Dystrophin Dp71 Expression Is Down-regulated during Myogenesis

ROLE OF Sp1 AND Sp3 ON THE Dp71 PROMOTER ACTIVITY*

Dp71 expression is present in myoblasts but declines during myogenesis to avoid interfering with the function of dystrophin, the predominant Duchenne muscular dystrophy gene product in differentiated muscle fibers. To elucidate the transcriptional regulatory mechanisms operating on the developmentally regulated expression of Dp71, we analyzed the Dp71 expression and promoter activity during myogenesis of the C2C12 cells. We demonstrated that the cellular content of Dp71 transcript and protein decrease in myotubes as a consequence of the negative regulation that the differentiation stimulus exerts on the Dp71 promoter. Promoter deletion analysis showed that the 224-bp 5′-flanking region, which contains several Sp-binding sites (Sp-A to Sp-D), is responsible for the Dp71 promoter basal activity in myoblasts as well as for down-regulation of the promoter in differentiated cells. Electrophoretic mobility shift and chromatin immunoprecipitation assays indicated that Sp1 and Sp3 transcription factors specifically bind to the Sp-binding sites in the minimal Dp71 promoter region. Site-directed mutagenesis assay revealed that Sp-A is the most important binding site for the proximal Dp71 promoter activity. Additionally, cotransfection of the promoter construct with Sp1- and Sp3-expressing vectors into Drosophila SL2 cells, which lack endogenous Sp family, confirmed that these proteins activate specifically the minimal Dp71 promoter. Endogenous Sp1 and Sp3 proteins were detected only in myoblasts and not in myotubes, which indicates that the lack of these factors causes down-regulation of the Dp71 promoter activity in differentiated cells. In corrobororation, efficient promoter activity was restored in differentiated muscle cells by exogenous expression of Sp1 and Sp3.

Duchenne muscular dystrophy (DMD)† is an inherited disorder characterized by progressive muscle degeneration due to the absence of dystrophin (1). Dystrophin is a 427-kDa protein consisting of four major domains as follows: an N-terminal actin-binding domain, a central spectrin-like rod domain consisting of 24 triple helix structures, a cysteine-rich domain, and a unique C-terminal domain (2). In skeletal muscle, dystrophin is associated with a group of sarcomembran proteins and glycoproteins known collectively as the dystrophin-associated proteins (DAP) (3). One of the proposed functions of dystrophin is to provide a structural link between the actin-based cytoskeleton and the extracellular matrix (4). The DMD gene exhibits a complex transcriptional regulation due to the presence of at least seven independent promoters that generate three full-length dystrophins (Dp427) and N-terminally truncated gene products (Dp260, Dp140, Dp116, and Dp71) (5–11). Dp71 contains a unique N-terminal sequence of seven amino acids and the cysteine-rich and C-terminal domains of dystrophin (12–14). Dp71 is expressed in all tissues tested so far, except for skeletal muscle, where its expression is exclusively confined to myoblasts (15–17). Conversely, Dp427 is not expressed until after the cells begin myogenic differentiation and is the major isoform expressed in mature fibers (18, 19).

Based on obvious structural differences between Dp71 and Dp427, it is unlikely that these proteins are functionally interchangeable. In fact, ectopic expression of Dp71 in skeletal muscle of mdx mice, which lack dystrophin, restored the normal levels of DAP but did not alleviate muscle damage (20, 21), and more surprisingly, ectopic expression of Dp71 in skeletal muscle of transgenic wild-type mice results in muscle damage, similar to that observed in mdx mice (22).

The performance of stage-specific tasks by Dp427 and Dp71 in muscle cells indicates that their expression is tightly controlled (23). The transcriptional regulation of Dp427 in muscle cells has been well established. It is known that high levels of YY1 protein down-regulate the Dp427 promoter in undifferentiated muscle cells, but upon the induction of muscular differentiation, YY1 protein levels are negatively controlled by the action of the protease m-calpain, allowing the dystrophin promoter bending factor to regulate positively the promoter activity (24). In contrast, regulation of Dp71 during myogenesis remains to be approached, and the Dp71 promoter region, which exhibits the structure of a typical housekeeping promoter, has been only partially characterized (11). In this study, we analyzed the expression of Dp71 and characterized the activity of its promoter region during muscle cell differentiation.

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* This work was supported in part by Consejo Nacional de Ciencia y Tecnología Grant 43285-M (to B. C.) and grants from the Muscular Dystrophy Association and Association Francaise contre les Myopathies (to U. N. and D. Y.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Ph.D. student supported by Consejo Nacional de Ciencia y Tecnología Scholarship 121176.

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The abbreviations used are: DMD, Duchenne muscular dystrophy; DAP, dystrophin-associated proteins; EMSA, electrophoretic mobility shift assay; ChIP, chromatin immunoprecipitation; FBS, fetal bovine serum; CAT, chloramphenicol acetyltransferase; RT, reverse transcription; r, ribosomal.
tion of the C2C12 cells, a sub-culture derived from the C2 cell line. Deletion analysis showed that the major Dp71 promoter activity in proliferating myoblasts depends on the proximal 224-bp promoter region, which contains several Sp-binding sites. Altogether, gel shift, chromatin immunoprecipitation, and site-directed mutagenesis assays, as well as transient transfection experiments in Drosophila SL2 cells, established that Sp1 and Sp3 transcription factors interact with the Sp-binding sites and transactivate the Dp71 promoter. In differentiating muscle cells, the Dp71 promoter activity is downregulated; the 224-bp proximal promoter region seems to be sufficient to exert such control, and a concomitant reduction in the endogenous Sp1 and Sp3 protein levels was observed. Restoration of significant promoter activity in differentiating cells was obtained after exogenous expression of Sp1 or Sp3 proteins. Our results indicate that the developmentally regulated expression of Dp71 in muscle cells during differentiation is based on the differential expression of Sp1 and Sp3 transcription factors.

**EXPERIMENTAL PROCEDURES**

**Cell Cultures—**C2C12 cells (ATCC CRL-1772), a sub-culture derived from C2 cell line (25), were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 4.5 g of glucose/liter, supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine (proliferation medium), and maintained at 37 °C in a humidified incubator with a 5% CO2 atmosphere. In some experiments, subconfluent C2C12 cells were induced to differentiate by lowering FBS to a final concentration of 1% (differentiation medium). Schneider’s Drosophila Line 2 (SL2, ATCC CRL-1963) cells were cultured in Schneider’s Drosophila Medium (Invitrogen) containing L-glutamine and were maintained at 25 °C without CO2. All culture media contained 100 units of penicillin and 100 μg/ml streptomycin.

**Real Time RT-PCR—**Dp71 transcript levels during C2C12 muscle cell differentiation were measured by real-time polymerase chain reaction (PCR) using the comparative C_{T} method described by Applied Biosystems. Total RNA was extracted using the TRIZol reagent (Invitrogen) from expression plasmids according to the manufacturer’s instructions. Decontamination of the C2C12 cells, a sub-culture derived from the C2 cell line, freshly grown cells from 3- to 4-day-old cultures were plated at density of 2.5 × 10^5 cells/40-mm dish and the day after were transfected using Cellfectin reagent (Invitrogen), according to the manufacturer’s instructions. Each plate was transfected previously with defined amounts of pRSV-β-gal, each Dp71 promoter-CAT reporter plasmids, and pPacSp1 and/or pPacUSp3 expression plasmids. After transfection, C2C12 and SL2 cells were maintained in their respective growth medium for 48 h, and the CAT and β-galactosidase activities were measured.

**CAT and β-Galactosidase Assays—**To prepare cell extracts for the CAT and β-galactosidase expression assays, cells were scraped into 1× phosphate-buffered saline and centrifuged at 2600 × g at 4 °C for 5 min. The cell pellet was resuspended in 150 μl of 0.25 M Tris and 1 mM EDTA, pH 8.0, and then subjected to six freeze-thaw cycles with dry ice. Cell debris was removed by centrifugation at 4 °C for 5 min at 3000 × g. The resulting supernatant was resuspended in an equal volume of 2× Laemmli sample buffer (10% glycerol, 125 mM Tris-HCl, pH 6.5, 0.1% sodium dodecyl sulfate), and aliquots were subjected to SDS-PAGE.

**DNA Amplification and Gel Electrophoresis—**Amplification of the C2C12 genomic DNA was performed by PCR using Taq DNA polymerase and dimethylsulfoxide (DMSO)-free PCR buffer in a 96-well plate format on a PTC-100 programmable thermal controller (MJ Research) and applying a touchdown PCR procedure. PCR primers were Dp71F 5'-TGTATTGGGTTCGTGGCGAGC-3' and Dp71R 5'-ATTCGATCGGGGCGGGGC-3'.

**Isolation of Nuclear Extracts—**Nuclear extracts were prepared from undifferentiated and differentiated C2C12 cells, as described previously (26). Briefly, cells were washed with cold phosphate-buffered saline and resuspended in 400 μl of lysis buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride). Cell lysates were incubated on ice for 15 min and then centrifuged for 3 min at 3000 × g at 4 °C. The pellet was resuspended in 20 μl of 1× lysis buffer (26 mM Tris-HCl, pH 7.9, 400 mM NaCl, 0.1% Nonidet-P40, 0.5 mM phenylmethylsulfonyl fluoride). After that, cells were vigorously shaken at 4 °C for 15 min and pelleted by centrifuging for 5 min at 12,000 × g at 4 °C. The supernatant was recovered as the nuclear extract, and its protein concentration was determined by the Bradford assay. Nuclear extracts were stored at −70 °C until use.

**Electrophoretic Mobility Shift Assay—**Synthetic complementary deoxyoligonucleotides corresponding to the pAP and Sp DNA elements of the Dp71 promoter were annealed and radioactively labeled by incubating 4 mM of dephosphorylated double-stranded oligonucleotides, 10 units of T4 polynucleotide kinase (Invitrogen), 5 μl of T4 polynucleotide kinase buffer, and 20 μCi of γ[^32]P-ATP (Amersham Biosciences) for 30 min at 37 °C and inactivated at 65 °C for 10 min. For the electrophoretic mobility shift assay (EMSA), 10 μg of nuclear extract proteins from either undifferentiated or differentiated cells were mixed in the binding buffer (12 μl HEPEs, pH 7.8, 4 mM MgCl2, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride). After that, cells were vigorously shaken at 4 °C for 15 min and pelleted by centrifuging for 5 min at 12,000 × g at 4 °C. The supernatant was recovered as the nuclear extract, and its protein concentration was determined by the Bradford assay. Nuclear extracts were stored at −70 °C until use.

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AP2, 5'-GATCGAATGACGTCCTGGCCGGCGCTT-3'; consensus OCT1 5'-TGGTCTTCCAATCATGAA-3'; mutated bases are underlined. For supershift analysis, 10 µg of anti-Sp1- or anti-Sp3-specific antibodies (Santa Cruz Biotechnology) were incubated with nuclear extract proteins for 12 h at 4 °C after addition of the DNA probe. Protein-DNA complexes were fractionated by electrophoresis in non-denaturing 6% polyacrylamide gels and visualized by autoradiography.

Antibodies and Western Blot Analysis—Antibodies directed to Sp1, Sp3, and myogenin were purchased from Santa Cruz Biotechnology. The anti-Dp71 polyclonal antibody 2166 was a donation of Dr. D. J. Blake (27). The anti-actin monoclonal antibody was generously provided by Dr. J. M. Hernandez. C2C12 cells cultured in 35-mm culture dishes were scraped, lysed as described previously (28), and centrifuged at 12,000 × g for 10 min at 4 °C. Protein samples were quantified by the Bradford assay and denatured at 100 °C for 5 min in a protein sample buffer containing 1% SDS and 1% dithiothreitol. One hundred µg of total protein extracts were loaded in each lane and subjected to 10% PAGE under denaturing conditions (SDS-PAGE) and transferred to a nitrocellulose membrane for immunoblotting. Immuneblots were probed with the corresponding primary antibodies and developed by using the ECL Western blotting system (Amersham Biosciences).

Chromatin Immunoprecipitation Assay—C2C12 cells (2 × 107) were treated with 1% formaldehyde to cross-link protein-DNA complexes. Immunoprecipitates of cross-linked complexes were prepared with anti-Sp1 and anti-Sp3 antibodies, treated with proteinase K for 2 h, and then incubated at 65 °C to eliminate cross-linking. DNA was purified by phenol/chloroform extraction and ethanol precipitation. DNA samples were quantified by the picogreen assay (Molecular Probes) and then analyzed by PCR amplification of the Dp71 promoter sequence located between -127 and +78 with 30 cycles of PCR using the following of 32P-labeled oligonucleotides: Dp71 (upper), 5'-CTATCTGAGTTCCAGGTC-3', and Dp71 (lower), 5'-CGGAGAGGAGCCCGGACG-3'. Consensus OCT1 oligonucleotides were purchased from Santa Cruz Biotechnology. The reaction was transferred into XL-1 Blue competent cells, and the transformation mixture was plated on LB ampicillin plates. The authenticity of the mutants was established by DNA sequencing.

RESULTS

Dp71 Expression Is Down-regulated during C2C12 Myogenesis—To evaluate the expression of Dp71 during C2C12 muscle cell differentiation, its mRNA and protein levels were determined. Dp71 mRNA expression was measured by real time RT-PCR using Dp71-specific primers, whereas Dp71 protein was assessed by Western blotting using the polyclonal antibody 2166, directed against its C-terminal domain. Myoblast cultures were induced to differentiate for 3, 6, and 9 days by lowering serum concentration, and cell-differentiated morphology was monitored by light microscopy analysis. As shown in lower panel, the expression of Dp71 transcript was regulated, at least in part, at a transcriptional level. In contrast, C2C12 maintained in differentiation medium resulted in up-regulation of myogenin, a myogenic gene marker; this protein was undetectable in myoblast cells but appeared by day 3 and remained steady throughout the rest of the differentiation treatment (Fig. 1B, lower panel), in agreement with previous reports (29–32). Thus, the concomitant decreases of Dp71 mRNA and protein levels were negatively correlated with the differentiation process of C2C12 cells and evaluated by the acquisition of a differentiated morphology and induction of myogenin expression. Furthermore, these results indicated that the reduction of Dp71 expression during skeletal muscle differentiation was regulated, at least in part, at a transcriptional level.

Dp71 Promoter Activity during Muscle Cell Differentiation—To characterize the mechanisms controlling the negative transcriptional regulation of Dp71 that occurred during myogenic differentiation, C2C12 myoblasts were transiently
cotransfected with pRSV-β-gal and p1.8CAT vectors; the latter contains 1.8 kb from the mouse Dp71 promoter region fused to the reporter gene CAT (11). Myoblast cultures were induced to differentiate for 2 days, and the Dp71 promoter function was evaluated by CAT assays using β-galactosidase activity to normalize transfection efficiency. The 1.8-kb Dp71 promoter fragment (p1.8CAT) drove the efficient expression of CAT in C2C12 myoblasts compared with promoterless pSV0CAT (Fig. 3). As expected from the mRNA results described above, reporter activity of the Dp71 promoter decreased by 70% in differentiated cells (Fig. 2). Because the induction of muscle cell differentiation was also modulated by the addition of cAMP (33, 34), we decided to evaluate the effect of this alternative inducer of differentiation on the Dp71 promoter activity. For that purpose, myoblasts, transiently transfected with the p1.8CAT vector, were cultured in growth medium for 48 h with or without cAMP. Fig. 2 shows that cAMP treatment resulted in a 40% inhibition of CAT activity, whereas a negative control experiment, in which the nerve growth factor (NGF) at 2 nm was added to cells cultured in proliferation medium. Data are expressed as the mean ± S.D. of at least three independent experiments, each performed in duplicate. Asterisks denote significant differences (p < 0.05).

Identification of cis-Elements Essentials for the Regulation of the Dp71 Promoter during Myogenensis—To define the cis-acting elements required for Dp71 promoter regulation in muscle cells, a series of mutant promoter constructs containing progressive 5'-end or internal deletions of the Dp71 promoter sequence, linked to the CAT reporter gene, were constructed and transfected into proliferating myoblasts. The cell cultures were then grown for 48 h under proliferation or differentiation conditions, and the promoter activity of each mutant plasmid was evaluated. In myoblast cells (Fig. 3, black bars), deletion of the promoter sequence encompassing positions −1834 to −1156 caused a near 70% reduction of the reporter activity, whereas further deletion beyond position −937 virtually recovered the transcriptional activity observed with the wild-type promoter. This suggest that positive and negative regulatory elements may be contained in these two regions, respectively. Further deletions to positions −709 (p709CAT) and −586 (p586CAT) did not significantly affect the transcriptional activity of the promoter constructs compared with the wild-type promoter. Vector p224CAT, which contains only 224-bp from the proximal promoter region, maintained ~50% of the wild-type promoter activity. Most interestingly, the removal of the 224-bp proximal promoter region abolished the reporter transcriptional activity of p1500CAT and p900CAT vectors, despite conserving most of the 5'-end distal promoter sequences (Fig. 3). Finally, as compared with wild-type promoter, plasmids carrying internal deletion between positions −1603 and −711 (pXbaIdelCAT), −1778 and −711 (p700CAT), and −1576 and −839 (pstuIdelCAT) did not modify substantially the reporter activity (Fig. 3). These findings suggest that the proximal 224-bp region is the core promoter essential for basal transcriptional activation of Dp71 in proliferating myoblast cells. When we analyzed the behavior of the different promoter constructs in differentiating C2C12 cells, we observed that all mutant constructs that already showed appreciable CAT activity in myoblast cells displayed substantial reductions in their reporter activity, ranging from 50 to 70% (Fig. 3, open bars). Because the minimal proximal promoter region (p224CAT vector) maintained the negative regulation displayed in response to the differentiation stimulus, we assumed that this sequence was also responsible for the negative modulation displayed by the Dp71 promoter during myogenensis.

Sp1 and Sp3 Specifically Bind in Vitro to the GC Boxes in the Dp71 Core Promoter—Sequence analysis of the proximal promoter region revealed the presence of four binding motifs for the Sp family of transcription factors located between positions −76/−71, −42/−37, −38/−33, and +26/+31 (termed Sp-A, Sp-B, Sp-C, and Sp-D boxes, respectively), and a single AP2 DNA element located in −29 to −23 (Fig. 4A). It should be noted that the Sp-B and Sp-C sequences overlap each other. To determine whether the consensus-binding sites present in the core promoter region interact with nuclear protein components, we performed EMSA with double-stranded 32P-labeled oligonucleotides spanning Sp-A, Sp-BC, Sp-D, and AP2 DNA elements and nuclear extracts from C2C12 myoblasts. Fig. 4B shows that both Sp-A and Sp-BC probes gave rise to several major DNA-protein complexes (C1, C2, C3, and C4) and additional diffuse retarded bands. To define the nature of the DNA-protein complexes formed, competitive binding experiments were performed (Fig. 4B). The four slower migrating complexes formed by Sp-A probe (C1 to C4) as well as the first, third, and fourth slower migrating complexes obtained with the Sp-BC oligonucleotide (C1, C3, and C4) were competed specifically by a 100-fold molar excess of either the respective unlabeled probe or a consensus Sp oligonucleotide. In contrast, the DNA-protein complexes remained unaltered after the addition of 100-fold molar excess of either a mutated oligonucleotide or an unrelated oligonucleotide Oct1 (complexes indicated in Fig. 4B). The remaining Sp-binding site, the Sp-D box, formed only a weakly diffused retarded band that did not show changes when challenged with a 100-fold molar excess of unlabeled oligonucleotides Sp-D, consensus Sp, and mutated Sp or Oct1 (Fig. 4B). Finally, the AP2 probe produced two major retarded bands (C1 and C2), and the formation was significantly reduced by adding a 100-fold molar excess of either an unlabeled AP2 probe or a consensus AP2 oligonucleotide; contrary, a 100-fold molar excess of either an unrelated Oct1 oligonucleotide or a mutated AP2 probe did not compete in binding (Fig. 4B).

Because functional analysis of the proximal Dp71 promoter region showed that the Sp-A site is the most important transcription factor binding site for activity of the minimal Dp71 promoter (see below), we decided to investigate whether complexes formed between the Sp-A probe and myoblast nuclear extracts consists of Sp1 and/or Sp3 proteins. Therefore, an
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Fig. 3. Deletion analysis of the Dp71 promoter in C2C12 muscle cells. A series of truncated promoter constructs (left) was cotransfected with the pRSV-β-gal standardization plasmid into C2C12 cells, and transfected cells were either grown under proliferation conditions (black bars) or induced to differentiate for 2 days by culturing in differentiation medium (open bars). Restriction enzyme sites used for the construction of CAT reporter constructs are shown on the top of the left side. Numbering indicates deletion end points of the promoter constructs relative to the Dp71 transcription initiation site (arrow). CAT activity of each construct was determined, and transfection efficiency was normalized with β-galactosidase activity. The mean value obtained with p1.8CAT was set at 100%. Values are presented as mean ± S.D. of at least three independent experiments, each performed in duplicate.

EMS A for the Sp-A probe in the presence of anti-Sp1 or anti-Sp3 antibodies was performed. Fig. 4C shows that the C1 and C2 complexes were shifted when Sp1 or Sp3 antibodies were included in the assay, respectively. Incubation of the binding reactions with an unrelated IgG did not affect the EMSA pattern confirming the specificity of the assay. Our results indicate that the Sp1 and Sp3 transcription factors bind in vitro to the Sp DNA elements in the Dp71 proximal promoter region.

The Dp71 Minimal Promoter Region Interacts with Sp1 and Sp3 In Vivo—In order to evaluate whether Sp1 and Sp3 proteins are indeed recruited in vivo to the Dp71 promoter, we employed a ChiP assay to cross-link the DNA with bound proteins in situ in C2C12 cells; the protein-DNA complexes were precipitated with antibody specific for Sp1 or Sp3. The DNA fragments containing the Dp71 minimal promoter region were then amplified by radioactive PCR. As shown in Fig. 5, PCR with primers flanking the Dp71 minimal promoter region produced a band from DNA coprecipitated with Sp1 or Sp3, and such a band migrated on an agarose gel to a position identical to the genomic DNA control (Fig. 5B, Input). In the positive control, we obtained an intense PCR product when the Sp1 and Sp3 immunoprecipitates were subjected to a PCR with primers flanking the telomerase promoter region (Fig. 5B, Tert), which contains multiple Sp-binding sites (35, 36). In the negative controls, Dp71 promoter primers did not generate any PCR product when the immunoprecipitation reaction was carried out with an unrelated IgG antibody. Likewise, PCR with primers flanking an irrelevant DNA region (a DMD gene region with no Sp boxes) did not produce any PCR products from the Sp1 and Sp3 immunoprecipitates. Our results indicate that the Sp1 and Sp3 transcription factors bind in vivo to the Dp71 proximal promoter region.

Sp1 and Sp3 Transcription Factors Transactivate the Dp71 Promoter—The EMSA and ChiP analyses described above indicate that Sp1 and Sp3 bind to the GC boxes present in the Dp71 proximal promoter. Therefore, to determine whether these transcription factors can functionally transactivate the Dp71 promoter, we employed a transfection system consisting of Drosophila SL2 cells, a cell line that naturally lacks the expression of endogenous Sp transcription factors, and vectors pPacSp1 (encoding Sp1), pPacUSp3 (encoding Sp3), and pPac (empty vector). The Dp71 minimal promoter vector (p224CAT) was transfected into SL2 cells with either pPacSp1 or pPacUSp3, and 48 h after transfection CAT activity of each promoter construct was evaluated. As shown in Fig. 6, cells transfected with p224CAT and 0.1 μg of pPacSp1 displayed a 150-fold augument in CAT activity; however, increasing amounts of Sp1 expression vector did not modify substantially such transactivation. On the other hand, relative to pPacUSp3 vector, a 2-fold molar excess of Sp3 expressing vector was required to obtain a significant increase in CAT activity controlled by the minimal promoter. Nevertheless, transfection with higher amounts of pPacSp3 (0.5 μg) caused a drastic reduction of 70% in the former promoter transactivation, which suggests that elevated amounts make Sp3 change from a positive to a negative transcriptional regulator. These findings strongly suggest that Sp1 and Sp3 play an important role in activating the Dp71 proximal promoter. In addition, it appears that precise amounts of each Sp protein are required to modulate positively the minimal promoter.

Functional Analysis of the Transcription Factor Binding Sites Present in the Proximal Dp71 Promoter—From the experiments described above, it seems that the Sp1/Sp3 proteins are key factors in modulating the Dp71 transcriptional activity in muscle cells. Hence, it is expected that disruption of the Sp1/Sp3-binding sites located in the Dp71 minimal promoter region would impair the activity of this promoter. To approach this hypothesis, we introduced point mutations that were shown to abolish nuclear protein binding in the EMSA competition experiments into Sp-A, Sp-B, Sp-C, Sp-D, and AP2 DNA ele-
ments. As shown in Fig. 7, the point mutation in the Sp-A site resulted in ~40% reduction of the CAT activity controlled by the minimal promoter region, whereas the mutation of Sp-B or Sp-D sites showed reductions of ~18 and ~27% of CAT activity, respectively. Finally, the mutation of the Sp-C site resulted in a slight increase of ~14% in the minimal promoter activity (Fig. 7). When point mutations were introduced into all of the potential Sp-binding sites located in the minimal promoter region, the reporter activity was reduced by ~54% compared with the wild-type construct; on the other hand, mutation of the AP2 site resulted in no significant alteration in the promoter activity (Fig. 7). These results suggest that the Sp-A site is the most crucial transcription factor-binding site in terms of basal Dp71 expression.

Endogenous Expression Levels of the Sp1 and Sp3 Transcription Factors—As Sp1/Sp3 transactivate significantly the Dp71 proximal promoter in Drosophila SL2 cells, it is likely that they are also involved in the molecular mechanism controlling the down-regulation of this promoter during myogenesis. Therefore, as a first step in clarifying this matter, we analyzed by Western blotting the endogenous expression of these transcription factors in nuclear extracts obtained from proliferating myoblasts and differentiated myotubes. In myoblast nuclear extracts, the expected protein bands for Sp1 (~100 kDa) and
Overexpression of the Sp3 Transcription Factor Restores the Activity of the Proximal Dp71 Promoter in Differentiated Cells—Because all of the experimental evidence shown before indicated that down-regulation of the Dp71 expression during myogenesis is caused by the scarcity of Sp1 and Sp3 found in differentiated myotubes, it is expected that the Dp71 promoter activity could be recovered by restoring the expression level of these transcription factors in differentiated muscle cells. To test this idea, the minimal promoter construct was introduced into C2C12 cells together with either pPac, pPacSp1, or pPacSp3 expression vectors, and the transfected cultures were induced to differentiate for 48 h. Fig. 9 shows that the expression of the empty vector (pPac) did not prevent the drop of the promoter activity that occurred in response to the differentiation stimulus. In contrast, increasing amounts of the Sp3-expressing vector recovered the reporter activity controlled by the minimal promoter at levels even higher than that observed in proliferating myoblasts. On the other hand, Sp1 overexpression provoked only modest increases in the promoter CAT levels that did not restore completely the activity shown by this promoter before differentiation. Simultaneous overexpression of Sp1 and Sp3 transcription factors did not have an additional effect on CAT activity obtained by the single overexpression of Sp3, indicating that the Sp1 and Sp3 proteins did not act synergistically over the Dp71 minimal promoter region. The participation of Sp1 and Sp3 in the recovery of the promoter activity appears to be specific because the overexpression of AP2 failed to rescue the reporter activity in the differentiated cells (data not shown).

**DISCUSSION**

Dp71 is expressed in a wide variety of tissues with the exception of skeletal muscle, where it is known that Dp71 protein levels decrease during myogenesis. On the contrary, dystrophin expression increases in differentiated muscle cells (13, 15–19, 37). The transcriptional regulation of dystrophin during myogenesis has been well established (24, 38), while the gene regulation of Dp71 during this cellular process remains to be approached. With this understanding, the purpose of the present study was to establish a muscular cell model that mimics the *in vivo* expression of Dp71 while allowing for definition of the molecular mechanisms controlling Dp71 expression during muscle cell differentiation. Here we demonstrated that expression of Dp71 is down-regulated during differentiation of C2C12 muscle cells as a consequence of decreases in their mRNA and protein levels (Fig. 1). The cellular content of Dp71 protein and mRNA decreased in parallel with the transcriptional activity of the Dp71 promoter in response to myogenesis (Fig. 2), indicating that the alteration in the transcription at the promoter level accounts for the elimination of Dp71 expression during muscle cell differentiation. Therefore, we characterized by deletion analysis the transcriptional activity of the mouse Dp71 promoter in C2C12 cells, and we determined that the Dp71 core promoter region, spanning from −224 to +65, is sufficient for the basal transcriptional activity of Dp71 in proliferating myoblasts as well as for the negative transcriptional modulation displayed by this gene in response to myogenesis (Fig. 2). The minimal promoter region of Dp71 lacks a TATA box and contains several potential cis-acting elements as follows: four Sp-binding sites (Sp-A, overlapping Sp-B and -C sites, and Sp-D) and a single AP2-binding site. To ascertain whether these transcription factor-binding sites are necessary for Dp71 transcriptional modulation in muscle cells, different molecular approaches were employed. By using EMSA, we detected several specific DNA-protein complexes formed by myoblast nuclear extracts with oligonucleotides corresponding to the Sp-A, Sp-BC, Sp-D, and AP2-binding sites; with the

**Fig. 5. Sp1 and Sp3 bind to the proximal Dp71 promoter in vivo.** The occupation of Sp1 and Sp3 on the proximal Dp71 promoter was determined by ChIP combined with PCR. A, schematic diagram of the proximal Dp71 promoter shows the Sp1/Sp3-binding sites (black boxes). Two open arrowheads indicate the position of the Dp71-specific primers used for PCR, and an arrow indicates the transcription start site. B, genomic DNA from C2C12 cells was immunoprecipitated with antibodies against Sp1 (α-Sp1) or Sp3 (α-Sp3) as described under “Experimental Procedures.” Input DNA, bound (B) and unbound (UB) chromatin fractions were amplified by using Dp71 32P-labeled oligonucleotides. As control, a DNA region from intron 63/exon 64 of the DMD gene with no canonical Sp-binding sites (Dys 63/64) and the telomerase gene promoter region that contains several canonical Sp-binding sites (Ter) were amplified with their respective 32P-labeled specific primers (see “Experimental Procedures”). An irrelevant IgG (α-IgG) was employed as a negative control for immunoprecipitation. Representative radiographies of PCR products are shown.

**Fig. 6. Transactivation of the proximal Dp71 promoter by Sp1 and Sp3 in Drosophila SL2 cells.** SL2 cells were transfected with 5 µg of p224CAT reporter plasmid (containing the proximal Dp71 promoter) with variable amounts of pPacSp1 (0.1, 0.3, and 0.5 µg) and pPacSp3 (0.1, 0.3, and 0.5 µg) plasmids. Empty pPac vector was used as negative control. In all cases, control plasmid pRSV-β-gal was included to normalize transfection efficiency. The CAT activity obtained with pPac was set at 1%, and all other CAT activities were represented relative to this value. Data represent the mean CAT activities ± S.E. of three independent experiments, each performed in duplicate.

Sp3 (~132 and ~70 kDa) were obtained after using their respective specific antibodies. Most interestingly, Sp1 and Sp3 protein bands disappeared from myotubes since the 6th and 3rd day of induced differentiation, respectively (Fig. 8A). These findings indicate that Sp1 and Sp3 protein expression is present but is extinguished as muscular differentiation proceeds. This pattern of protein expression resembled that of Dp71 (Fig. 1). In support of these findings, we observed that DNA-protein complexes formed in vitro between the Sp-A probe and myoblast extracts (complexes C1, C2, and C3) disappeared completely when protein extracts from differentiated cells of 6 days were employed in the EMSA (Fig. 8B).
exception of Sp-D, the rest of these DNA-protein complexes were competed efficiently by their respective consensus oligonucleotides (Fig. 4B). We have also identified the binding of Sp1 and Sp3 to the Sp-A box by performing supershift assays with Sp1- and Sp3-specific antibodies (Fig. 4C). Furthermore, by ChIP assays, we demonstrated that transcription factors Sp1 and Sp3 are indeed recruited in vivo to the Dp71 core promoter (Fig. 5). Consequently, by using the Drosophila SL2 cell line, we demonstrated that these two transcription factors behave as activators of the Dp71 promoter region (Fig. 6). Altogether, these findings indicate that binding of Sp1 and Sp3 to the Dp71 core promoter has a functional role in myoblast cells. The transcription factors Sp1 and Sp3 are ubiquitously expressed proteins that bind their recognition sequence (GC boxes) with similar affinity. Sp1 is thought to play a primary role in the regulation of a large number of genes including constitutive housekeeping genes and inducible genes (39), whereas Sp3 contains a transcriptional repression domain and can act as an activator or as a repressor of Sp1-mediated activation (40–43). In fact, we observed that elevated levels of Sp3 change its role from activator to repressor of Dp71 expression in Drosophila SL2 cells (Fig. 6), whereas in differentiating C2C12 cells this transcription factor always acts as an activator regardless of its protein levels (Fig. 9). Because repression function of Sp3 depends on additional proteins which act as corepressors (42), it is likely that the differential behavior of Sp3 observed in our study is determined by the cellular context.

Functional reporter gene studies revealed that the Sp-A site is the most important transcription binding site for the activity of the minimal Dp71 promoter, and cancellation of this Sp site caused a marked decrease of 40% in reporter activity, whereas cancellation of all of the Sp sites resulted in only 54% reduction of reporter activity. The apparent redundancy due to the presence of multiple Sp-binding sites in the Dp71 promoter may constitute a mechanism by which expression of Dp71 is guaranteed in myoblast; hence, if an Sp-binding site is disrupted, alternative Sp-binding sites may participate in nuclear protein binding to maintain sufficient gene expression. The fact that mutation of all Sp1-binding sites reduced the promoter activity in /H11011 by 54% suggests that additional transcription factor-binding sites, other than Sp1/Sp3 DNA elements, maintain a residual
activity. Because overexpression of the AP2 protein and the cancellation of the AP2-binding site cause no changes in the Dp71 promoter activity (Fig. 7), it seems that the AP2 DNA element is irrelevant for the function of this promoter in muscle cells. Downstream from the transcription start site, from +43 to +52, a previously unnoted NFκB element was identified, which might participate in the Dp71 promoter function. Recently, it was demonstrated that Sp1 and Sp3 transactivate several promoters by interacting directly with NFκB-like elements (44, 45). Therefore, it is possible that, in the absence of intact consensus Sp DNA-binding sites, Sp1 and Sp3 might act on the Dp71 promoter by binding to the NFκB element. Additional work is required to test this hypothesis.

The presence of multiple potential Sp transcription factor binding sites proximal to the start of transcription is a feature of a number of promoters lacking a consensus TATA box, including the promoter regions for utrophin, epidermal growth factor receptor, and insulin-like growth factor genes (46–48). It has been proposed that such multiple Sp-binding sites and associated proteins may stabilize the transcriptional machinery and establish a site of transcription start in TATA-less promoters.

As Sp1/Sp3 factors seem to be crucial for the transcriptional activity of the Dp71 minimal promoter region in myoblasts, we envisaged that the drop in the activity of this promoter during myogenesis could be caused by impairment in the functioning of these transcription factors. In this direction, we observed that Sp-specific DNA-protein complexes obtained with myoblast extracts disappeared when nuclear extracts from differentiated muscle cells were employed in the EMSA (Fig. 8B). These findings indicate that the protein levels and/or binding activities of the Sp1 and Sp3 proteins are altered in myotubes. Further immunoblotting experiments clarified this matter; we revealed that Sp1 and Sp3 proteins are present in proliferating myoblasts but their levels decrease drastically as differentiation proceeds (Fig. 8A). Most interestingly, the expression pattern of Dp71 resembles that of Sp proteins; Dp71 protein is present in proliferating myoblasts but disappears in myotubes since the 3rd day of induced differentiation (Fig. 1B). These results are consistent with the conclusion that both Sp1 and Sp3 directly regulate the expression of Dp71 in C2C12 muscle cells by interacting with the promoter region in myoblast cells, and the lack of these factors appears to be sufficient to cause down-regulation of the Dp71 expression in myotubes (Fig. 10). Supporting our conclusion, we observed that exogenous expression of Sp1 and Sp3 restores the transcriptional activity of the proximal Dp71 promoter in differentiated muscle cells (Fig. 9).

Although these results could not rule out completely the possibility that additional unrevealed repressor factors participate in Dp71 down-regulation, they do indicate that the action of Sp1 and Sp3 on the Dp71 promoter constitute the main, if not the unique, regulatory mechanism working in differentiating muscle cells to modulate Dp71 expression.

Because the extensive distribution of Sp1 and Sp3 in mammalian tissues is consistent with the expression profile of Dp71 (44, 45), it is plausible to propose that the positive regulation exerted by these transcription factors on the Dp71 promoter is not a specific phenomenon restricted to myoblasts but a general regulatory mechanism present in different cell types.

Muscle cell differentiation is mediated by the transcription factor MyoD, which acts as a master regulator leading to the activation of many muscle-specific genes (49, 50). Recent studies (51, 52) have shown that the overexpression of MyoD leads to the repression of Sp1 and Sp3. In this context, although MyoD may not directly participate in decreasing Dp71 transcription in our experimental system, it might indirectly suppress the Dp71 promoter transcription in vivo by repressing genes for Sp1 and Sp3.

The modulation of Dp71 expression during myogenesis has a noticeable relevance in the physiology of muscle tissue. In early myogenesis, Dp71 is expressed to participate in cytoskeletal remodeling (23), whereas in mature muscle fibers its expression must be extinguished to allow dystrophin to be the predominant DMD gene product. It has been proposed that elevated Dp71 expression provokes the Dp71 protein competition in muscle sarcolemma with dystrophin for the available binding sites in the DAP complex. This event could interfere with the normal formation of the linkage between the actin cytoskeleton and the DAP complex (20, 21). In fact,
the ectopic expression of Dp71 in skeletal muscle of transgenic mice with normal dystrophin expression causes a muscular dystrophy phenotype (22).

Acknowledgments—We thank Dr. Guntram Suske (Institute of Molecular Biology and Tumor Research, Philips-University Marburg, Marburg, Germany) for providing us with pPacSp1, pPacSp2, and pPacUSp3 plasmids and Dr. Ronald J. Weigel (Department of Surgery, Stanford University, Stanford, CA) for AP2/pcDNA3.1(+) plasmid. We are grateful to Derek J. Blake (Department of Human Anatomy and Genetics, University of Oxford, Oxford, UK) for Dp71-specific antibody (2166) and to Dr. Ramon Menéndez (Department of Pediatrics, University of Pittsburgh School of Medicine, Pittsburgh, PA) for Drosophila SL-2 cells. We are indebted to María Guadalupe Aguilar González and Carlos Cruz Cruz for their technical assistance with the nucleotide sequencing. We also thank Francisco Depardon Benitez for valuable technical assistance.

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Dystrophin Dp71 Expression Is Down-regulated during Myogenesis: ROLE OF Sp1 AND Sp3 ON THE Dp71 PROMOTER ACTIVITY

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J. Biol. Chem. 2005, 280:5290-5299.
doi: 10.1074/jbc.M411571200 originally published online November 18, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M411571200

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