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Myogenesis and MyoD Down-regulate Sp1

A MECHANISM FOR THE REPRESSION OF GLUT1 DURING MUSCLE CELL DIFFERENTIATION*

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Muscle cell differentiation caused a reduction of glucose transport, GLUT1 glucose transporter expression, and GLUT1 mRNA levels. A fragment of 2.1 kilobases of the rat GLUT1 gene linked to chloramphenicol acetyltransferase drove transcriptional activity in myoblasts, and differentiation caused a decrease in transcription. Transient transfection of 5′ and 3′ deletion constructs showed that the fragment −99/−33 of the GLUT1 gene drives transcriptional activity of the GLUT1 gene and participates in the reduced transcription after muscle differentiation. Electrophoretic mobility shift assays showed the binding of Sp1 protein to the fragment −102/−37 in the myoblast state but not in myotubes, and Sp1 was found to transactivate the GLUT1 promoter. Western blot analysis indicated that Sp1 was drastically down-regulated during myogenesis. Furthermore, the forced over-expression of MyoD in C3H10T1/2 cells mimicked the effects observed during myogenesis, Sp1 down-regulation and reduced transcriptional activity of the GLUT1 gene promoter.

In all, these data suggest a regulatory model in which MyoD activation during myogenesis causes the down-regulation of Sp1, which contributes to the repression of GLUT1 gene transcription and, therefore, leads to the reduction in GLUT1 expression and glucose transport.

The formation of skeletal muscle during embryogenesis involves, first, commitment of mesodermal stem cells to the myogenic lineage. Myoblast cells, although undifferentiated and capable of continued proliferation, differentiate when they receive the appropriate environmental signals, fuse, and form multinucleate myotubes. At the same time as this morphological differentiation, a battery of adult muscle-specific genes whose products are required for the unique contractile and metabolic properties of the muscle fiber are activated (1, 2). The factors that regulate the expression of muscle-specific genes following commitment to terminal differentiation are well established. The best characterized are the members of the myogenic basic helix-loop-helix (bHLH)[1] protein family or MyoD family, that function as master regulators of muscle cell fate during development (2, 3). Four members of the family have been cloned: MyoD (4), Myf5 (5), myogenin (6, 7), and Mrf4 (8–10). Each of these factors is expressed exclusively in skeletal muscle, and when expressed ectopically in a variety of non-muscle cell types, they activate the complete program of myogenic differentiation (2). All the members of the myogenic bHLH family activate the transcription of muscle-specific genes by binding to the E-box consensus sequence (CANNTG) in muscle gene promoters and enhancers. However, not all muscle genes contain functional E boxes in their regulatory promoter regions, and myogenic bHLH proteins can also activate transcription of muscle-specific genes that lack E boxes in their control regions (3). As expected, from these data, other muscle-specific transcription factors have been described to function as intermediates in the activation of gene expression during myogenesis, such as the M-CAT binding factor (11), and the myocyte enhancer factor 2 (MEF2) (12, 13).

Myogenesis is also associated with down-regulation of several growth-regulated myoblast proteins, including c-Fos (14), β- and γ-actins (15, 16), and the differentiation inhibitor (Id) (17). In contrast to the wealth of information regarding the mechanisms that activate genes participating in the myotube phenotype, relatively little is known regarding regulatory sequences or factors involved in the control of the repression of the muscle embryo genes during muscle cell differentiation. Thus, it has been described that an activating transcription factor site is required for the expression of the Id2A gene in muscle cells, and that the binding of nuclear factors to the activating transcription factor site is decreased during myogenic differentiation (18).

Glucose transporter expression is developmentally regulated in skeletal muscle (19, 20). Thus, during fetal and early postnatal life, GLUT1 is highly expressed in heart and skeletal muscles. Postnatal life is characterized by GLUT1 repression in muscle, which is concomitant with the induction of GLUT4 expression (19). Similarly, it has been reported that myogenesis leads to induction of GLUT4 expression and repression of GLUT1 expression (21, 22). Based on the fact that congenital hypothyroidism partially blocks GLUT1 repression associated with neonatal life (23) and that denervation up-regulates GLUT1 in skeletal muscle (20, 24–26), it is likely that thyroid hormones and muscle innervation play a role in the regulation of muscle GLUT1 expression *in vivo*. However, the detailed mechanisms that contribute to GLUT1 repression during peri-

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[1] The abbreviations used are: bHLH, basic helix-loop-helix; CAT, chloramphenicol acetyltransferase; EMSA, electrophoretic mobility shift assay; Id, differentiation inhibitor; DMEM, Dulbecco's modified Eagle's medium; CMV, cytomegalovirus; PBS, phosphate-buffered saline; bp, base pair(s).

natal development and myogenesis are largely unknown. Here we have examined the mechanisms that repress GLUT1 expression during myogenesis.

EXPERIMENTAL PROCEDURES

Materials—^{125}I-labeled protein A and [α-^{32}P]-dCTP were purchased from ICN. 2-Deoxy-D-[^{3}H]glucose was obtained from DuPont NEN. Hybond N was from Amersham Corp., and random primed DNA labeling kit was from Boehringer Mannheim. Immobilon was obtained from Millipore Corp. γ-Globulin and most commonly used chemicals were from Sigma. Dulbecco's modified Eagle's medium (DMEM), fetal-bovine serum, glutamine, and antibiotics were obtained from Whittaker (Walkersville, MD). L6E9 rat skeletal muscle cell line was kindly provided by Dr. B. Nadal-Ginard (Harvard University). C3H10T1/2 mouse cells stably transfected with MyoD were obtained from Dr. V. Andrés (St. Elizabeth's Medical Center, Boston).

The plasmid containing the −2106/+134 region of the rat GLUT1 genomic sequence was obtained from Dr. M. Birnbaum (University of Pennsylvania). pCAT-basic vector was obtained from Promega (Madison, WI). pCMV-β-galactosidase vector was obtained from Dr. N. Brand (National Heart & Lung Institute, London). Plasmid CMV-Sp1 was a generous gift of Dr. R. Tjian (University of California, Berkeley).

Cell Culture and Preparation of Membrane Fractions—Rat skeletal muscle L6E9 myoblasts were grown in monolayer culture in DMEM supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) antibiotics (10,000 units/ml penicillin G and 10 mg/ml streptomycin), 2 mM glutamine, and 25 mM Hepes, pH 7.4. Confluent myoblasts were differentiated by lowering fetal bovine serum to a final concentration of 2% (v/v). Mouse C3H10T1/2 fibroblasts stably transfected with MyoD were grown as L6E9 cells in the presence of 0.5 mg/ml geneticin and differentiated in DMEM containing 5% (v/v) horse serum with antibiotics and geneticin.

Cells were washed 2 times with PBS, scraped, and homogenized in 2 ml of ice-cold buffer containing 25 mM Hepes, 250 mM sucrose, 4 mM EDTA, 1 trypsin inhibitor unit/ml of aprotinin, 25 mM benzamidine, 0.2 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, and 1 μM pepstatin, pH 7.4, using a Dounce A homogenizer. Homogenates were processed as previously reported (27). Proteins were measured by the method of Bradford (28) using γ-globulin as a standard.

Glucose Uptake—Before transport experiments, cells were incubated for 2 h in DMEM containing 0.2% bovine serum albumin. After this time, cells were washed, and transport solution (20 mM Hepes, 150 mM NaCl, 5 mM KCl, 5 mM $MgSO_4$, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 2 mM pyruvate, pH 7.4) was added, together with 100 μM 2-deoxy-D-[^{3}H]glucose (96 mCi/mmol). After 20 min, transport was stopped by addition of 2 volumes of ice-cold 50 mM glucose in PBS. Cells were washed 3 times in the same solution and disrupted with 0.1 M NaOH, 0.1% SDS. Radioactivity was determined by scintillation counting. Protein was determined by the Bradford method (28). Each condition was run in duplicate, and the nonspecific uptake (t = 0) was determined by incubation of the 2-deoxy-D-[^{3}H]glucose in stop solution (50 mM glucose in PBS) instead of transport solution. In all cases, the value at t = 0 represented 4% of the basal transport activity at t = 20 min.

Electrophoresis and Immunoblotting—SDS-polyacrylamide gel electrophoresis was performed in accordance with the method of Laemmli (29). Proteins were transferred to Immobilon as reported (30). Transfer was confirmed by Coomassie Blue staining of the gel after the electroblot. Rabbit Bb antiserum raised against the purified human erythrocyte glucose transporter (a gift of Dr. C. Carter-Su, University of Michigan) was used at a 1:400 dilution and was incubated with transferred protein overnight at room temperature in 1% nonfat dry milk, 0.02% sodium azide in PBS to detect GLUT1. An anti-Sp1 affinity-purified rabbit polyclonal antibody (PEP-2, Santa Cruz Biotechnology) was used at a 5 μg/ml dilution in 1% nonfat dry milk, 0.02% sodium azide in PBS and incubated overnight at 4 °C. Detection of the immune complexes with the rabbit polyclonal antibodies was accomplished using ^{125}I-protein A for 4 h at room temperature or using the ECL Western blot detection system (Amersham Corp.). Immunoblots were performed under conditions in which autoradiographic detection was in the linear response range.

RNA Isolation and Northern Blot Analysis—Total RNA was extracted using the acid guanidinium thiocyanate/phenol/chloroform method as described by Chomczynski and Sacchi (31). All samples had a A_{260}/A_{280} ratio above 1.8. After quantification, total RNA (30 μg) was denatured at 65 °C in the presence of formamide, formaldehyde, and ethidium bromide to allow the visualization of RNA. RNA was separated on a 1.2% agarose-formaldehyde gel and blotted on Hybond N filters. The RNA in gels and in filters was visualized with ethidium bromide by UV transillumination to ensure the integrity of RNA, to check the loading of equivalent amounts of total RNA, and to confirm proper transfer. Northern blot was performed as reported (27). The rat cDNA probe for GLUT1 was a 2,521 fragment obtained from Dr. M. Birnbaum (University of Pennsylvania) and was labeled with [^{32}P]dCTP by random oligonucleotide priming.

GLUT1 CAT Reporter Constructs—Plasmid 2,106/+134-CAT was constructed by inserting a 2,240-bp *Eco*RI-*Xho*I fragment containing the rat GLUT1 promoter region from positions −2,106 to +134 (relative to the transcription start site) into the *Xba*I site of pCAT-basic vector (Promega). pCAT-basic was digested with *Xba*I, filling in of the ends with dNTPs in the presence of the Klenow fragment, and treated with alkaline phosphatase. The GLUT1 promoter DNA fragment was filled in the presence of dNTPs and Klenow fragment and ligated to the pCAT-basic vector. 5′ deletions were generated by cleaving with *Hin*dIII at −1672 (−1672/+134-CAT), with *Hin*dIII and *Bst*EII (−1203/+134-CAT), with *Hin*dIII and *Ban*II (−812/+134-CAT), with *Hin*dIII and *Sma*I (−201/+134-CAT), and with *Hin*dIII and *Aoc*I (−99/−33-CAT), adding Klenow and T4 DNA ligase. The −33/+134-CAT construct was generated by obtaining a 28-bp *Hin*dIII-*Bss*HII DNA containing the fragment −38/−15 from a 106-bp *Ava*II fragment (position −38/+68) of the GLUT1 promoter subcloned in Bluescript. The 28-bp fragment was subcloned into the *Bss*HII site of the *Bss*HII-G1CAT construct (−15/+134). The 3′ deletion constructs were generated by cleaving with *Xba*I and *Bfr*I (−2106/+46-CAT) and with *Xba*I and *Bss*HII (+2106/−15-CAT).

Transient Transfection—250,000 L6E9 cells were grown in 10-mm diameter plates for 2 days in DMEM with 10% fetal bovine serum. Monolayers were washed, and DNA transfection was performed by using the $CaPO_4$ coprecipitation procedure (32). One ml of calcium phosphate DNA precipitate containing 10 μg of various deletion promoter-chloramphenicol acetyltransferase constructs, 5 μg of pCMV-β-galactosidase control vector, and 20 μg of Bluescript DNA (pSK$^-$, Stratagene), was added dropwise to the plate, and medium was added 15 min later. After 16 h, the cells were washed and incubated with 1 ml of 15% glycerol in Hepes-buffered saline for 3 min, washed with DMEM, and incubated with fresh complete medium for 72 h. For the myotubes, the medium was changed to differentiation medium (DMEM supplemented with 2% fetal bovine serum) after the 15 min incubation with the DNA precipitate.

Preparation of Cell Extracts and Measurement of CAT Activity—The cells were washed 2 times with PBS and were harvested by scraping in 1 ml of STE (10 mM Tris-HCl, pH 7.5, containing 100 mM NaCl, and 1 mM EDTA). The cells were collected by centrifugation in a microcentrifuge for 10 min, and the pellet was resuspended in 200 μl of 0.25 M Tris, pH 7.5. The cells were lysed by 3 cycles of freezing and thawing at 37 °C. After centrifugation in a microcentrifuge for 5 min at 4 °C, the supernatant was stored at −20 °C.

The CAT activity of 75 μl of cytoplasmic extract was measured by incubating 0.1 μCi of ^{14}C-chloramphenicol, 1.3 mM acetyl-CoA, 200 mM Tris-HCl, pH 7.5, and the soluble extract for 3.5 h at 37 °C. At the end of the incubation, extraction into ethyl acetate and thin layer chromatography (33) were performed. The CAT activity was quantitated using an InstantImager (Packard). β-Galactosidase activity was measured as described (34).

Electrophoretic Mobility Shift Assay (EMSA)—Preparation of the nuclear protein extracts was performed as described by Ausubel *et al.* (35). The DNA probe (fragment −102/−37) was obtained by digesting the −201/+134-CAT construct with *Ava*II, purifying the 66-bp fragment, and ^{32}P-end-labeled using the Klenow fragment of the DNA polymerase. The gel mobility shift assays were performed in a 12-μl reaction volume, containing 2 μg of double-stranded poly(dI-dC), 15,000 cpm of labeled DNA probe, 5–10 μg of protein of nuclear extracts, 10 mM Hepes, pH 7.9, 10% glycerol, 50 mM KCl, 0.1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, and 0.25 mM dithiothreitol. The mixture (without the labeled DNA) was incubated for 15 min at room temperature. After the addition of the labeled DNA, the reaction mixture was incubated for another 10 min at 4 °C, immediately loaded on a 7% polyacrylamide gel (30:0, 8 acrylamide-bis-acrylamide), and run in 0.5 × TBE buffer (45 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8) for 3–4 h at 8 V/cm. The gels were dried and autoradiographed.

For competition assays, varying concentrations of unlabeled probe (fragment −102/−37) or the following oligonucleotides were used in the reaction mixture prior to addition of extract: oligonucleotide I (−100/−82), 5′-CCTCAGGCCCCGCCCCCCG-3′; oligonucleotide I mutated, 5′-CCTCAGGCCCCGTACCCCG-3′; oligonucleotide −55/−42, 5′-GCGCGGGCCAATGG-3′; and oligonucleotides containing the consen-

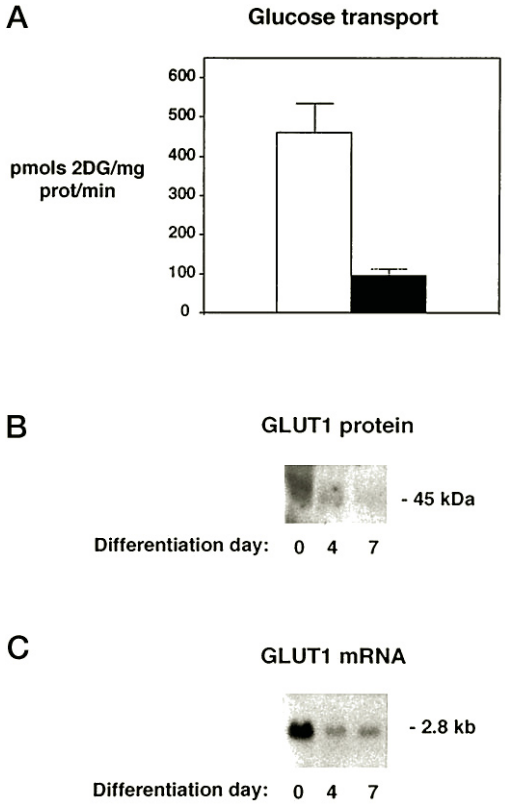

FIG. 1. **Effect of muscle cell differentiation on glucose transport and GLUT1 expression.** *A*, after serum deprivation, L6E9 myoblasts and myotubes after 7 days of differentiation were incubated in the presence of 0.1 mM 2-deoxy-[^{3}H]glucose. The cellular hexose uptake at $t = 20$ min was measured as described under "Experimental Procedures." Differences between myoblasts (*open bars*) and myotubes (*black bars*) were statistically significant at $p < 0.05$. *B*, membrane proteins were purified from L6E9 myoblasts or myotubes after 4 or 7 days of differentiation. 20 μg of membrane proteins were laid on gels. After blotting, GLUT1 protein was detected by incubation with a polyclonal antibody against the COOH terminus of the carrier. A representative autoradiogram is shown. *C*, total RNA was purified from L6E9 myoblasts or myotubes after 4 or 7 days of differentiation. 20 μg of total RNA were laid on gels. The integrity and the relative amounts of RNA in each sample were checked by ethidium bromide staining on the same gel. After blotting, GLUT1 mRNA was detected after hybridization with a 2,521-base pair *Eco*RI fragment as a cDNA probe and as described under "Experimental Procedures." A representative autoradiogram is shown.

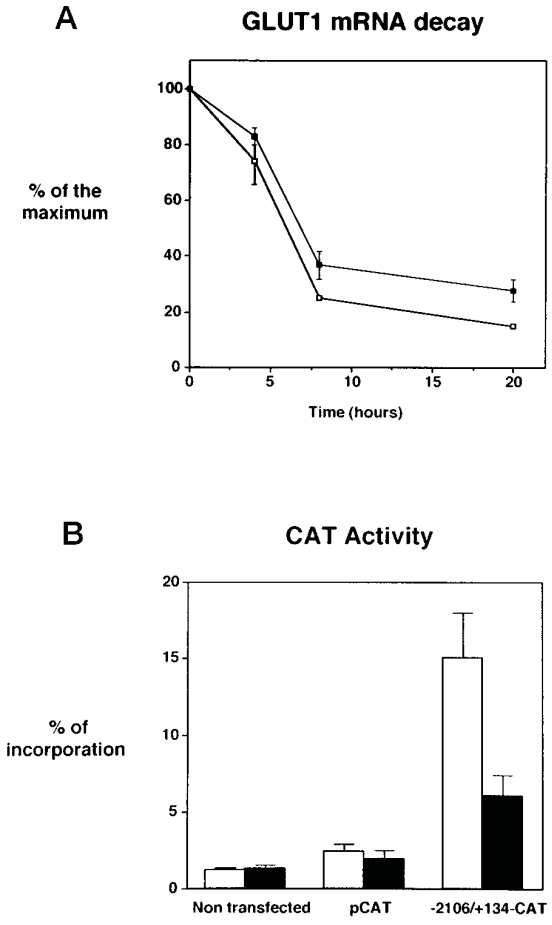

FIG. 2. **Myogenesis reduces the transcriptional activity of the GLUT1 gene promoter in the absence of alterations in the half-life of GLUT1 mRNA.** *A*, L6E9 myoblasts (*open symbols*) and myotubes (*closed symbols*) were incubated for different time periods in the presence of 5 μg/ml actinomycin D. After different times, total RNA was obtained, and GLUT1 mRNA levels were determined by Northern blot (see details in legend to Fig. 1). Results were quantified and expressed as a percentage of values at time 0. *B*, L6E9 myoblasts (*open bars*) or myotubes (*black bars*) were transiently co-transfected by calcium phosphate precipitation with the −2106/+134-CAT construct (10 μg) or with the promoterless pCAT-basic vector (10 μg) as indicated. 72 h after transfection, cells were harvested and homogenized, and CAT activity was determined. Data of CAT activity are expressed as a percentage of acetylated chloramphenicol (mean ± S.E.) from five to six experiments performed in duplicate using at least two preparations of each DNA construct.

sus site for Sp1 or AP2 (Promega).

Supershift experiments were performed by incubating nuclear extracts or commercial human recombinant Sp1 protein (Promega), poly(dI-dC), and end-labeled probe as detailed above and then incubated for 30 min at 4 °C in the presence of 1 μg of Sp1 antibody (PEP-2, Santa Cruz Biotechnology) or with an irrelevant antibody. The samples were loaded on a 4% polyacrylamide gel, dried, and autoradiographed.

RESULTS

Myogenesis Diminishes Glucose Transport and Represses GLUT1 Expression and the Transcriptional Activity of the GLUT1 Promoter—Differentiation of L6E9 myoblasts into myotubes was associated with a diminished rate of basal glucose transport (near 80% decrease) (Fig. 1). Under these conditions, the total cellular content of GLUT1 glucose transporter protein was also markedly reduced (levels in myotubes accounted for 22 ± 6% of values found in myoblasts) (Fig. 1). This is in keeping with previous observations performed in L6 muscle cells (21, 22). A reduction in GLUT1 mRNA levels was also detected in L6E9 myotubes compared with myoblast cells (levels in myotubes accounted for 33 ± 3% of values found in myoblasts) (Fig. 1). The reduction in GLUT1 protein and mRNA occurred under conditions in which no changes in the cellular content of β1-integrin protein or rRNA were detected (data not shown).

To determine the basis for the repression of GLUT1 expression, myoblasts or myotubes were incubated in the presence of actinomycin D (5 μg/ml) for different time periods, and the levels of GLUT1 mRNA were assessed (Fig. 2*A*). Results indicate that the half-life of GLUT1 mRNA species was near 5 h, and no differences between myoblast and myotube cells were detected (Fig. 2*A*). Therefore, the reduction in GLUT1 mRNA levels during muscle cell differentiation is not due to alterations in the stability of GLUT1 mRNA.

Next, myoblast or myotube cells were transiently transfected with a fragment of the GLUT1 gene promoter (−2106/+134) fused to the reporter gene CAT. Cells transfected with the reporter gene showed very low levels of CAT activity, similar to those shown by non-transfected cells (Fig. 2*B*). Transfection with the construct −2106/+134-CAT in L6E9 myoblasts caused a 11.5-fold increase in CAT activity (Fig. 2*B*). Furthermore,

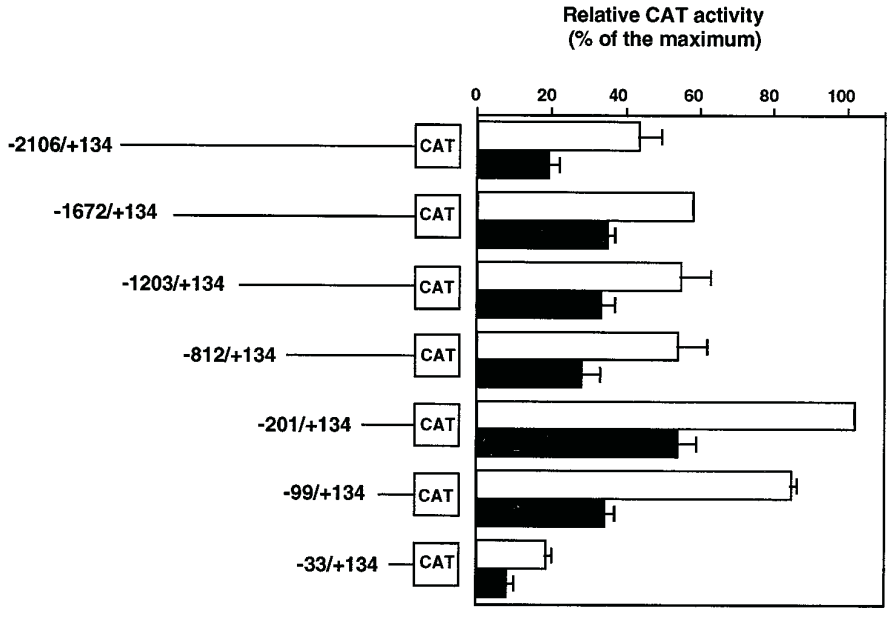

FIG. 3. **Effect of myogenesis on the expression of GLUT1 5′ deletion constructs in L6E9 cells.** A series of 5′ deletions of the upstream region of the GLUT1 gene from −2106 to −33 bp relative to the transcription start site were generated as indicated. L6E9 myoblasts (*open bars*) or myotubes (*black bars*) were transiently transfected with the various constructs. 96 h after transfection, cells were harvested. The data are expressed as relative CAT activity/β-galactosidase activity ± S.E. from four to six experiments with the −201/+134-CAT construct being set to a value of 100 (in the myoblast group). These results were obtained from transfection experiments performed in duplicate using at least two preparations of DNA. Differences between myoblasts and myotubes were significant at $p < 0.05$.

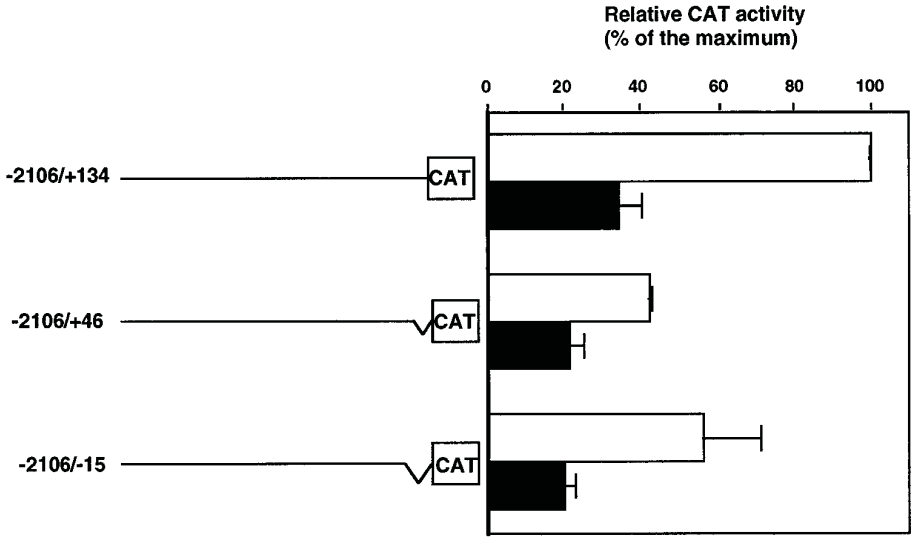

FIG. 4. **Effect of myogenesis on the expression of GLUT1 3′ deletion constructs in L6E9 cells.** A series of 3′ deletions of the upstream region of the GLUT1 gene from +134 to −15 bp relative to the transcription start site were generated as indicated. L6E9 myoblasts (*open bars*) or myotubes (*black bars*) were transiently transfected with the various constructs. 96 h after transfection, cells were harvested. The data are expressed as relative CAT activity/β-galactosidase activity ± S.E. from four to six experiments with the −2104/+134-CAT construct being set to a value of 100 (in the myoblast group). These results were obtained from transfection experiments performed in duplicate using at least two preparations of DNA. Differences between myoblasts and myotubes were significant at $p < 0.05$.

CAT activity detected in myotubes was substantially reduced (60% decrease) compared with values in myoblasts (Fig. 2*B*). These results indicate that the fragment −2106/+134 of the GLUT1 gene contains information that is relevant to transcriptional activity in the myoblast and that allows repression in response to myogenesis.

Cis-elements Responsible for the Transcriptional Activity of the GLUT1 Promoter and Effects of Myogenesis—To determine the cis-elements involved in the transcriptional activity of the GLUT1 promoter, 5′ deletion constructs of the GLUT1 promoter fused to the CAT reporter gene were generated and transiently transfected in myoblast and myotube L6E9 cells (Fig. 3).

Deletion from −2106 to −812 of the GLUT1 gene caused no significant alterations in CAT activity (Fig. 3), and deletion from −812 to −201 caused nearly 60% stimulation of CAT activity, suggesting a repressor element (Fig. 3). The transcriptional activity of the −201 construct was maximal and only a slight decrease was noted after deletion from −201 to −99. However, deletion of a further 66 base pairs (from −99 to −33) led to a marked reduction (80%) in the transcriptional activity.

The repression of transcriptional activity due to myogenesis was maximal in the −99/+134 construct although some differences were still found in the −33/+134 construct (Fig. 3). The −33/+134 construct contains the TATA box, located at −32/−27 relative to the transcription start site (36).

To rule out the participation of the 3′-end of the fragment of the GLUT1 promoter which lies 3′ of the transcription start site, additional constructs were generated by 3′ deletion (Fig. 4). Deletion of 88 base pairs lying between +134 and +46 caused a 60% reduction in transcriptional activity (Fig. 4), suggesting elements important for the transcriptional activity. Under these conditions, myogenesis reduced the transcriptional activity of all constructs studied (Fig. 4).

These data indicate that the fragment −99/−33 is responsible for the transcriptional activity of the GLUT1 promoter. Furthermore, this fragment, together with the fragment containing the TATA box, seems to confer sensitivity to muscle cell differentation.

Nuclear Proteins Bind to the −99/−33 Fragment of the GLUT1 Promoter in a Differentiation-dependent Manner—The −99/−33 fragment of the GLUT1 gene contains one consensus Sp1 site, two AP-2-like sites, and one CAAT box (Fig. 6). To determine whether nuclear proteins bind to the fragment −99/−33 of the GLUT1 promoter, a DNA fragment encompassing the sequence −102/−37 was radioactively labeled, and EMSA

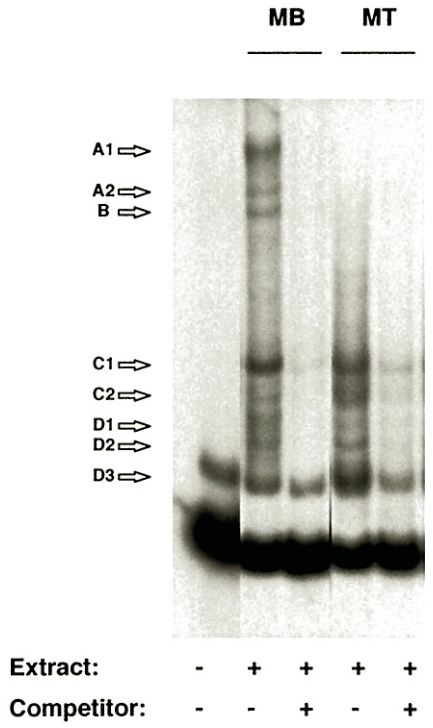

FIG. 5. **Specific binding of nuclear factors from L6E9 myoblasts and myotubes to the −102/−37-bp GLUT1 DNA fragment.** Labeled *Ava*II-*Ava*II fragment (−102/−37) was incubated with 5 μg of nuclear extracts either from L6E9 myoblasts (*MB*) or myotubes (*MT*) after 7 days of differentiation and analyzed on a 7% polyacrylamide gel. The specificity of the complex formation was examined by addition of the 100 M excess of the unlabeled −102/−37 fragment used as a competitor. A representative autoradiogram is shown. *Arrows* indicate specific complexes.

assays were performed in the presence of nuclear extracts obtained from L6E9 myoblasts or myotubes (Fig. 5). A number of specific bands was detected in nuclear extracts (Fig. 5). Some of them, named A1, A2 and B, were restricted to myoblasts, and others (complexes D1 and D2) were more abundant in myoblasts than in myotubes. In contrast, complexes C2 and D3 were more abundant in myotubes than in myoblasts (Fig. 5). C1 was the only complex to show a similar abundance in extracts from myoblasts and from myotubes (Fig. 5).

To map the DNA elements that allowed the binding of the different complexes, EMSA assays were performed in the presence of an excess of unlabeled oligonucleotides (Fig. 6). EMSA assays performed in the presence of unlabeled oligonucleotide −100/−82 (oligonucleotide I), which contains the canonical Sp1 site, and an overlapping AP-2-like site displaced, in a concentration-dependent manner, complexes A1 and A2 found in myoblast extracts (Fig. 7*A*). No bands were displaced in the presence of the canonical sequence corresponding to the AP-2 site or with oligonucleotide −55/−42, which contains the CAAT box (data not shown).

Sp1 Binds and Transactivates the GLUT1 Promoter and Is Repressed during Myogenesis—Next, we focused on the nature of the complexes A1 and A2, characteristic of myoblast extracts. Based on the presence of a canonical Sp1 site in position −92/−87, we searched whether binding of factors to this site might be responsible for complexes A1 and A2. EMSA assays were performed in the presence of an excess of unlabeled oligonucleotides containing a canonical Sp1 site (Fig. 7*A*). Under these conditions, complexes A1 and A2 were displaced very efficiently in the presence of 100-fold molar excess of the oligonucleotide Sp1 (Fig. 7*A*). Furthermore, the incubation in the presence of an excess of oligonucleotide −100/−82 in which the

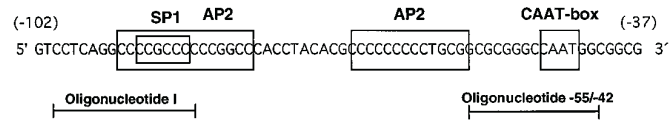

FIG. 6. **Scheme of the fragment −102/−37 of the GLUT1 promoter and oligonucleotides used as competitors.** *Boxes* indicate the consensus sequences for Sp1, AP-2, and CAAT boxes.

Sp1 site was mutated (Fig. 7*A*, oligonucleotide *Imut*) failed to displace complexes A1 and A2 (Fig. 7*A*). Sp1 protein belongs to a family of zinc-finger transcription factors (37, 38), and the formation of complexes in band-shift assay is sensitive to the presence of Zn^{2+} or EDTA in the medium (39). Based on this, gel-retardation analyses were performed in the absence or presence of Zn^{2+} or EDTA. The addition of Zn^{2+} to the medium increased the formation of complexes A1 and A2 in a concentration-dependent manner (Fig. 7*B*). In contrast, addition of EDTA caused a concentration-dependent inhibition of complexes A1 and A2 (Fig. 7*B*).

To confirm the binding of Sp1 protein, super shift assays were also performed. To this end, EMSA assays were carried out in the presence of an anti-Sp1 antibody. Due to the utilization of a different percentage of polyacrylamide and due to a longer electrophoresis run, the complex A1 previously seen as a single broad band was resolved as two distinct complexes (named A1 and A1′) (Fig. 8). In these studies, recombinant Sp1 protein was also incubated with labeled fragment −102/−37 (Fig. 8). Results indicate that recombinant Sp1 forms a complex with fragment −102/−37, which shows a retardation similar to complex A1, and the formation of this complex was prevented in the presence of an excess of oligonucleotide I (−100/−82) (Fig. 8). In addition, anti-Sp1 antibody eliminated part of complex A1 and generated a complex showing a greater retardation. A similar super-retarded band was observed when recombinant Sp1 was incubated with anti-Sp1 antibody (Fig. 8).

These results indicate that endogenous Sp1 present in extracts from L6E9 myoblasts binds to the GLUT1 promoter. To determine whether Sp1 modulates the transcriptional activity of the GLUT1 promoter, L6E9 myoblasts or myotubes were co-transfected with the construct −2106/+134-CAT and the cDNA coding for Sp1. Sp1 caused a large transactivation of the GLUT1 promoter activity (4.9-fold increase) in myoblasts (Fig. 9). In addition, Sp1 activated the GLUT1 promoter activity in myotubes; however, the transcriptional activity detected in myoblasts was much greater than in myotubes (Fig. 9).

We have found that Sp1 protein binds to the GLUT1 gene promoter in myoblasts but not in myotubes. To determine the nature of the mechanisms involved, we determined the level of Sp1 protein in nuclear extracts obtained from L6E9 myoblasts and myotubes (Fig. 10). Sp1 protein was observed in Western blot as two bands with an apparent molecular masses of 105 and 95 kDa, which is in keeping with previous observations (40, 41). The content of Sp1 protein in myoblasts was much greater than in myotubes (Fig. 10) (levels of Sp1 protein in myotubes accounted for 27 ± 10% of values found in myoblasts). This effect was specific since the abundance of the transcription factor STAT-1 was similar in preparations from myoblasts or myotubes (data not shown).

Over-expression of MyoD Represses Sp1 and Inhibits the Transcriptional Activity of the GLUT1 Gene Promoter—The best characterized factors that regulate the terminal differentiation of the muscle cells are the members of the MyoD family. To determine whether MyoD plays a role in the regulation of GLUT1 gene expression during myogenesis, we studied the effect of the stable over-expression of MyoD in C3H10T1/2 cells. The stable expression of MyoD in these cells caused a marked reduction in the transcriptional activity of the GLUT1 pro-

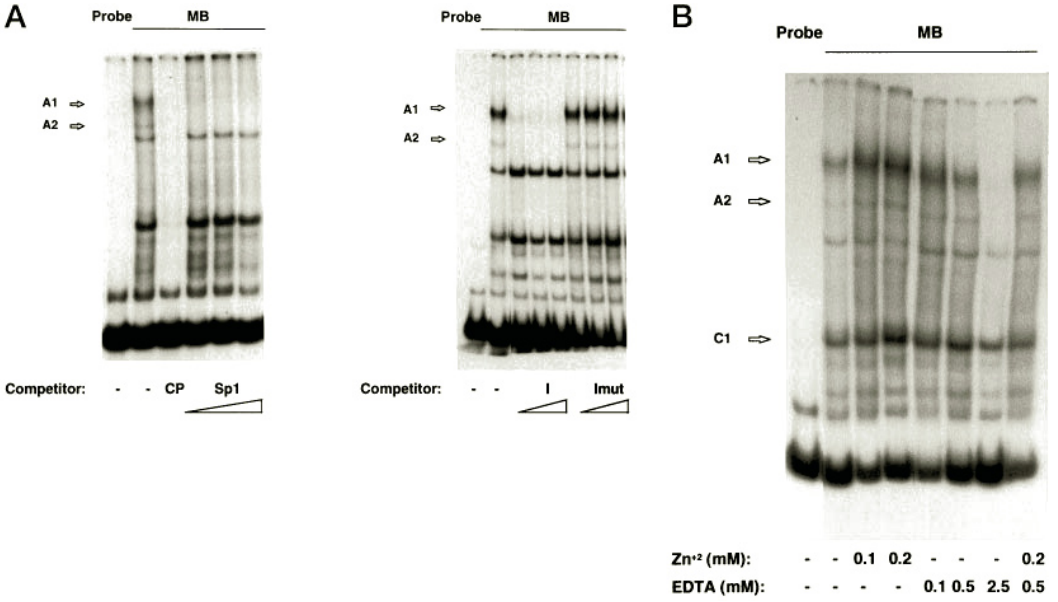

FIG. 7. **Characterization of factors binding to the Sp1 element of the GLUT1 gene.** Labeled *Ava*II-*Ava*II fragment (−102/−37) was incubated with 5 μg of nuclear extracts from L6E9 myoblasts (*MB*) and analyzed on a 7% polyacrylamide gel. *A*, the specificity of the complexes A1 and A2 formation was examined by addition of a molar excess of unlabeled competitors containing a canonical Sp1 site (*Sp1*, molar excesses of 100, 200, and 400), oligonucleotide *I* (−100/−82), oligonucleotide −100/−82 containing a mutated Sp1 site (*Imut*, molar excesses of 500, 1000, and 2500), or the unlabeled −102/−37 fragment (*CP*, 100-fold M excess). *B*, binding of nuclear factors to the labeled fragment was performed after the addition of different concentrations of Zn^{2+} or EDTA. Representative autoradiograms are shown.

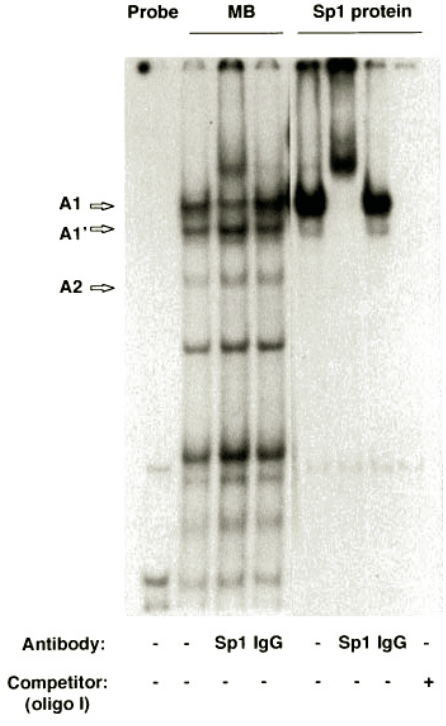

FIG. 8. **Sp1 binds to the −102/−37-bp GLUT1 DNA fragment.** Labeled *Ava*II-*Ava*II fragment (−102/−37) was incubated with 5 μg of nuclear extracts from L6E9 myoblasts in the presence of an Sp1 antibody (*Sp1*) or an irrelevant antibody (*IgG*) and analyzed on a 4% polyacrylamide gel. The recombinant Sp1 protein (0.5 μg) was also incubated with the labeled *Ava*II-*Ava*II fragment (−102/−37) in the presence of an Sp1 antibody, an irrelevant antibody, or oligonucleotide I (−100/−85) at a 1,000-fold M excess. Due to the utilization of a different percentage of polyacrylamide and due to a longer electrophoresis run, the complex A1 previously seen as a single broad band was resolved as two distinct complexes (named *A1* and *A1′*). A representative autoradiogram is shown.

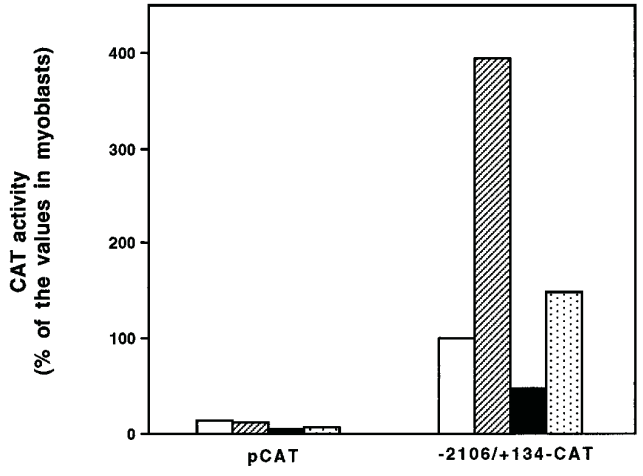

FIG. 9. **Sp1 transactivates the GLUT1 promoter.** L6E9 myoblasts or myotubes were transiently co-transfected by calcium phosphate precipitation with the −2106/+134-CAT construct (10 μg) or with the promoterless pCAT-basic vector (10 μg) in the absence or presence of an expression vector coding for Sp1 (CMV-Sp1) (10 μg). 96 h after transfection, cells were harvested and homogenized, and CAT activity was determined. Data are expressed as CAT activity/μg of protein (mean ± S.E.) from three experiments performed in duplicate using at least two preparations of each DNA construct. *Open bars*, myoblast; *hatched bars*, myoblasts + Sp1; *black bars*, myotubes; *gray bars*, myotubes + Sp1.

moter as assessed by transient transfection of the −2106/+134-CAT construct (CAT activity levels in C3H10T1/2 wild type and C3H10T1/2-MyoD were 43 ± 3 and 15 ± 3, respectively, expressed as arbitrary units and corrected per β-galactosidase activity).

EMSA assays revealed the presence of highly retarded complexes in nuclear extracts from C3H10T1/2 cells that showed similar mobility to complexes A1 and A2 from L6E9 myoblasts. Furthermore, these complexes were competed with an oligonucleotide containing the consensus sequence for Sp1 binding (Fig. 11) and with oligonucleotide I (−100/−82) (data not shown). Stable over-expression of MyoD caused the disappearance of the complex binding to the Sp1 element (Fig. 11) and the formation of low-retarded complexes (Fig. 11). Furthermore, Western blot assays of Sp1 protein from nuclear extracts

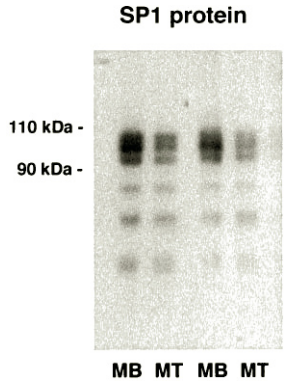

FIG. 10. **Expression of Sp1 protein in nuclear extracts from myoblasts and myotubes.** Nuclear factors were obtained from L6E9 myoblasts (*MB*) or myotubes (*MT*). 20 μg of nuclear extracts were laid on gels. After blotting, Sp1 protein was detected by incubation with a specific polyclonal antibody. A representative autoradiogram is shown.

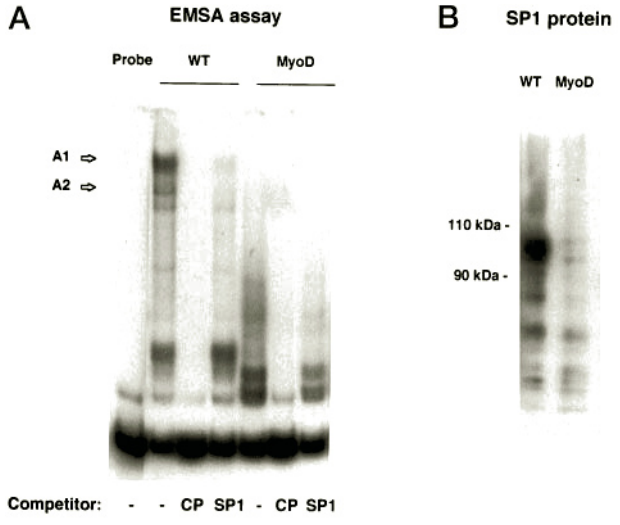

FIG. 11. **Over-expression of MyoD represses Sp1 expression and formation of complexes with the −102/−37 bp of the GLUT1 gene.** *A*, labeled *Ava*II-*Ava*II fragment (−102/−37) was incubated with 5 μg of nuclear extracts either from wild-type C3H10T1/2 cells (*WT*) or C3H10T1/2 cells stably transfected with MyoD (*MyoD*) and analyzed on a 7% polyacrylamide gel. The specificity of the complex formation was examined by addition of a 100 M excess of the unlabeled −102/−37 fragment (*CP*) or a 200 M excess of unlabeled oligonucleotide containing a canonical Sp1 site used (*SP1*) as competitors. *B*, nuclear factors were obtained from wild-type C3H10T1/2 cells (*WT*) or C3H10T1/2 cells stably transfected with MyoD (*MyoD*). 20 μg of nuclear extracts were laid on gels. After blotting, Sp1 protein was detected by incubation with a specific polyclonal antibody. Representative autoradiograms are shown.

obtained from wild-type C3H10T1/2 and C3H10T1/2-MyoD cells indicated a dramatic down-regulation of Sp1 protein after MyoD over-expression (Fig. 11). These results suggest a role of MyoD in the down-regulation of Sp1 associated with myogenesis.

DISCUSSION

In this study, we have demonstrated that GLUT1 is repressed in muscle cells during differentiation as a consequence of alterations in transcriptional activity of the GLUT1 gene, which seems to involve the fragment −99/−33 and the fragment 5′ proximal to the transcription start site containing the TATA box. Furthermore, the levels of GLUT1 mRNA and the transcriptional activity of the GLUT1 promoter were similarly reduced in response to myogenesis; this suggests that the alterations in the transcriptional activity of the GLUT1 promoter are sufficient to account for the GLUT1 repression. In the

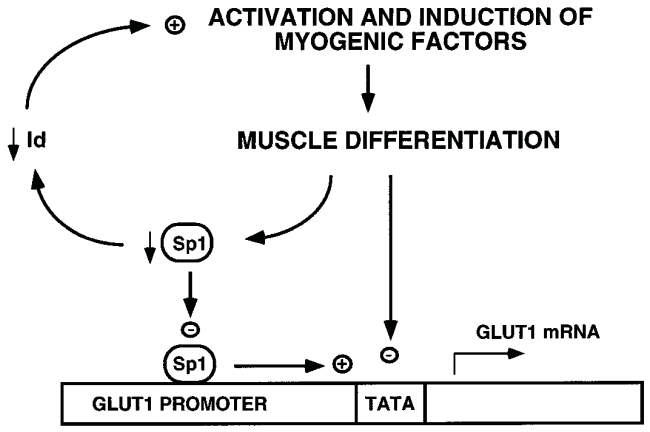

FIG. 12. **Hypothetical scheme of the mechanisms responsible for the repression of GLUT1 gene expression during myogenesis.**

region −99/−33 of the GLUT1 gene, we have identified the binding of Sp1 to the GLUT1 gene promoter; this seems to be important from a functional viewpoint since Sp1 transactivates, in transient transfection assays, the transcriptional activity of GLUT1 promoter. In contrast to the current view stating that Sp1 is a ubiquitous factor, we have found that myogenesis leads to a drastic reduction in the formation of a DNA-protein complex involving Sp1, which is due to a marked down-regulation of Sp1 expression. Our results also indicate that MyoD over-expression down-regulates Sp1 expression in cells, which is in parallel to a reduction in the transcriptional activity of the GLUT1 gene. Based on this, we propose the model depicted in Fig. 12. According to this, the transcriptional activity of the GLUT1 gene is high in proliferating myoblasts, in part due to a high expression of the activator Sp1. Muscle cell differentiation is associated with activation of MyoD transcription factors, which act as master regulators leading to activation of many muscle-specific genes. In our model, Myo D activation leads to the repression of Sp1 expression in muscle cells. In turn, Sp1 down-regulation causes inactivation of the transcriptional activity of the GLUT1 gene and, therefore, leads to GLUT1 down-regulation and to a diminished rate of glucose transport. A prior report indicates that an Sp1-site is required for the expression of Id (an inhibitory factor of MyoD function) in muscle cells (18). Based on this, we additionally postulate that Sp1 down-regulation contributes to the repression of Id found during myogenesis and which is known to participate in the activation of myogenic transcription factors (17, 42).

Prior studies on the regulation of the GLUT1 gene have exclusively focused on the functional role of two enhancer elements found in the mouse GLUT1 gene. The first enhancer has been located 2.7 kilobases upstream of the transcription start site, whereas the second is in the second intron of the gene (43). These enhancers permit the activation of the transcription in response to growth factors, insulin, or hypoxia (43–45). Here, we have analyzed the properties of the proximal promoter of the rat GLUT1 gene. Our results indicate the presence of a repressor element located in the fragment −812/−201 that is active both in myoblasts and in myotubes. Our data also suggest that the fragment close to the TATA box or the TATA box itself participates in the repression of GLUT1 transcription during myogenesis. In this regard, it has been reported that factors such as TEF-1 or NC2 block the formation of TBP-TATA complexes and inhibit gene transcription (46, 47). Whether some of these factors play a role through the TATA box in the regulation of the GLUT1 gene during myogenesis remains unknown.

Furthermore, we have identified the fragment −99/−33 of the GLUT1 gene that is responsible for the transcriptional activity of the GLUT1 promoter. We have also identified the presence of different complexes found in myoblasts or myotube nuclear extracts and that bind to the −99/−33 region. Specifically, we have identified high-retardation complexes (named A1 and A2) that are restricted to myoblasts. Based on the selective competition of these bands to oligonucleotides containing the Sp1 binding site, the sensitivity of these complexes to Zn^{2+} and EDTA, the fact that they show a similar retardation to recombinant Sp1, and that there is a super-shift in the presence of an antibody against Sp1, we propose that Sp1 participates in the formation of these complexes.

In our study, we have found that Sp1 stimulates the transcriptional activity of the GLUT1 gene 5-fold in transient transfection assays. These data, together with the fact that there is a high expression level of Sp1 protein in nuclear extracts obtained from myoblasts and that Sp1 binds to the fragment −99/−33 selectively in myoblasts, strongly support the hypothesis that Sp1 regulates GLUT1 transcription in muscle cells. Interestingly, the binding of Sp1 protein to the GLUT1 promoter correlates with a high GLUT1 gene expression in a variety of experimental conditions.[2] The transient transfection of Sp1 into myotubes led to a stimulation of the GLUT1 rate of transcription that was markedly lower than that obtained in myoblasts. These results suggest a defect of Sp1 action in myotube cells. In this regard, it has been shown that Sp1 forms heteromeric complexes with several cellular proteins. The TATA-binding protein protein-associated protein TAF110 binds Sp1 and functions as a co-activator in Sp1-dependent transcription (37). Sp1 also interacts with the cellular protein YY1 (48, 49), with the RelA subunit of NF-κB (50), and with the bovine papilloma virus (51). Based on the fact that the cotransfection of a retinoblastoma expression vector is able to modulate the transactivation of responsive genes by Sp1, the function of Sp1 has been linked to that of the retinoblastoma protein (52, 53) through retinoblastoma control elements. Some mechanisms of inhibition of Sp1 action have also been reported. Thus, the cell-cycle-regulatory protein 107 can be found endogenously associated with Sp1 and, in cotransfection assays, p107 specifically represses Sp1-dependent transcription (41). Furthermore, G10BP protein or Sp3 competes with Sp1 for G-rich sequences and inhibits Sp1 action (54–56). Based on all these items of information, it might be postulated that myogenesis not only leads to Sp1 down-regulation but also causes alteration in the biological potency of Sp1, which might be explained either by alterations in the proteins that allow the formation of active complexes or due to the presence of Sp1-binding inhibitory proteins.

Sp1 is thought to be a ubiquitously expressed transcription factor that plays a primary role in the regulation of a large number of genes, including constitutive housekeeping genes and inducible genes (57). Recent studies indicate that Sp1 is a limiting factor in cultured cells and that overexpression of Sp1 increases expression from promoters containing GC-box elements (58). Additionally, there is evidence that Sp1 expression is regulated. Thus, Sp1 levels increase during SV40 infection of the CV1 cells (58). Furthermore, it has been reported that Sp1 expression varies greatly in different cell types and changes in Sp1 occur during development *in vivo* (59). Thus, high levels of Sp1 expression were found in spermatids, T cells, epithelial cells and hematopoietic cells (59). Interestingly, the expression of Sp1 mRNA in heart and skeletal muscles was higher in early neonatal mice than in adult animals (59). Along these lines, we have found in this study that myogenesis greatly alters the expression of Sp1 in muscle cells. Furthermore, our results support the view that MyoD causes down-regulation of Sp1 in stable transfection assays. In summary, we propose that activation of MyoD transcription factors causes down-regulation of Sp1 protein, and this might be one the mechanisms that lead to GLUT1 repression and accomplishment of the differentiation program in the muscle cell. The mechanisms underlying Sp1 down-regulation in response to MyoD transcription factors are currently under study in our laboratory.

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[2] F. Viñals, C. Fandos, T. Santalucía, J. Ferré, X. Testar, M. Palacín, and A. Zorzano, unpublished observations.

REFERENCES

1. Lassar, A., and Münsterberg, A. (1994) *Curr. Opin. Cell Biol.* **6,** 432–442
2. Olson, E. N., and Klein, W. H. (1994) *Genes & Dev.* **8,** 1–8
3. Edmondson, D. G., and Olson, E. N. (1994) *J. Biol. Chem.* **268,** 755–758
4. Davis, R. L., Weintraub, H., and Lassar, A. B. (1987) *Cell* **51,** 987–1000
5. Braun, T., Buschhausen-Denker, G., Bober, E., Tannich, E., and Arnold, H. H. (1989) *EMBO J.* **8,** 701–709
6. Edmondson, D. G., and Olson, E. N. (1989) *Genes & Dev.* **3,** 628–640
7. Wright, W. E., Sassoon, D. A., and Lin, V. K. (1989) *Cell* **56,** 607–617
8. Braun, T., Bober, E., Winter, B., Rosenthal, N., and Arnold, H. H. (1990) *EMBO J.* **9,** 821–831
9. Miner, J. H., and Wold, B. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 1089–1093
10. Rhodes, S. J., and Konieczny, S. F. (1989) *Genes & Dev.* **3,** 2050–2061
11. Mar, J. H., and Ordahl, C. P. (1990) *Mol. Cell. Biol.* **10,** 4271–4283
12. Gosset, L. A., Kelvin, D. J., Sternberg, E. A., and Olson, E. N. (1989) *Mol. Cell. Biol.* **9,** 5022–5033
13. Yu, Y.-T., Breitbart, R. E., Smoot, L. B., Lee, Y., Mahdavi, V., and Nadal-Ginard, B. (1992) *Genes & Dev.* **6,** 1783–1798
14. Trouche, D., Grigoriev, M., Lenormand, J. L., Robin, P., Leibovitch, S. A., Sassone-Corsi, P., and Harel-Bellan, A. (1993) *Nature* **363,** 79–82
15. Hayward, L. J., and Schwarts, R. J. (1986) *J. Cell Biol.* **102,** 1485–1493
16. Paterson, B. M., and Eldridge, J. D. (1984) *Science* **224,** 1436–1438
17. Benezra, R., Davis, R. L., Lockshon, D., Turner, D. L., and Weintraub, H. (1990) *Cell* **61,** 49–59
18. Kurabayashi, M., Dutta, S., and Kedes, L. (1994) *J. Biol. Chem.* **269,** 31162–31170
19. Santalucía, T., Camps, M., Castelló, A., Muñoz, P., Nuel, A., Testar, X., Palacín, M., and Zorzano, A. (1992) *Endocrinology* **130,** 837–846
20. Castelló, A., Cadefau, J., Cussó, R., Testar, X., Hesketh, J. E., Palacín, M., and Zorzano, A. (1993) *J. Biol. Chem.* **268,** 14998–15003
21. Mitsumoto, Y., Burdett, E., Grant, A., and Klip, A. (1991) *Biochem. Biophys. Res. Commun.* **175,** 652–659
22. Mitsumoto, Y., and Klip, A. (1992) *J. Biol. Chem.* **267,** 4957–4962
23. Castelló, A., Rodríguez-Manzaneque, J. C., Camps, M., Pérez-Castillo, A., Testar, X., Palacín, M., Santos, A., and Zorzano, A. (1994) *J. Biol. Chem.* **269,** 5905–5912
24. Block, N. E., Menick, D. R., Robinson, K. A., and Buse, M. G. (1991) *J. Clin. Invest.* **88,** 1546–1552
25. Coderre, L., Monfar, M. M., Chen, K. S., Heydrick, S. J., Kurowski, T. G., Ruderman, N. B., and Pilch, P. F. (1992) *Endocrinology* **131,** 1821–1825
26. Henriksen, E. J., Rodnick, K. J., Mondon, C. E., James, D. E., and Holloszy, J. O. (1991) *J. Appl. Physiol.* **70,** 2322–2327
27. Kaliman, P., Viñals, F., Testar, X., Palacín, M., and Zorzano, A. (1996) *J. Biol. Chem.* **271,** 19146–19151
28. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254
29. Laemmli, U. K. (1970) *Nature* **227,** 680–685
30. Camps, M., Castelló, A., Muñoz, P., Monfar, M., Testar, X., Palacín, M., and Zorzano, A. (1992) *Biochem. J.* **282,** 765–772
31. Chomczynsky, P., and Sacchi, N. (1987) *Anal. Biochem.* **162,** 156–159
32. Graham, F. L., and Van der Eb, A. J. (1973) *Virology* **52,** 456–457
33. Gorman, C. M., Moffat, L. F., and Howard, B. H. (1982) *Mol. Cell. Biol.* **2,** 1044–1051
34. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
35. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. M., Seidman, J. G., Smith, J. A., and Struhl, K. (1987) *Current Protocols in Molecular Biology*, Greene Publishing Associates & Wiley-Interscience, New York
36. Williams, S. A., and Birnbaum, M. J. (1988) *J. Biol. Chem.* **263,** 19513–19518
37. Hoey, T., Weinzierl, R. O., Gill, G., Chien, J. L., Dynlacht, B. D., and Tjian, R. (1993) *Cell* **72,** 247–260
38. Kadonaga, J. T., Jones, K. A., and Tjian, R. (1986) *Trends Biochem. Sci.* **11,** 20–23
39. Jensen, D. E., Rich, C. B., Terpstra, A. J. Farmer, S. R., and Foster, J. A. (1995) *J. Biol. Chem.* **270,** 6555–6563
40. Briggs, M. R., Kadonaga, J. T., Bell, S. P., and Tjian, R. (1986) *Science* **234,** 47–52
41. Datta, P. K., Raychaudhuri, P., and Bagchi, S. (1995) *Mol. Cell. Biol.* **15,** 5444–5452
42. Sun, X.-H., Copeland, N. G., Jenkins, N. A., and Baltimore, D. (1991) *Mol. Cell. Biol.* **11,** 5603–5611

43. Murakami, T., Nishiyama, T., Shirotani, T., Shinohara, Y., Kan, M., Ishii, K., Kanai, F., Nakazuru, S., and Ebina, Y. (1992) *J. Biol. Chem.* **267,** 9300–9306
44. Todaka, M., Nishiyama, T., Murakami, T., Saito, S., Ito, K., Kanai, F., Kan, M., Ishii, K., Hayashi, H., Shichiri, M., and Ebina, Y. (1994) *J. Biol. Chem.* **269,** 29265–29270
45. Ebert, B. L., Firth, J. D., and Ratcliffe, P. J. (1995) *J. Biol. Chem.* **270,** 29083–29089
46. Goppelt, A., Stelzer, G., Lottspeich, F., and Meisterernst, M. (1996) *EMBO J.* **15,** 3105–3116
47. Jiang, S.-W., and Eberhardt, N. L. (1996) *J. Biol. Chem.* **271,** 9510–9518
48. Lee, J.-S., Galvin, K. M., and Shi, Y. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 6145–6149
49. Seto, E., Lewis, B., and Shenk, T. (1993) *Nature* **365,** 462–464
50. Sif, S., Capobianco, A. J., and Gilmore, T. D. (1993) *Oncogene* **7,** 2501–2509
51. Li, R., Knight, J. D., Jackson, S. P., Tjian, R., and Botchan, M. R. (1991) *Cell* **65,** 493–505
52. Kim, S. J., Onwuta, U. S., Lee, Y. I., Li, R., Botchan, M. R., and Robbins, P. D. (1992) *Mol. Cell. Biol.* **12,** 2455–2463
53. Udvadia, A. J., Rogers, K. T., Higgins, P. D. R., Murata, Y., Martin, K. H., Humphrey, P. A., and Horowitz, J. M. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 3265–3269
54. Birnbaum, M. J., van Wijnen, A. J., Odgren, P. R., Last, T. J., Suske, G., Stein, G. S., and Stein, J. L. (1995) *Biochemistry* **34,** 16503–16508
55. Suzuki, M., Kuroda, C., Oda, E., Tsunoda, S., Nakamura, T., Nakajima, T., and Oda, K. (1995) *Mol. Cell. Biol.* **15,** 5423–5433
56. De Luca, P., Majello, B., and Lania, L. (1996) *J. Biol. Chem.* **271,** 8533–8536
57. Courey, A. J., and Tjian, R. (1988) *Cell* **55,** 887–898
58. Saffer, J. D., Jackson, S. J., and Thurston, S. J. (1990) *Genes & Dev.* **4,** 659–666
59. Saffer, J. D., Jackson, S. P., and Annarella, M. B. (1991) *Mol. Cell. Biol.* **11,** 2189–2199