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Expression and Subcellular Localization of Myogenic Regulatory Factors During the Differentiation of Skeletal Muscle C2C12 Myoblasts

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ABSTRACT

It is known that the MyoD family members (MyoD, Myf5, myogenin, and MRF4) play a pivotal role in the complex mechanism of skeletal muscle cell differentiation. However, fragmentary information on transcription factor-specific regulation is available and data on their post-transcriptional and post-translational behavior are still missing. In this work, we combined mRNA and protein expression analysis with their subcellular localization. Each myogenic regulator factor (MRF) revealed a specific mRNA trend and a protein quantitative analysis not overlapping, suggesting the presence of post-transcriptional mechanisms. In addition, each MRF showed a specific behavior in situ, characterized by a differentiation stage-dependent localization suggestive of a post-translational regulation also. Consistently with their transcriptional activity, immunogold electron microscopy data revealed MRFs distribution in interchromatin domains. Our results showed a MyoD and Myf5 contrasting expression profile in proliferating myoblasts, as well as myogenin and MRF4 opposite distribution in the terminally differentiated myotubes. Interestingly, MRFs expression and subcellular localization analysis during C2C12 cell differentiation stages showed two main MRFs regulation mechanisms: (i) the protein half-life regulation to modulate the differentiation stage-dependent transcriptional activity and (ii) the cytoplasmic retention, as a translocation process, to inhibit the transcriptional activity. Therefore, our results exhibit that MRFs nucleo-cytoplasmic trafficking is involved in muscle differentiation and suggest that, besides the MRFs expression level, also MRFs subcellular localization, related to their functional activity, plays a key role as a regulatory step in transcriptional control mechanisms. J. Cell. Biochem. 108: 1302–1317, 2009. © 2009 Wiley-Liss, Inc.

KEY WORDS: MyoD; Myf5; MYOGENIN; MRF4; MRFs SUBCELLULAR LOCALIZATION; MRFs NUCLEO-CYTOPLASMIC TRAFFICKING

Skeletal muscle specification and differentiation are controlled by a family of transcription factors known as myogenic regulatory factors (MRFs) [Pownall et al., 2002; Blais et al., 2005], including MyoD [Ishibashi et al., 2005; Tapscott, 2005; Cao et al., 2006], Myf5 [Ishibashi et al., 2005], myogenin [Cao et al., 2006], and MRF4 [Kassar-Duchossoy et al., 2004]. These molecules, exclusively expressed in myogenic cell lineage, become active in a space- and time-correlated manner during embryogenesis [Pownall et al., 2002]. Each of the MRFs proteins contains the helix-loop-helix domain, which recognizes a simple consensus sequence of CANNTG, termed E-box, present in the regulatory regions of many muscle-specific genes and heterodimerizes with the ubiquitously expressed E proteins, to achieve an efficient binding to DNA [Tapscott, 2005].

MRFs are sufficient to induce myogenesis when introduced into a variety of non muscle cells in culture [Dedieu et al., 2002; Dodou et al., 2003] and directly activate the myocyte enhancer factor 2 (MEF2) family of MADS box factors, which participates to the regulation of most muscle-specific genes by a combinatorial activity with the MRFs [Dodou et al., 2003].

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Although these MRFs have been considered genetically redundant, since their expression patterns overlap during myogenic differentiation [Rudnicki et al., 1992, 1993], it has been demonstrated that these factors play distinct roles during myogenesis [Pownall et al., 2002], as shown by targeted gene inactivation experiments [Arnold and Braun, 1996]. The coordinated action of each MRFs family member is required for the expression of the specific-muscle genes [Blais et al., 2005; Tapscott, 2005] and each of the MRFs plays a specific role in regulating a specific subset of muscle genes at the onset of fusion [Dedieu et al., 2002]. Moreover, MRFs unexpected wide-ranging roles, in directing the assembly and function of neuromuscular junction and in inducing myoblasts to different type of stress responses, have been also demonstrated [Blais et al., 2005].

MyoD is able to induce a variety of cell types to differentiate in muscle cells, since it is able to activate the transcription program of muscle specific genes [Tapscott, 2005]. Indeed, it is a transcription factor with binding sites in the regulatory regions of many genes, which are expressed throughout the myogenic program [Blais et al., 2005; Tapscott, 2005]. Moreover, MyoD induces cell cycle withdrawal both by cross-talking with the cell cycle regulators [Kitzmann and Fernandez, 2001; Ishibashi et al., 2005; De Falco and De Luca, 2006] and by inhibiting cytokine signals [Kataoka et al., 2003]. MyoD and Myf5 are activated in different muscle precursor cells [Braun and Arnold, 1996], suggesting that they have a distinct role in determining different muscle cell lineages [Braun and Arnold, 1996; Pownall et al., 2002]. Indeed, they have a specific target gene activation and it has been reported that Myf5 enhances myoblast proliferation, whereas MyoD promotes cell cycle withdrawal and induction of myoblast differentiation [Ishibashi et al., 2005]. MyoD and Myf5 have been shown to regulate skeletal muscle commitment, whereas myogenin mediates the differentiation process [Dedieu et al., 2002; Cao et al., 2006]. Indeed, myogenin is involved in the myotube formation [Venuti et al., 1995] and the contractile apparatus protein synthesis [Sánchez and Robbins, 1994]. MRF4 acts both as a determination factor, since it is expressed in a subset of myocytes in the early somite [Kassar-Duchossoy et al., 2004] and as a differentiation factor involved in the final maturation of myotubes [Zhang et al., 1995]. It also plays an important role in the regulation of muscle fiber phenotype in the postnatal life, especially in maintaining the slow phenotype [Walters et al., 2000].

MRFs have been shown not only to regulate the transcription of numerous muscle-specific genes but also to autoregulate each other's transcription. MyoD is subject to positive autoregulation and it induces myogenin expression, which in turn is able to induce MyoD expression; therefore, MyoD and myogenin appear to be subject to a positive autoregulatory loop, which may amplify the level expression of both genes above a critical threshold necessary for the activation of the myogenic program [Thayer et al., 1989]. Moreover, Myf5 expression is inhibited by MyoD [Rudnicki et al., 1992] and MRF4 is required for the down-regulation of myogenin expression [Zhang et al., 1995].

C2C12 are murine myoblasts derived from satellite cells and their behavior corresponds to that of progenitor lineage. C2C12 are a subclone of C2 myoblasts, which spontaneously differentiate in culture after serum removal and provide an useful experimental model to study myogenesis and muscle differentiation [Burattini et al., 2004].

Although a number of transcription factors and signal transduction pathways involved in muscle differentiation have been identified, the picture remains far from complete. MRFs have been widely investigated along the differentiation of skeletal muscle cells and tissues [Pownall et al., 2002]; however, fragmentary information on transcription factor-specific regulation is available and data on their post-transcriptional and post-translational behavior combined with their subcellular localization during myoblast differentiation stages are still missing. To address this issue, in this study, we analyzed MRFs behavior during the differentiation of skeletal muscle C2C12 myoblasts by different technical approaches. The real-time RT-PCR and Western blotting techniques were used to quantify the MRFs transcripts and their protein expression level, whereas protein subcellular localization was investigated by immunofluorescence and immunoelectron microscopy.

MATERIALS AND METHODS

CELL CULTURE

C2C12 mouse adherent myoblasts were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with heat-inactivated 10% fetal bovine, 2 mM glutamine, 1% antibiotics, 0.5% anti-mycoplasma and 25 mM Hepes (pH 7.5), were maintained in a 5% CO_2 atmosphere at 37°C and cell viability was assessed by the Trypan Blue exclusion test. To induce myogenic differentiation, when about 80% cell confluence was attained, the medium containing 10% fetal calf serum was changed with a 1% new one, as previously described [Burattini et al., 2004].

DIFFERENTIATION MORPHOLOGICAL ANALYSIS

Cells were analyzed at the undifferentiated stage and at the early, middle and late differentiation stage for each investigation. In order to eliminate discrepancies in the differentiation time points analyzed, we chose to assess some differentiation landmarks. The cells, grown with the containing 10% fetal calf serum medium and attained 80% cell confluence, were analyzed and considered undifferentiated cells at the day 0 of the differentiation process (T0). To induce myogenic differentiation, cells at T0 were switched to differentiation medium containing 1% fetal calf serum. Cells in an early differentiation stage were analyzed 24 h after serum removal (T1). To check the differentiation progression the estimation of myoblast fusion, beginning 2 days after serum removal (T2), was carried out. Subsequently cells were analyzed in the middle differentiation time, that is, 3–5 days after serum removal, when myotubes containing more of two nuclei appeared (T3–5), and in the late differentiation time, that is, 7–10 days after serum removal, when the presence of long multinucleated myotubes frequently overpassed the underlying mononucleated myoblasts (T7–10).

The cell fusion was assessed by determining the fusion index as previously described [Dedieu et al., 2002; Sestili et al., 2009]. C2C12 cells were grown in dishes containing coverslides; at the different phases of differentiation they were rinsed twice with phosphate

buffer saline (PBS; 0.1 M, pH 7.4), methanol fixed for 10 min at room temperature and air dried. Cells were then stained with water 1:10 May Grunwald-Giemsa solution for 30 min, washed with water and mounted to evaluate cell fusion by a TE 2000-S reverted light microscope, equipped with a DN 100 Nikon digital system. As described in a previous work [Sestili et al., 2009], cells were considered fused if they contained two nuclei within one cytoplasmic continuity. The fusion percentage was evaluated as number of nuclei in myotubes divided by the total number of nuclei in myoblasts and myotubes multiplied for 100 (40× objective). Twenty optical fields were randomly chosen. Data were expressed as mean ± SEM.

IMMUNOFLUORESCENCE

Immunofluorescence was directly carried out in dishes containing a cover slide, where cells had been seeded. Cells were fixed with 4% paraformaldehyde in PBS for 30 min at room temperature, washed with PBS and stored at 4°C until all time points were collected. Cells were permeabilized with 0.2% Triton X-100 in PBS for 10 min at room temperature and then processed for immunofluorescence.

For MyoD detection, cells were treated with 5% normal horse serum (D.B.A., Vector) and 2% bovine serum albumin (BSA) in PBS for 30 min at room temperature and then incubated with a mouse anti-MyoD antibody (D.B.A., Novocastra; 1:50 in PBS) overnight at 4°C, followed by an incubation with a FITC-conjugated horse anti-mouse secondary antibody (D.B.A., Vector; 1:50 in PBS) for 1 h at room temperature.

To investigate Myf5, myogenin or MRF4 expression, cells were treated with 5% normal goat serum (D.B.A., Vector) and 2% BSA in PBS for 30 min at room temperature and then incubated with a rabbit anti-Myf5 (Santa Cruz Biotech, USA; 1:200 in PBS), a rabbit anti-myogenin (Santa Cruz Biotech; 1:200 in PBS) or a rabbit anti-MRF4 (Santa Cruz Biotech; 1:200 in PBS), respectively, overnight at 4°C, followed by a FITC-conjugated goat anti-rabbit (D.B.A., Vector; 1:50 in PBS) for 1 h at room temperature.

All specimens were observed and photographed with a fluorescence microscope (VANOX Olympus MI, Italy): for viewing FITC fluorescence a combination of BP 490 and EY 455 excitation filters was used. In order to investigate MyoD expression in myotubes a combination of phase contrast and confocal microscopy (ZEISS, LSM 510 Meta) was also utilized.

IMMUNOGOLD ELECTRON MICROSCOPY

In order to maintain cell morphology in each differentiation time point, C2C12 cells were cultured in dishes containing a coverslide and directly processed for electron microscopy. Cells were washed with PBS, fixed with 1% glutaraldehyde in phosphate buffer 0.1 M for 1 h, partially dehydrated to 70% alcohol and embedded directly on cover slides in London Resin White (LRW) acrylic resin (TAAB, England, UK) at 0°C.

For immunogold technique, thin sections were collected on 400 mesh nickel grids. After distilled water washing, grids were rinsed with TBS1 (0.1% BSA and 10% Tris–HCl buffer 0.05 M, pH 7.6 in saline) and treated with normal goat serum (Sigma; 1: 20 in TBS1) for 30 min. Subsequently, cells were incubated with a mouse anti-MyoD (D.B.A., Novocastra; 1:5 in TBS1), a rabbit anti-Myf5 (Santa Cruz Biotech; 1:20 in TBS1), a rabbit anti-myogenin (Santa Cruz Biotech; 1:20 in TBS1) or a rabbit anti-MRF4 (Santa Cruz Biotech; 1:40 in TBS1), respectively, overnight at 4°C. After TBS2 washing (0.1% BSA and 10% Tris–HCl buffer 0.02 M, pH 8.2 in saline), the specimens were incubated with a 10 nm colloidal gold particle-conjugated anti-mouse antibody (Amersham; 1:25 in TBS2) or a 10 nm colloidal gold particle-conjugated anti-rabbit antibody (Amersham; 1:50 in TBS2), respectively, for 1 h at 37°C. The grids were rinsed with distilled water, stained with uranyl acetate in water for 2 min and with lead citrate for 1 min and analyzed by a Philips CM10 electron microscope.

ELECTROPHORESIS AND WESTERN BLOTTING

The C2C12 cells were homogenized in 200 μl of ice-cold lysis buffer [50 mM Tris–HCl, pH 7.8, 250 mM NaCl, 1 μg/ml pepstatin, 10 μg/ml leupeptin, 2 mM sodium orthovanadate, 10 mM NaF, 5 mM EDTA, 40 μg/ml PMSF (phenylmethylsulfonyl fluoride), and 0.1% (w/v) Triton X-100] and sonicated for 60 s at 100 W. Samples were then centrifuged for 10 min at 14,000*g* to remove insoluble debris. Supernatants were mixed 1:1 (v/v) with sample buffer (0.5 M Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 4% 2-mercaptoethanol and 0.05% bromophenol blue) and 50 μg of sample proteins were loaded onto 12% SDS–polyacrylamide slab gels and subjected to electrophoresis. Pre-stained molecular weight markers (BioRad, Milan, Italy) were run on adjacent lanes. The gels were electroblotted and stained with Coomassie blue. Blots were probed with the specific primary antibodies: a mouse anti-MyoD (D.B.A., Novocastra; 1:50 in Tris–HCl buffer), a rabbit anti-Myf5 (Santa Cruz Biotech, 1:200 in Tris–HCl buffer), a rabbit anti-myogenin (Santa Cruz Biotech, 1:200 in Tris–HCl buffer) or a rabbit anti-MRF4 (Santa Cruz Biotech; 1:200 in Tris–HCl buffer) overnight at 4°C. Horseradish-peroxidase-conjugated goat anti-mouse or horseradish-peroxidase-conjugated goat anti-rabbit IgG (BioRad, Milan, Italy, 1:3,000 in Tris–HCl buffer) were used as secondary antibodies, respectively. After antibody probing, nitrocellulose membranes were stripped for 30 min at 50°C with a stripping buffer (62.5 mM Tris–HCl, pH 6.7, containing 10 mM β-mercaptoethanol and 2% SDS) and re-probed with a mouse anti-tubulin (Sigma, St. Louis, MO; 1:10,000 in Tris–HCl buffer) followed by a secondary hybridization as above described. Immune complexes were visualized using an enhanced chemioluminescence Western blot analysis system (Amersham-Pharmacia, Milan, Italy), following the manufacturer's specifications. Blot images were digitized (Chemidoc, BioRad, Milan, Italy) and the optical density of bands was quantified using the computerized imaging system (Quantity One, BioRad). Quantitative data were expressed as mean ± SEM.

RNA EXTRACTION AND REAL-TIME QUANTITATIVE RT–PCR

Three plates for each differentiation step were washed with PBS and total RNA was extracted using RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. One microgram of total RNA was reverse transcribed using Omniscript RT (Qiagen) and random hexamers (Promega) in a final volume of 20 μl.

The PCR real-time approach was used to quantify the expression levels of the MRFs RNA transcripts. Oligonucleotide primers for the *MyoD*, *Myf5*, *myogenin*, and *Mrf4* transcription factors were previously tested in a parallel study [Sestili et al., 2009]. Specific primers for the ribosomal protein S16 were used as housekeeping gene. Preliminary experiments showed that S16 mRNA was stable during the differentiation process as previously reported [Sestili et al., 2009].

Measurements reported were made by an iCycler machine (BioRad) and QuantiTect SYBR green PCR kit (Qiagen). The real-time RT-PCR was carried out in 96-well rt-RT-qPCR plate (BioRad). For each well, the 25 μl reaction medium contained: 12.5 μl of 2× QuantiTect SYBR green PCR master mix, 0.3 μM each forward and reverse primer, 7.5 μl of RNase-free H_2O and 2.5 μl cDNA template. The cycling conditions were: 95°C for 15 min followed by 50 cycles of 94°C for 30 s, 60°C for 30 s for 40 cycles. Primer efficiency was checked and reported (see Results Section). Real-time RT-PCR products were confirmed by melting curves and sequencing. The amount of the target transcript was related to that of the reference S16 gene by the method described in Sestili et al. [2009]. Each sample was tested in triplicate by quantitative PCR, and samples obtained from at least four independent experiments were used to calculate the means and standard errors.

STATISTICS

Results from real-time RT-PCR and Western blotting analysis were expressed as mean ± SEM. The data were compared with the ANOVA test, followed by a post-hoc test using Tukey's multiple comparison test. The threshold of significance for the ANOVA and the Tukey's test was fixed at $P \leq 0.05$.

RESULTS

DIFFERENTIATION MORPHOLOGICAL ANALYSIS

Undifferentiated myoblasts, analyzed at T0, were recognizable as flat, fusiform or star-shaped cells, which appeared scattered on the substrate and rigorously mononucleated (Fig. 1A). Twenty-four hours after serum removal (T1), cells were considered myoblasts in an early differentiation stage and they were characterized by myoblast orientation change, as well as cell lengthening and thickening (Fig. 1B). Myoblast cells began to fuse after 2 days in differentiation medium forming binucleated cells (T2, fusion index = 8 ± 3%) (Fig. 1C). By day 3–5 after serum removal, myotube genesis progressively increased and the appearance of myotubes containing more than two nuclei was considered the middle differentiation stage (T3–5, fusion index = 35 ± 5%) (Fig. 1D). The

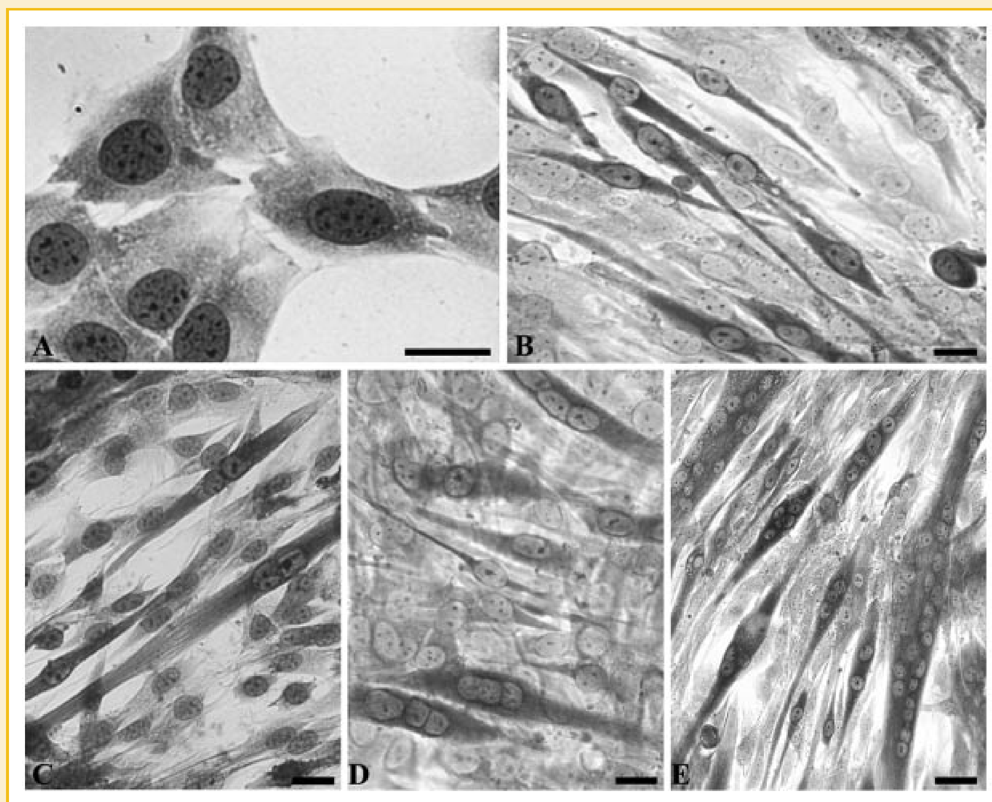

Fig. 1. Differentiation morphological analysis. A: Undifferentiated cells, analyzed at T0, are flat, fusiform or star-shaped; they scatter on the substrate and are rigorously mononucleated; bar = 40 μm. B: Cells in the early differentiation time (T1, 24 h after serum removal) are characterized by myoblast orientation change, cell lengthening and thickening; bar = 40 μm. C: Cells, analyzed at T2, are characterized by binucleated cell appearance; bar = 40 μm. D: Cells in the middle differentiation stage (T3–5, analyzed 3–5 days after serum removal) are characterized by the presence of myotubes containing more than two nuclei; bar = 40 μm. E: Cells in the late differentiation stage (T7–10, analyzed 7–10 days after serum removal) are characterized by the presence of long multinucleated myotubes which frequently overpass the underlying mononucleated myoblasts; bar = 40 μm.

phenomenon progressively increased and reached the maximum level of fusion 7–10 days after serum removal, when the presence of long multinucleated myotubes frequently overpassed the underlying mononucleated myoblasts, indicating the late differentiation stage (T7–10, fusion index = 60 ± 6%) (Fig. 1E).

IMMUNOFLUORESCENCE AND IMMUNOGOLD ANALYSIS

In undifferentiated cells, MyoD-positive cells were present (Fig. 2A). MyoD-positive cells increased 24 h after differentiation induction (Fig. 2B) and then decreased at the middle and late differentiation time (Fig. 2C,D). Along differentiation process, MyoD was always expressed and heterogeneously distributed in the nucleus (Fig. 2E,F). MyoD-positive cells with different degrees of labeling intensity could be observed in each differentiation stage (Fig. 2F). Mitotic myoblast cells were MyoD-positive: MyoD was present in the cytoplasm, whereas condensed chromosomes were not labeled (Fig. 2G).

At the late differentiation stage, myotubes resulted MyoD-positive: MyoD labeling was displayed in the nuclei, whereas no staining was present in the cytoplasm, as observed by the combined confocal and phase contrast microscopy analysis (Fig. 2H,I). Moreover, in the same myotube, the simultaneous existence of MyoD-positive nuclei with different degree of labeling could be detected (Fig. 2I, →).

Immunogold analysis confirmed and extended the immunofluorescence results. Along differentiation process, MyoD labeling was present almost exclusively in the nucleus (Fig. 2L,M), where it was localized in interchromatin domains (Fig. 2L,M, *), and nucleoli were negative (Fig. 2L,M, n and inset).

Along differentiation Myf5 was always expressed, but its localization changed during the different time points. Indeed, at the undifferentiated time, all cells resulted Myf5-positive (Fig. 3A,B), including mitotic myoblast cells, which were more intensely labeled than surrounding cells (Fig. 3A, →). Myf5 staining was present both in the cytoplasm and in the nucleus, as demonstrated by immunofluorescence (Fig. 3A,B) and immunogold analysis (Fig. 3C). After differentiation induction, Myf5 appeared mainly concentrated in the nucleus with respect to the cytoplasm, both in the middle (Fig. 3D,E) and in the late differentiation time, as demonstrated by the myotube staining (Fig. 3F). Immunogold analysis confirmed and extended these observations. In the nucleus, Myf5 labeling was especially localized in interchromatin domains (Fig. 3C,E,G, *), whereas nucleoli were negative (Fig. 3C,E, n).

Myogenin was expressed in all undifferentiated cells (Fig. 4A) mainly in the cytoplasm with a perinuclear distribution, as observed by immunofluorescence (Fig. 4A) and immunogold analysis (Fig. 4B). After differentiation induction, intensely labeled myogenin-positive cells, where myogenin progressively concentrated in the nucleus, appeared (Fig. 4C–E). Subsequently, at the late differentiation stage, myogenin was strongly expressed in the cytoplasm but especially in the nuclei, both in mononucleated cells and in myotubes (Fig. 4F,G,I,L). In addition, there were some myotubes showing an intense myogenin staining in the cytoplasm, but with myogenin-negative nuclei (Fig. 4H). Nevertheless, at this stage, many myogenin-negative cells were present (Fig. 4F), related to quiescent myoblastic cells, previously identified as "resting cells" [Yoshida et al., 1998]. Immunogold analysis confirmed these observations: in undifferentiated cells myogenin labeling was mainly localized in the cytoplasm (Fig. 4B), whereas, after differentiation induction, myogenin labeling was preferentially distributed in the nucleus (Fig. 4E,I,L). In all differentiation stages, in the nucleus myogenin labeling was localized in interchromatin domains (Fig. 4E,I,L, *) and was not present in nucleoli (Fig. 4B,E,I,L, n).

At the undifferentiated time, all cells were MRF4-positive (Fig. 5A). MRF4-positive cells were characterized by the presence of the protein in the nucleus, but mainly in the cytoplasm with a perinuclear distribution, as observed by immunofluorescence analysis (Fig. 5A,B). Immunogold analysis evidenced a MRF4-positive cytoplasm, even in the cell filopodia (Fig. 5C). Soon after differentiation induction, some very intensely MRF4-labeled cells appeared: these elonged cells displayed the MRF4 staining in the nucleus but especially in the cytoplasm, where it showed a perinuclear distribution (Fig. 5D). In the middle differentiation time, MRF4-positive myotubes with MRF4 labeling especially localized in the cytoplasm appeared (Fig. 5E). Immunogold analysis confirmed a stronger MRF4 staining in the cytoplasm than in the nucleus (Fig. 5F,G), where it was present in interchromatin domains (Fig. 5G, *) and nucleoli were not labeled (Fig. 5F,G, n). In the late differentiation stage, myotubes showed a very intense MRF4 staining, especially in the cytoplasm, as displayed by immunofluorescence (Fig. 5H); at this time, some mononucleated cells weakly labeled could be observed (Fig. 5H). In the late differentiation time, immunogold analysis (Fig. 5I,L) confirmed a stronger MRF4 labeling in the cytoplasm with respect to the nucleus (Fig. 5I,L), where it was localized in interchromatin domains (Fig. 5I,L, *) and it was not present in the nucleoli (Fig. 5L, n).

ELECTROPHORESIS AND WESTERN BLOTTING

MyoD protein was expressed in the undifferentiated cells at T0 (Fig. 6A). Subsequently MyoD significantly increased after the differentiation induction reaching the highest level 24 h after serum removal and then decreasing in the middle and late differentiation time (ANOVA test: $P < 0.05$; Tukey's test: 1 vs. 0, $P < 0.05$; 1 vs. 3, $P < 0.05$; 1 vs. 7, $P < 0.05$; 0 vs. 3, $P > 0.05$; 0 vs. 7, $P > 0.05$; 3 vs. 7, $P > 0.05$) (Fig. 6A,B).

The Myf5 protein was expressed in undifferentiated cells at T0 and significantly decreased after differentiation induction keeping its expression level constant during the middle and late differentiation time (ANOVA test: $P < 0.05$; Tukey's test: 1 vs. 0, $P < 0.05$; 1 vs. 3, $P > 0.05$; 1 vs. 7, $P > 0.05$; 0 vs. 3, $P < 0.05$; 0 vs. 7, $P < 0.05$; 3 vs. 7, $P > 0.05$) (Fig. 6A,C).

Myogenin was already expressed in undifferentiated cells at T0; afterwards it significantly increased after the differentiation induction reaching the highest level in the early differentiation time and then significantly decreased both in the middle and in the late differentiation stages (ANOVA test: $P < 0.05$; Tukey's test: 1 vs. 0, $P < 0.05$; 1 vs. 3, $P < 0.05$; 1 vs. 7, $P < 0.05$; 0 vs. 3, $P < 0.05$; 0 vs. 7, $P > 0.05$; 3 vs. 7, $P < 0.05$) (Fig. 6A,D).

MRF4 protein was scarcely present in undifferentiated cells at T0, being almost undetectable (Fig. 6A). Even though it did not change soon after the differentiation induction, it significantly increased

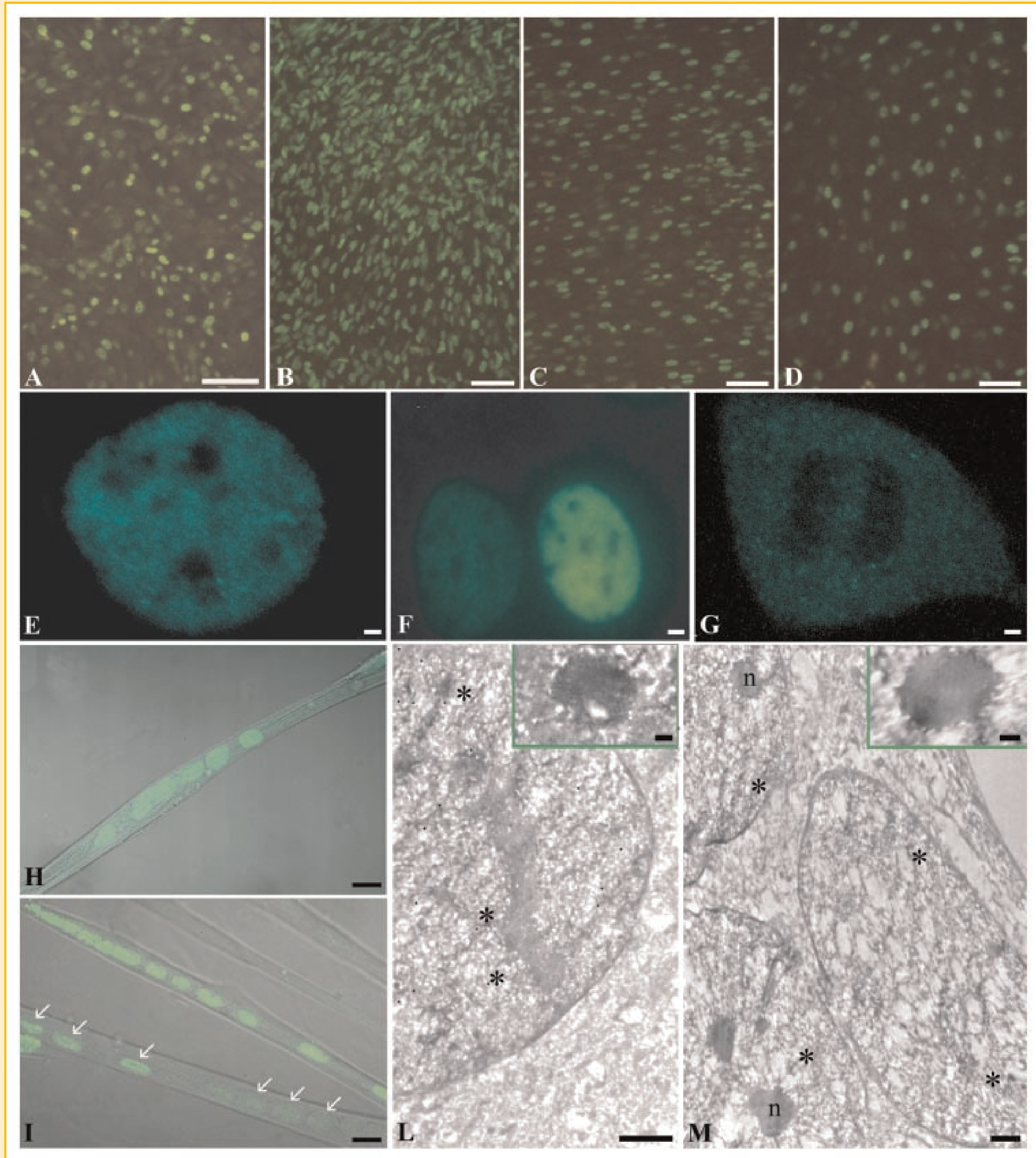

Fig. 2. MyoD staining. A–D,F: Fluorescence microscopy. E,G: Confocal microscopy. H,I: Confocal and phase contrast microscopy combination. L,M: Electron microscopy. A: MyoD-positive cells at the undifferentiated time; bar = 500 μm. B: Increase in MyoD-positive cells in the early differentiation stage; bar = 500 μm. C: Decrease in MyoD-positive cells at the middle differentiation stage; bar = 500 μm. D: Decrease in MyoD-positive cells at the late differentiation stage; bar = 500 μm. E: Nuclear MyoD labeling; bar = 50 μm. F: Coexistence of MyoD-positive cells with different labeling degree; bar = 60 μm. G: A MyoD-positive mitotic cell: MyoD labeling is present in the cytoplasm, whereas condensed chromosomes are not labeled; bar = 50 μm. H: A MyoD-positive myotube; bar = 100 μm. I: MyoD-positive myotubes with differently labeled nuclei (→); bar = 100 μm. L: MyoD-positive cell in the early differentiation time with MyoD labeling concentrated in the nucleus; in the nucleus MyoD labeling is localized in interchromatin domains (*) and nucleolus is MyoD-negative (inset). Bar = 1 μm; inset: bar = 0.5 μm. M: A MyoD-positive myotube in the late differentiation time with MyoD labeling concentrated in the nuclei; in the nuclei MyoD labeling is localized in interchromatin domains (*) and nucleoli are MyoD-negative (n, inset). Bar = 1 μm; inset: bar = 0.25 μm.

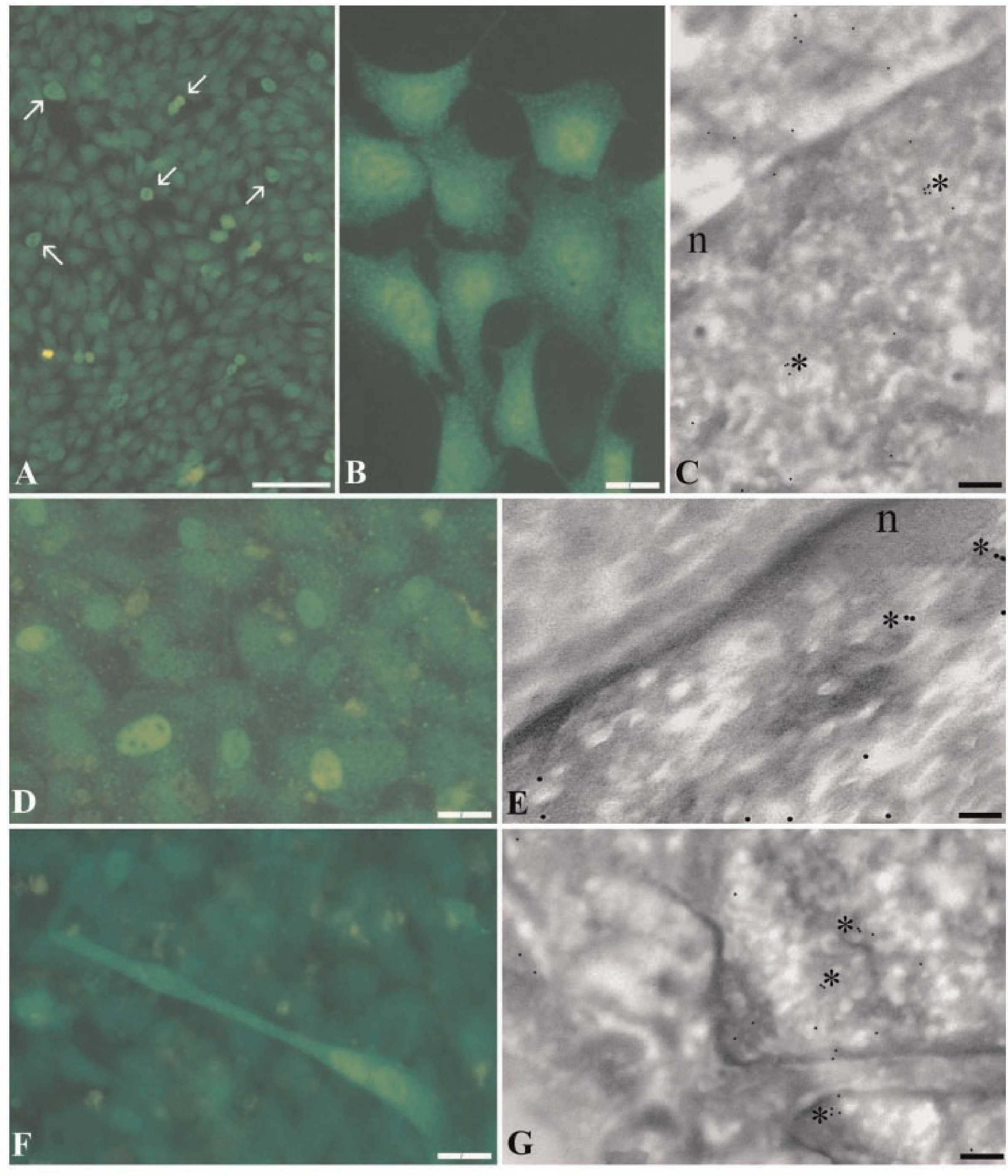

Fig. 3. Myf5 staining. A,B,D,F: Fluorescence microscopy. C,E,G: Electron microscopy. A: Myf5-positive undifferentiated cells, including proliferating myoblasts (→) more intensely labeled than surrounding cells, at low magnification; bar = 700 μm. B: Myf5-positive undifferentiated cells with Myf5 labeling both in the cytoplasm and in the nucleus; bar = 25 μm. C: Undifferentiated cell with Myf5 labeling both in the cytoplasm and in the nucleus; in the nucleus Myf5 labeling is localized in interchromatin domains (*) and nucleolus (n) is Myf5-negative; bar = 0.3 μm. D: Myf5-positive cells in the middle differentiation time with Myf5 labeling in the cytoplasm but especially in the nucleus; bar = 25 μm. E: Myf5-positive cell in the middle differentiation time with Myf5 labeling especially concentrated in the nucleus; in the nucleus Myf5 labeling is localized in interchromatin domains (*) and nucleolus (n) is Myf5-negative. Bar = 0.15 μm. F: Early myotube with Myf5 labeling in the cytoplasm but especially in the nuclei; bar = 25 μm. G: Myf5-positive cell in the late differentiation time with Myf5 labeling in the cytoplasm but especially in the nucleus; in the nucleus Myf5 labeling is localized in interchromatin domains (*). Bar = 0.3 μm.

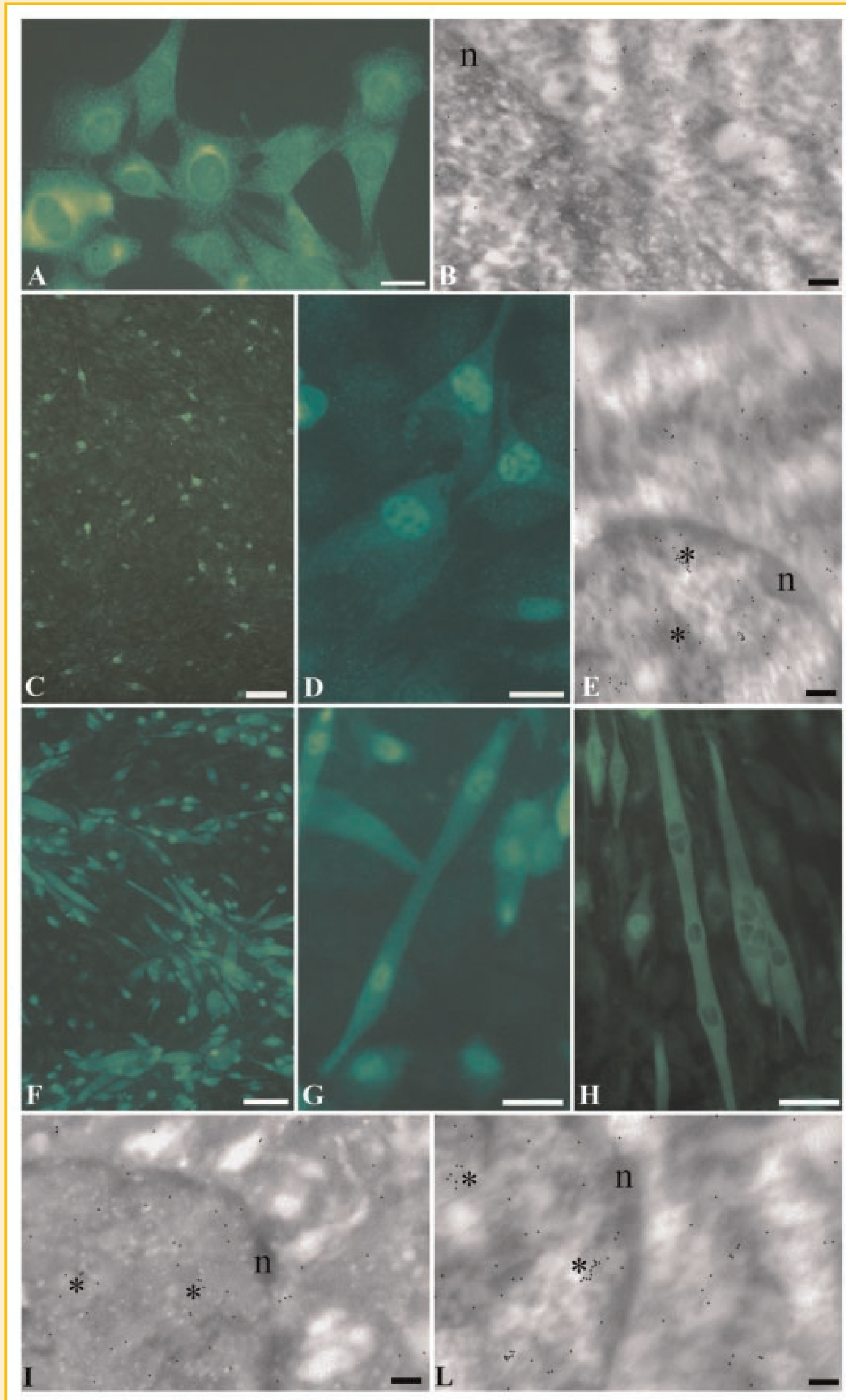

Fig. 4. Myogenin staining. A,C,D,F,G,H: Fluorescence microscopy. B,E,I,L: Electron microscopy. A: Myogenin-positive undifferentiated cells with myogenin labeling in the nucleus but mainly in the cytoplasm with a perinuclear distribution; bar = 25 μm. B: Myogenin-positive undifferentiated cell with myogenin labeling in the nucleus but mainly in the cytoplasm with a perinuclear distribution; nucleolus (n) is myogenin-negative. Bar = 0.2 μm. C: C2C12 in the early differentiation time: myogenin-positive cells with myogenin labeling especially concentrated in the nucleus are present, at low magnification; bar = 500 μm. D: Myogenin-positive cells in the early differentiation time with myogenin labeling especially concentrated in the nucleus; bar = 25 μm. E: Myogenin-positive cell in the early differentiation time with myogenin labeling especially concentrated in the nucleus; in the nucleus myogenin labeling is localized in interchromatin domains (*) and nucleolus (n) is myogenin-negative. Bar = 0.2 μm. F: C2C12 in the late differentiation time: myogenin-positive myotubes and myogenin-positive cells with myogenin labeling especially concentrated in the nucleus and myogenin-negative cells are present, at low magnification; bar = 500 μm. G: myogenin-positive myotubes in the late differentiation time with myogenin labeling in the cytoplasm but especially in the nuclei; myogenin-positive cells with myogenin labeling especially concentrated in the nucleus and myogenin-negative cells are also present. Bar = 25 μm. H: myogenin-positive myotubes in the late differentiation time with myogenin labeling in the cytoplasm but with myogenin-negative nuclei; myogenin-positive cells with myogenin labeling especially concentrated in the nucleus and myogenin-negative cells are also present. Bar = 25 μm. I: myogenin-positive cell in the middle differentiation time with myogenin labeling in the cytoplasm but especially in the nucleus; in the nucleus myogenin labeling is localized in interchromatin domains (*) and nucleolus (n) is myogenin-negative. Bar = 0.2 μm. L: myogenin-positive cell in the late differentiation time with myogenin labeling in the cytoplasm but especially in the nucleus; in the nucleus myogenin labeling is localized in interchromatin domains (*) and nucleolus (n) is myogenin-negative. Bar = 0.2 μm.

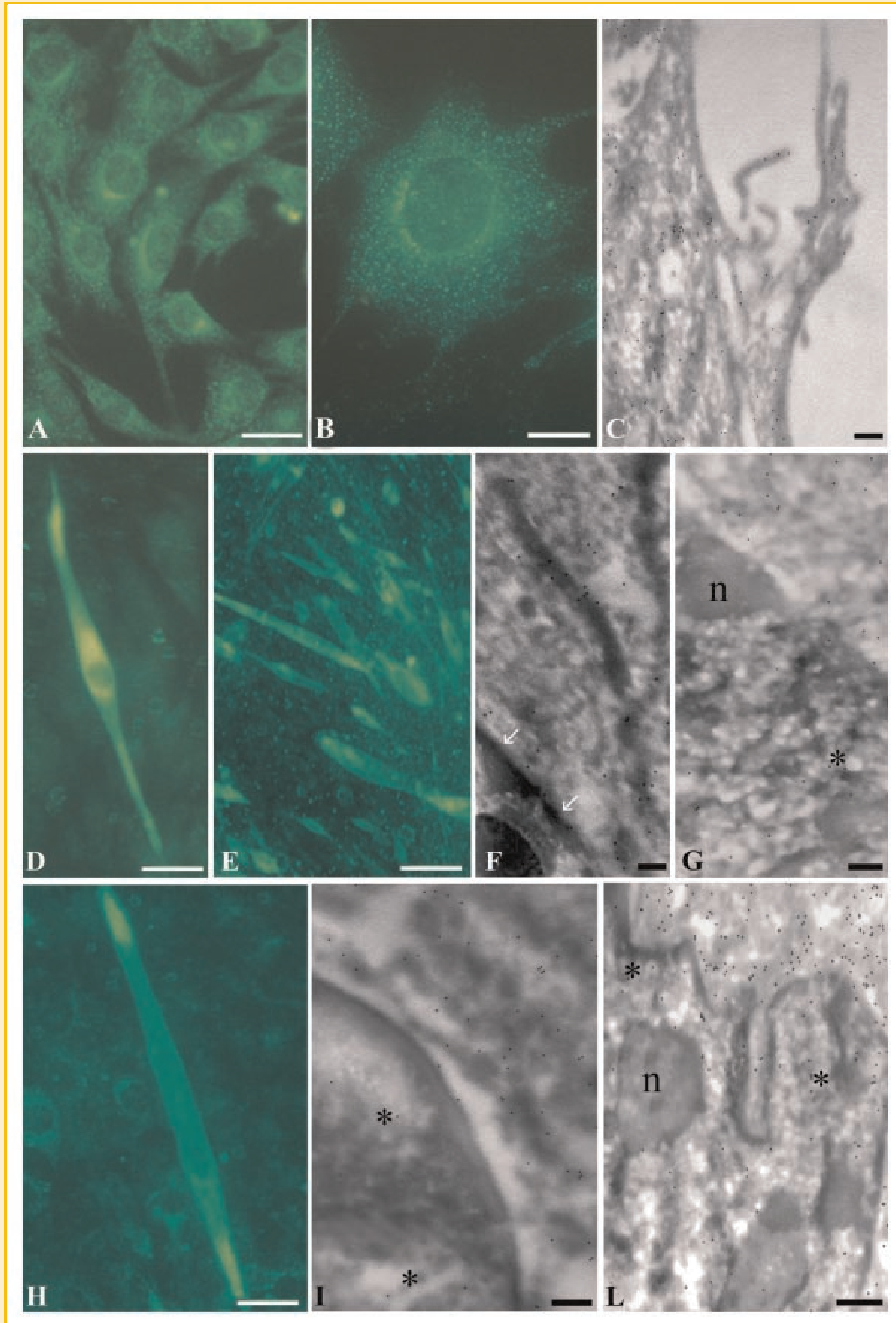

Fig. 5. MRF4 staining. A,B,D,E,H: Fluorescence microscopy. C,F,G,I,L: Electron microscopy. A: MRF4-positive undifferentiated cells with MRF4 labeling in the nucleus but mainly in the cytoplasm with a perinuclear distribution; bar = 25 μm. B: A MRF4-positive undifferentiated cell with MRF4 labeling in the nucleus but mainly in the cytoplasm with a perinuclear distribution, at high magnification; bar = 5 μm. C: MRF4-positive cell filopodia in undifferentiated cells; bar = 0.2 μm. D: An elongated mononucleated cell in the early differentiation time with MRF4 labeling mainly concentrated in the nucleus with a perinuclear distribution; bar = 25 μm. E: C2C12 in the middle differentiation time: mononucleated cells and early myotubes with MRF4 labeling in the nucleus but mainly in the cytoplasm, at low magnification; bar = 25 μm. F: MRF4-positive cell in the early differentiation time with MRF4 labeling in the nucleus (nuclear envelope: →) but mainly in the cytoplasm; bar = 0.2 μm. G: MRF4-positive cell in the middle differentiation time with MRF4 labeling in the nucleus but mainly in the cytoplasm; in the nucleus MRF4 labeling is localized in interchromatin domains (*) and nucleolus (n) is MRF4-negative. Bar = 0.2 μm. H: MRF4-positive myotube in the late differentiation time with MRF4 labeling in the nuclei but especially in the cytoplasm; weakly MRF4-positive cells are also present. Bar = 25 μm. I: MRF4-positive cell in the late differentiation time with MRF4 labeling in the nucleus but mainly in the cytoplasm; in the nucleus MRF4 labeling is localized in interchromatin domains (*). Bar = 0.2 μm. L: MRF4-positive cell in the late differentiation time with MRF4 labeling in the nucleus but mainly in the cytoplasm; in the nucleus MRF4 labeling is localized in interchromatin domains (*) and nucleoli (n) are MRF4-negative. Bar = 0.5 μm.

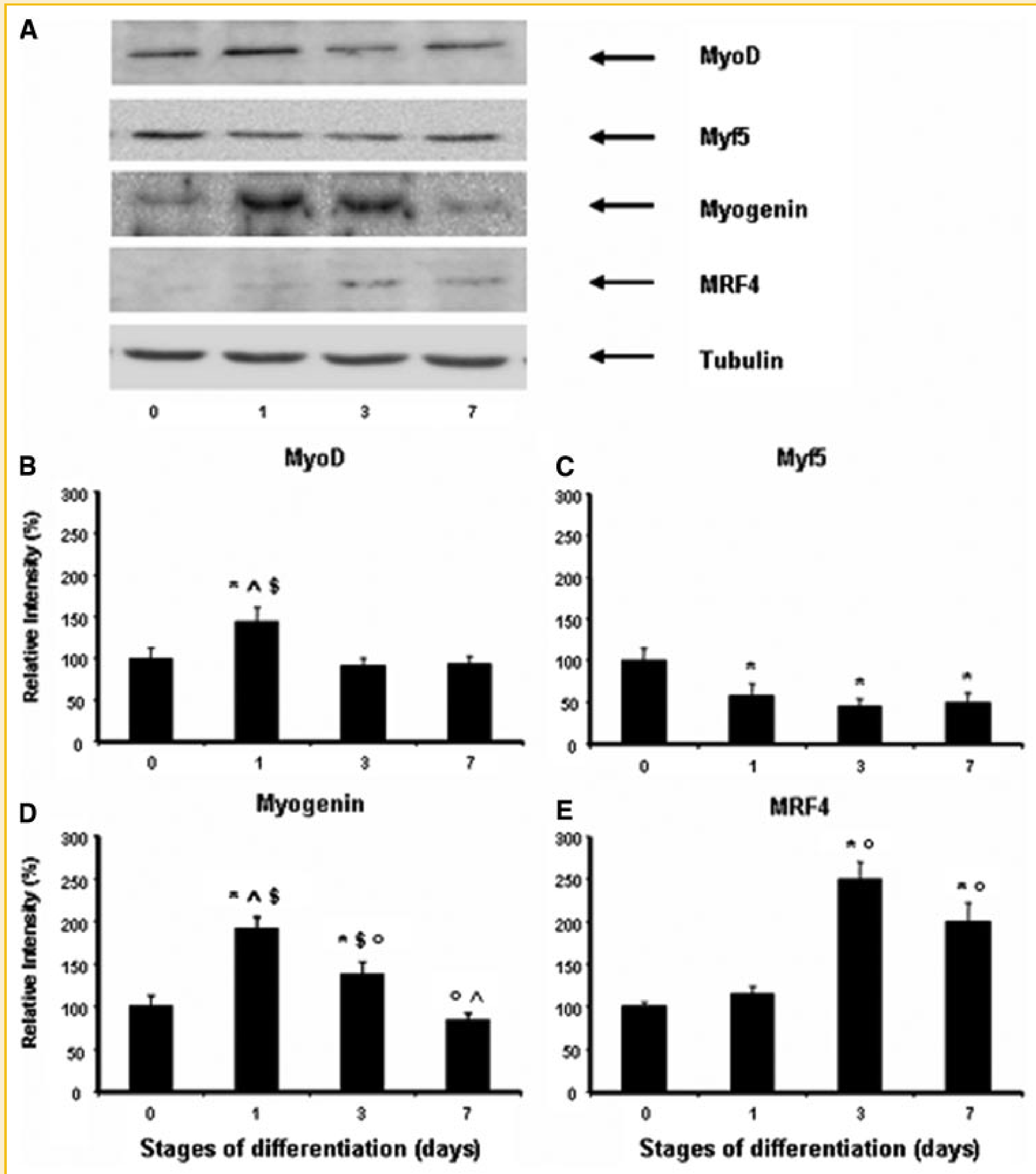

Fig. 6. Western blotting analysis. A: Western blotting of MyoD, Myf5, myogenin, MRF4 and tubulin protein levels in C2C12 during the differentiation course. Anti-tubulin blots are shown as loading controls. Bands were detected using enhanced chemioluminescence reagents (see Materials and Methods Section). Results are representative of three independent experiments. B: densitometric analysis of blots of MyoD during C2C12 differentiation course (ANOVA test: $P < 0.05$; Tukey's test: 1 vs. 0, $P < 0.05$; 1 vs. 3, $P < 0.05$; 1 vs. 7, $P < 0.05$; 0 vs. 3, $P > 0.05$; 0 vs. 7, $P > 0.05$; 3 vs. 7, $P > 0.05$). C: densitometric analysis of blots of Myf5 during C2C12 differentiation course (ANOVA test: $P < 0.05$; Tukey's test: 1 vs. 0, $P < 0.05$; 1 vs. 3, $P > 0.05$; 1 vs. 7, $P > 0.05$; 0 vs. 3, $P < 0.05$; 0 vs. 7, $P < 0.05$; 3 vs. 7, $P > 0.05$). D: densitometric analysis of blots of myogenin during C2C12 differentiation course (ANOVA test: $P < 0.05$; Tukey's test: 1 vs. 0, $P < 0.05$; 1 vs. 3, $P < 0.05$; 1 vs. 7, $P < 0.05$; 0 vs. 3, $P < 0.05$; 0 vs. 7, $P > 0.05$; 3 vs. 7, $P < 0.05$). E: densitometric analysis of blots of MRF4 during C2C12 differentiation course (ANOVA test: $P < 0.05$; Tukey's test: 1 vs. 0, $P > 0.05$; 1 vs. 3, $P < 0.05$; 1 vs. 7, $P < 0.05$; 0 vs. 3, $P < 0.05$; 0 vs. 7, $P < 0.05$; 3 vs. 7, $P > 0.05$); Tukey's test: $P < 0.05$: * vs. 0; $^{\circ}$ vs. 1; ^ vs. 3; § vs. 7. Protein levels are normalized on tubulin expression in the same lane.

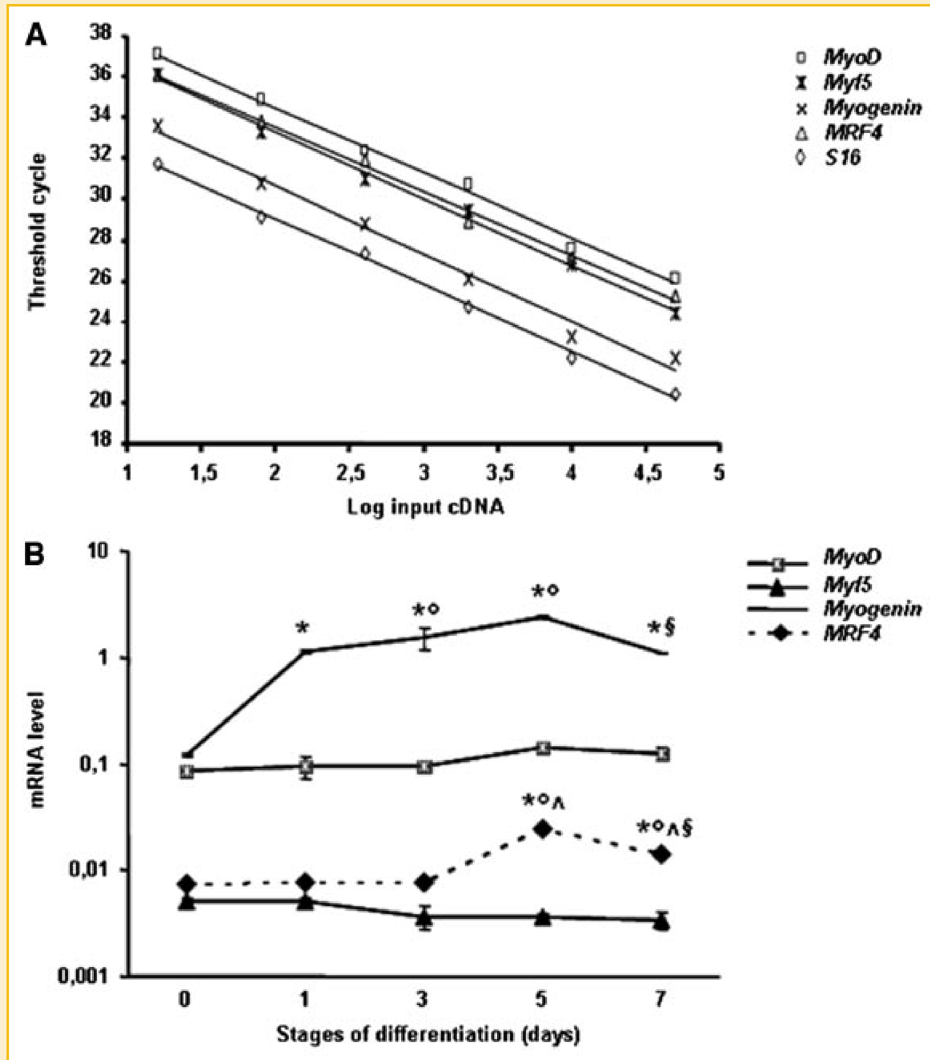

Fig. 7. PCR real-time analysis. A: Determination of the efficiency reaction for each mRNA level of target gene. B: Time course change of mRNA level of MyoD, Myf5, myogenin and MRF4 during myoblast differentiation (For MyoD mRNA, ANOVA test: $P > 0.05$; for Myf5 mRNA, ANOVA test: $P > 0.05$; for myogenin mRNA, ANOVA test: $P < 0.05$; Tukey's test: 1 vs. 0, $P < 0.05$; 1 vs. 3, $P < 0.05$; 1 vs. 5, $P < 0.05$; 1 vs. 7, $P > 0.05$; 0 vs. 3, $P < 0.05$; 0 vs. 5, $P < 0.05$; 0 vs. 7, $P < 0.05$; 3 vs. 5, $P > 0.05$; 3 vs. 7, $P > 0.05$; 5 vs. 7, $P < 0.05$; for MRF4 mRNA, ANOVA test: $P < 0.05$; Tukey's test: 1 vs. 0, $P > 0.05$; 1 vs. 3, $P > 0.05$; 1 vs. 5, $P < 0.05$; 1 vs. 7, $P < 0.05$; 0 vs. 3, $P > 0.05$; 0 vs. 5, $P < 0.05$; 0 vs. 7, $P < 0.05$; 3 vs. 5, $P < 0.05$; 3 vs. 7, $P < 0.05$; 5 vs. 7, $P < 0.05$; * vs. 0; $^{\circ}$ vs. 1; $^{\wedge}$ vs. 3; § vs. 5). The expression level of target genes are related to S16 mRNA gene level.

and reached the highest level in the middle and late differentiation time (ANOVA test: $P < 0.05$; Tukey's test: 1 vs. 0, $P > 0.05$; 1 vs. 3, $P < 0.05$; 1 vs. 7, $P < 0.05$; 0 vs. 3, $P < 0.05$; 0 vs. 7, $P < 0.05$; 3 vs. 7, $P > 0.05$) (Fig. 6A,E).

REAL-TIME RT-PCR

The mRNA of the four MRFs analyzed by real-time RT-PCR was detectable and quantifiable at each point investigated. The efficiency reaction for each set of primers was near-equal to 100% (Fig. 7A).

The MyoD mRNA was expressed in undifferentiated cells at T0. The level of MyoD mRNA did not change significantly after differentiation induction remaining stable during the myotube formation and the late differentiation time (ANOVA test: $P > 0.05$) (Fig. 7B).

The molecular analysis of *Myf5* gene showed the presence of its transcript since the undifferentiated stage (T0). Although Myf5 mRNA did not change significantly (ANOVA test: $P > 0.05$) and its expression profile was essentially constant during differentiation process, at the final differentiation time its mRNA level was decreased of 25% with respect to the initial level (Fig. 7B).

The myogenin mRNA was already expressed at the undifferentiated time (T0). Twenty-four h after differentiation induction, the level of myogenin mRNA significantly increased up to two orders of magnitude at the intermediate maturation period, and then decreased at the final stage (ANOVA test: $P < 0.05$; Tukey's test: 1 vs. 0, $P < 0.05$; 1 vs. 3, $P < 0.05$; 1 vs. 5, $P < 0.05$; 1 vs. 7, $P > 0.05$; 0 vs. 3, $P < 0.05$; 0 vs. 5, $P < 0.05$; 0 vs. 7, $P < 0.05$; 3 vs. 5, $P > 0.05$; 3 vs. 7, $P > 0.05$; 5 vs. 7, $P < 0.05$) (Fig. 7B).

The MRF4 mRNA was already present in undifferentiated cells (T0) and its level remained stable during the first steps of differentiation. Nevertheless, in the middle differentiation time MRF4 mRNA significantly increased its expression, to significantly decrease at the final differentiation stage (ANOVA test: $P < 0.05$; Tukey's test: 1 vs. 0, $P > 0.05$; 1 vs. 3, $P > 0.05$; 1 vs. 5, $P < 0.05$; 1 vs. 7, $P < 0.05$; 0 vs. 3, $P > 0.05$; 0 vs. 5, $P < 0.05$; 0 vs. 7, $P < 0.05$; 3 vs. 5, $P < 0.05$; 3 vs. 7, $P < 0.05$; 5 vs. 7, $P < 0.05$) (Fig. 7B).

DISCUSSION

Muscle differentiation is a complex process which involves the combinatorial action of a relatively small number of transcription factors acting both temporally and spatially in a coordinated manner to generate a high degree of diversity. Although a great deal has been elucidated concerning the mechanisms regulating muscle differentiation, little is known about transcription factor-specific regulation and few studies have investigated the relationship between MRFs protein expression levels and their subcellular distribution. In order to gain insights about MRFs functional regulation by post-transcriptional and post-translational events, we combined biochemical and morphological approaches. Indeed, Western blotting is a technique which allows to show quantitative changes of proteins at different time points of differentiation, however it does not provide any information about either cell type or compartments which express the MRFs. Therefore, we coupled Western blotting with immunocytochemistry in order to analyze MRFs behavior in situ.

It is commonly accepted that MyoD and Myf5 are already highly expressed in undifferentiated myogenic cells before the differentiation program is activated, whereas myogenin and MRF4 are late-acting factors, expressed during the middle and late differentiation [Dedieu et al., 2002; Pownall et al., 2002; Cao et al., 2006].

MyoD EXPRESSION AND SUBCELLULAR LOCALIZATION

Our results demonstrate a constant level of MyoD mRNA along the differentiation process, as previously reported [Shimokawa et al., 1998; Tomczak et al., 2004]. Accordingly with previous findings [Puri and Sartorelli, 2000], Western blotting analysis shows that MyoD protein is also expressed both in the undifferentiated time and in the differentiated one. Indeed, MyoD acts to negatively regulate the transcription of some genes in myoblasts before differentiation induction and prior to initiating chromatine remodeling, whereas it switches from its association with repressive factors to positively regulate gene expression, when muscle differentiation is initiated [Tapscott, 2005]. Therefore, along differentiation, MyoD is active throughout the entire process of muscle gene expression, binding directly to the regulatory elements of genes expressed early and late during the differentiation program [Blais et al., 2005], as confirmed by MyoD-positive nucleus appearance also in myotubes. Indeed, it is known that during the differentiation progression, MyoD controls the expression of early genes, encoding adhesion and extracellular matrix molecules, of intermediate genes, including transcription factors, and of the latest genes, comprehending the myofibril and cytoskeletal proteins [Tapscott, 2005]. Consistently, previous findings reported that the expression of MyoD in differentiating C2C12 cells defines two populations: the first includes myoblasts and myotubes in which MyoD is highly expressed and the other is represented by the quiescent "reserve" cells where it appears to be down-regulated [Yoshida et al., 1998].

Our results show that, in each differentiation stage, MyoD is localized in the nucleus, accordingly with previous data [Sun et al., 2005], whereas it is not detectable in the cytoplasm, suggesting that, after its synthesis, it is rapidly transported across the nuclear envelope and degraded.

Consistently with its role as transcription factor, nuclear MyoD labeling is heterogeneous. Indeed, MyoD labeling pattern resembles to the hyperphosphorylated large subunit of RNA polymerase II immunofluorescence labeling one, which detects the "active" transcriptional sites [Stenoien et al., 1998]. Our electron microscopy results confirm these data revealing that MyoD labeling, like other MRFs labeling pattern in the nucleus, is concentrated in interchromatin domains, where transcriptional activity takes place. In particular, it is able to initiate chromatine remodeling at binding sites in muscle gene enhancers and then to activate transcription activity at previously silent loci, acting as a "master switch" gene, converting cells of many different lineages and differentiation stages to skeletal muscle cells [Tapscott, 2005].

Notably, our results describe MyoD expression in myoblast cytoplasm during mitosis. Indeed, a stable and inactive MyoD mitotic form following the phosphorylation by Cdk2, which is required for the release from condensed chromosomes and the inhibition of its DNA-binding, has been described [Batonnet-Pichon et al., 2006]. The MyoD phosphorylation by Cdk2 is a crucial event to allow G2/M transition during cell cycle progression [Batonnet-Pichon et al., 2006]. Subsequently, MyoD transcriptional activity reaugments in the beginning of G1 phase thanks for dephosphorylation events [Batonnet-Pichon et al., 2006]. Accordingly, Kitzmann et al. [1998] described that MyoD is absent in G0, peaks in G1, falls to its minimum level at G1/S transition and reaugments from S to M transition [Kitzmann et al., 1998]. Then, similarly to Myf5, MyoD expression is regulated in myoblasts by phosphorylation events related to cell cycle progression, which may be responsible for its degradation in proliferating cells and may induce its rapid turnover in undifferentiated myoblasts, preventing its accumulation which in turn could stop the cell cycle [Kitzmann et al., 1999]. Therefore, it has been proposed that MyoD phosphorylation may be linked to the control of MyoD activity by modulation of its half-life [Puri and Sartorelli, 2000]. In addition, although MyoD mRNA doesn't significantly change after differentiation induction, Western blotting analysis shows that MyoD protein significantly increases in the early differentiation time, as also confirmed by the increase in MyoD-positive cell number detected by the immunofluorescence assay. This result is consistent with the MyoD role in the differentiation induction involving the cell cycle withdrawal and the muscle-specific gene expression [Ishibashi et al., 2005] and suggests a MyoD protein turnover regulation by multiple degradation pathways [Batonnet et al., 2004]. Indeed, besides the cell cycle progression-related degradation [Batonnet-Pichon et al., 2006], recent findings demonstrated that MyoD half-life is also regulated by the lysine 133, which plays a

crucial role in the ubiquitination and degradation of MyoD in the nucleus [Batonnet et al., 2004].

Interestingly, MyoD-positive cells with a different nuclear labeling degree can be observed in each differentiation stage. These data are consistent with a different transcriptional activity pattern, which in turn may be related to a MyoD protein level regulation dictated by relative rates of differentiation stage-dependent protein synthesis [Sun et al., 2005] and degradation, as already described for MyoD itself [Lingbeck et al., 2005] and other myogenic transcription factors, such as the E2A and Id proteins [Sun et al., 2007]. The detection of different degree of MyoD labeling can also be observed among the nuclei of the same myotube, where it is suggestive of the presence of myonuclear domains [Gundersen and Bruusgaard, 2008], characterized by a specific gene expression and behavior [Rosser et al., 2002; Gundersen and Bruusgaard, 2008].

Myf5 EXPRESSION AND SUBCELLULAR LOCALIZATION

According to previous data [Shimokawa et al., 1998], our results show a constant level of Myf5 mRNA, which does not significantly change along differentiation, although it decreases in the middle and late differentiation. Nevertheless, Western blotting analysis shows a significant decrease in Myf5 protein expression after differentiation induction, consistent with the in vivo data reporting that Myf5 is the earliest of the factors expressed in the mouse embryo but its expression, which is required to maintain the determined state of muscle precursor cells, is transient [Lindon et al., 1998].

In contrast to MyoD, Western blotting analysis shows that Myf5 protein significantly decreases after differentiation induction and then it keeps constant during the late differentiation time. The MyoD and Myf5 different behavior is consistent with the Myf5 expression inhibition by MyoD [Rudnicki et al., 1992] and reflects the Myf5 ability to promote myoblast proliferation contrarily to the MyoD role in cell cycle exit [Kitzmann and Fernandez, 2001; Kataoka et al., 2003; Ishibashi et al., 2005]. In fact, whereas Myf5 mRNA level is not affected by the cell cycle, our results describe a Myf5 protein expression downregulation by the cell cycle progression according to previous findings reporting Myf5 phosphorylation by mitosis-specific kinases and its proteolytic degradation by cell cycle-associated events [Lindon et al., 1998; Kitzmann and Fernandez, 2001; Doucet et al., 2005]. This is consistent with the Myf5 protein significant decrease following serum removal, when serum proliferative signals cease [Yoshida et al., 1998]. Although mitotic myoblast cells are Myf5-positive, Kitzmann et al. [1998] described a Myf5 distinct and contrasting expression profile during cell cycle progression with respect to MyoD: indeed, Myf5 protein is high in G0, decreases during G1 and reappears at the end of G1 to remain stable until mitosis, during which it is subject to mitotic degradation by proteolytic events under the control of cell cycle apparatus [Kitzmann and Fernandez, 2001; Doucet et al., 2005]. Therefore, the opposite cell cycle-specific expression patterns of MyoD and Myf5 may be related to the maintenance of the proliferative status and/or the commitment to the myogenic lineage [Doucet et al., 2005].

Taking into account the Myf5 protein short half-life [Lindon et al., 1998], Myf5 mRNA and protein levels, detected in the late differentiation phase, may reflect the presence of residual myoblasts in the culture [Kitzmann et al., 1998; Lindon et al., 1998], identified as quiescent "reserve cells" [Yoshida et al., 1998] and may be related to the proposal Myf5 role in myoblast fusion [Dedieu et al., 2002]. Accordingly, our results describe the presence of Myf5 staining also in early myotubes, as previously described [Lindon et al., 1998].

Immunocytochemistry results demonstrate that, in undifferentiated cells, Myf5 is highly expressed both in the nucleus and in the cytoplasm. After differentiation induction, when the Myf5 protein level significantly decreases, it remains still present in the cytoplasm but it progressively concentrates and enters the nucleus. This relocation may be related to its transcriptional activity, which might underlie its ability to repress the transcription of muscle gene related to the cell cycle progression and differentiation onset, as previously proposed [Lindon et al., 1998].

The Myf5 nuclear distribution, which reflects the Myf5 transcriptional activity, closely resembles to MyoD and other MRFs labeling one in the nucleus. In particular, like MyoD, Myf5 is able to recruit histone-modifying and chromatin remodeling enzymes [Tapscott, 2005]. However, although MyoD and Myf5 have been considered genetically redundant for a long time because they both are expressed in the early phase of differentiation [Rudnicki et al., 1992, 1993; Braun and Arnold, 1996], they are expressed in different muscle precursor cells [Braun and Arnold, 1996]; accordingly, the codetection of Myf5 and MyoD identifies two cell populations including MyoD-positive cells which will undergo the fusion into myotubes and Myf5-positive cells, representing quiescent satellite cells, which will fail to differentiate [Kitzmann et al., 1998; Lindon et al., 1998; Yoshida et al., 1998; Kitzmann and Fernandez, 2001].

MYOGENIN EXPRESSION AND SUBCELLULAR LOCALIZATION

According to previous data [Shimokawa et al., 1998; Delgado et al., 2003], our results describe a significant increase in myogenin mRNA after differentiation induction reaching the highest level in the middle differentiation time. Western blotting analysis reveals a corresponding significant increase in myogenin protein soon after differentiation induction followed by a decrease in the middle and late differentiation time. Therefore, both myogenin transcript and protein significantly increase after serum removal, when cell proliferative activity arrests, as previously described [Edmondson et al., 1991]. However, our results report that myogenin mRNA reaches the highest level in the middle differentiation time, whereas myogenin protein begins to decrease. Although the possibility of an accumulation of unprocessed myogenin transcript has been described during embryogenesis [Sánchez and Robbins, 1994] this apparent discrepancy between myogenin transcript and protein trend may be explained considering that mRNA and protein turnover are differentially regulated. For example, it should be taken into account that myogenin mRNA half-life [Figueroa et al., 2003] is longer than myogenin protein half-life [Edmondson et al., 1991] and myogenin mRNA stability is modulated by the RNA binding protein HuR in a differentiation-dependent manner: therefore, the longest mRNA half-life reached after differentiation induction [Figueroa et al., 2003] might explain the RNA transcript accumulation in the

middle differentiation time, when myogenin protein levels begin to significantly decrease. In addition, myogenin protein half-life might be regulated by the relative rates of differentiation stage-dependent protein synthesis and degradation. This regulation mechanism could affect both myogenin itself, as already described for MyoD [Lingbeck et al., 2005; Sun et al., 2007], and/or other myogenic factors able to modulate myogenin half-life, such as MyoD or Id proteins [Viñals and Ventura, 2004; Sun et al., 2005]. In particular, it should be considered that myogenin expression is enhanced by MyoD [Thayer et al., 1989], whereas it is down-regulated by MRF4 [Zhang et al., 1995].

Western blotting analysis shows a myogenin protein pattern very similar to MyoD one; this behavior reflects that MyoD and myogenin are subjected to a positive autoregulatory loop [Thayer et al., 1989]. These data are consistent with myogenin expression induction by MyoD [Thayer et al., 1989] and with myogenin appearance simultaneously or soon after Myf5 downregulation [Lindon et al., 1998], which in turn is induced by MyoD [Rudnicki et al., 1992]. Consistently, the establishment of a positive autoregulatory loop between MyoD and myogenin may be crucial to either initiate activation of the myogenic program and/or secure terminal differentiation. Therefore, the findings reporting that both MyoD and myogenin proteins have short half-lives may be relevant to explain their auto- and cross-regulation and be interpreted as a means that the two proteins have devised to finely regulate their own expression [Edmondson et al., 1991; Puri and Sartorelli, 2000]. Moreover, this hypothesis supports the findings reporting that MyoD and myogenin control similar set of target genes but have distinct regulatory roles and that the role of myogenin in the terminal differentiation is, at least in part, to enhance expression of a subset of genes previously initiated by MyoD, since myogenin does not efficiently bind to DNA without MyoD [Cao et al., 2006].

Therefore, the rapid induction of myogenin expression and its fast increase after serum removal confirm that myogenin is a pivotal factor in inducing myoblast differentiation in C2C12 cells [Delgado et al., 2003; Figueroa et al., 2003; Blais et al., 2005]. Indeed, previous data demonstrated that, both in vitro and in vivo, myogenin expression precedes terminal differentiation and controls the synthesis of proteins making up the contractile apparatus [Sánchez and Robbins, 1994; Blais et al., 2005].

Myogenin is already expressed in undifferentiated cells, where it is especially detected in cytoplasm. After differentiation induction myogenin translocates into the nucleus. The shuttling between the nucleus and the cytoplasm is a feature already described for other myogenic transcription factors, such as I-mfa and HDACs [Chen et al., 1996; Miska et al., 2001]. Cytoplasmic retention is a mechanism to regulate the biological activity of a protein, as revealed by the well-known example provided by NFkB [Chen et al., 1996]. The cytoplasmic retention of myogenin before differentiation induction might be necessary for a correct regulation of C2C12 myoblast differentiation, suggesting that myogenin might be held in the cytoplasm to prevent the differentiation from beginning and it might enter the nucleus when proliferative signals cease and its protein level significantly increases [Edmondson et al., 1991]. Once myogenin enters the nucleus, it may exert its regulatory transcriptional activity to a subset of myogenic promoters and thus establish a fully differentiated state, consistent with its prevalent localization in the nuclei rather than in the cytoplasm of myotubes. In our experimental model, also myotubes with myogenin-negative nuclei can be observed: these findings might be related to terminally differentiated myotubes expressing high levels of MRF4 protein, which significantly increases after the myogenin highest level and inhibits myogenin expression [Zhang et al., 1995].

MRF4 EXPRESSION AND SUBCELLULAR LOCALIZATION

Our results display a significant increase in MRF4 mRNA in the middle and late differentiation time revealing a MRF4 mRNA behavior perfectly consistent with MRF4 protein expression level. Indeed, Western blotting analysis demonstrates that MRF4 protein expression significantly increases in the middle and late differentiation, when myoblast fusion has already occurred, confirming its role in the myotube maturation [Zhang et al., 1995].

In each differentiation phase, MRF4 protein is mainly concentrated in the cytoplasm with respect to the nucleus. In particular, both immunofluorescence and immunogold electron microscopy reveal a protein distribution especially in a perinuclear area, where protein synthesis mostly occurs. As previously hypothesized for myogenin, MRF4 predominantly cytoplasmic retention might be required to allow maturation of myotubes to take place. Accordingly, in myotubes myogenin and MRF4 localizations appear to change reciprocally: this might suggests that myogenin and MRF4 regulate a distinct subset of target genes involved in myotube formation and maturation. Western blotting analysis confirms that MRF4 protein significantly increases when myogenin protein is downregulated, consistently with myogenin expression inhibition by MRF4 [Zhang et al., 1995]. Moreover, although MRF4 functions as a positive transcriptional regulator involved in the myotube maturation during terminal differentiation, the MRF4 protein is subject to a negative regulation because of the phosphorylation by PKA and PKC both in vivo and in vitro [Puri and Sartorelli, 2000], which can justifies its preferentially cytoplasmic localization. In addition, more recently it has been demonstrated that MRF4 activity inhibition following the phosphorylation by the differentiation-dependent p38 MAPK is responsible for the transcriptional activity repression of a specific subset of genes during late stages of muscle differentiation [Suelves et al., 2004].

The PCR real-time quantitative analysis shows that the expression level of MRF4 mRNA is comparable to the one of Myf5. Indeed, MRF4 gene is linked to Myf5 in the same locus and a recent analysis of an allelic series of mutants in the MRF4-Myf5 locus evidences that both genes act as determination factors at the onset of myogenesis differentiation [Chang et al., 2004], suggesting that their comparable expression level could be explained by an event of co-regulation. These data support a recent hypothesis revising the epistatic relationship of the MRFs and proposing MRF4 as a determination gene: MyoD, Myf5, and MRF4 define the muscle identity of multipotent progenitor cells and then MyoD, myogenin and MRF4 sustain the muscle differentiation process [Kassar-Duchossoy et al., 2004].

CONCLUSIONS

The study of MRFs activity regulation may have important implications in understanding the mechanisms underlying several myopathies and regulating muscle growth and tissue regeneration. In fact, the MRFs behavior during muscle differentiation highlights that the irreversible withdrawal from the cell cycle is a prerequisite for further expression of tissue specific genes. In addition, the property of terminally differentiated myofiber nuclei to not reactivate the DNA synthesis in response to mitogenic stimuli explains the lack of a regenerative response. Finally, it has been reported that the impairment of MRFs transcriptional activity is involved in age-related skeletal muscle dysfunctions [Degens, 2007] and that MRFs are selectively expressed in human congenital myopathies [Weise et al., 2006] and different muscular disorders, such as Duchenne and Becker muscular dystrophies and polymyositis [Olivé et al., 1997].

Our results confirm that MyoD and Myf5 are involved in myoblast determination, whereas myogenin and MRF4 are required later during differentiation. In addition, our data providing a description of the MRFs subcellular localization during C2C12 differentiation stages, suggest that besides the MRFs protein expression level, also the MRFs subcellular localization plays a key role in muscle differentiation, being related to their functional activity, as reported for other myogenic transcription factors [Chen et al., 1996; Miska et al., 2001]. In particular, these results demonstrate that each MRF shows a specific behavior in situ and they reveal a MyoD and Myf5 contrasting expression profile in proliferating myoblasts, as well as a myogenin and MRF4 opposite distribution in myotubes and mononucleated cells in the late differentiation time. Interestingly, MRFs expression level and subcellular localization analysis during C2C12 differentiation stages highlights two main MRFs activity modulation mechanisms: (i) the MRFs protein half-life regulation dictated by the relative rates of differentiation stage-dependent protein synthesis and degradation and (ii) the MRFs functional control by the cytoplasmic retention, as a translocation process, in order to inhibit their transcriptional activity. Therefore, taking into account that the MRFs relocation during the differentiation phases may be due to their different rate of nuclear import and export, our results exhibit that the MRFs nucleo-cytoplasmic trafficking is involved in the control of muscle differentiation and they allow us to propose a role of MRFs subcellular localization as a regulatory step in transcriptional control mechanisms.

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