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# Down-Regulation of Transcription Factors AP-1, Sp-1, and NF-κB Precedes Myocyte Differentiation

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Terminal differentiation of myocytes involves withdrawal from the cell cycle, induction of myogenin expression, and finally formation of myotubes. To study the factors that regulate the initial phase of muscle differentiation, we analyzed the binding activities of transcription factors AP-1, Sp-1, and NF-κB in L6, C2C12, and rhabdomyosarcoma BA-Han-1C cells. Temporal changes in transcription factor binding activities were compared to the activation of myogenin promoter-driven CAT reporter gene and the expression level of myogenin, a master gene of myogenic differentiation. We observed a prominent decrease in the nuclear binding activities of AP-1, Sp-1, and NF-κB already 12 to 24 h after the transfer of cells to differentiation medium. The response was very similar in L6 and C2C12 myocytes and in BA-Han-1C rhabdomyosarcoma cells. The down-regulation clearly preceded the activation of myogenin promoter and the induction of myogenin and retinoblastoma expression, as well as the initiation of myocyte fusion. Cholera toxin and okadaic acid, established inhibitors of myogenin expression and muscle differentiation, strongly up-regulated the binding activities of AP-1, Sp-1, and NF-κB in differentiation medium. Myogenin expression and myocyte fusion were also inhibited. Levels of nuclear c-Fos and c-Jun proteins, components of the AP-1 complex, showed a prominent decrease already after 12 h in differentiation medium. These results show that the down-regulation of the proliferation-promoting transcription factors is a prerequisite to the initiation of myocyte differentiation. 

Determination and differentiation phases of skeletal muscle development are regulated by MyoD gene family of myogenic regulators (1, 2). Terminal differentiation of myocytes involves a withdrawal from the cell cycle and the induction of myogenin and MEF-2 gene expression (3). Several growth factors and oncoproteins inhibit the myogenin expression and muscle differentiation (1, 4). The proliferation and differentiation of myocytes seem to be exclusive processes although the mechanisms regulating the balance are still unknown.

Recently, retinoblastoma protein Rb (5) and cell cycle inhibitor p21 (6) have been proposed to have a central role in the initiation of myogenic differentiation program. However, the slow induction of these proteins compared to the withdrawal from the cell cycle implies their role as a mediator in the differentiation process and that the initiation phase could be regulated by transcription factors, such as the early response genes. Elevation of *c-fos* expression, for instance, inhibits the myocyte differentiation (7). Furthermore, the activation of protein kinase cascades, *e.g.* by protein kinase A and growth factor tyrosine kinases (1, 3) inhibit the withdrawal of myocytes from the cell cycle even in the low-serum differentiation medium.

Here we demonstrate that the initiation phase of the myogenic differentiation of L6, C2C12, and rhabdomyosarcoma BA-Han-1C cells involves a down-regulation of proliferation promoting transcription factors AP-1, Sp-1, and NF-κB, prior to the activation of myogenin promoter

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driven CAT reporter gene and myogenin expression. Cholera toxin and okadaic acid, inhibitors of myogenesis (4, 8), strongly up-regulated the binding activities of these transcription factors and opposed the withdrawal from the cell cycle, myogenin expression, and the fusion of myocytes to myotubes. These results support the model that the down-regulation of proliferation promoting transcription factors is a prerequisite to the myocyte differentiation.

## MATERIALS AND METHODS

*Cell culture and myocyte differentiation.* Rat L6 myocytes and mouse C2C12 myocytes were from American Type Culture Collection (Rockville, MD). The isolation and characterization of the rat rhabdomyosarcoma cell line BA-Han-1C has been described previously (9, 10). L6, C2C12, and BA-Han-1C cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum and antibiotics. BA-Han-1C medium was also supplemented with L-arginine and L-asparagine (10).

The differentiation of L6 myocytes was induced with 2% Viable AC-2 medium supplement (Valio Bioproducts Ltd). C2C12 myocytes were induced to differentiate with 2% horse serum, and BA-Han-1C cells with 2% horse serum supplemented with 15 mM suramin (Bayer AG) (10). Differentiation of BA-Han-1C cells was induced by 1 $\mu$M retinoic acid (11) or 50 ng/ml pertussis toxin (10).

Differentiation of myocytes was inhibited with okadaic acid (8) or cholera toxin (4). Okadaic acid concentration used was 50 nM for L6 and C2C12 myocytes and 25 nM for BA-Han-1C cells. The concentration of cholera toxin was 2.5 mg/ml for all cells. The effects of okadaic acid and cholera toxin were followed up to 48 h. Culture media were not changed during treatments.

*Isolation of nuclear proteins.* Nuclear proteins were isolated using the modified method of Dignam et al. (12). We have described the modifications in detail recently (14). Protein concentrations were measured using the Protein Assay Reagent of BioRad.

*Electrophoretic mobility shift assays (EMSA).* The double-stranded oligonucleotides containing consensus and mutated binding sites of transcription factors AP-1, Sp-1, and NF-$\kappa$B were from Santa Cruz. Oligonucleotides were labelled with T4 polynucleotide kinase according to the manufacturer's protocol. The labelled oligonucleotides were purified using 4% native PAGE.

EMSA method was used to assay the binding activities of AP-1, Sp-1, and NF-$\kappa$B transcription factors in nuclear extracts (13), basicly as described recently (14). The binding activity of NF-$\kappa$B was also analyzed in cytoplasmic extracts after the activation of inactive cytoplasmic NF-$\kappa$B with deoxycholate (15). Protein-DNA binding reactions were carried out with 10 $\mu$g of nuclear protein or with 25 $\mu$g of cytoplasmic protein. Binding assays and electrophoretic separation with 4% native PAGE are described earlier (14).

*Western blot and immunocytochemistry.* The protein levels of c-Fos and c-Jun, as well as the retinoblastoma protein (Rb) were assayed using Western blots with specific primary antibodies against c-Fos (Pharmingen), c-Jun (Oncogene Sci.), and Rb (C-15, Santa Cruz). Proteins were separated on 12% SDS-PAGE and Western blot assay used was described recently (14). Prestained SDS-PAGE molecular weight standard mixture (BioRad) was used in gel electrophoresis. Results were visualized using Renaissance Western Blot Chemiluminescence Reagent (Du Pont) according to the protocoll of the manufacturer.

Differentiation level of myocytes was followed by the immunocytochemical staining of myogenin protein using the same antibody and protocol as described earlier (10, 11). Sp-1 protein expression was assayed immunocytochemically with anti-Sp-1 (PEP 2) (Santa Cruz) using the same protocol.

*Reporter gene techniques and RNA hybridization.* Production and characterization of the stable C2C12 myocyte line harboring the 1.1 kb myogenin promoter linked to CAT (chloramphenicol acetyltransferase) reporter gene as described earlier (4). CAT activity of stable myocyte line was measured in supernatant fraction normalized to protein concentration using Boehringer Mannheim's ELISA kit for CAT activity. As a control, the effect of differentiation was studied using a stable C2C12 myocyte line harboring $\beta$-actin-CAT vector (4).

Northern blot assays of myogenin and GAPDH (glyceraldehyde phosphate dehydrogenase) mRNA were done as previously (4). The specific activity of muscle creatine kinase was measured in supernatant fraction using the kit from Sigma.

## RESULTS

*Down-regulation of AP-1, NF-$\kappa$B, and Sp-1 binding activities precedes myogenic differentiation.* The transfer of proliferating myocytes to differentiation medium activates the expression of myogenin, which is the main regulator of the terminal differentiation in myogenic lineage (2, 4). We have earlier observed that the activation of myogenin promoter precedes the expression of myogenin protein as well as the expression of muscle specific proteins, such as creatine

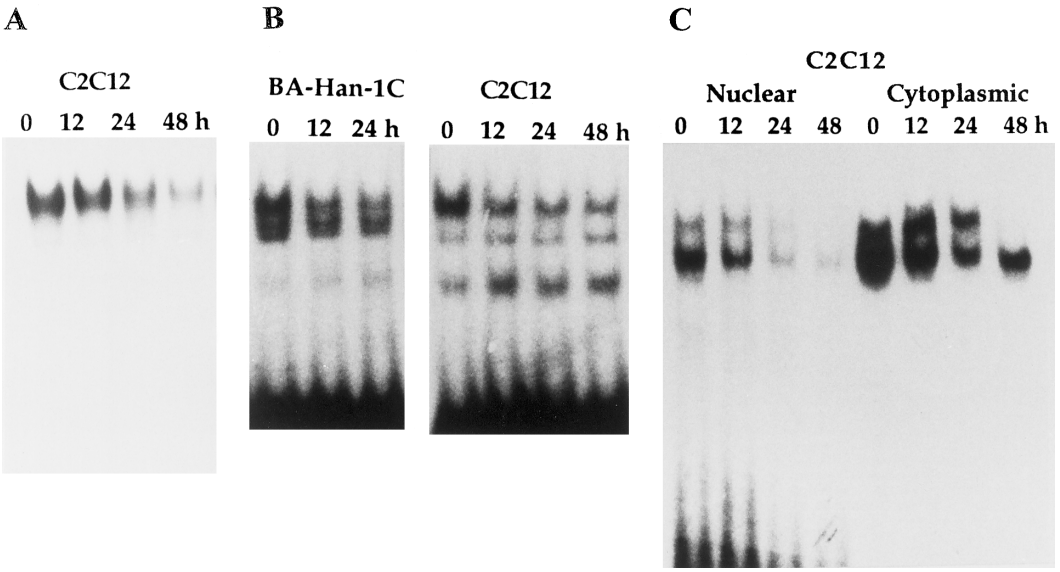


**FIG. 1.** Down-regulation of transcription factor AP-1, Sp-1, and NF-$\kappa$B binding activities during myocyte differentiation. Electrophoretic mobility shift assays of AP-1 binding activities in C2C12 cells [A], Sp-1 activities in BA-Han-1C and C2C12 cells [B], and NF-$\kappa$B binding activities in nuclear and cytoplasmic fractions [C] 12, 24, and 48 h after the transfer of myocytes to the low-serum differentiation medium [see Materials and Methods]. Cells at zero hour are the proliferating, nearly confluent myocyte cultures before the switch. Mutated oligonucleotide probe controls for AP-1, Sp-1, and NF-$\kappa$B did not show any specific binding activity. The addition of 10-100 times excess of competing unlabelled probe to binding assays inhibited each of the specific bindings [data not shown].

kinase (4). In the present experiments, the immunostaining of myogenin was nearly absent in proliferating myocytes but myogenin positive nuclei were present already 24 h after transfer to the differentiation medium and the fusion of myocytes appeared after 48 h differentiation. In confluent BA-Han-1C cells, the differentiation process induced *e.g.* by pertussis toxin was even more vigorous than in L6 and C2C12 myocytes and fusion appeared already after 24 h in differentiation medium (10, data not shown).

Myocyte transfer to differentiation medium strongly reduced the nuclear binding activities of transcription factors AP-1, Sp-1, and NF-$\kappa$B both in L6 and C2C12 myocytes, as well as in BA-Han-1C rhabdomyosarcoma cells (Fig. 1-4). Decrease in DNA-binding activities appeared already 12 h after the switching cells to differentiation medium (Fig. 1). The level and timing of down-regulation slightly varied in separate experiments (Fig. 1-4), perhaps due to culture conditions, such as the density of myocytes during the switching. After 48 h in differentiation medium, the binding activities of AP-1, Sp-1, and NF-$\kappa$B were very low in all myogenic cell lines, coincidentally with the most abundant nuclear staining of myogenin. Interestingly, the most dramatic decreases in the binding activities of AP-1 and NF-$\kappa$B occurred in BA-Han-1C rhabdomyosarcoma cells (Fig. 1 and 3) that also show more effective fusion than L6 and C2C12 myocytes when the differentiation is induced by, *e.g.*, pertussis toxin (10).

Differentiation of BA-Han-1C rhabdomyosarcoma cells affected the gel retardation properties of AP-1 binding complex (Fig. 3B). Differentiation induced the assembly of a faint AP-1 complex which was considerably smaller than in proliferating cells. The remodeling of AP-1 complex during differentiation appeared whether BA-Han-1C cells were induced to differentiate with suramin, retinoic acid, or pertussis toxin (data not shown). Differentia-

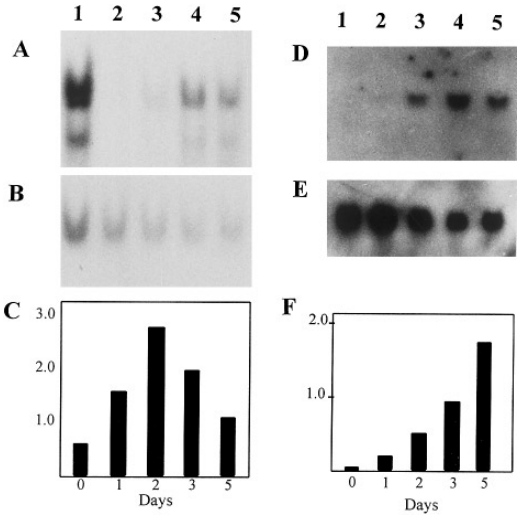


**FIG. 2.** Down-regulation of AP-1 and Sp-1 binding activities precedes myogenin induction. Electrophoretic mobility shift assays of Sp-1 [A] and AP-1 [B] binding activities in C2C12 myocytes carrying myogenin-CAT reporter gene before the transfer to differentiation medium (lane 1) and 1 day (lane 2), 2 days (lane 3), 3 days (lane 4), and 5 days after the switch. [C] CAT activity of myogenin CAT reporter gene in C2C12 myocyte supernatant fraction [ng/(mg protein × min)]. [D] Northern blot hybridization of myogenin mRNA. Lanes are as in [A]. [E] GAPDH mRNA expression in myocytes, lanes are as in [D]. [F] Creatine kinase activity (units/mg protein) in C2C12 myocytes during differentiation.

tion also down-regulated the cytoplasmic, deoxycholate-activated NF-$\kappa$B binding activities (Fig. 1C).

C2C12 myocytes harboring the 1.1 kb myogenin promoter linked to CAT reporter gene were used to compare the kinetics of AP-1 and Sp-1 down-regulation to the activation of myogenin promoter and the expression of myogenin during differentiation process. Figure 2 shows that the binding activities of Sp-1 (Fig. 2A) and AP-1 (Fig. 2B) are strongly down-regulated already 24 h after the switch to differentiation medium. However, the activity of myogenin promoter driven CAT reporter gene is increasing and reaches the highest level after 48 h (Fig. 2C). Myogenin mRNA (Fig. 2D) shows the highest expression on the third day and the target gene to myogenin, creatine kinase, on the fifth day (Fig. 3F). Retinoblastoma protein level was increased 48 h after the switch (data not shown). Interestingly, the binding activities of Sp-1 and AP-1 remained low during the differentiation process (Fig. 1-4). Differentiation only slightly reduced the expression level of GAPDH (Fig. 2E) and $\beta$-actin-CAT (data not shown).

*Cholera toxin and okadaic acid up-regulate AP-1, Sp-1, and NF-$\kappa$B binding activities and inhibit muscle differentiation.* Earlier studies have shown that cholera toxin (4) and okadaic acid (8) down-regulate myogenin expression and inhibit the myogenic differentiation program. In our present experiments, the treatment of differentiating myocytes with cholera toxin and okadaic acid strongly increased the DNA-binding activities of transcription factors AP-1, Sp-1, and NF-$\kappa$B (Fig. 3, 4). The binding activities were highest after 12 h of treatment and decreased later, perhaps due to the inactivation of drugs in culture or due to the opposing down-regulation effect induced by myocyte differentiation in low-serum medium. Cholera toxin and okadaic acid induced very similar up-regulation of these transcription factors both in L6 and C2C12 myocytes and in BA-Han-1C rhabdomyosarcoma cells (Figs. 3, 4).

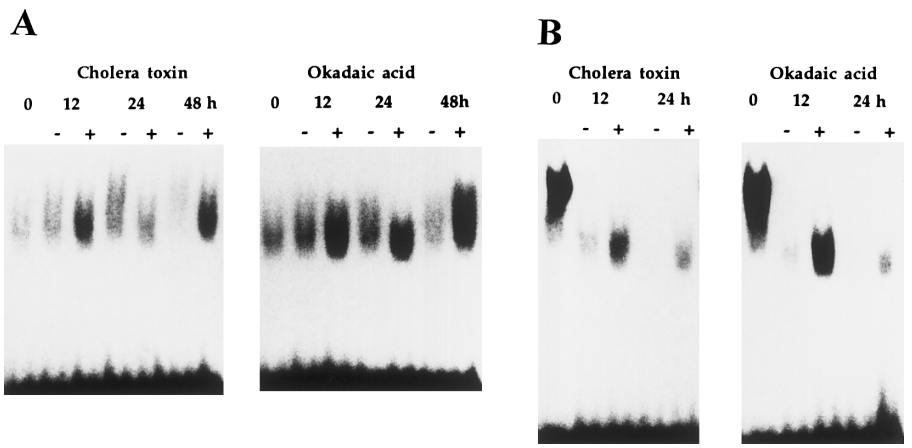


**FIG. 3.** Up-regulation of AP-1 binding activity and inhibition of myocyte differentiation by okadaic acid and cholera toxin. Electrophoretic mobility shift assays of AP-1 binding activities in L6 myocytes [A] and BA-Han-1C rhabdomyosarcoma cells [B] after 12, 24, and 48 h in the differentiation medium without [−] and with [+] okadaic acid and cholera toxin. Differentiation conditions are described under Materials and Methods. The concentration of okadaic acid was 50 nM for L6 and 25 nM for BA-Han-1C cells. The concentration of cholera toxin was 2.5 mg/ml for both.

Interestingly, cholera toxin and okadaic acid treatments induced in differentiating BA-Han-1C cells the smaller, differentiation type of AP-1 complex and not the larger AP-1 complex present in proliferating cells (Fig. 2B).

Cholera toxin and okadaic acid treatments inhibited the up-regulation of myogenin expression and myocyte fusion (data not shown), in accordance with our earlier studies (10) and those of Kim et al. (8). The withdrawal of myocytes from cell cycle was also inhibited and a sustained proliferation occurred. Immunocytochemical study of Sp-1 protein showed that the proteins are located in the nuclei both in proliferatig and differentiating myocytes but the level of staining was not affected by cholera toxin or okadaic acid treatments. However, occasionally the forming myotubes showed Sp-1 negative nuclei (data not shown).

We also studied whether differentiation affects the nuclear levels of the major components

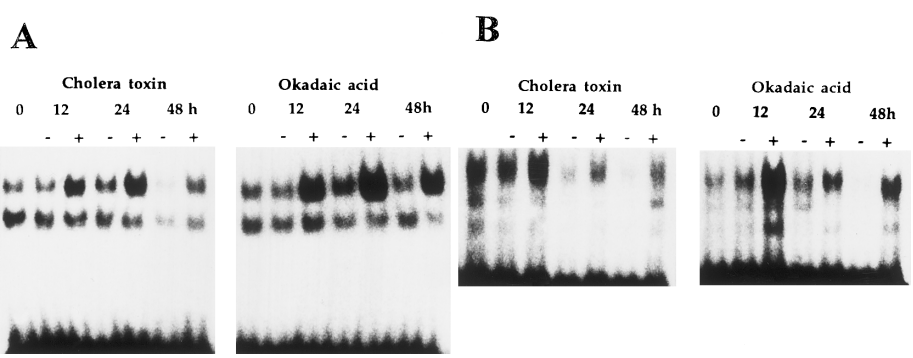


**FIG. 4.** Up-regulation of NF-$\kappa$B and Sp-1 binding activities and inhibition of myocyte differentiation by okadaic acid and cholera toxin. Electrophoretic mobility shift assay of nuclear NF-$\kappa$B [A] and Sp-1 [B] binding activities in L6 myocytes during differentiation [0-48 h] without [−] or with [+] okadaic acid or cholera toxin. The concentration of okadaic acid was 50 nM and that of cholera toxin 2.5 mg/ml. Free probe is on the bottom.

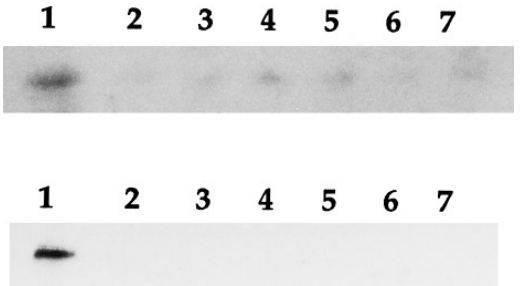


**FIG. 5.** Down-regulation of c-Fos and c-Jun protein levels during the induction of myocyte differentiation in BA-Han-1C rhabdomyosarcoma cells. Western assays for c-Jun (upper) and c-Fos (lower) proteins are shown. Lanes in both blots are [1] control cells, [2] suramin induction for 12 h, [3] retinoic acid induction for 12 h, [4] pertussis toxin induction for 12 h, [5] suramin induction for 24 h, [6] retinoic acid induction for 24 h, and [7] pertussis toxin induction for 24 h. The following concentrations were used: 15 mM for suramin, 1 $\mu$M for retinoic acid, and 50 ng/ml for pertussis toxin.

of AP-1. Figure 5 shows that the protein levels of c-Fos and c-Jun are down-regulated already after 12 h in differentiation medium in BA-Han-1C cells, and simultaneously the AP-1 binding activity disappears [Fig. 3B].

## DISCUSSION

Terminal differentiation of skeletal muscle cells involves the withdrawal of myoblasts from the cell cycle, the induction of myogenin expression, and finally the fusion of cells to myotubes. The mechanisms regulating the initiation phase, the cell cycle withdrawal, are still poorly defined compared the mechanisms regulating MyoD gene family driven differentiation (1, 2). After determination to muscle lineage, myoblasts can proliferate without differentiation, although they express MyoD and Myf-5 genes, which are able to induce myogenin expression and can even induce muscle differentiation in non-muscle cells (16). Several extracellular factors, positive and negative, are known to regulate balance between proliferation and differentiation (1, 2).

Several mechanisms have been suggested to cause the inability of MyoD and Myf-5 proteins to induce myogenin expression and to initiate myogenic program during the proliferation phase of myocytes (1-3). Phosphorylation of threonine in the basic domain of myogenic factors, *e.g.* by protein kinase C, could inhibit the DNA binding (17). However, this kind of phosphorylation has not been verified *in vivo* (18). The products of Id gene family, helix-loop-helix proteins without the basic domain, can dimerize with myogenic factors and thus neutralize them in proliferating myocytes. This is an intriguing possibility since Id protein levels are high in proliferating myocytes and, furthermore, they can be induced by growth factors and down-regulated by low-serum differentiation medium (19, 20). However, Id as well as Rb proteins probably are mediators rather than inducers of the withdrawal of myocytes from the cell cycle and to the subsequent differentiation.

Here we observed that the transfer of growth medium to differentiation medium quickly and strongly down-regulated the DNA binding activities of AP-1, Sp-1, and NF-$\kappa$B transcription factors. Interestingly, the down-regulation occurred clearly prior to the activation of myogenin promoter driven CAT reporter gene and prior to the induction of myogenin mRNA expression. The response also preceded the up-regulation of the retinoblastoma protein. Li et al. (17) showed that c-Fos and c-Jun can repress the transcriptional activation of myogenin and MyoD, most probably via direct binding to these proteins (21). This represents a functional

antagonism between these transcriptional pathways (1). However, the down-regulation of AP-1 binding activity during myocyte differentiation clearly preceded the up-regulation of myogenin and, furthermore, the protein levels of c-Fos and c-Jun appeared to be down-regulated, *e.g.* in BA-Han-1C rhabdomyosarcoma cells. This suggests that the down-regulation of AP-1 components leaves room for the positive signals to activate myogenin promoter and thus induce myogenin expression.

The functional properties of AP-1, Sp-1, and NF-κB, both the binding and transactivation mechanisms, are very divergent. However, all these are induced by serum mitogens (for examples, see 22-24) and down-regulated by the withdrawal of serum mitogens, as observed in this study. On the other hand, cholera toxin and okadaic acid, well-known inhibitors of myocyte differentiation (4, 8), up-regulated the DNA binding activities of AP-1, Sp-1, and NF-κB, even in the low-serum differentiation medium. Protein kinase A pathway has been known to regulate the AP-1 binding activity (23). However, the DNA binding activities of Sp-1 and NF-κB were also up-regulated both by cholera toxin and okadaic acid. The activity of transcription factor AP-1 is required *e.g.* for the initiation of DNA synthesis during the cell proliferation (25). The role of Sp-1 and NF-κB factors during myocyte proliferation and differentiation are still unknown. However, Sp-1, for instance, regulates the promoter activity of several cell cycle dependent genes, such as histone H4 gene (26), and can associate with E2F factor (27). On the contrary to proliferation, Leggett et al. (28) showed that Sp-1 protein is phosphorylated and its binding activity down-regulated during terminal differentiation in liver cells.

Our results suggest the differentiation model in which the down-regulation of proliferation promoting transcription factors is an obligatory step that precedes the activation of myogenin promoter and the induction of myogenin in the initiation of myocyte differentiation.

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