid was constructed in a similar manner using the mutant oligos, 5′-GTGCCCA-GGGCGGGC**TA**GGGTGGACCC-3′ (M9) in which the consensus Egr-1 binding site GGGCGGG was changed to GGGCTAG. All constructs containing PCR-amplified fragments were verified by sequencing to make sure that only the intended mutations were incorporated.

2.9. Western immunoblot analysis of Sp protein expression in C2C12 cultures

Undifferentiated cultures of myogenic C2C12 cells maintained in DMEM containing 10% FBS were harvested before they reached confluence. Differentiated C2C12 cultures were obtained following a 6-day exposure to DMEM containing 5% HS. Cytosine arabinoside (40 μM) was added for the last 4 days to eliminate undifferentiated cells. Nuclear extracts were prepared from both sets of cultures using the NE-PER kit (Pierce) according to manufacturer's suggested protocol. Protein concentrations in the extracts were determined using the micro BCA assay (Pierce). Equal amounts of proteins were fractionated by SDS polyacrylamide gel electrophoresis and blotted onto nitrocellulose membranes. The protein blots were incubated in Tris buffered saline containing 0.05% Tween 20 to block non-specific protein binding and exposed to the appropriate antibody solution (Santa Cruz). After extensive washing and incubation with horseperoxidase-linked secondary antibody, the immunblots were developed with the West Pico chemiluminescent substrate (Pierce) and exposed to X-ray films.

3. Results

3.1. Identification of nuclear protein binding sites in the proximal −180 bp Id3 promoter region using DNase I footprinting analysis

Previously, we have identified a 0.18-kb region in the Id3 proximal promoter by deletional analysis as sufficient for mediating high level of transcriptional activity in proliferating myogenic C2C12 cells [29]. To determine the specific sites of DNA-protein interaction within this region, we incubated end-labeled Id3 promoter probes with nuclear extracts from proliferating C2C12 cells, and looked for protein binding sites protected from in vitro DNase I digestion. When compared to free probes subjected to DNase I digestion alone, pre-incubation with nuclear extracts from C2C12 cells protected two to three putative binding sites within the −180-bp region on both the + and the − strands of the Id3 promoter (Fig. 1A). The first of these footprints extends from approximately −37 to the −59 position (footprint 1). The second footprint extends from approximately −70 to −95 (footprint 2), while the third footprint extends from approximately −95 to −113 (footprint 3). The precise demarcation between the second and the third footprints is not very clear and could in fact represent one extended protected region. The footprint protected regions 2 and 3 are also less pronounced compared to footprint protected region 1, which may reflect a difference in the amount or activity of the relevant transcription factor(s) in the nuclear extracts. Mapping the footprints to the Id3 promoter sequence shows that footprint 2 contains overlapping Sp1/Egr 1 sites that had been previously reported by Christy et al. [7] and identified by us using the Transfac database [32]. Similar analysis revealed an AP-1 like site (TGAATAA) and a GC box-like element (GGGGGTGTGTCCT) within footprint 1 and a number of overlapping potential GC box and Sp1-like elements within footprint 3. Computer based alignment of the murine proximal mouse Id3 promoter with the human Id3 promoter indicates that sites 1 and 2 are largely conserved between the two species, but interestingly, the sequence corresponding to site 3 is missing from the human Id3 promoter. However, careful comparison of the sequences reveals that sites 2 and 3 in the mouse promoter are flanked by similar sequences that also flanked the putative site 2 region of the human promoter (Fig. 1B). It is possible that

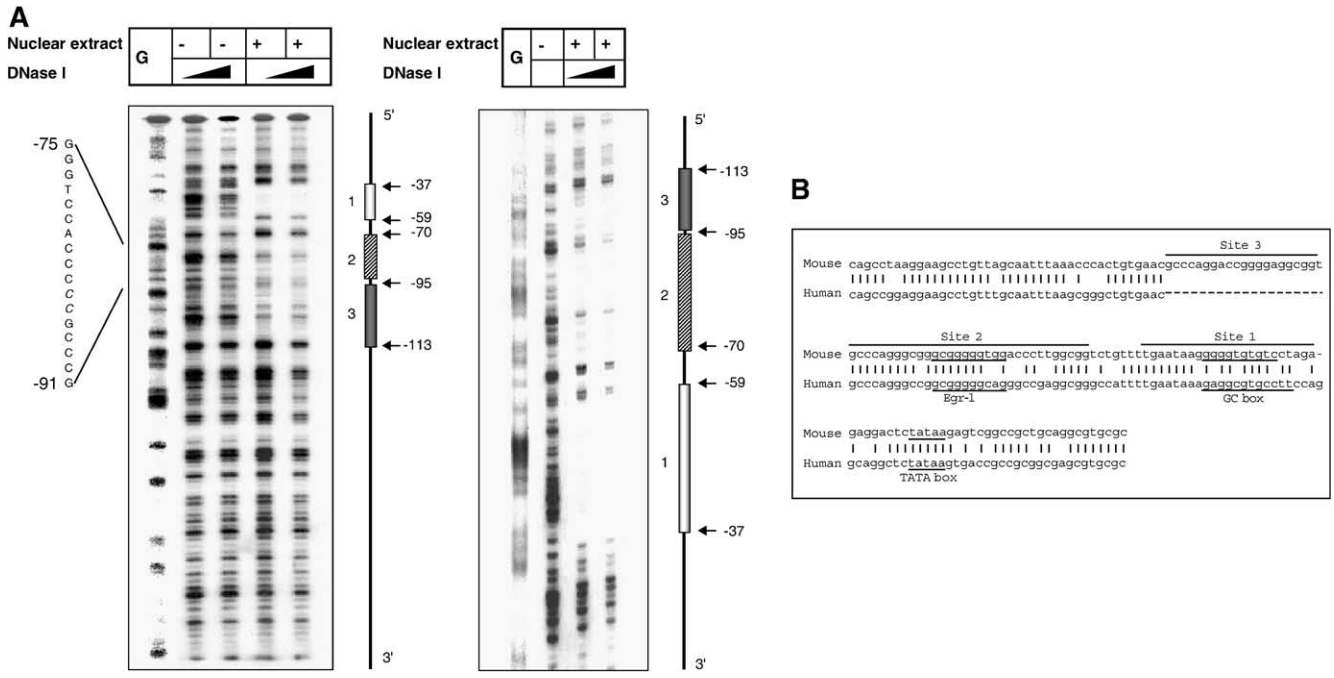

Fig. 1. Identification of transcription factor binding sites in the −180 bp of murine Id3 proximal promoter region using DNase I Footprinting assay. (A) Double stranded DNA fragment containing 180 bp of the Id3 promoter was labeled with [γ-^{32}P]dCTP at the 3′ end of either the non-coding "−" strand (left panel) or the coding "+" strand (right panel) and incubated either alone (−) or with nuclear extracts from proliferating C2C12 cells (+) as indicated on top of the figure. The samples were then treated with increasing concentrations of DNase I (indicated by the triangles) and subsequently analyzed by electrophoresis on denaturing 6% acrylamide sequencing gel. The first lane of each panel (labeled as G) represents G-specific sequencing reaction products of the labeled DNA fragment. Promoter regions protected from DNase I digestion by C2C12 nuclear extracts are indicated schematically on the right side of each panel and numbered according to the nucleotide positions relative to the transcription start site (+1) of Id3 gene. A representative sequence corresponding to footprint 2 on the "−" strand is shown to the left of the figure. (B) Alignment of the relevant region of the murine Id3 promoter surrounding the footprint sites with the corresponding region of the human Id3 promoter. The mouse and human Id3 promoter was aligned using the BLAST2 program from NCBI. The approximate locations of the 3 footprints in the mouse Id3 promoter are indicated by overlines. The putative egr-1 binding site, GC box and TATA box motifs are indicated by underlines. Two separate computer-aligned fragments were merged together manually by introducing the extended gap corresponding roughly to the mouse footprint 3 in the human Id3 promoter. Nucleotide sequence at the beginning and the end of the apparently "duplicated" regions in the mouse Id3 promoter are indicated by bold italics.

site 3 in the mouse promoter might have arisen by duplication following the evolutionary divergence of the two species.

3.2. Sequence definition of the nuclear protein binding sites by EMSA

To more precisely define the sequence requirement of the regulatory element(s) within the footprints, the 180 bp of Id3 promoter was subdivided into three fragments, a 30-bp fragment S1 (−39/−68, encompassing footprint 1), a 27-bp fragment S2 (−75/−101, roughly corresponding to footprint 2,) and a 41-bp fragment S3 (−96/−136, encompassing footprint 3). Mutant oligos mutated at one or more specific residues within the putative protein-binding site(s) in the first two fragments (S1 and S2) were generated and used as unlabeled competitors in competitive EMSAs. As shown in Fig. 2, mutations in the last two residues in the AP-1 like site (TGAATAA) did not affect the ability of the mutant oligo (M3) to compete with the S1 probe for binding to nuclear proteins from proliferating C2C12 cells. The Ap-1 like site is thus not likely to be involved. In contrast, mutant oligos with either single (M6) or multiple nucleotide substitutions (M1, M4, M8) within the core motif (GGGT) of the putative GC box element (GGGGGTGTGTCCT) were unable to compete for protein binding to S1. Changing the first G, corresponding to a flanking residue in the consensus GC box motif (R**GGGGCGGGGC**NK), from G to T (M5) had only a minor effect. Although transversion mutations (G to T and T to G) within the core GGGT motif (M1, M4, M6, M8) were unacceptable, transition mutations (G to A and T to C) in one or more nucleotides within the core motif (M2 and M7) were quite well tolerated. Indeed, the M7 oligo sequence which was designed to mimic the corresponding region of the human Id3 promoter (AGAGGCGTG) competed very effectively with S1 for protein binding. Thus, a functional "GC box" appears to be present at the human promoter even though the precise nucleotide sequence might have diverged substantially. We have not yet analyzed the 3′ end of the Id3 "GC box" in detail. While the nucleotide sequence in this region differs significantly from the consensus motif, such sequence variations appear to be well tolerated in multiple eukaryotic promoters [33].

To define the DNA sequence required for nuclear factor binding to footprint site 2, we focused our attention on the putative Egr-1 and Sp1 sites previously identified. As shown in Fig. 3, a mutant oligo containing mutations that disrupted the core Egr-1 binding site (GG to TA at −86/85, M9) was as ineffective in competing for protein binding to S2 as an

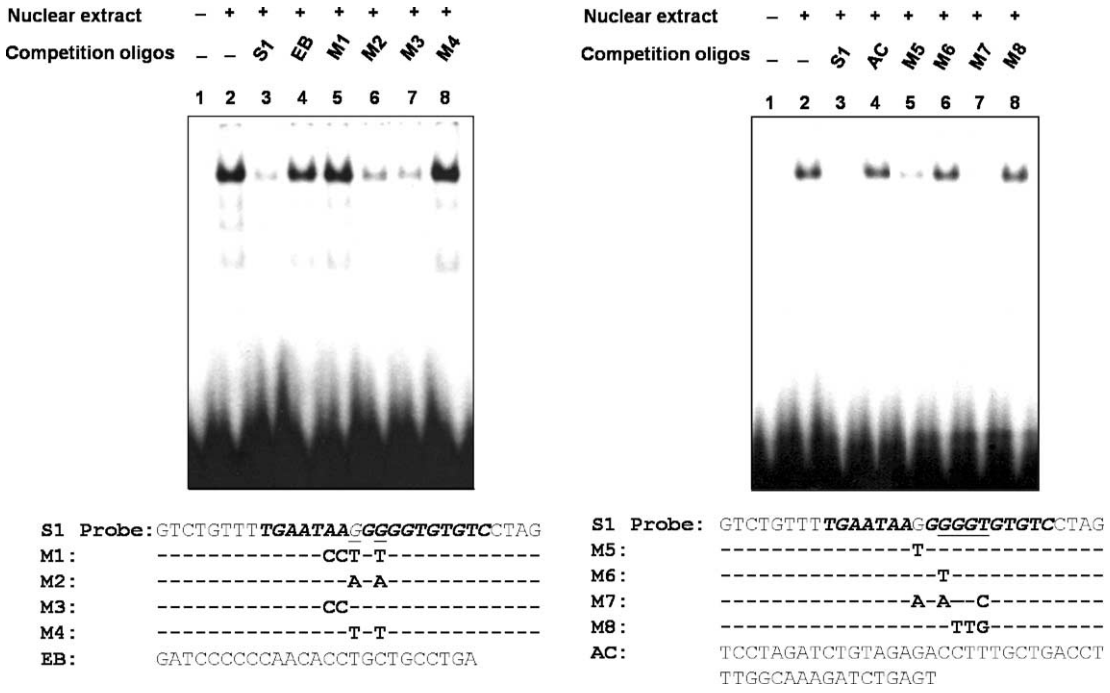

Fig. 2. Definition of C2C12 nuclear protein-binding site within the footprint site 1 region of the mouse Id3 promoter. EMSAs were performed with the ^{32}P-labeled 30 bp double-stranded site 1 Id3 probe S1. Two to three μg of nuclear extract proteins were incubated with the labeled probe for 20 min at 25 °C with 2 μg of salmon sperm DNA either in the absence or presence of a 50-fold molar excess of the indicated unlabeled competitor oligonucleotides. The reaction mixtures were electrophoresed in a 5% non-denaturing polyacrylamide gel, fixed, dried and exposed to X-ray films. The nucleotide sequences of the S1 probe and the various competitor oligos are shown at the bottom of the figure. The putative AP1-like and the core GC box-like motifs in the S1 probe are indicated by italics, with residues shown to be critical for binding to C2C12 nuclear proteins underlined. The EB and AC oligos are non-specific competitors containing, respectively, an E-box element from the muscle creatine kinase (MCK) enhancer and a fragment of the acetylcholine receptor γ subunit promoter.

unrelated oligo containing a consensus E-box element (EB). In contrast, both the unlabeled wild-type S2 probe and an oligo with mutations in the putative Sp1 binding site (from GGG to TTG at −94/−92, M10) prevented the formation of the major DNA–protein complex. A minor faster-migrating DNA protein complex was also observed but none of the oligos including the unlabeled wild-type S2 probe interfered with its formation, indicating that it probably resulted from a non-sequence-specific DNA–protein interaction. Taken together, the data suggest the Egr-1 site to be the relevant motif in the second footprint.

3.3. Sp2 and Egr-1 bind to footprint sites 1 and 2, respectively, in the murine Id3 promoter

The Sp family of transcription factors has been implicated to interact with GC boxes in other eukaryotic promoters and could potentially interact with Egr-1 like sites. To identify the transcription factor(s) binding to the footprint sites 1 and 2 in vitro, we performed antibody supershift analysis using antibodies to Sp1, Sp2, Sp3, Sp4 and Egr-1 (Fig. 4A and B). Of the four Sp family antibodies, only the anti-Sp2 antibody produced a "supershift" when incubated with footprint site 1 probe (Fig. 4A). In contrast, none of the Sp family antibodies we used affected protein binding to the site 2 probe (Fig. 4B), but 1 or 2 μl of anti-Egr-1 antibody produced a dramatic "supershifting" of the major S2 DNA–protein complex to slower-migrating forms (indicated by the arrows in Fig. 4B, lanes 3 and 4). These data confirm that it is Egr-1 that binds to the footprint site 2 and suggest that the transcription factor Sp2 is at least partly responsible for binding to the GC box in footprint site 1. Since the anti-Sp2 antibody did not "supershift" all the DNA–protein complexes that were formed, other related transcription factors might contribute to the binding to this region of the Id3 promoter. It should be noted also that not all the antibodies we used in the experiment produced changes in electrophoretic mobility of the complexes, indicating that the observed "supershifts" did not result from non-specific interactions with "irrelevant" IgG molecules.

3.4. The GC Box is important for regulating the −180-bp Id3 promoter activity in proliferating muscle cells, but the Egr-1 element is largely dispensable under such experimental conditions

To determine the biological significance of the GC box and Egr-1 binding sites in regulating Id3 expression, we mutated each of these two sites individually in the −180 bp Id3 promoter and examined the activity of the wild-type and mutant Id3 promoters in undifferentiated C2C12 cells (Fig. 5). The same mutations shown by EMSA to destroy in vitro DNA–protein interactions (M6 and M9) were inserted into the Id3 promoter to generate, respectively, the −180 mut-Sp2 and the −180 mut-Egr1 Id3 promoter constructs. Results from multiple independent experiments demonstrated that the

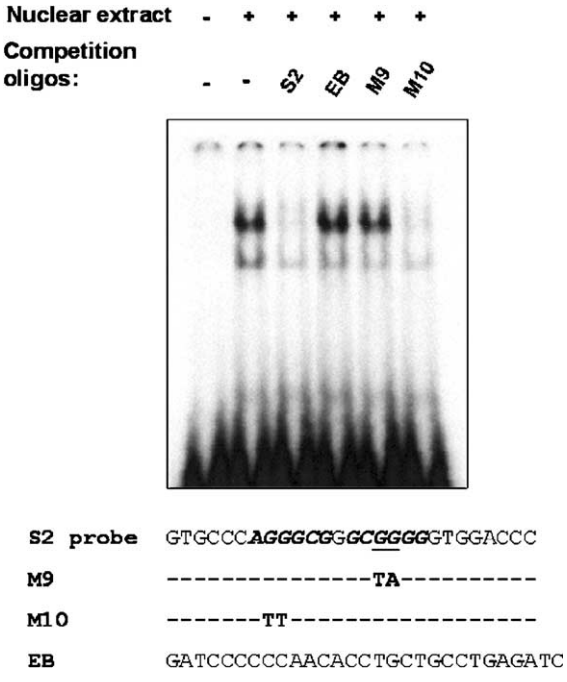

Fig. 3. Definition of C2C12 nuclear protein-binding site in the footprint site 2 region of the mouse Id3 promoter. EMSAs were performed with the ^{32}P-labeled 27 bp double-stranded site 2 Id3 probe S2. Two to three μg of nuclear extract proteins were incubated with the probe for 20 min at 25 °C together with 2 μg of salmon sperm DNA either in the absence or presence of a 50-fold molar excess of the indicated unlabeled oligonucleotide. Nucleotide sequences of the S2 probe and the various competitor oligonucleotides are shown below the figure. The putative Sp1-like and Egr1-like core motifs in the S1 probe are indicated by italic, with residues shown to be critical for protein binding underlined. The M9 oligo contains a mutation (GG to TA) at the Egr1-like motif and the M10 oligo contains a mutation (GG to TT) at the Sp1-like motif. The EB oligo containing a consensus E-box element from the muscle creatine kinase (MCK) enhancer is used as a non-specific competitor.

single base pair mutation from 'G' to 'T' in the GC box resulted in an approximately 50% decrease in CAT activity compared with the wild-type promoter. Statistical analysis indicates the difference to be highly significant ($P<0.01$). In contrast, the mutation in the Egr-1 binding site resulted in a less than 10% reduction in promoter activity that did not appear to be statistically significant. We conclude that binding of Sp2 to the GC box is important in regulating Id3 transcription in proliferating C2C12 cells but that the binding of Egr-1 to the footprint site 2 may be dispensable under the experimental condition we tested.

3.5. Transfection of Sp2 expression plasmid fails to affect muscle differentiation and expression of endogenous Id3 gene

If Sp2 binding to the Id3 promoter is controlling the transcription of Id3, one might expect that overexpression of Sp2 would induce the expression of the endogenous Id3 gene and inhibit muscle differentiation even under culture conditions that favor the differentiation process. We therefore transfected C2C12 cells with the pPacSp2 expression vector [34] together with another plasmid carrying a neomycin resistance selectable marker. The transfected cells were then either exposed directly to differentiation-inducing conditions to test the effect on differentiation or treated with the antibiotic geneticin to select for stable transfectants. Cells that survive the geneticin selection were tested for their ability to differentiate, and the expression level of the endogenous Id3 gene in these cells was measured by reverse transcriptase-coupled PCR (RT-PCR) assay. Contrary to our expectation, neither the transient nor stable transfectants exhibit a reduction in myogenicity relative to cells transfected with

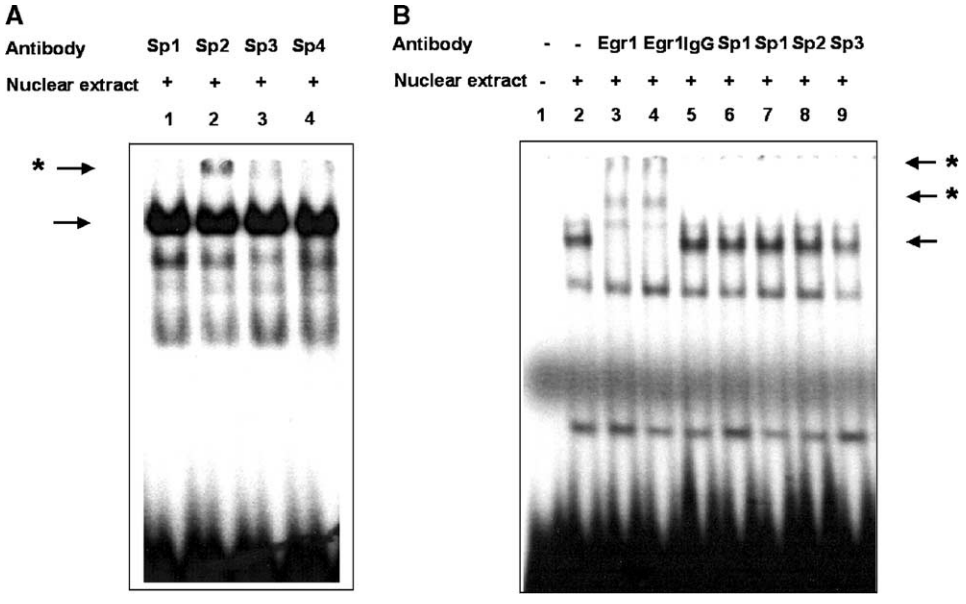

Fig. 4. Antibody-mediated supershift analysis of nuclear factors binding to the S1 and S2 probes. EMSAs were performed as in Figs. 2 and 3, except that the S1 probe was pre-incubated as indicated with antibodies to Sp1, Sp2, Sp3 or Sp4 (A) and the S2 probe with antibodies to Egr-1 (1 or 2 μl), Sp1 (1 or 2μl), Sp2, Sp3 or with non-immune rabbit IgG (IgG) (B) for 10 min prior to the addition of the radioactive probes. Protein–DNA complexes were separated by non-denaturing electrophoresis as described under Fig. 2. The specific DNA–protein complex containing the S1 or S2 probe, respectively, in each panel is indicated by an arrow. The antibody-supershifted complexes are indicated by an asterisk (*).

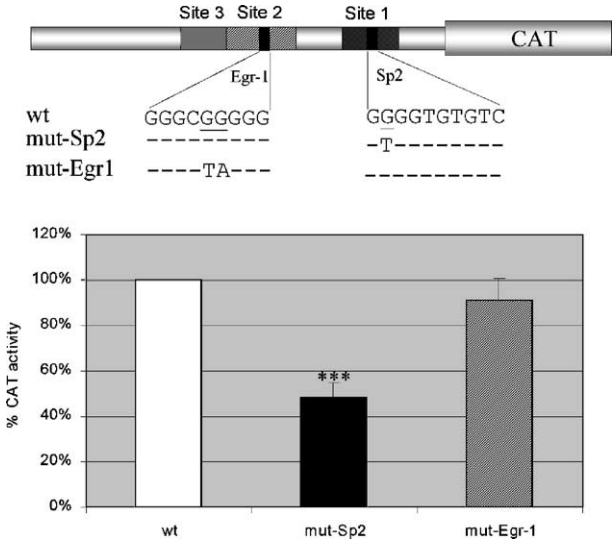

Fig. 5. Mutation that disrupts protein interaction with the Sp2 but not the Egr-1 binding site interferes with Id3 promoter activity in proliferating C2C12 cells. C2C12 cells were transfected with CAT reporter genes driven by the wild type −180 bp Id3 promoter (wt) or mutant promoters in which the core Sp2 binding site (mut-Sp2) or Egr-1 binding site (mut-Egr1) have been mutated. CAT activities were assayed after normalizing for differences in transfection efficiency according to the β-galactosidase activity from a co-transfected SV40-βgal plasmid used as internal control. Values represent the mean ± S.E. from four independent experiments ($n=4$) in which each transfection was performed with triplicate samples. ANOVA test indicated that the mut-Sp2 promoter activity differs significantly from that of the wild-type promoter ($P<0.01$). A schematic representation of the Id3-CAT reporter gene is shown above the graph with the three DNase-protected footprint sites indicated. The nucleotide sequences corresponding to the Sp2 and Egr-1 binding sites in the wild type Id3 promoter are also shown with the nucleotide(s) mutated in the mut-Sp2 (G to T) and mut-Egr-1 (GG to TA) promoters underlined. The nucleotide sequences for the two mutant promoters are identical to that of the wild type except for the indicated substitutions.

the empty expression vector, pPac0 (negative data not shown). RT-PCR analysis of the stable transfectants also detected only minimal increase in the transcript level of the endogenous Id3. Thus, Sp2 expression by itself might be insufficient to significantly increase Id3 expression or alter the differentiation property of the transfected cells.

3.6. DNA binding activity to the GC box is downregulated during myogenic differentiation

Expression of the Id3 gene is downregulated as skeletal muscle cells differentiate [11,23]. Since the GC box appeared to have major importance in driving the expression of Id3 in proliferating muscle cells, we wished to investigate if the downregulation of Id3 expression during muscle differentiation might be related to reduced protein binding to this motif. Nuclear extracts from proliferating and differentiating C2C12 myogenic cells were made and incubated separately with radioactively labeled S1 probes containing the GC box element. As shown in Fig. 6A, proteins in the nuclear extract prepared from differentiated cells bound much less avidly to the S1 probe than proteins extracted from proliferating cells (left panel). To make sure that the differentiated nuclear extract retained the ability to interact appropriately with target DNA sequences, we also incubated the same extract with a radioactive probe (EB) containing the consensus E-box element from the mouse muscle creatine kinase (MCK) enhancer (right panel). A differentiation-specific DNA–protein complex (indicated by the lower arrow) was clearly evident. While this complex was absent with extracts from proliferating cells, a proliferation-specific and slower migrating complex (upper arrow) could be seen instead. A similar complex has been observed previously with extracts from non-muscle cells although the identity of the complex is not known. The reduced binding to the S1 probe is thus not attributable to either an overall decrease in DNA binding activities the nuclear extracts from differentiated cells or an artificial loss in activity because of improper sample handling. Fig. 6B indicates that the decrease in DNA binding is accompanied by apparent reduced expression of the Sp2 protein.

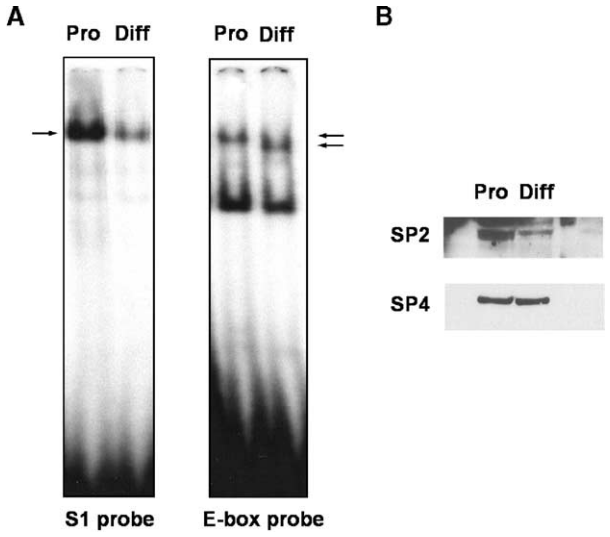

Fig. 6. Downregulation of DNA binding activities to the Sp2 binding site and reduction of Sp2 protein level occur following myogenic differentiation. (A) EMSAs were performed as described under Fig. 2 with nuclear extracts prepared from proliferating C2C12 myoblasts (Pro) and differentiated C2C12 myotubes (Diff). The resulting DNA–protein complexes were electrophoresed on 5% polyacrylamide gel under non-denaturing conditions and analyzed by autoradiography. A radioactively labeled S1 probe containing the Sp2 binding site was used in the left panel. The E-box probe containing the E-box motif from the mouse CK enhancer (right panel) was used to confirm that differentiated nuclear extracts retained appropriate DNA binding activity. The migratory position of the putative Sp2-containing complex is shown by the arrow in the left panel. The upper arrow in the right panel indicates the position of a proliferation-specific complex seen when the EB probe was incubated with nuclear extracts from undifferentiated muscle cells. The identity of this protein complex is unknown but similar complexes have been observed with extracts from non-muscle cells [52]. The lower arrow points to a differentiation-specific complex seen only with nuclear extracts from differentiated cells. A third apparently non-specific DNA–protein complex was detected with both proliferating and differentiated nuclear extracts. (B) Western immunoblot of nuclear protein extracts from proliferating (Pro) and differentiated (Diff) C2C12 cells probed with either the anti-Sp2 antibody K-20 (Santa Cruz) or the anti-Sp4 antibody V-20 (Santa Cruz) used in the EMSA assay described in Fig. 4. The two antibodies recognized protein bands with slightly different mobility and distinct patterns of expression.

4. Discussion

4.1. Multiple transcription factor binding sites exist at the proximal mouse Id3 promoter but an atypical GC box element has major importance in regulating the promoter activity in proliferating skeletal muscle cells

We have identified three regions within the proximal 180 bp of the murine Id3 promoter that interact in a sequence-specific manner with nuclear proteins from proliferating skeletal muscle C2C12 cells. The putative nuclear protein binding sites include an Egr-1 recognition motif within footprint 2 and an atypical GC box motif within footprint 1. Further experiments indicated that the GC box motif could account for about 50% of the −180 Id3 promoter activity in proliferating muscle cells, but that even though the Egr-1 site could interact with Egr-1 in C2C12 nuclear extracts in vitro, the site did not contribute significantly to the activity of the Id3 promoter under our assay conditions. Other as yet unidentified regulatory motif(s) are presumably responsible for the remaining activity of the −180 proximal promoter. It is important to note also that the corresponding region of the proximal promoter is largely conserved in human suggesting that similar regulatory mechanisms might act on the human promoter.

4.2. Is Egr-1 involved in the regulation of Id3 expression?

The presence of the putative Egr-1 site in the mouse Id3 promoter has long been known. Christy et al. were the first to identify the putative binding site at the −88 position of the mouse Id3 promoter (corresponding to our footprint site 2) and to demonstrate that it could indeed bind recombinant Egr-1 in vitro [7]. However, whether this site is important for Id3 promoter activity inside the cell has never been established. In a more recent study, Bain et al. implicated the involvement of Egr-1 in regulating Id3 expression in T lymphocytes by showing that ectopic expression of Egr-1 upregulated the expression of the endogenous Id3 gene [35]. Whether Egr-1 acted directly on the Id3 promoter was not addressed. In addition, Egr-1 has been implicated in the serum induction of Id1 in skeletal muscle cells [36]. In contrast, our study does not support a direct involvement of Egr-1 in regulating Id3 transcription in proliferating skeletal muscle cells. Mutation of the Egr-1 binding site had little effect on Id3 promoter activity and Egr-1 protein present in the proliferating cell nuclear extract did not bind to the footprint 1 probe that contains the critical GC box (data not shown). It could be that Egr-1 regulates Id3 expression indirectly or perhaps interacts with distal sites on the endogenous Id3 promoter. Alternatively, Egr-1 might participate in Id3 regulation only in lymphoid cells. Another interesting possibility is that Egr-1 might mediate the acute activation of Id3 expression in response to extracellular stimuli whereas the GC box motif we identified might be driving the chronic and maintained, moderate level of Id3 expression detected in cycling cells.

4.3. Importance of Sp family or related transcription factors in Id3 regulation

In contrast to the Egr-1 site, the existence of the GC box motif in footprint 1 has never before been reported. GC boxes are generally targets for the Sp family proteins and the related "Kruppel-like factors" (KLF)—a superfamily of transcription factors implicated in modulating genes important in cell growth, development and carcinogenesis (reviewed in [37]). Different family members exhibit preference for slightly different GC box sequences. In particular, the atypical GC box motif in the Id3 promoter resembles the so-called GT box preferred by Sp2 and the KLFs. Only a handful of potential target genes have been identified to date as being regulated by Sp2 [38,39]. Our data indicate that Id3 may be another Sp2 target. While other Sp family members and GC box binding proteins have been implicated in regulating the expression of the other Id family genes [36, 40, 41], the involvement of Sp2 appeared to be unique for the Id3 gene. Interestingly, GKLF (gut-enriched Kruppel-like factor) – one of the KLFs – was reported recently to mediate the downregulation of Id3 expression in response to specific oxidative stress in vascular smooth muscle cells [42]. A putative GKLF binding site is present in the region surrounding the translational initiation codon of the human Id3 gene, but the functional significance of that particular binding site has not been documented. Since the GC or GT box we identified might be interacting with multiple proteins in the C2C12 extract, it would be interesting to see if our GC box could bind GKLF.

There are several explanations of why transfection of Sp2 failed to alter substantially the differentiation potential or the expression level of the endogenous Id3 gene. Some of these might be purely technical, such as inadequate transfection efficiency or the selective silencing of the cotransfected Sp2 gene due to differential integration into the heterochromatic regions, etc. Others might have more physiologically relevant basis. For example, Sp2 might be subjected to additional posttransciptional regulation that limits its effectiveness in activating gene expression under the non-proliferative conditions used in the assay (in order to reduce the background level of Id3 expression). Alternatively, optimal Sp2 function might require cooperation with additional factors that could be limiting. In this regard, it is interesting that others have reported that Sp2 functions as a rather weak transactivator in transfection experiments in other systems [43]. Since it is well known that Id3 protein function can also be modulated by posttranslation control [44], a slight activation of the endogenous Id3 gene by the transfected Sp2 might not result in the accumulation of sufficient "active" Id3 to affect the differentiation process. Thus, rather than proving that Sp2 protein is irrelevant, the negative results only underscore the complexity of the regulatory process that needs to be further explored.

4.4. Transcriptional regulation of Id3 genes by other transcription factors

Other transcription factors have been implicated either directly or indirectly in regulating expression of Id3. Both

Id1 and Id3 are inducible by transforming growth factor β family members and Smad proteins have been implicated in their regulation [45–47]. Although overexpression of MyoD induced Id3 in proliferating muscle cells [48], no consensus bHLH binding motif was found within the −180-bp Id3 promoter or up to approximately 800 bp region upstream. Whether MyoD binds to sites outside of the region of the Id3 gene, we have characterized or exert its regulation on the Id3 promoter indirectly through other transcription factors remains to be determined. Fully understanding the regulation of the endogenous Id3 gene would require more detailed analysis of the Id3 promoter under different physiological context.

4.5. Downregulation of GC box binding activity in differentiated skeletal muscle cells

The relevance of the GC box motif in the physiological regulation of Id3 expression is also supported by our observation that the binding of nuclear protein(s) to the site was dramatically and specifically reduced following muscle differentiation. Such reduction in binding could contribute to the downregulation of Id3 transcription in differentiated muscle cells. Since some degree of DNA binding persists, additional mechanisms might be involved in shutting down Id3 expression following muscle differentiation.

Reduced nuclear protein binding to GC box-like-motifs has been implicated in the decreased expression of at least three other genes following muscle differentiation—the glucose transporter gene GLUT1 [49], the avian fibroblast growth factor receptor 1 gene FGFR1 [50], and the murine telomerase gene TERT [51]. In every case, the proteins believed to interact with the respective GC boxes were Sp1 and/or Sp3. In contrast, the GC box in Id3 appears to bind Sp2 but not Sp1 or Sp 3. A coordinated reduction in the DNA binding activity of the Sp family transcription factors might thus be a common theme in reducing or silencing specific gene expression in differentiated skeletal muscle cells, with distinct family members affecting specific individual genes.

We do not yet know what causes the diminution of the GC-box binding activities following muscle differentiation or whether this occurs by transcriptional or posttranscriptional mechanisms. Future studies will be necessary to distinguish such alternatives and uncover other factors that might be involved in silencing Id3.

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