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 is expressed leading to the acquisition of the adult muscle phenotype, relatively little is known regarding regulatory sequences or factors involved in the control of the repression of the muscle embryonic 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 perinatal development are still to be elucidated.

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Myogenesis and GLUT1 Gene Repression

EXPERIMENTAL PROCEDURES

Materials—[3H]-Labeled protein A and [a-32P]-DCTP were purchased from ICN. 2-Deoxy-D-[3H]glucose was obtained from DuPont NEN. Hybrid 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 cultures were grown in monolayer culture in DMEM supplemented with 10% fetal bovine serum, glutamine, and antibiotics. Monolayers were washed twice with PBS, scraped, and homogenized in 2 ml of ice-cold buffer containing 25 mM Hepes, 150 mM NaCl, 5 mM KCl, 2.5 mM CaCl2, 1.2 mM MgSO4, 10 mM NaHCO3, 1 mM spermidine, 10 mM spermine, 5 mM 2-mercaptoethanol, 0.02% sodium azide, and 0.02% antimycotic. The homogenate was centrifuged at 15,000 × g at 4 °C for 10 min. The supernatant was stored at −80 °C. After quantification, total RNA (30 μg) was extracted from 2 × 107 cells with RNAzol B (CinnaGene) by the CTAB method.

Cell fractionation was performed as described by Tyrrell and Schubach (31). After lysis, the cell suspension was subjected to 100,000 × g for 20 min at 4 °C. The supernatant was stored at −80 °C. After quantification, total RNA (30 μg) was extracted from 2 × 107 cells with RNAzol B (CinnaGene) by the CTAB method.

Glucose Uptake—2-deoxy- D-[3H]glucose in stop solution (50 mM glucose in PBS) was incubated with cells at room temperature or using the ECL Western blot detection system (Amersham Corp.). Immunoblots were performed using 125I-protein A for 4 h at room temperature or using the ECL Western blot detection system. Rabbit polyclonal antibodies was accomplished using 125I-protein A and 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 antibody—purified rabbit polyclonal antibody (PEP-2, Santa Cruz Biotechnology) was used at 2 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 125I-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 guanidium thiocyanate/phenol/chloroform method as described by Chomczynski and Sacchi (31). All samples had a A260/A280 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 32P-CTP by random oligonucleotide priming.
blasts or myotubes after 4 or 7 days of differentiation. A representative autoradiogram is shown.

Antibody against the COOH terminus of the carrier. A representative blotting, GLUT1 protein was detected by incubation with a polyclonal antibody against the COOH terminus of the carrier. A representative autoradiogram is shown. GLUT1 protein was detected by incubation with a polyclonal antibody against the COOH terminus of the carrier.

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). 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). 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. 2A). 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. 2A). Transfection with the construct −2106/+134-CAT in L6E9 myoblasts caused a 11.5-fold increase in CAT activity (Fig. 2B). Furthermore,
CAT activity detected in myotubes was substantially reduced (60% decrease) compared with values in myoblasts (Fig. 2B). 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 −33/+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 differentiation.

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
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 myotubes than in myoblasts. In contrast, complexes C2 and D3 were more abundant in myotubes than in myoblasts. In addition, 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 specific complexes, EMSA assays were performed in the presence of an excess of unlabeled oligonucleotides (Fig. 6). EMSA assays were performed in the presence of unlabeled oligonucleotides, 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. 7A). 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 site was mutated (Fig. 7A, oligonucleotide Imut) failed to displace complexes A1 and A2 (Fig. 7A). 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 Zn2+ or EDTA in the medium (39). Based on this, gel-retardation analyses were performed in the absence or presence of Zn2+ or EDTA. The addition of Zn2+ to the medium increased the formation of complexes A1 and A2 in a concentration-dependent manner (Fig. 7B). In contrast, addition of EDTA caused a concentration-dependent inhibition of complexes A1 and A2 (Fig. 7B).

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 (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 or 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-
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. 7. Characterization of factors binding to the Sp1 element of the GLUT1 gene. Labeled AvaII-AvaII 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 Zn2+ or EDTA. Representative autoradiograms are shown.

FIG. 8. Sp1 binds to the −102/−37-bp GLUT1 DNA fragment. Labeled AvaII-AvaII 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 AvaII-AvaII 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.
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 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, MyoD 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 myogenic transcription factors, which act as master regulators leading to activation of many muscle-specific genes. In our model, MyoD activation leads to the repression of Sp1 associated with myogenesis.

FIG. 10. Expression of Sp1 protein in nuclear extracts from myoblasts and myotubes. Nuclear extracts 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 AvaII-AvaII 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 100x excess of the unlabelled −102/−37 fragment (CP) or a 200x 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.

FIG. 12. Hypothetical scheme of the mechanisms responsible for the repression of GLUT1 gene expression during myogenesis.
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 Zn²⁺ 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. 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 myotubes. 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 p107 specifically represses Sp1-dependent transcription (41).

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 alteration in the biological potency of Sp1, which might be genesis not only leads to Sp1 down-regulation but also causes these items of information, it might be postulated that myo-

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