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Growth and Differentiation of C2 Myogenic Cells Are Dependent on Serum Response Factor

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In order to study to what extent and at which stage serum response factor (SRF) is indispensable for myogenesis, we stably transfected C2 myogenic cells with, successively, a glucocorticoid receptor expression vector and a construct allowing for the expression of an SRF antisense RNA under the direction of the mouse mammary tumor virus long terminal repeat. In the clones obtained, SRF synthesis is reversibly down-regulated by induction of SRF antisense RNA expression by dexamethasone, whose effect is antagonized by the anti-hormone RU486. Two kinds of proliferation and differentiation patterns have been obtained in the resulting clones. Some clones with a high level of constitutive SRF antisense RNA expression are unable to differentiate into myotubes; their growth can be blocked by further induction of SRF antisense RNA expression by dexamethasone. Other clones are able to differentiate and are able to synthesize SRF, MyoD, myogenin, and myosin heavy chain at confluency. When SRF antisense RNA expression is induced in proliferating myoblasts by dexamethasone treatment, cell growth is blocked and cyclin A concentration drops. When SRF antisense RNA synthesis is induced in arrested confluent myoblasts cultured in a differentiation medium, cell fusion is blocked and synthesis of not only SRF but also MyoD, myogenin, and myosin heavy chain is inhibited. Our results show, therefore, that SRF synthesis is indispensable for both myoblast proliferation and myogenic differentiation.

Muscle development is characterized by different steps: the first step involves determination of the cells which give rise to myoblasts; later, these proliferating myoblasts withdraw from the cell cycle, align, and fuse to form multinucleate myotubes which then mature to form myofibers (27, 30).

Numerous studies have tried to decipher the mechanisms by which cells withdraw from the cell cycle to differentiate. Several proteins thought to play a role in this process are known to be differentially expressed before and after this step (27). Some of them are down-regulated during the differentiation process, such as Id, an inhibitor of helix-loop-helix (HLH) myogenic factors, growth factor receptors, and cyclin D1 (44), while others such as cyclin D3 or the cyclin-dependent kinase inhibitor $p21^{WAF1/Cip1}$ are up-regulated (17, 19, 25, 34, 40). Some proteins also have different functions before and after cell fusion, such as the retinoblastoma protein Rb (31, 61), or are already present but inactive at the myoblast stage and become activated during differentiation, such as MyoD1 (47).

In contrast to all of these proteins, the serum response factor (SRF) is present and active throughout the differentiation process (58); it seems to be involved in the transcriptional regulation of genes specific not only to cell proliferation but also to muscle differentiation. Indeed, SRF-binding sites are *cis*-active elements involved in both (i) response of immediate-early genes, e.g., the c-*fos* proto-oncogene, to serum and growth factors (54), and (ii) muscle-specific transcriptional activation of muscle-specific genes, e.g., the cardiac and skeletal α-actin genes (32, 37, 42, 49), the dystrophin gene (13), and the myosin light chain 1/3 gene (10). In addition, SRF binding sites of the regulatory regions of immediate-early genes, coined serum response elements, or of muscle-specific genes, coined CArG boxes, are interchangeable (56).

The SRF protein seems itself to be involved in these transcriptional regulation processes. Microinjected anti-SRF antibodies block fibroblasts in phase G_1 (11) and inhibit fusion and expression of the myogenic program of L6 or C2 myoblasts. Also, similarly injected myogenic cells do not express specific muscle proteins such as troponin T and myogenin (58).

SRF is a 67-kDa ubiquitous phosphoprotein which binds the core sequence of serum response elements or CArG boxes as an homodimer. It is a member of the MADS box (MCM1-agamous–Arg-80–deficiens–SRF) transcription factor family (46). DNA binding and dimerization domains were mapped to amino acids 133 to 222 and 168 to 222, respectively (39), and the transcriptional activation domain was mapped to amino acids 339 to 508 (22). This protein exhibits a half-life of more than 12 h and is predominantly nuclear (33). The SRF gene, itself a member of the immediate-early gene family (33, 39), is highly conserved throughout evolution and produces two transcripts differing in the lengths of their 3′ untranslated regions (35, 39).

We have previously established that CArG-dependent gene activation is a very early phenomenon during ex vivo myogenesis, occurring approximately at the same time as MyoD gene activation in 10T1/2 cells treated with 5-azacytidine (57). Since this gene activation through CArG boxes involves SRF (48), we asked to what extent and, if so, at which stage SRF is indispensable for myogenesis.

To answer these questions, we created myogenic cell lines in which SRF synthesis could be blocked at will in order to study the effect of the lack of functional SRF at different steps of muscle differentiation. In this aim, the dexamethasone-induc-

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ible mouse mammary tumor virus long terminal repeat (MMTV LTR) was used to direct the expression of an SRF antisense RNA. Dexamethasone-induced accumulation of the antisense RNA led to decrease in SRF levels, blocking of myoblast proliferation and fusion, and decrease in cyclin A, MyoD, myogenin, and myosin heavy chain levels. All of these phenotypic changes were reversible upon dexamethasone withdrawal or addition of the RU486 anti-hormone.

MATERIALS AND METHODS

Plasmids. Vector pSO1 contains human glucocorticoid (Gc) receptor cDNA under the control of the Rous sarcoma virus promoter and harbors the hygromycin resistance gene.

Vector pSO4 bears the neomycin resistance gene.

Vector pSO4-antisense SRF was constructed by cloning the human SRF cDNA from nucleotide 1092 to nucleotide 576 (corresponding to the DNA binding and dimerization domains) between the *Xba*I and *Eco*RV sites of the pSO4 vector. This vector allows for the expression of an SRF antisense RNA under the control of the MMTV LTR.

Vector pFc31 Luc contains the luciferase gene under the control of the MMTV promoter (15).

Cell culture and transfections. C2C12 cells (2), derived from the mouse myogenic C2 cell line (63), were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) fetal calf serum (FCS). Cells were grown at 37°C under a humidified atmosphere of air plus 7% (vol/vol) CO_2. Myogenic differentiation was induced by a low-serum medium (DMEM plus 2% FCS).

C2C12 cells (150,000 cells plated per 90-mm diameter dish) were transfected by the calcium phosphate precipitation method with 15 μg of pSO1 vector. Stably transfected cells were selected in culture medium containing 300 μg of hygromycin B per ml. Foci of hygromycin-resistant cells were isolated and propagated before conservation.

After transient transfection of each stably transfected clone with 10 μg of pFc31 Luc plasmid and dexamethasone induction (10^{-7} M during 24 h), one clone, named C2CL2, was selected for further experiments.

Clone C2CL2 was subsequently subcloned after transfection with 10 μg of pSO4-antisense SRF plasmid. Hygromycin B and G418 (150 and 400 μg/ml, respectively) were added to the medium 48 h after transfection in order to select doubly stably transfected cells. Twenty-one different clones of hygromycin- and G418-resistant cells were then isolated. Two of them, designated anti6 and anti9, were selected for further studies.

Cells were plated at low density, 140,000 cells per 90-mm-diameter dish or 60,000 cells per 60-mm-diameter dish, in DMEM plus 10% FCS. Dishes were divided into three shares as follows: the first one was treated with dexamethasone at a final concentration of 10^{-6} M, the second one was treated with RU486 (which is a Gc antagonist) at a final concentration of 10^{-8} M, and the third one was untreated. When cells were treated with drugs (dexamethasone or RU486), fresh drugs were added in the medium every 36 h.

For proliferation studies, cells were plated with or without drugs and grown for 3 days.

For myogenic differentiation studies, cells were first grown for 3 days in DMEM plus 10% FCS. Drugs were added (or not) to the proliferating medium 12 h before cell confluency. Medium was then replaced by a differentiation medium (DMEM plus 2% FCS) with or without drugs for 3 more days.

Immunoblotting. Cells were grown in proliferation or differentiation culture conditions with or without dexamethasone. About 10^6 cells were homogenized in 1 ml of a solution containing 40 mM Tris-HCl (pH 6.8), 1% (wt/vol) sodium dodecyl sulfate (SDS), 7.5% (vol/vol) glycerol, 5 mM dithiothreitol, and 0.03% (vol/vol) bromophenol blue. The homogenized cells were then boiled for 5 min, sonicated, and centrifuged for 10 min at 10,000 × *g*. Supernatants (25 μl) were resolved in SDS-polyacrylamide gels and transferred to a nitrocellulose filter (53). Nitrocellulose transfers were blocked for 1 h in TBST (10 mM Tris-HCl [pH 8.0], 0.15 M NaCl, 0.05% [vol/vol] Tween 20) containing 5% dry milk and subsequently incubated for 2 h with primary antibody. The following antibodies were used: rabbit polyclonal antibodies directed against SRF (33), diluted 1:10,000; annexin (45), diluted 1:2,000; MyoD (M318 from Santa Cruz Biotechnology), obtained at a concentration of 1:200; cyclin A (9), diluted 1:2,000; mouse monoclonal antibodies directed against myogenin (clone F5D) (62), diluted 1:100; and myosin heavy chain (clone RNMy2/9D2 from Novocastra), diluted 1:100. Immunoblots were then washed in TBST and incubated for 45 min with a peroxidase-conjugated secondary antibody (Dako S.A.) at a dilution of 1:2,000. After washing the nitrocellulose blots with TBST, the blots were incubated for 1 min in chemiluminescence reagents according to the manufacturer's instruction (Amersham) and exposed to film for detection by autoradiography. Antigen concentration was appreciated by scanning the different specific autoradiographic bands with a Shimadzu microdensitometer, each value being standardized by the intensity of the annexin band observed with the same sample.

Immunofluorescence. Cells were fixed during 5 min at room temperature in 3.7% (vol/vol) formaldehyde diluted in phosphate-buffered saline (PBS) which was followed by a 30-s immersion in ice-cold acetone before rehydration in PBS containing 1% bovine serum albumin. The cellular distribution of myogenin protein and either SRF or MyoD protein was analyzed simultaneously by indirect immunofluorescence by using a monoclonal anti-myogenin antibody and either the rabbit antiserum $p67^{SRF2}$ already described (11) (diluted 1:10) or a polyclonal anti-MyoD antibody (diluted 1:100). Immunofluorescence experiments were performed as previously described (58).

Northern (RNA) blot analysis. Poly(A)$^+$ RNAs were isolated from at least 10^8 cells with a Fast Track Kit (Invitrogen). Northern blot analysis was performed as previously described (6). Membranes were successively hybridized with different randomly labeled probes. The 1.15-kbp human SRF C-terminal cDNA fragment was used as a probe. For standardization, the blots were probed with an R45 cDNA probe (corresponding to a fragment of human 18S rRNA).

Hybridized membranes were scanned with a PhosphorImager (Molecular Dynamics).

RESULTS

Establishment of C2C12 myoblasts with dexamethasone-inducible SRF antisense RNA expression. The Gc-inducible promoter-enhancer of the MMTV LTR was chosen to express the antisense SRF RNA upon treatment of the cells by Gcs. This promoter can be induced by dexamethasone complexed with the Gc receptor through Gc response elements. First, we stably transfected C2C12 myoblasts with an expression vector for the Gc receptor. The Gc receptor function was tested in isolated clones by checking for dexamethasone-dependent induction of a transiently transfected plasmid containing the luciferase reporter gene under the control of the MMTV promoter (pFc31 Luc). We selected for further experiments the clone in which induction of the MMTV promoter by dexamethasone was maximal (about eightfold) (clone C2CL2). It should be noticed that this induction level is much lower than when both Gc receptor expression vector and reporter gene are transiently cotransfected. In the latter case, the reporter gene activity in the absence of added dexamethasone was very low, and it was induced more than 200-fold by the hormone. The Gc receptor was most likely less intensively expressed from the integrated expression vector in the C2CL2 clone than from the multiple nonintegrated copies after transient transfection.

Subsequently, the C2CL2 clone was further transfected with the inducible SRF antisense expression vector (pSO4-antisense SRF vector). Twenty-one doubly stably transfected clones were isolated and characterized. We have distinguished two types of clones: (i) clones harboring an SRF expression level strongly diminished compared with the parental C2CL2 clone, even in the absence of dexamethasone induction (e.g., the anti9 clone, see below); and (ii) clones showing an SRF expression level only moderately lower than that of C2C12 or C2CL2 control cells but which was significantly decreased by dexamethasone induction (e.g., the anti6 clone; see below). Anti9 and anti6 clones, representative of both patterns, were selected for further experiments in different culture conditions (see Materials and Methods).

RU486 is a Gc antagonist competing with the hormone for binding to the receptor (1). SRF mRNA analysis by Northern blot showed that SRF expression was similar when cells were grown either with or without RU486 (data not shown), but that, as expected, RU486 in excess (10^{-5} M RU486 and 10^{-6} M dexamethasone) inhibited the dexamethasone effect. These observations confirmed that the SRF-decreased expression level upon dexamethasone treatment was due to activation of the MMTV LTR promoter mediated by the Gc receptor. In clones with constitutively low SRF synthesis levels, it is likely that the antisense construct is constitutively expressed at a high level, probably because of the chromatin environment at the integration site; this basal expression in the absence of dexa-

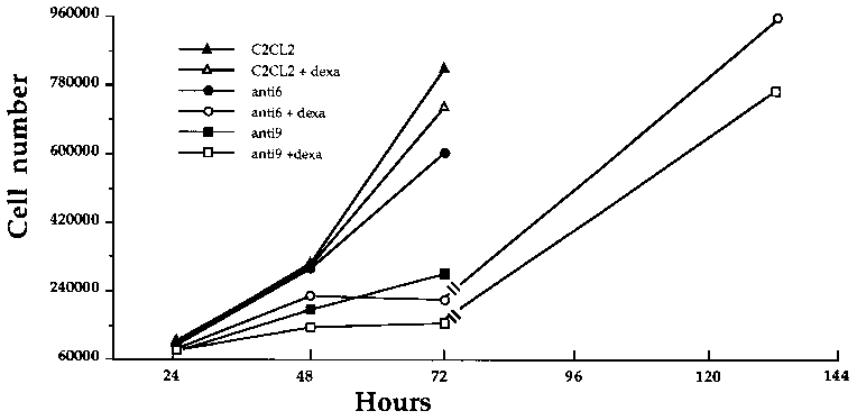

FIG. 1. Effect of dexamethasone (dexa) on proliferation of C2CL2, anti6, and anti9 cell lines. Cell number was counted for each clone after 1, 2, or 3 days of culture either with or without dexamethasone treatment. After 3 days of treatment with 10^{-6} M dexamethasone, the growth medium was changed for a dexamethasone-free medium, and cells were grown for 62 h more.

methasone or Gc induction was particularly important for the anti9 clone.

Effect of SRF depletion in proliferating myoblasts. In order to study the effect of SRF antisense RNA expression on myoblast proliferation, cells were plated at low density and treated (or not) with 10^{-6} M dexamethasone for 3 days. Cell phenotype was observed during these 3 days, after which time cells were either fixed for immunofluorescence experiments or collected for either Northern blot analysis or protein preparation and Western blot (immunoblot) analysis. In other experiments, cells were plated at low density and treated (or not) with 10^{-6} M dexamethasone, and the cell number was counted each day.

Figure 1 shows the results of the experiments of cell counting. Growth of the C2CL2 parental cells was totally insensitive to dexamethasone and to the anti-hormone RU486 (not shown). Growth of the anti6 cells was only slightly lower than that of the C2CL2 cells in the absence of dexamethasone and was totally blocked after the second day in the presence of the hormone. Growth resumed after hormone withdrawal or RU486 addition (not shown). Finally, growth of anti9 cells was slow even in the absence of the dexamethasone and was blocked by the hormone; growth resumed upon dexamethasone withdrawal.

In the absence of dexamethasone treatment, the SRF mRNA level was already very diminished in anti9 cells compared with that of the parental cell line C2CL2 (Fig. 2). After 3 days of dexamethasone treatment, the two isoforms of SRF mRNAs were nonmodified in C2CL2 cells, were clearly reduced in the anti6 clone (as shown in Fig. 2, the SRF/R45 ratio decreased two- to threefold after hormone induction), and were at the limit of detectability in anti9 cells (data not shown).

Figure 3 shows a Western blot experiment detecting SRF, cyclin A, MyoD, and annexin in the various cell lines cultured with or without dexamethasone. SRF, cyclin A, and MyoD intensities standardized by the annexin intensity are shown in Table 1, expressed as percentages of the values for C2CL2 cells in the absence of dexamethasone. Expression of these proteins was insensitive to dexamethasone treatment in C2CL2 cells. Compared with C2CL2 cells, nontreated anti6 cells contained threefold less SRF and similar amounts of cyclin A and MyoD. Treatment of these cells with dexamethasone resulted in a 5-fold decrease of SRF, a 6-fold decrease of cyclin A, and an 18-fold decrease of MyoD. In anti9 cells, SRF and MyoD levels were decreased in the absence of hormone by 12- and 3-fold with respect to C2CL2 cells, respectively. The cyclin A level, in contrast, was normal. In dexamethasone-treated anti9 cells, SRF and MyoD levels were further reduced by two- to threefold, and cyclin A was reduced by ninefold.

Immunofluorescence experiments (Fig. 4) confirmed these results. In nontreated cells, SRF expression was intense in C2CL2 myoblasts (Fig. 4A) and in anti6 cells (Fig. 4I) and seemed to be lower in anti9 cells (Fig. 4Q). MyoD was ex-

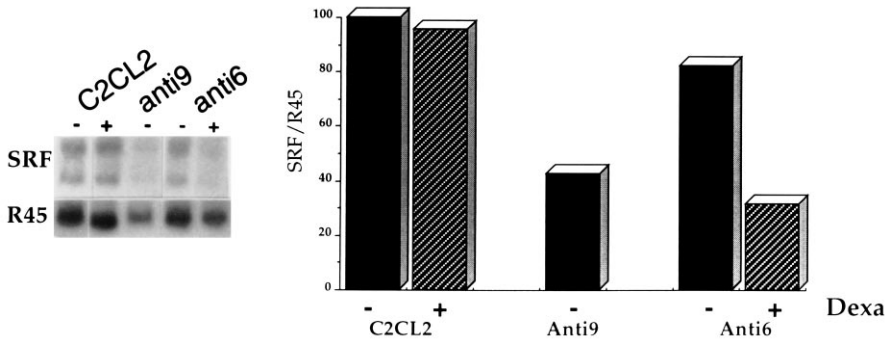

FIG. 2. Northern blot analysis of SRF transcripts in C2CL2, anti6 and anti9 cells; effect of dexamethasone. Cells were grown in proliferation culture condition with (+) or without (−) dexamethasone (dexa) for 3 days; cells were then collected and poly(A)$^+$ RNA was prepared. Northern blot analysis was performed as described in Materials and Methods. On the left, autoradiogram after hybridization with SRF and R45 probes. On the right, ratios of the SRF to R45 radioactivities measured with a PhosphorImager; the ratio obtained with C2CL2 cell poly(A)$^+$ RNAs was taken as 100%.

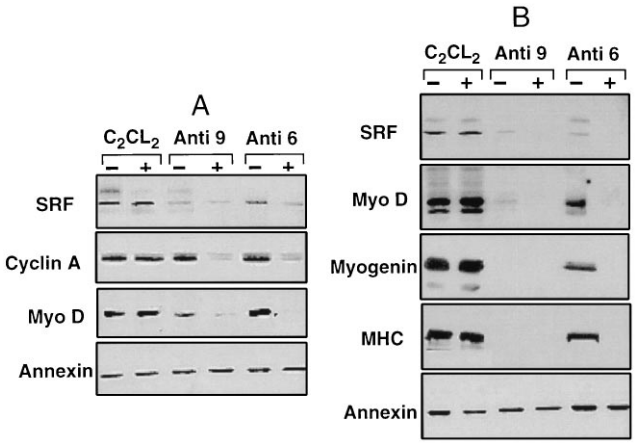

FIG. 3. Immunoblotting analysis of SRF, cyclin A, MyoD, myogenin, and myosin heavy chain proteins in C2CL2, anti6, and anti9 cells; effect of dexamethasone. Cells were grown in proliferation (A) or differentiation (B) culture conditions with (+) or without (−) dexamethasone. Immunoblot analysis was performed as described in Materials and Methods. Blots in panel A were labelled with anti-SRF, anti-cyclin A, anti-MyoD, and anti-annexin antibodies. Blots in panel B were labelled with anti-SRF, anti-MyoD, anti-myogenin, anti-myosin heavy chain, and anti-annexin antibodies. SRF, cyclin A, MyoD, myogenin, and myosin heavy chain intensities standardized by annexin intensity, expressed in arbitrary units, are shown in Table 1. Western blot images were processed by the Adobe Photoshop 3.05 program on a Macintosh IIVx (Apple Computer Inc.).

pressed in about 75% of the C2CL2 myoblasts (C), in 55% of the anti6 cells (K), and was not detected in anti9 cells (S). Myogenin was detected in only a few C2CL2 myoblasts and in still fewer anti6 cells and was undetectable in anti9 cells. Dexamethasone treatment had no effect on C2CL2 cells (E and G), except for a slight increase of myogenin expression (F and H), which is probably due to the myogenic influence of this hormone (41). In contrast, treated anti6 cells expressed much less SRF than nontreated cells (compare M and I) and appeared to cease expressing MyoD (O) and myogenin (N and P).

To determine whether the blocking effect of SRF antisense RNA expression on cell growth was reversible, we stopped inducing SRF antisense RNA synthesis after 3 days of dexamethasone treatment either by addition of RU486 (10^{-5} M) or by changing the medium for a fresh one without dexamethasone. Cells were then maintained in culture for at least 3 more days. Two days after RU486 addition or dexamethasone withdrawal, the cells recovered their normal growth rate (Fig. 1) and phenotype (not shown).

Effect of SRF depletion in differentiating myoblasts. In order to study the effect of SRF depletion on the differentiation of confluent myoblasts into myotubes, cells were grown in 10% FCS and treated (or not) with dexamethasone just before confluency. After 12 h, the medium was changed for a differentiation medium (DMEM plus 2% FCS) with or without dexamethasone and cells were allowed to differentiate for 3 days (Fig. 5).

Under these conditions, treated as well as nontreated C2CL2 parental cells differentiate to form myotubes (Fig. 5A and B). Differentiation of anti6 cells in myotubes was only partial (C) and was totally blocked by dexamethasone (D). Anti9 cells never fuse into myotubes, even in the absence of dexamethasone (Fig. 5F). These effects of SRF synthesis inhibition on terminal differentiation of myogenic C2CL2 cells were further analyzed by immunoblotting and in situ immunofluorescence. Western blots (Fig. 3 and Table 1) showed that dexamethasone seemed to increase the expression of SRF and MyoD in C2CL2 cells by three- to fourfold and, in parallel, the expression of the terminal differentiation markers, myogenin and myosin heavy chain. This probably reflects the previously mentioned differentiating effect of Gcs on myogenic cells (41). In untreated anti6 cells, all of the markers analyzed were reduced with respect to C2CL2 cells. The relative levels of reduction were about fourfold for SRF and MyoD, sixfold for myogenin, and threefold for myosin heavy chain. After treatment with dexamethasone, SRF further declined about fivefold while MyoD and both terminal differentiation markers myogenin and myosin heavy chain became undetectable, consistent with the total blockage of myotube formation. Anti9 cells were unable to differentiate either without or with dexamethasone and were therefore unable to synthesize myogenin and myosin heavy chain.

Immunofluorescence studies (Fig. 6) show the same type of results. SRF, MyoD, and myogenin proteins are intensely expressed in C2CL2 cells, whether the cells or not were treated with dexamethasone (compare Fig. 6E, F, G, and H with 6A, B, C, and D). Untreated anti6 cells express MyoD and myogenin in approximately 55% of the cells (Fig. 6J, K, and L) and express SRF protein (I). Dexamethasone-treated anti6 cells expressed SRF, MyoD, and myogenin proteins at a very reduced, practically undetectable, level (M, N, O, P).

DISCUSSION

The role of SRF in cell proliferation is well established. By itself or in a ternary complex with an *ets*-type partner (e.g., elk-1 [7, 8, 20]), SRF is involved in transcriptional activation of immediate-early genes, among which c-*fos* constitutes an archetype. The *ets* partner of SRF in the complex assembled on the c-*fos* SRE is the target of signalling pathways issued from

TABLE 1. Quantification of antigen signals standardized with annexin signals under proliferation and differentiation conditions[a]

Development phase	C2CL2		Anti9 cells		Anti6 cells	
	Untreated	Treated	Untreated	Treated	Untreated	Treated
Proliferation						
SRF	100	103	8	4	31	6
Cyclin A	100	78	72	8	79	13
MyoD	100	78	32	12	132	7
Differentiation						
SRF	100	440	14	4	25	5
MyoD	100	470	6	0	25	0
Myogenin	100	375	0	0	17	0
Myosin heavy chain	100	328	0	0	37	0

[a] Antigen/annexin ratios are expressed relative to untreated C2CL2 cells (100 arbitrary units). Untreated, without dexamethasone; treated, with dexamethasone.

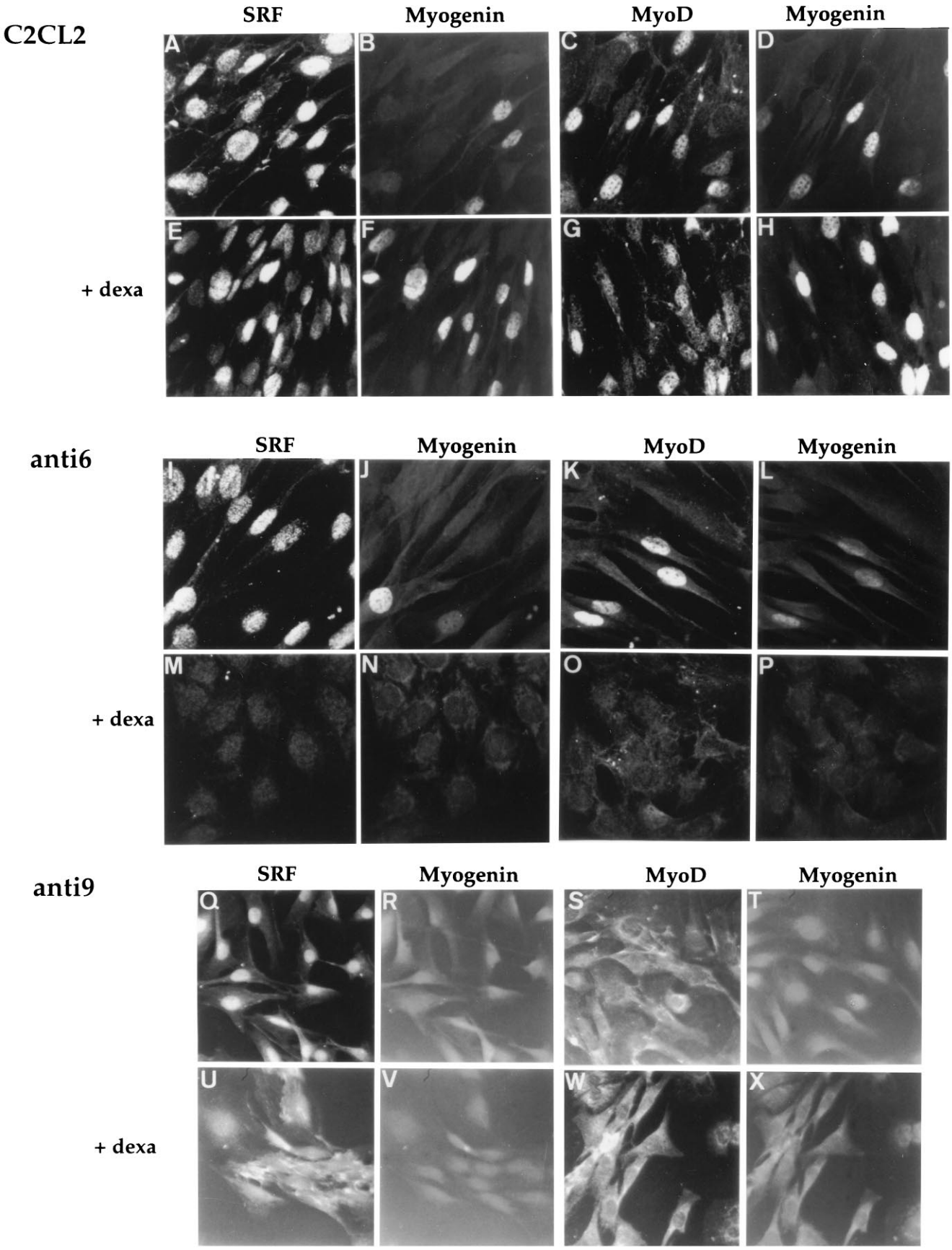

FIG. 4. Immunolocalization of SRF, MyoD, and myogenin proteins during proliferation of C2CL2, anti6, and anti9 cells grown without or with (+dexa) 10^{-6} M dexamethasone. Double immunofluorescence was performed by using a monoclonal anti-myogenin antibody (B, D, F, H, J, L, N, P, R, T, V, and X) and either rabbit antiserum $p67^{SRF2}$ (A, E, I, M, Q, and U) or a polyclonal anti-MyoD antibody (C, G, K, O, S, and W).

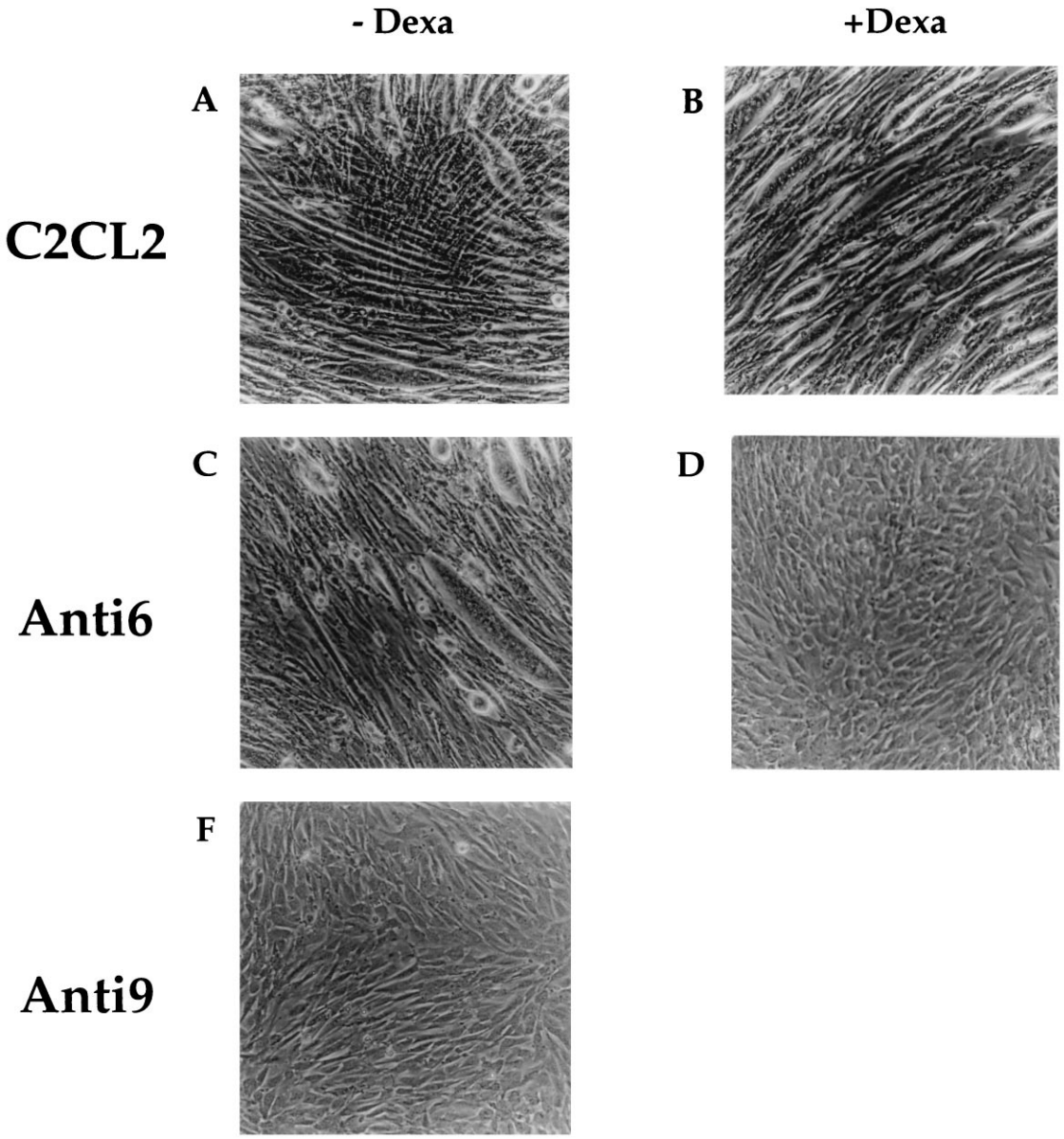

FIG. 5. Effect of dexamethasone (Dexa) on the phenotypes of C2CL2, anti6, and anti9 cell lines in differentiation culture conditions. Phase-contrast staining of each clone after 3 days of culture in DMEM plus 2% FCS either without (A, C, and F) or with (B and D) 10^{-6} M dexamethasone is shown.

growth factor and cytokine receptors and mediated by $p21^{ras}$ and either extracellular signal-regulated kinases (ERKs) or *Jun N*-terminal domain kinase (JNK) (4, 23, 59, 60). In comparison, the role of SRF in myogenic differentiation is less firmly established. This role was initially suspected from the discovery of CArG boxes in several muscle-specific genes (10, 11, 32, 37, 42, 49) and the demonstration that these boxes were able to bind SRF (3, 18, 42, 50). In addition, it was shown that CArG boxes of muscle-specific genes and c-*fos* SRE were interchangeable; in particular, the c-*fos* SRE was capable of mediating the muscle-specific activation of CArG-dependent promoters as efficiently as authentic muscular CArG boxes (50, 56). This promoter activation mediated by CArG or SRE boxes appeared to be a very early phenomenon during ex vivo myogenesis, i.e., as early as the first appearance of MyoD mRNA in 10T1/2 cells induced to differentiate by 5-azacytidine treatment (57). However, several CArG box-binding proteins, among which some are ubiquitous and others muscle specific, have been reported (10, 28, 51), so that the participation of SRF itself in CArG box-mediated activation of muscle-specific genes, and to myogenesis, was not clear. Very recently, we observed that a minimal promoter limited to a functional TATA box and ligated to four oligomerized CArG boxes, very active in myogenic C2C12 cells, was further activated by cotransfection with an expression vector for complete SRF and inhibited by an expression vector for a negative transdominant SRF mutant limited to the DNA binding and dimerization domains (48), which strongly argued for a direct participation of SRF in the muscle-specific activation complex interacting with CArG boxes. As for the participation of SRF in myogenesis, it was strongly supported by the microinjection data of Vandromme et al. (58), i.e., an anti-SRF antibody microinjected into C2 and L6 myogenic cells blocked all cell fusion and expression of muscle-specific markers.

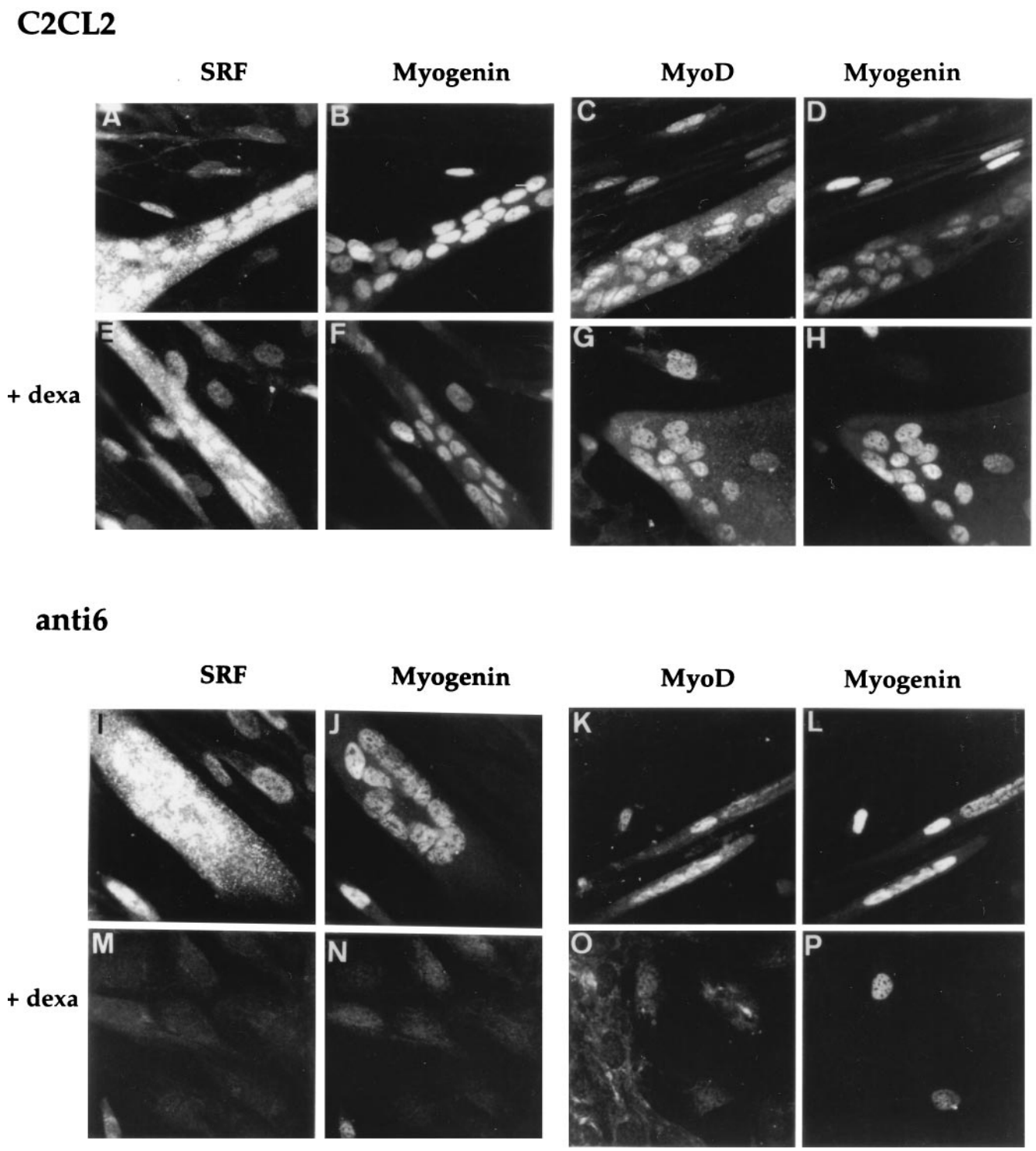

FIG. 6. Immunolocalization of SRF, MyoD, and myogenin proteins in C2CL2 and anti6 cells grown in differentiation culture conditions without or with (+dexa) 10^{-6} M dexamethasone. Double immunofluorescence was performed by using a monoclonal anti-myogenin antibody (B, D, F, H, J, L, N, and P) and either rabbit antiserum $p67^{SRF2}$ (A, E, I, and M) or a polyclonal anti-MyoD antibody (C, G, K, and O).

To analyze in further detail the role of SRF in the different stages of ex vivo myogenesis, we decided to create myogenic cell lines in which SRF synthesis could be turned off and on at will. First, we attempted to use an inducible system for a negative transdominant SRF mutant. This attempt did not succeed because we were unable to obtain any stable clone containing a functional transdominant SRF construct (57a). Retrospectively, this failure can easily be explained by the leakiness of the system, i.e., by the dexamethasone-independent activity of the MMTV LTR promoter, as well docu-

mented by the antisense constructs. Most likely, constitutive synthesis of the negative transdominant SRF mutant precluded any possibility of cell division and subsequent cloning. In contrast, accumulation of SRF antisense RNA at a sufficiently low level does not totally block SRF synthesis. The anti9 clone grows slowly and is unable to fuse into myotubes. Its levels of SRF mRNA and protein are very low, it is severely deficient in MyoD, and under differentiation conditions, it was unable to synthesize markers of myogenic terminal differentiation, e.g., myogenin and myosin heavy chain. This phenotype is most likely explained by a high level of antisense RNA expression in noninduced cells, which strong inhibits SRF synthesis. Residual SRF expression could be sufficient for a slow cell growth but not for myogenesis. Upon dexamethasone treatment, however, cells cease to grow and cyclin A declines, indicating that a further reduction of SRF synthesis totally inhibits cell division. This could indicate that less SRF is required to allow cells to grow than to allow myoblasts to differentiate into myotubes.

The anti6 clone, compared with the C2CL2 clone containing only the Gc receptor expression vector, is also partially impaired in SRF accumulation and myogenesis, which is most likely a consequence of the dexamethasone-independent transcription of the antisense construct, as discussed before. This antisense RNA could be clearly detected by reverse-transcriptase PCR, but it is not easy to quantify, probably because of its untranslatability and instability, as is generally observed with constructs for antisense RNAs (38). In fact, its expression is more easily analyzed by its effect on SRF (mRNA and protein) and cell phenotype. Since these effects depend on the presence of the antisense constructs, they are exacerbated by dexamethasone and decreased by the anti-hormone RU486, and they can be ascribed without any ambiguity to antisense RNA synthesis. The effect of dexamethasone on SRF mRNA content of anti6 cells is only partial (50 to 60% decrease as standardized by rRNA), which contrasts with a more dramatic effect on SRF expression detected by immunofluorescence and Western blot analysis (fivefold decrease). One hypothesis to explain this discrepancy is that the antisense-dependent inhibition of SRF synthesis could be due to both degradation of the mRNA and inhibition of its translatability, as previously described (21, 24).

In anti6 cells, the antiproliferative effect of dexamethasone-dependent inhibition of SRF synthesis observed with anti9 cells, associated with down-regulation of cyclin A, was found again. This effect cannot be ascribed to the hormone since neither dexamethasone nor RU486 had any effect on cyclin A abundance and growth of the C2CL2 cells, stably transfected with the Gc receptor expression vector. The proliferation inhibitory effect of antisense RNA induction was reversible upon hormone withdrawal or anti-hormone addition, indicating that cells were arrested in their growth but were not committed to die as, for instance, by apoptosis.

The effect of SRF antisense accumulation on ex vivo myogenesis can only be explored with anti6 cells at confluency, which partially fuse into myotubes and synthesize myogenin and myosin heavy chain in the absence of dexamethasone. Dexamethasone treatment totally blocks cell fusion and, in addition to SRF, inhibits MyoD, myogenin, and myosin heavy chain expression. In contrast, dexamethasone has rather a differentiating effect on the parental C2CL2 clone, increasing the abundance of all markers studied, in particular of myogenin and myosin heavy chain. As already discussed for antisense-dependent growth arrest, inhibition of differentiation was fully reversible upon dexamethasone withdrawal or RU486 addition (not shown). In addition, the antisense construct used was specific to SRF and, as shown by Northern blot analysis, did not recognize other MADS box protein mRNA, such as MEF2 mRNA (not shown). In fact, murine SRF and MEF2 mRNA sequences encoding MADS boxes were only 45% identical (29, 39, 43).

Therefore, SRF is indispensable for muscle terminal differentiation of confluent, arrested myoblasts as well as for myoblast proliferation. The mechanism of the growth arrest probably involves inhibition of growth factor-dependent induction of the c-*fos* gene and other immediate-early genes (5). We have used in this work cyclin A as a marker of proliferative cells and of the S phase (26, 64). However, this cyclin is also required for the onset of DNA replication (14, 64), and whatever the mechanism of its decrease in cells induced to accumulate anti-SRF antisense RNA, down-regulation of cyclin A could contribute to cell cycle inhibition. In contrast, the mechanism of the SRF action on myogenesis is totally unknown. We can suppose that the myogenic action of SRF is mediated by its effect on CArG-dependent genes which are activated early during myogenesis (57). Whether one of the previously described HLH differentiation factors of the MyoD family is a direct target of SRF, or whether these factors are induced indirectly by SRF, cannot be determined from the current experiments and is the subject of a paper published elsewhere (12). In C2CL2 cells, MyoD is a determination factor expressed in myoblasts and is required for myogenic terminal differentiation (12, 17, 19, 27) and expression of its early markers (e.g., myogenin) as well as its late markers (e.g., myosin heavy chain). Therefore, down-regulation of MyoD expression by the anti-SRF antisense RNA is most likely sufficient to explain the subsequent differentiation blockage (12).

Another unsolved question concerns the mechanism of the SRF-dependent activation of muscle-specific genes through CArG boxes. Further experiments will be needed to determine whether this muscle-specific activation of SRF-containing CArG complexes involves particular posttranslational modifications of SRF or its muscle-specific association with other partners, which could constitute the actual target of the muscle differentiation signal, much as Elk proteins mediate the growth factor-dependent induction of the c-*fos* gene by an SRF-containing complex (55). In fact, these hypotheses are not mutually exclusive since posttranslational modification of SRF could be necessary for its muscle-specific partnership. The muscle-specific SRF partners could be factors of the MyoD family. Indeed, Groisman et al. have recently shown that SRF could physically interact with myogenin-E12 and MyoD1-E12 heterodimers in vitro and ex vivo (16). However, the role of SRF cannot be only to recruit myogenic factors of the MyoD family to CArG boxes; indeed, we have shown that a truncated SRF mutant limited to the MADS box, although being capable of binding basic HLH (bHLH) heterodimers (16), behaves as a negative transdominant regulator of CArG box-dependent promoter activity in myogenic cells (47). However, it could be that binding of a heterodimeric bHLH myogenic factor to full-length SRF increases either its affinity for CArG boxes or the transactivating efficiency of the complex. Conversely, we can hypothesize that recruitment of SRF to E boxes of the MyoD gene regulatory sequences (52) could be involved in the effect of SRF on MyoD gene expression. Such a scheme, in which both SRF and myogenic bHLH proteins might interact between themselves and act on gene transcription through the cognate binding site of their partner, is reminiscent of the similar interplay recently described between MyoD-E12 heterodimers and MEF2, a MADS box protein as is SRF (36). Although these problems remain unsolved at present, our experiments demonstrate clearly that SRF plays at least a twofold role in myogenesis: it is required both for myoblast proliferation and, after confluency, for fusion into myotubes and

expression of the muscle-specific genetic program. The availability of a cell model in which proliferation and myogenic differentiation can be both blocked at will and reversed through induction of an antisense SRF RNA will facilitate future investigations on the molecular processes associated with different stages of myogenesis and on the role of SRF in these processes.

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