Serum Response Factor p67SRF Is Expressed and Required during Myogenic Differentiation of Both Mouse C2 and Rat L6 Muscle Cell Lines

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Abstract. The 67-kD serum response factor (p67SRF) is a ubiquitous nuclear transcription factor that acts by direct binding to a consensus DNA sequence, the serum response element (SRE), present in the promoter region of numerous genes. Although p67SRF was initially implicated in the activation of mitogen-stimulated genes, the identification of a sequence similar to SRE, the CArG box motif, competent to interact with SRE binding factors in many muscle-specific genes, has led to speculation that, in addition to its function in cell proliferation, p67SRF may play a role in muscle differentiation. Indirect immunofluorescence using affinity-purified antibodies specifically directed against p67SRF reveals that this factor is constitutively expressed and localized in the nucleus of two skeletal muscle cell lines: rat L6 and mouse C2 myogenic cells during myogenic differentiation. This result was further confirmed through immunoblotting and Northern blot analysis. Furthermore, specific inhibition of p67SRF in vivo through microinjection of purified p67SRF antibodies prevented the myoblast–myotube transition and the expression of muscle-specific genes such as the protein troponin T. We further showed that anti-p67SRF injection also inhibited the expression of the myogenic factor myogenin, implying an early requirement for p67SRF in muscle differentiation. These results demonstrate that p67SRF is involved in the process of skeletal muscle differentiation. The potential action of p67SRF via CArG sequences is discussed.

Skeletal myogenesis constitutes a good model to study biochemical mechanisms regulating cellular differentiation. Exposure of proliferating myoblasts to media lacking mitogen induces withdrawal from the proliferative cell cycle; cells fuse to form polynucleate myotubes. With fusion, terminal differentiation leads to the activation of many muscle-specific genes, including those encoding the sarcomeric proteins such as myosin light and heavy chains, α-actin, troponin I, and troponin T (Nadal-Ginard, 1978; Caravatti et al., 1982).

Various reports have examined the mechanisms governing tissue-specific expression of sarcomeric proteins and led to the identification of potential cis regulatory sequences conserved among many muscle genes and of different trans-acting factors that could interact at these consensus sequences. The muscle-determining factors (MyoD, myogenin, Myf-5, MFR4 [also called Herculin or Myf6]) have recently been identified and bind to a consensus sequence CANNTG (the “E box”) present in the promoter of many muscle-specific genes. Those proteins are members of the family of helix-loop-helix DNA–binding proteins, which includes the oncoprotein c-myc (Braun et al., 1989; Edmonson and Olson, 1989; Wright et al., 1989; Rhodes and Konieczny, 1989; Tapscott et al., 1988; Brennan and Olson, 1990; Braun et al., 1990; Miner and Wold, 1990). Ectopic expression of these factors in nonmyogenic cell lines is sufficient to convert them into muscle-specific lineage. Cotransfection experiments have demonstrated that MyoD and myogenin are able to transactivate muscle-specific genes such as muscle creatine kinase, acetylcholine receptor α-subunit, or α-actin (Lassar et al., 1989; Piette et al., 1990; Yutzey et al., 1990; Wentworth et al., 1991).

In addition to the “E-box,” another DNA regulatory sequence, CC(A/T)6CG, has been identified in a number of muscle-specific genes, including cardiac and skeletal muscle actins (Minty and Kedes, 1986), dystrophin (Klamut et al., 1990), and myosin light chain 1/3 (Ernst et al., 1991). This element, called the CArg box motif, is related to the serum response element (SRE),1 first identified in the upstream region of the c-fos gene and implicated in the induction of c-fos transcription in response to cell stimulation by serum or mitogens (Treisman, 1985). In muscle cells, this consensus sequence has been implicated in the regulation of cardiac and skeletal actin genes from different species, as demonstrated by deletion and mutational analysis (Miwa and

1. Abbreviations used in this paper: p67SRF, 67-kD serum response factor; SRE, serum response element.
Kedes, 1987; Mohun et al., 1989; Chow and Sharwst, 1990).

A ubiquitous 67-kD nuclear factor, termed the serum response factor (p67SRR) interacts with the c-fos SRE and has been isolated as a polypeptide of molecular mass 62–67 kD (Treisman, 1987; Norman et al., 1988). Numerous studies have reported that CARG and SRE can compete for the same binding activity in C2 cells (Gutafson et al., 1988; Phan-Dinh-Tuy et al., 1988) and in FAP2 cells (Taylor et al., 1989). A protein called CBF, expressed in all cell types and immunologically and biochemically indistinguishable from p67SRR, has been shown to bind to the actin CARG boxes (Boxer et al., 1989). Furthermore, Taylor et al. (1989) demonstrated that purified p67SRR from HeLa cells binds to the Xenopus cardiac actin CARG box 1 in vitro, although with an affinity fourfold lower than for the cytoskeletal actin SRE. In addition, several investigations have demonstrated that CARG and SRE are functionally interchangeable, showing that these two motifs could also bind the same factor in vivo (Taylor et al., 1989; Tuil et al., 1990); this factor is probably p67SRR. Other data implicate the binding of specific factors distinct from p67SRR. For example, the existence of two muscle actin factors, MAPF1 and MAPF2, has been described (Walsh and Schimmel, 1987). Each of these factors binds to the actin CARG box sequence in preference to the c-fos SRE. MAPF1 or p62 is the prominent binding activity in nonmuscle cells and embryonic chicken skeletal muscle. MAPF2, a 35-kD protein, is restricted to muscle cells and found in nuclear extracts prepared from different myogenic cell lines such as L6, C2, and SoI8 (Walsh and Schimmel, 1988, 1989; Ernst et al., 1991). More recently, a family of four embryo CARG box binding factors (ECF), which differ from p67SRR, have been identified in Xenopus embryos. One of these, ECF1, seems to be related to MAPF1 and MAPF2 and is the predominant binding activity in cardiac actin-expressing tissues (Taylor, 1991). Nevertheless, other reports have suggested that p67SRR is implicated both in muscle-specific gene expression and serum-responsive transcription in Xenopus embryos (Mohun et al., 1991).

Using specific antibodies against p67SRR, we have examined the presence and implication of this protein during myogenesis. We describe p67SRR mRNA and protein expression during the differentiation of two cell lines—the rat myogenic L6 line and the mouse myogenic cell line C2—revealing both expression and specific nuclear localization of p67SRR in both myoblasts and differentiated myotubes. Moreover, we show that p67SRR is implicated in skeletal muscle differentiation, because anti-p67SRR antibodies microinjected into L6 or C2 cells specifically prevented the myoblast–myotube transition and expression of both the myogenic factor, myogenin, and the skeletal muscle protein, troponin T, a marker of the differentiated state. These results suggest that p67SRR, in addition to its role in cell proliferation, is also implicated in the transmission of the differentiation signal.

Materials and Methods

Cell Culture

The L6G7 subclone was obtained by limited dilution of the myogenic rat L6 line, initially isolated by Yaffe (1968). L6G7 cells and myogenic mouse C2 line (Yaffe and Saxel, 1977) were routinely grown in growth medium (Ham F12/DME [ratio, 2:1] supplemented with 10% FCS and 2 mM glutamine). To induce terminal differentiation, L6 cells were plated at a density of 1 to 5 x 10⁴ cells/cm² on plastic dishes in growth medium. After 2 d, growth medium was replaced with differentiation medium consisting of DME supplemented with 1 μg/ml insulin and 100 μg/ml transferrin. C2 cells were plated at a density of 5 x 10⁵ to 10⁶ cells/cm² on plastic dishes and grown for 2 d before replacing growth medium with DME containing 2% FCS.

From the time of their initial passage into the appropriate differentiation medium, differentiation was effectively complete within 6 d for L6 cells and 3 d for C2 cells. At this time, >60–90% of the cells were differentiated into myotubes as assessed by phase microscope analysis.

Immunofluorescence Microscopy

Cells growing on 35-mm dishes were fixed in 3.7% (wt/vol) formaldehyde in PBS followed by a 30-s extraction in −20°C acetone before rehydration in PBS containing 1% BSA. The cellular distribution of p67SRR protein and either troponin T or myogenin was analyzed simultaneously by indirect immunofluorescence using a rabbit antisera raised against a synthetic peptide derived from the amino acid sequence of p67SRR (anti-p67SRR diluted 1:100; Gauthier-Rouvière et al., 1991) and a mouse monoclonal antibody against troponin T diluted 1:500 (Amersham, Les Ulis, France) or a monoclonal antimyogenin antibody generously provided by W. Wright (University of Dallas, Dallas, TX). Primary antibodies diluted in PBS/BSA were incubated for 1 h at 37°C. After a 15-min wash in PBS, cells were incubated with biotinylated anti–rabbit antibody (Amersham). Staining for p67SRR was revealed by a 30-min incubation with Texas red streptavidin (Amersham) together with affinity-purified fluorescein-conjugated goat anti-mouse antibodies (Cappel, Organon Technica, Paisens, France) to visualize either troponin T or myogenin staining. Stained cells mounted in Airvo 205 (15% Airvo 205, Air Products, Utrecht, the Netherlands, 33% glycerol, 0.1% Na3P, in PBS, pH 7) were observed on a microscope (Axiohot; Carl Zeiss, Inc., Thornwood, NY) using a planapochromat 40× objective. Fluorescent images were recorded onto TriXpan 400 film (Eastman Kodak Co., Rochester, NY) and developed in Diafine (Acufine, Inc., Chicago, IL).

One-Dimensional Gel Electrophoresis and Western Immunoblotting

To analyze the protein expression in C2 or L6 cells before or after differentiation, cells growing on 35- or 60-mm dishes were rinsed twice in PBS and solubilized into Laemmli sample buffer (40 mM Tris-Cl, pH 6.8; 5 mM DTT, 1% SDS, 7.5% glycerol; 0.01% bromophenol blue) by direct addition to the dish. After scraping and boiling, the sample (~10⁶ cells) was loaded on a 12.5% polyacrylamide gel. After electrophoresis, proteins were transferred onto nitrocellulose and blotted with anti-p67SRR or antitroponin T antibodies (used at dilutions of 1:100 and 1:1000, respectively). Primary antibodies were identified with horseradish peroxidase–conjugated anti–rabbit antibody or anti–mouse antibodies and developed with chloronaphthol as previously described (Gauthier-Rouvière et al., 1991).

Northern Blot

Total RNA was extracted using the guanidium isothiocyanate/LiCl procedure of Cathala et al. (1983). Total RNA (20 μg) was fractionated on 1% agarose gels containing 2 M formaldehyde, transferred, and bound to nylon membranes (Hybond; Amersham) as described by the supplier. Filters were hybridized using the following cDNA probes labeled by random priming: human SRF, generously provided by R. Treisman (ICFR, London, UK), mouse myogenin (Edmonson and Olson, 1989), and rat GAPDH (Port et al., 1987). Filters were washed twice for 30 min with 0.2× SSC, 0.1% SDS at 65°C and autoradiographed for 1–5 days at −70°C.

Microinjection

For microinjection studies, cells growing on plastic dishes were microinjected with anti-p67SRR antibodies or, as a control, the anti-p67SRR antibodies preincubated with the immunogen peptide or the preimmune serum. In all cases, injection solutions contained additional rabbit inert IgG antibodies (1 mg/ml) to serve subsequently in identifying injected cells. After microinjection, cells were placed in differentiation media and returned to the incubator for >72 h to obtain a maximal level of differentiation. Cells were then fixed with formalin, and effects were analyzed by immunofluorescence using biotinylated anti–rabbit antibodies followed by subsequent reaction with Texas red streptavidin to visualize injected cells and anti-troponin T or antimyogenin antibodies to probe for differentiating cells.
Results

p67SRF Expression and Intracellular Localization during Myogenesis

The expression of p67SRF was investigated in two different cell lines, a subclone of the rat myogenic cells L6 (Yaffe, 1968) and the mouse myogenic cell line C2 (Yaffe and Saxel, 1977). In media containing 10% FCS, these cells proliferate rapidly without expressing the muscle phenotype. On withdrawal of the mitogen, they undergo differentiation such that within 3 d for C2 cells or 6 d for L6 cells, 60–90% of cells fuse to form plurinucleate myotubes expressing muscle-specific proteins. The expression and intracellular localization of p67SRF protein was analyzed by immunofluorescence using anti-p67SRF antibodies. The specificity of this antibody has been described elsewhere (Gauthier-Rouvière et al., 1991) and gives a specific nuclear staining in a variety of nonmyogenic cell lines, showing that p67SRF is a constitutive nuclear protein present throughout the cell cycle.

The two myogenic cell lines L6 and C2 were examined both in the proliferative myoblast stage and after they differentiated into myotubes. We performed anti-p67SRF staining together with antitroponin T staining, which constitutes a good marker of the differentiated state (Fig. 1). Fig. 1, A and E shows the immunofluorescence staining for p67SRF in L6 and C2 myoblasts, respectively, where all cells are stained throughout the nucleus. Simultaneous staining for troponin T in these cells confirms an absence of expression of this marker of differentiation (Fig. 1, B and F). In Fig. 1, C and G, where the cells are in the differentiated myotube stage, all the nuclei in both the L6 (C) and the C2 (G) myotubes show positive staining for p67SRF, while at the same time extensive cytoplasmic expression of troponin T is now present (D and H). These data show that p67SRF staining is nuclear in the two cell lines and present in all the cells, both in myoblasts and myotubes, and that the level of p67SRF seems similar between these two stages. Using immunofluorescence staining with anti-p67SRF, we have also examined the expression of p67SRF in L6 and C2 cells during the course of differentiation (from day 1, when they were placed in differentiation medium, to day 4 (C2) or 6 (L6), when differentiation was nearly complete). In all cases, we were unable to detect significant changes or differences in p67SRF staining level (data not shown). Because the protein level is difficult to appreciate accurately by immunofluorescence, we also performed immunoblot analysis on the two cell lines, before and after differentiation. Like in REF 52 cells (an embryonic rat fibroblast cell line; Gauthier-Rouvière et al., 1991), the antibody recognizes a 67-kD band in L6 and C2 muscle cells and reveals a similar level of SRF expression between myoblasts (Fig. 2 A, lanes L6 mb and C2 mb) and myotubes (Fig. 2 A, lanes L6 MT and C2 MT). This band is abolished by preincubation with the immunogen peptide (data not shown). These data also show the specificity of the anti-p67SRF antibody, which recognizes only p67SRF in the two myogenic cell lines used in this study.

Together, these results demonstrate that p67SRF protein is always present throughout the differentiation of the two cell lines C2 and L6 without detectable changes in its level. These data are in agreement with a previous report of Taylor et al. (1989) showing that the amount of CArG box binding activity, biochemically and immunologically similar to HeLa cells p67SRF, was the same in both FAF2 myoblasts and myotubes.

p67SRF mRNA Level in Myoblasts and Myotubes

To study p67SRF expression at the mRNA level, we performed Northern blot analysis in proliferative myoblasts and differentiated myotubes. Total mRNA isolated from L6 or C2 myoblast cultures before or after differentiation was subjected to RNA blot analysis by probing membranes with the human SRF cDNA (a gift from R. Treisman) and with the myogenin cDNA as a marker of differentiation (Edmonson and Olson, 1989). Myogenin expression occurs when myoblasts are triggered to differentiate. The GAPDH cDNA was used to control levels of loading.

SRF probe reveals two mRNA species at ~2.5 and 4.5 kb in C2 and L6 growing myoblasts (Fig. 3, lane C2 mb and L6 mb) and C2 differentiated myotubes (Fig. 3, lane C2 MT; 3 d in differentiation medium composed of DME plus 2% FCS). Similar results have been reported in HeLa cells (Norman et al., 1988) and mouse NIH3T3 fibroblasts (Misra et al., 1991). The two mRNA were shown to have identical coding sequences differing in the length of their 3' untranslated regions (Norman et al., 1988). Surprisingly, only the 4.5-kb mRNA species is detected in differentiated L6 myotubes (Fig. 3, lane L6 MT1). To ensure that the difference observed between C2 and L6 myotubes (two mRNA species for C2 and only one for L6) was not artifactual, for example, resulting from differences in composition of the differentiation medium used for the two cell lines (see Materials and Methods), differentiation of L6 cells was also induced by placing L6 cells in C2 differentiation medium (DME containing 2% serum). Under these conditions, differentiation occurred in 5–6 days, with 50–70% of cells forming myotubes. We still observed only a single 4.5-kb mRNA band (Fig. 3, lane L6 MT2). The reason for this difference between the two cell lines (C2 and L6) is unclear. However, the absence of detectable expression of the smaller transcript for p67SRF in L6 myotubes is unlikely to be a significant feature of the differentiated stage since both species are clearly present in C2 myotubes. Therefore, this difference between C2 and L6 myotubes might be related to a difference in the expression of myogenic factors (Myf5 in L6 cells, Myf5 and MyoD in C2 cells).

Microinjected Anti-p67SRF Antibodies Block the Myoblast–Myotube Transition

Because the results described above show the continuous expression of both p67SRF protein and mRNA through the process of muscle cell differentiation, we have undertaken microinjection experiments to investigate the potential requirement for p67SRF during the myoblast–myotube transition. Previous experiments showed that p67SRF activity in vivo could be specifically abolished by microinjection of anti-p67SRF antibodies (Gauthier-Rouvière et al., 1991). Effects of such an injection were analyzed in both L6 and C2 cells. C2 or L6 myoblasts were seeded onto plastic dishes and allowed to proliferate in growth medium for 2–3 d, when they nearly reach confluence. At this stage, anti-p67SRF antibodies were microinjected into myoblasts; 30–60 cells were
Figure 1. Immunolocalization of p67SPF during differentiation of L6 and C2 myoblasts. Using rabbit anti-p67SPF serum, the intracellular localization of p67SPF was examined by indirect immunofluorescence in L6 and C2 cells at both the proliferative myoblast and differentiated myotube stages. Double immunofluorescence was performed using rabbit anti-p67SPF serum (diluted 1:100) and mouse monoclonal antitroponin T antibodies (diluted 1:500) as primary antibodies on L6 (A-B, D) or C2 (E-H) cells before (A, B, E, F) or after differentiation (C, D, G, H). Shown are immunofluorescence micrographs of growing (A, B) and differentiated (C, D) L6 cells stained with anti-p67SPF (A, C) and antitroponin T (B, D). Growing (E, F) and differentiated (G, H) C2 cells stained with anti-p67SPF and antitroponin T are shown (E, G and F, H, respectively). Bar, 10 μm.
Figure 2. Western blot analysis of p67SRF in myoblasts and myotubes. Total cell extracts from \( \times 10^6 \) proliferative or differentiated C2 or L6 cells were subjected to electrophoresis for immunoblotting with rabbit anti-p67SRF antibodies (A) and monoclonal mouse antitroponin T antibodies (B) as described in Materials and Methods. The arrow in A indicates the band corresponding to p67SRF. Lane L6mb shows proliferative L6 myoblasts, lane L6MT shows differentiated L6 myotubes, lane C2mb shows proliferative C2 myoblasts, and lane C2MT shows differentiated C2 myotubes. The arrow in B indicates staining for troponin T.

Figure 3. Northern blot analysis of p67SRF transcripts in muscle cells. 20 \( \mu \)g total RNA samples were loaded per lane. Cultures were harvested either during exponential growth (mb) or after differentiation (MT); Northern blots were performed using human SRF, mouse myogenin, or rat GAPDH cDNA as probes. Shown are autoradiographs of hybridization on total RNA from proliferative (lane C2, mb) and differentiated (lane C2, MT) C2 cells, proliferative L6 myoblasts (lane L6, mb), and L6 cells differentiated into L6 differentiation medium (lane L6, MT1) or C2 differentiation medium (lane L6, MT2) using SRF, myogenin, and GAPDH cDNA probes.

Microinjected in the same area. After microinjection, the differentiation of the cells was induced by replacing the growth medium with the appropriate differentiation medium. Cells were further incubated for \( \geq 72 \) h to allow optimal differentiation; then they were fixed and processed for immunofluorescence. Microinjection of preimmune serum followed by an identical period of incubation served as a control for this experiment. Microinjected cells were subsequently located by staining with anti-rabbit antibodies as described in Materials and Methods, and cell differentiation was evaluated by monitoring the expression of troponin T using monoclonal antibodies against this myotube-specific protein.

C2 cells constitute a better model than L6 cells for these microinjection experiments essentially because they differentiate more rapidly, and at the time of fixation (72 h after injection), only a minor percentage of cells, evaluated by expression of troponin T and phase contrast, remained undifferentiated. As shown in Fig. 4, in control experiments, in which myoblasts were microinjected with preimmune antiserum, rabbit fluorescent staining (A) was observed in myotubes and mononucleated cells, with 50–60% of injected
Figure 4. Anti-p67SRF injection into myoblasts prevents myoblast–myotube transition and expression of the skeletal muscle protein tropo-
nin T in C2 cells. To examine the potential role of p67SRF in the process of muscle differentiation, we injected subconfluent C2 cells
with anti-p67SRF antibodies. After injection, cells were placed in differentiation medium and incubated for 72 h before fixation and stain-
ing for microinjected rabbit antibodies and troponin T, as described in Materials and Methods. Shown are fluorescent images of the injected
rabbit antibodies (A, C) or the staining for troponin T (B, D). (A–B) Cells injected with preimmune rabbit antibodies before induction
of differentiation: the asterisk indicates anti-rabbit staining found in differentiated myotubes. The same result was obtained when p67SRF
antibodies preincubated with the immunogen SRF peptide were used as injection control (data not shown). (C, D) Cells injected with
affinity-purified rabbit anti-p67SRF before induction of differentiation. The injected cells, marked by arrows, all remained in the myoblast
stage. Bar, 10 μm.

cells differentiating into myotubes and/or expressing tropo-
nin T (B). In contrast, the majority of C2 myoblasts micro-
jected with antibodies to p67SRF (90–95% of injected
cells) remained in a mononucleated form (C) without ex-
pressing the muscle-specific marker troponin T (D). These
results were observed four times with 30–60 cells injected
in each experiment (see Fig. 6 for quantitation).

The experiment was also done in L6 cells. The same
period of incubation after antibody injection was chosen be-
fore fixation of the cells because 72 h corresponds to a mid-
point between the half-life of the injected antibodies and the
time required for differentiation. In contrast with C2 cells,
72 h was not sufficient to obtain optimal differentiation in L6
cells. Nevertheless, the use of conditioned medium as L6
differentiation medium allowed 40–50% of L6 myoblasts to
differentiate during this period. As observed for C2 cells,
when L6 myoblasts were injected with preimmune antibod-
ies, anti-rabbit fluorescent staining was observed both in
myotubes and mononucleated cells, with 40–50% of injected
cells incorporated into myotubes (Fig. 5, A and B). In all
cases (>100 cells observed in three experiments), >95% of
L6 cells injected with anti-p67SRF antibodies remained in
mononucleated stage, although most of them were in close
proximity to or in contact with a myotube, as observed by
anti-troponin T staining (Fig. 5, C and D; see Fig. 6 for
quantitation).

We further investigated this inhibitory effect of anti-
p67SRF antibodies by looking at their effect on the expres-
sion of the myogenic factor myogenin, considered one of
the earliest markers for terminal differentiation. For that
purpose, we used monoclonal antmyogenin to monitor the
expression of myogenin in injected myoblasts. Again, the in-
Figure 5. Anti-p67SRF injection into L6 myoblasts prevents their differentiation into myotubes. Subconfluent L6 cells were injected and processed as described in Fig. 4. Shown are immunofluorescent images of the injected rabbit antibodies (A, C) or the staining for troponin T (B, D). (A, B) L6 cells, injected with preimmune serum before induction of differentiation, showing a differentiated myotube (asterisk) containing injected marker antibodies. (C, D) L6 cells injected with anti-p67SRF before induction of differentiation. The injected cells (arrows) are mononucleated and in close proximity to a noninjected differentiated myotube. Bar, 10 μm.

Injectected cells were identified, after 72 h of incubation in differentiating medium, through immunofluorescence against the injected rabbit antibodies. Fig. 7 shows the results of such an experiment in L6 myoblasts. Injection of anti-p67SRF antibodies before the induction of differentiation effectively prevented the expression of myogenin in injected cells (D, E, and F), whereas nuclear myogenin is detected in surrounding noninjected cells both in mononucleated myoblasts and differentiating myotubes (E). In contrast, when control preimmune antibodies were injected, no inhibitory effect was observed on the expression of myogenin, which was detected in 50–60% of the injected cells (A, B, and C), a figure similar to the percentage observed in noninjected cells. These results were reproduced and averaged from four different sets of microinjection experiments involving 30–50 injected cells per experiment and show that injection of anti-p67SRF prevented the expression of myogenin in >90% of the injected cells. The same results have been obtained with C2 cells, where injected anti-p67SRF antibodies also blocked the expression of myogenin. More than 60% of control cells (injected with the preimmune antiserum) expressed myogenin, whereas <10% of anti-p67SRF–injected C2 cells were positive for this myogenic factor (see Fig. 8 for quantitative representation of these data).

Having shown the ability of anti-p67SRF antibodies to produce an early block in myogenesis, it was important to check that such an effect was not resulting from nonspecific long-term cytotoxicity of the injected antibodies. In that respect, we found that injection of anti-p67SRF antibodies did not alter viability of cells because no cellular death was observed; >95% of the injected cells were still alive 72 h after injection, a figure similar to the value observed with injection of the control antibodies. Furthermore, as an additional control experiment, we addressed the ability of injected cells to retain such functions as adherence, which usually implies a good viability of cells. For that purpose, C2 cells were inoculated in a small square delineated on a 35-mm dish, injected with anti-p67SRF antibodies, and placed into differentiation medium before being returned to the incubator for 72 h. After this period, cells were trypsinized, reseeded on a 35-mm dish, and allowed to reattach for 8 h before fixation and staining for injected antibodies (anti–rabbit staining) and troponin T. Again, >90% of the injected cells reattached properly and did not express the troponin T differentiation marker, showing that injection of anti-p67SRF did not impair the ability of the injected cells to reattach following trypsinization (data not shown). In addition, we observed that anti-p67SRF injection into REF52 fibroblasts at the G1 phase did not prevent the expression of cyclin A (this protein is expressed at the onset of S phase; Gauthier-Rouvière, C., unpublished results) showing that such an injection of anti-p67SRF did not interfere with essential functions such as transcription and translation.

Taken together, these results clearly show that inhibition
of p67sRF in vivo, through microinjection of anti-p67sRF antibodies, specifically prevented the process of myogenic differentiation in L6 and C2 myoblasts.

Discussion

This study shows that p67sRF is expressed constitutively at both mRNA and protein levels throughout skeletal myogenesis, with a nuclear localization both in myoblasts and myotubes, as demonstrated by immunofluorescence staining with anti-p67sRF antibodies. Furthermore, we showed that p67sRF is required for muscle differentiation, because microinjection of anti-p67sRF antibodies into myoblasts blocks the myoblast–myotube transition. These results, reproduced in mouse C2 and rat L6 myogenic cells, demonstrate the general implication of this transcription factor in the process of muscle differentiation.

p67sRF Is Required for Myoblast–Myotube Transition

The CArG sequence CC(A/T)6GG was first identified in the c-fos gene promoter as the core of the SRE element (Treisman, 1985; Gilman et al., 1986). It was subsequently found in many other genes, including cytoskeletal β- and γ-actins, interleukin-2 receptor, Krox 20/1, Krox 20/2, dystrophin, myosin light chain 1/3, and the family of muscle-specific α-actins (Taylor et al., 1988; Treisman, 1990). In addition to its well-known function in immediate early gene expression, previous work has implicated the CArG boxes in muscle-specific transcription (Miwa and Kedes, 1987; Phan-Dinh-Tuy et al., 1988; Walsh and Shimmel, 1988). p67sRF participates in the activation of c-fos transcription by direct binding to the SRE element. Using band-shift assays, several studies demonstrated that p67sRF can bind to CArG box sequences from muscle actin promoters, suggesting that this factor may also play a role in muscle differentiation (Boxer et al., 1989; Taylor et al., 1989; Tuil et al., 1990). Our results extend these observations further and clearly demonstrate that p67sRF is implicated in myogenesis:

(a) p67sRF expression was not reduced and its cellular localization was not affected in the course of the differentiation process. It is well documented that other components of the proliferative pathways are down-regulated during the course of differentiation (for example, cdc2 kinase [Akhurst et al., 1989], p21ras [Olson et al., 1987; Sternberg et al., 1989], c-myc [Denis et al., 1987], and c-fos [Rahm et al., 1989]).

(b) Inhibition of p67sRF activity following injection of anti-p67sRF antibodies into myoblasts prevented their differentiation into myotubes and expression of both myogenin and the muscle-specific marker troponin T. The inhibition of p67sRF activity in fibroblasts has previously been shown to prevent cell cycle progression by stopping cells during the G1 phase, the period that precedes DNA synthesis (Gauthier-Rouvière et al., 1991). The transition from the proliferative to the differentiated state is characterized by withdrawal of myocytes from the cell cycle in the G1 phase (Nadal-Ginard, 1978; Compton and Konigsberg, 1988). If p67sRF was exclusively implicated in cell proliferation, a likely prediction would be that inactivation of cellular p67sRF will induce the cells to commit into the differentiation pathway. We found that this was not the case; instead, it resulted in an inhibition of the differentiation pathway.

The fact that all cells microinjected with anti-p67sRF remained in a mononucleated state and did not express myogenin, which marks the activation of the muscle differentiation program (Edmonson and Olson, 1989; Wright et al., 1989), suggests that p67sRF is required early in the differentiation process. One potential CArG box has been identified in the myogenin promoter. However, the (A/T)6 stretch in that case is interrupted in the middle by a C/G pair, and it has been shown in a minimal test promoter that the myogenin CArG element, isolated from its promoter and cloned upstream from the c-fos TATA element with a reporter gene, was not functional both in muscle and nonmuscle cells (Santoro and Walsh, 1991). This may imply that the inhibitory effect of anti-p67sRF on myogenin expression results from an indirect pathway whereby p67sRF function would be implicated upstream.

How Could a Single Protein Account for the Regulation of Serum-responsive and Muscle-specific Transcription?

Our results show that, in addition to its role in cell cycle
Figure 7. Inhibition of myogenin expression in L6 myoblasts injected with anti-p674RF antibodies. Cells were injected and processed as described in Fig. 4 except that they were stained for expression of myogenin using a monoclonal antimyogenin antibody, as described in Materials and Methods. Shown are immunofluorescent images of the injected antibodies (A, D), nuclear staining for myogenin (B, E), and DNA staining with HOECHST (C, F). (A–C) L6 cells injected with preimmune serum before induction of differentiation with, in A, anti-rabbit staining found in differentiated myotubes expressing myogenin (asterisk) in B. (D–F) L6 cells injected with anti-p674RF. The injected cells, marked by white arrows (D), remained mononucleated and did not express myogenin, whereas other noninjected mononucleate cells, marked by black arrows (E), express this myogenic factor. Bar, 10 μm.
progression, p67SRF participates in the expression of the muscle-specific program. The following question thus arises: How can the same factor be involved in both transcriptional activation of proliferative immediate early genes such as c-fos or β-actin, and in the expression of muscle-specific genes such as skeletal and cardiac α-actin, a differentiation-dependent process that requires the downregulation of genes implied in cellular growth? A simple view is that p67SRF activity is altered in a tissue-specific fashion. p67SRF is a phosphoprotein; phosphorylation mediated by casein kinase II is thought to play a role in the regulation of p67SRF binding to the SRE sequence throughout growth factor stimulation. Differentiation in culture is triggered by depletion of growth factors from the culture medium. This implies dramatic modifications in signal transduction, with particular changes in protein kinase activities (Adamo et al., 1989; Kelvin et al., 1989; Tollefsen et al., 1989). Differential posttranslational modifications of p67SRF may thus confer different transcriptional specificities to this protein, particularly by changing its affinity to the CArG sequence and/or its binding with various cofactors. In this respect, transcriptional activation acting at the CArG box may involve interactions of SRF with different nuclear factors. One such factor, p62CF, binds p67SRF at the c-fos SRE and has been shown to be required for full transcriptional activation of the c-fos gene (Shaw et al., 1989). p62CF has been proposed to be a physiological substrate for the protein kinase C pathway, providing an additional level of control for SRF transcriptional activity (Graham and Gilman, 1991). According to this view, different accessory proteins binding to p67SRF may be involved in the tissue-specific expression acting at the CArG boxes. It will be of interest to determine whether such a heteromultimeric complex with p67SRF could be detected in a state- or tissue-specific fashion. In addition, the sequence flanking the CC(A/T)GG core sequence (Shaw et al., 1989; Graham and Gilman, 1991). Furthermore, in light of the numerous CArG binding factors actually described, a complementary mechanism of regulation could involve competition between p67SRF and other proteins for binding to the CArG element. In this respect, a recent study reported that F-ATC1 (also called MAPF1 or p62) may act as a repressor of skeletal α-actin gene transcription, whereas SRF acts as a positive transcription factor (Lee et al., 1991). As described, F-ATC1 is abundantly present in myoblasts; its level reduces during myogenic differentiation, whereas the level of p67SRF remains constant throughout myogenesis (Taylor et al., 1989). Therefore, the change in this ratio in favor of p67SRF during the course of differentiation may allow p67SRF to transactivate muscle-specific genes.

Finally, the CArG box sequences may act in concert with other cis-acting sequences to accomplish coordinate transcriptional regulation during myogenesis. A number of muscle-specific sequences have been described, including the CANNTG box (the E-box motif). The E box is the binding site for the recently identified family of MyoD1 myogenic factors. In contrast to p67SRF, these factors (MyoD, Myf5, myogenin, MRF4) are expressed only in skeletal muscle cells. Their role in muscle-specific expression is well documented (Weintraub et al., 1991). Functional E-box sequences are localized in proximity to the CArG box in many muscle-specific genes, suggesting that the CArG box may function as a constitutive and prerequisite promoter element and, as such, does not directly contribute to the absolute muscle-specific regulation. However, such a model does not exclude the implication of p67SRF in muscle differentiation, and, as shown by Sartorelli et al. (1990), both MyoD, Spl (a ubiquitous transcription regulatory factor), and p67SRF (or SRF-related proteins) bind specifically to the human cardiac α-actin gene and are involved in its activation.

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