Identification of a New Hybrid Serum Response Factor and Myocyte Enhancer Factor 2-binding Element in MyoD Enhancer Required for MyoD Expression during Myogenesis

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MyoD is a critical myogenic factor induced rapidly upon activation of quiescent satellite cells, and required for their differentiation during muscle regeneration. One of the two enhancers of *MyoD*, the distal regulatory region, is essential for *MyoD* expression in postnatal muscle. This enhancer contains a functional divergent serum response factor (SRF)-binding CArG element required for *MyoD* expression during myoblast growth and muscle regeneration in vivo. Electrophoretic mobility shift assay, chromatin immunoprecipitation, and microinjection analyses show this element is a hybrid SRF- and MEF2 Binding (SMB) sequence where myocyte enhancer factor 2 (MEF2) complexes can compete out binding of SRF at the onset of differentiation. As cells differentiate into postmitotic myotubes, *MyoD* expression no longer requires SRF but instead MEF2 binding to this dual-specificity element. As such, the *MyoD* enhancer SMB element is the site for a molecular relay where *MyoD* expression is first initiated in activated satellite cells in an SRF-dependent manner and then increased and maintained by MEF2 binding in differentiated myotubes. Therefore, SMB is a DNA element with dual and stage-specific binding activity, which modulates the effects of regulatory proteins critical in controlling the balance between proliferation and differentiation.

INTRODUCTION

Skeletal muscle repair is fulfilled by satellite cells, quiescent myogenic progenitors located between the basement membrane and myofibers in both growing and mature muscle (Mauro, 1961; Schultz *et al.*, 1978; Muir *et al.*, 1965; Bischoff and Heintz 1994; Yablonka-Reuveni, 1995). They undergo mitogenic activation in response to exercise or damage-induced signals proliferate, differentiate, and fuse into pre-existing or newly formed myofibers during muscle regeneration (Grounds and Yablonka-Reuveni, 1993; Schultz and McCormick, 1994).

Among basic helix-loop-helix (bHLH) myogenic regulatory factors (MRFs), *MyoD* is the earliest to be induced and detected both in vivo upon muscle regeneration with activation of satellite cells and in ex vivo in cell lines derived from such precursors (Füthbauer and Westphal, 1992; Grounds *et al.*, 1992). Indeed, after isolation, activated satellite cells enter the cell cycle and express *MyoD* concomitantly with proliferating cell nuclear antigen (Smith *et al.*, 1994; Yablonka-Reuveni and Rivera, 1994). This finding suggested a possible role for *MyoD* during satellite cell activation in regeneration (Yablonka-Reuveni and Rivera, 1994; Yablonka-Reuveni *et al.*, 1999). In agreement with this, we have reported an early induction of *MyoD* 4–6 h after entry of

quiescent myoblasts into the cell cycle (Kitzmann *et al.*, 1998). In addition, muscles from *MyoD*^{–/–} mutant mice are severely deficient in regenerative capacity after injury, supporting an essential role of *MyoD* in adult muscle (Megeney *et al.*, 1996). Both in vivo and ex vivo studies using isolated myofibers and primary cultured myoblasts from *MyoD*^{–/–} mice show that without *MyoD*, satellite cells undergo enhanced self-renewal rather than giving rise to progeny that can differentiate (Sabourin *et al.*, 1999; Yablonka-Reuveni *et al.*, 1999; Cornelison *et al.*, 2000).

To better understand how postnatal skeletal muscle regeneration is controlled, it is essential to investigate the mechanisms that regulate *MyoD* expression in activated satellite cells, proliferating myoblasts, and postmitotic differentiating myoblasts. Previous studies have shown that a 24-kbp 5'-flanking region of mouse and human *MyoD* is sufficient to recapitulate endogenous *MyoD* expression during mouse muscle development and in mature postnatal muscles (Chen *et al.*, 1999, 2001). Three regions were characterized among these 24-kbp in humans and mice: a minimal 275-base pair promoter called proximal regulatory region (PRR) (Goldhamer *et al.*, 1992; Tapscott *et al.*, 1992), and two muscle-specific enhancers with distinct but overlapping specificity, a 750-base pair distal regulatory region (DRR) at –5 kb (Tapscott *et al.*, 1992; Asakura *et al.*, 1995; Chen *et al.*, 2002), and a 300-base pair core enhancer at –20 kb (Goldhamer *et al.*, 1992, 1995; Chen *et al.*, 1999, 2001). Whereas the core enhancer has been implicated in *MyoD* gene expression during development (Goldhamer *et al.*, 1995; Chen *et al.*, 1999, 2001), it is inactive after birth (Faerman *et al.*, 1995). In contrast, targeted inactivation of the DRR showed it to be dispensable during development and essential in mature

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muscle (Chen *et al.*, 2002). Recently, we reported that the DRR enhancer is sufficient to induce a reporter gene expression with the same kinetics as endogenous *MyoD* expression in vivo during satellite cell activation linked to muscle regeneration (L'honore *et al.*, 2003). Sequence analysis of the human and mouse DRR *MyoD* gene revealed a conserved but noncanonical serum response factor (SRF)-binding sequence [CC(A/T)₆AG]. Using satellite cell-derived myoblasts and in vivo muscle regeneration assays, we showed that this divergent *MyoD*-CArG-box binds SRF-containing complexes during proliferation and that it is required for transcriptional activation of *MyoD* in growing myoblasts and during regeneration (L'honore *et al.*, 2003).

In the present study, we further analyzed the potential role of this DRR CArG-like element, and we show it binds an additional complex that does not contain SRF but instead myocyte enhancer factor 2 (MEF2) proteins during differentiation. Thus, the divergent CArG sequence in *MyoD* DRR represents a new hybrid element composed of binding sites for both SRF and MEF2 factors, and it was named SMB element for SRF/MEF2/Binding element. Analyzing *MyoD* expression after microinjection and reporter expression in stably transfected myoblasts, we show that this *MyoD*-SMB element enables a molecular relay from SRF-driven to MEF2-driven activation of *MyoD* transcription when progressing from myoblast proliferation to differentiation into myotubes.

MATERIALS AND METHODS

Cell Culture

C2 mouse myoblast cells were grown in DMEM with 15% (vol/vol) fetal bovine serum (FBS), 2 mM L-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin at 37°C in a humidified 5% CO₂ incubator. For differentiation, confluent C2 cells were reseeded with DMEM (2% FBS) for indicated times. Stable transfections were performed with Lipofectamine reagent (Invitrogen, Carlsbad, CA).

Overexpression and Purification of His-tagged Proteins

His-tagged proteins were expressed in bacteria His-MEF2C plasmid (kind gift from A. Lassar, Harvard Medical School, Boston, MA) and purified as described previously (Janknecht *et al.*, 1991) by using nickel-nitrilotriacetic acid beads (QIAGEN, Valencia, CA). The integrity of proteins was analyzed by SDS-polyacrylamide gel electrophoresis and Coomassie blue staining, along with known amounts of bovine serum albumin to calculate the yield of tagged proteins.

Oligonucleotides and Electrophoretic Mobility Shift Assay (EMSA)

High-performance liquid chromatography-purified oligonucleotides (MWG Biotech, Roissy CDG, France) were labeled and purified as described previously (L'honore *et al.*, 2003). Nuclear extracts from proliferated and differentiated C2 cells were prepared as described previously (Dignam *et al.*, 1983).

For EMSA, 5 µg of nuclear extract was incubated for 15 min on ice in 1X retardation mix (10 mM Tris-Cl, pH 8, 0.1 mM EDTA, 10 mM MgCl₂, 2 mM dithiothreitol, 15% glycerol, and 2 mg/ml bovine serum albumin [BSA]) and 250 ng of single-stranded DNA. Labeled probe was added to the mixture and incubated at 30°C for 10 min. For supershift and in vitro competition analysis, 0.2 µg of rabbit polyclonal antibody (SRF G20, sc-335X; MEF2 H300, sc-10794X; Santa Cruz Biotechnology, Santa Cruz, CA) or 10–100 ng of His-tagged proteins was included before probe addition and incubated with the mixture for 1 h on ice. Samples were resolved on a nondenaturing 5% polyacrylamide gel.

Chromatin Immunoprecipitation (ChIP) in C2

Proliferative or differentiated (3 d) C2C12 cells were prepared for ChIP as described previously (Thomas *et al.*, 2001). Sonicated chromatin corresponding to 5 × 10⁶ cells was subjected to immunoprecipitation at 4°C with 4 µg of antibodies against SRF (G-20; Santa Cruz Biotechnology), MEF2 (C-21; Santa Cruz Biotechnology), or matched nonimmune immunoglobulin G (IgG) (Sigma-Aldrich, St. Louis, MO) as negative control, in the presence of 0.5 mg/ml yeast tRNA and 10 mg/ml BSA. Immunoprecipitates were collected with protein A-Sepharose beads (GE Healthcare, Little Chalfont, Buckingham-

hamshire, United Kingdom) and washed 5-fold in immunoprecipitation buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, and 1% NP-40). Immunoprecipitated DNA was isolated using Chelex-100 (Bio-Rad, Hercules, CA) as described previously (Nelson *et al.*, 2006). Quantitative real-time polymerase chain reaction (PCR) (LC480; Roche Diagnostics, Mannheim, Germany) was performed using the IQ SYBR Green Supermix (Bio-Rad). Quantitation of proopiomelanocortin third exon was used as internal control to normalize *MyoD* DRR enrichment. -Fold recruitment was calculated relative to IgG control sample.

Microinjection and Immunofluorescence

Proliferating or 3-d differentiated C2 cells were microinjected with *MyoD*-wtCArG, -csCArG, -csMEF2, and -mutCArG oligonucleotides or buffer at concentration: 50 µg/ml in 50% phosphate-buffered saline in water (vol/vol). In all cases, injection solutions contained inert rabbit immunoglobins (1 mg/ml) to serve subsequently in identifying injected cells. Three hours after microinjection, cells were fixed with Formalin, and expression of *MyoD* in the injected cells was analyzed by double immunofluorescence. Cells were stained with Alexa Red-conjugated anti-rabbit antibodies (Molecular Probes Europe, Leiden, The Netherlands) to visualize injected cells and monoclonal anti-MyoD (5.8A; BD Biosciences PharMingen, San Diego, CA) followed by Alexa Green 488-conjugated anti-mouse (Molecular Probes Europe) to probe for *MyoD* expression.

Site-directed Mutagenesis

Construct 17.11wt containing the mouse DRR and PRR regions of *MyoD* gene fused to the β-galactosidase gene was generously provided by Dr. S. J. Tapscott (Fred Hutchinson Cancer Research Center, Seattle, WA) (Tapscott *et al.*, 1992). Constructs 17.11m and 17.11cs were described previously (L'honore *et al.*, 2003). Mutation of the CArG-box in the DRR of 17.11MEF2 plasmid was generated using the QuikChange mutagenesis kit (Stratagene La Jolla, CA) with the following oligonucleotide: CCAAAAAGCCAGCTCTCTATTTAT-AGCACCT (and its corresponding lower strand oligonucleotide).

For *MyoD*-green fluorescent protein (GFP) reporter constructs, the region containing the DRR and PRR of mouse *MyoD* was excised from p17.11 (15) and cloned into pBS. The SMB element in the DRR region was subsequently mutated to MEF2 or SRF consensus by the QuikChange system (Stratagene). After excising the intragenic region separating DRR and PRR, the shortened regulatory region was subcloned in place of the cytomegalovirus promoter in pEGFP-C1-*MyoD*.

Reporter Assays

Stably transfected C2 myoblasts at the indicated stages were either used for β-galactosidase activity assays, as described previously (L'honore *et al.*, 2003), or fixed for immunofluorescence analysis after 2 d in differentiation to monitor for *MyoD*-GFP expression (via enhanced green fluorescent protein [EGFP] fluorescence) together with the expression of myosin heavy chain, a marker of differentiation, using monoclonal MF20 from the Developmental Hybridoma Study Bank (University of Iowa, Iowa City, IA), followed by Alexa 555-conjugated anti-mouse IgGs (Molecular Probes Europe).

RESULTS

The *MyoD*-DRR Enhancer Contains a Divergent CArG Element, CCATTTATAG, Required In Vivo for DRR-driven Induction of *MyoD* during Regeneration

We have previously identified in *MyoD* DRR, a divergent CArG motif, CCATTTATAG, that binds SRF from myoblast nuclear extracts and is functionally required for induction of a DRR-driven reporter gene in transgenic mice during regeneration (L'honore *et al.*, 2003). To further confirm and analyze the role of this newly identified CArG element, we have examined the induction of endogenous *MyoD* together with that of a *MyoD*-DRR-driven β-galactosidase (β-Gal) reporter transgene in mice Tibialis muscle (TA) after induction of regeneration by notexin injury. Shown in Figure 1 is an immunohistochemical analysis of β-Gal and *MyoD* expression on cryosections from 4-d regenerating TA muscle in mice bearing a transgene with either wild-type (WT) or a CArG-mutated DRR enhancer. Staining in noninjured contralateral muscle from the WT-DRR transgenic mouse is also shown. *MyoD* is absent in sections from control muscle, and it is induced in regenerating muscle as shown in the top panels. Together with induction of endogenous *MyoD*, β-Gal transgene expression is also induced in the case of the

Figure 1. Requirement for the CARg element in *MyoD*-DRR for MyoD induction during muscle regeneration. Transgenic mice carrying either the wild-type 17.11 *MyoD*-DRR-PRR- β -galactosidase reporter construct (Asakura *et al.*, 1995) (A–F) or the same construct mutated in the CARg-box present in the DRR enhancer (G–I) were subject to notexin injury of one of their TA muscle. Four days later, mice were killed, and both TA muscles were taken for cryosections and analysis of endogenous MyoD (A, D, and G) and β -galactosidase reporter expression (B, E, and H) by double immunofluorescence. A–C show control staining in contralateral uninjured TA muscle.

transgene carrying a wild type CARg-box in the DRR, showing this construct reliably mimics the expression of endogenous MyoD (middle panel). However, when the CARg-box in the DRR was mutated to a nonfunctional element (no longer able to bind SRF), the corresponding transgene did not show any induction of the reporter upon induction of regeneration, even though endogenous MyoD was effectively induced. These results show that the CARg element we identified in *MyoD* DRR enhancer is essential for MyoD induction in vivo in postnatal myogenesis.

MyoD-CARg Element: A Unique Hybrid Sequence Composed of Binding Sites for Both SRF and MEF2 Factors

To investigate whether SRF binding to the *MyoD*-CARg element could regulate *MyoD* gene expression during differentiation, we tested the capacity of *MyoD*-CARg-containing oligonucleotides to bind nuclear factors from proliferative and differentiated cell extracts by EMSAs. Specific probes spanning the CARg-box region (Figure 2A) were incubated with C2 myoblast or myotube nuclear extracts. Whereas with extracts from proliferative C2, the *MyoD*-wtCARg probe formed a major slow-migrating complex that was fully supershifted with anti-SRF (Figure 2B, complexes A and sA), in differentiated C2 extracts, an additional slower migrating complex occurred, complex B, that was not supershifted by anti-SRF antibody (marked by an asterisk). Because *MyoD*-CARg-box is characterized by a 3'-end divergence (AG instead of GG pair), we investigated whether complex B could be related to this divergence. This C-to-A divergence creates a stretch of 7 A/T that forms a motif close to a MEF2-binding site: *MyoD*-wtCARg is CCA(A/T)₄TAG, whereas MEF2 consensus sequence is CTA(A/T)₄TAG (Figure 2A).

Using specific anti-MEF2 antibodies and *MyoD*-wtCARg, we assayed for the presence of MEF2 in complex B. As shown Figure 2B, anti-MEF2 antibodies supershifted complex B to complex sB, demonstrating MEF2 factors are present in the complex.

Addition of both anti-SRF and anti-MEF2 antibodies supershifted all complexes from differentiated extracts, confirming that these two proteins are present in two different complexes, A and B, respectively (Figure 2C). Because the

G-to-A 3'-end divergence in *MyoD*-CARg is similar to that reported previously in *MLC1A*-CARg-box, shown to be functional in binding SRF (Catala *et al.*, 1995), we investigated whether *MLC1A*-CARg also binds MEF2 proteins during differentiation. Anti-MEF2 supershift experiments with *MLC1A* probe show that *MLC1A*-CARg only bound SRF but not MEF2 proteins (Figure 2B, right lanes). Therefore, the A in position 3 of the decapeptide is essential for MEF2 binding, confirming previous analyses of MEF2-binding consensus.

To further investigate the possible roles of SRF and MEF2 in *MyoD*-CARg activity during differentiation, we searched for mutations of this *MyoD*-DRR sequence that would allow either only SRF binding or only MEF2 binding. As shown in Figure 2C, a single nucleotide mutation to restore the consensus CARg GG pair (instead of AG) at the 3' end of this element (Figure 2C, SRFcs), results in both stronger SRF binding in complex A and no MEF2 binding as complex B. In contrast, when the *MyoD*-CARg motif was mutated at its 5' end from CC to CT (Figure 2A, MEF2cs), the binding of SRF as complex A was abolished, and only MEF2 was bound as complex B all supershifted to sB by anti MEF2 antibodies.

These results identify a decanucleotide consensus sequence in *MyoD*-CARg required to generate a unique hybrid sequence that includes both SRF and MEF2 divergent binding sites: CCA(A/T)₄TAG. This new hybrid element was named SMB for SRF and MEF2 Binding element.

Competitive Binding of SRF and MEF2 to *MyoD*-SMB Element in Differentiated C2 Cells

To investigate whether SRF and/or MEF2 proteins were bound to the SMB element in *MyoD* promoter in living cells, we performed chromatin immunoprecipitation by using nuclear extracts from proliferating or differentiated C2 myoblasts. Results in Figure 3A show a three- to fourfold higher recruitment of SRF to *MyoD*-SMB element in proliferating than in differentiated myoblasts. In contrast, the level of MEF2 proteins recruitment is the same as control nonimmune Ig level in proliferation and twice the level of SRF recruitment in differentiated cells. These data show that SRF is bound to *MyoD*-SMB in myoblasts, whereas either MEF2 or SRF can be bound in myotubes. It is known that differentiated myoblasts contains two populations of cells:

Figure 2. The CARG element in *MyoD* gene DRR is an hybrid sequence composed of binding sites for both SRF and MEF2 factors. (A) Comparison of the classical CARG sequence to the divergent CARG elements present in *MyoD*-DRR and MLC1A promoters. (B) *MyoD*-wt-CARG and *MyoD*-MLC1A sequence-containing oligonucleotides were radiolabeled and used as probes in EMSA with nuclear extracts from growing (Pro) and differentiated (Dif) C2 myoblasts. Rabbit polyclonal antibodies raised against SRF or MEF2 were added or not before the DNA binding reaction. Two complexes A and B, and anti-SRF or anti-MEF2 supershifted (complex sA and sB), are indicated by arrowheads. An asterisk marks the complex specifically formed with differentiated cell extracts and not supershifted by anti-SRF antibodies. (C) Single point mutations of the *MyoD*-wt-CARG probes as shown were used in EMSA with nuclear extracts from differentiated C2 cells. Antibodies to SRF, MEF2, or both were added or not before DNA binding. The complexes formed are indicated with arrows in B.

"reserve" cells that become quiescent but do not differentiate (15–20% of total nuclei) and differentiated multinucleate myotubes. To examine whether the binding of SRF and MEF2 to *MyoD*-SMB was different in these two subpopulations, we have analyzed their binding by chromatin immunoprecipitation performed on each subpopulation isolated from differentiated C2 myoblasts as described previously (Kitzmann *et al.*, 1998). Figure 3B shows that whereas only little SRF and no MEF2 is found recruited on the *MyoD*-SMB in quiescent reserve cells, four- to fivefold more MEF2 than SRF is recruited to the *MyoD*-SMB element in differentiated myotubes.

MyoD-SMB element contains both SRF and MEF2 binding sequences that overlap, a situation that has not been previously described for these two factors. Because SRF interacts

with DNA by point contacts with the helix minor groove and MEF2 with the major groove of DNA (Mueller and Nordheim, 1991; Wynne and Treisman, 1992), both factors could, in principle, occupy the SMB site simultaneously. However, interactions between the two members of the MADS-box family have not been described previously (our unpublished data; Shore and Sharrocks, 1995). The steric hindrance of high molecular weight (MW) complexes associated with either SRF (L'honore *et al.*, 2003) or MEF2 (~600 kDa; our unpublished observation) suggests a mutually exclusive binding of SRF- and MEF2-containing complexes to SMB element. To test this hypothesis, we performed DNA binding competition studies between the two factors by using His-tagged MEF2 proteins. The experiment was performed using constant amounts of nuclear extract from pro-

Figure 3. Competitive Binding of SRF and MEF2 to MyoD-SMB element in differentiated C2 cells. (A) Chromatin immunoprecipitation assay of SRF and MEF2 binding to the MyoD-DRR SMB element in proliferating (empty bars) and differentiated (full bars) C2 cells. (B) Using C2 cells differentiated for 4 d, myotubes were separated from quiescent undifferentiated reserve cells by limited trypsinization (Kitzmann *et al.*, 1998), and each population was analyzed by ChIP for binding of SRF and MEF2 to the SMB element. (C) Competition of endogenously formed SRF-containing complex by increasing amounts of His-tagged-MEF2: *MyoD*-wtCARG oligonucleotides were radiolabeled and used as probes in EMSA in presence of 2 μ g of proliferating C2 nuclear extracts and increasing amounts of His-MEF2 protein (10–100 ng). SRF (A) and YY1 complexes, and MEF2 shift, are indicated by arrowheads. (D) Binding of SRF complex (A) and His-MEF2 protein was measured by quantification of each shift. Shown is the MEF2-competed down SRF complex.

liferative C2 cells (where only SRF is present and bound as complex A), in the presence of labeled *MyoD*-wtCARG (SMB) probe and increasing quantities of His-MEF2 protein (0–100 ng). EMSA analysis in Figure 3, C and D, shows that adding MEF2 proteins displaced endogenous SRF-containing complexes bound to *MyoD*-SMB element. In addition, YY1-containing complexes, described previously with proliferative cell extracts (L'honore *et al.*, 2003), were also reduced with increased MEF2 binding. A similar reduction of the YY1-containing complex was observed in differentiated extracts in comparison to proliferative extracts (Figure 2B). These data show SRF and MEF2 proteins compete for binding to the *MyoD*-CARG SMB element.

Endogenous *MyoD* Expression Is Rapidly Suppressed in Differentiated Myotubes Microinjected with *MyoD*-SMB- and MEF2-binding Oligonucleotides

To determine the functional importance of the SMB element, and particularly the contribution of an interaction with SRF and/or MEF2 to endogenous *MyoD* expression in growing myoblasts and in differentiated myotubes, we microinjected oligonucleotides that can bind and titrate out endogenous transcription factors, thereby preventing their binding to endogenous sequences (Lamb *et al.*, 1996). *MyoD*-wtSMB (SRF and MEF2 binding), -mutCARG (binding none), -csCARG (SRF-binding only), and -csMEF2 (MEF2-binding only) oligonucleotides were injected in proliferative myoblasts or in differentiated myotubes, and cells were fixed for analysis of *MyoD* expression 2–3 h after injection of the oligonucleotides, a time sufficient to allow for the turnover of endogenous *MyoD* protein and RNA but not sufficient for any indirect effect on other potential targets of either SRF or MEF2 transcription factors. Results, shown for injections in myotubes Figure 4A, are summarized for both stages in Figure 4B. Microinjection of the injection marker alone (control) had little or no effect on *MyoD* gene expression with 55 and 92% *MyoD*-positive nuclei in microinjected myoblasts

and myotubes, respectively. Microinjection of *MyoD*-wtSMB oligonucleotides (that can bind SRF or MEF2), induced a significant decrease of the number of *MyoD*-positive myonuclei, with only 40% of myonuclei expressing *MyoD* in microinjected myotubes. An even stronger reduction was observed when *MyoD*-csMEF2 oligonucleotides—that bind only MEF2 protein—were microinjected, with <5% *MyoD*-positive nuclei in injected myotubes. Interestingly, the same MEF2-cs oligonucleotides had no effect on *MyoD* expression when injected in proliferative myoblasts (Figure 4B, gray bars). In contrast, consensus *MyoD*-csCARG—that binds only SRF—did not affect *MyoD* expression in differentiated myotubes (Figure 4, A and B), whereas the same *MyoD*-csCARG oligonucleotides strongly reduced *MyoD* gene expression in myoblasts (Figure 4B, black bars). As a control, a mutated *MyoD*-CARG-SMB that no longer binds SRF nor MEF2 had no effect on *MyoD* expression in myoblasts or myotubes. These results show that although the endogenous expression of *MyoD* requires SRF in myoblasts, it no longer does in myotubes where MEF2 is involved.

MyoD DRR Reporter Activity in C2 Cells during Transition from Proliferation to Differentiation by Using Wild-Type and Mutated SMB Sequences

To investigate whether *MyoD*-SMB element was required for specific activation of *MyoD* gene during differentiation, and the respective contributions of SRF and MEF2 binding to SMB in this process, we used a reporter construct 17.11wt described previously by Tapscott *et al.* (1992), which is specifically expressed after chromatin integration in myoblast cell lines but not in 10T1/2 fibroblast cells. This construct containing the 720 base pairs of the DRR fused to the PRR and β -Gal is specifically activated in muscle from transgenic mice during regeneration (Figure 1). Two mutations in the SMB sequence of the DRR of this construct were introduced to transform *MyoD*-wtSMB into either a consensus *MyoD*-csCARG that shows enhanced SRF binding and no MEF2

Figure 4. MEF2-binding and not SRF-binding oligonucleotides inhibit MyoD expression when injected into differentiated myotubes. (A) Differentiated C2 myotubes were microinjected with oligonucleotides corresponding to MyoD-wtCARG, -csCARG, -csMEF2, and -mutCARG (SMB) sequences (see Figure 2) at 50 µg/ml. Cells were fixed 3 h after injection, and expression of MyoD was analyzed in microinjected cells by double immunofluorescence staining. (B) Percentage of MyoD-positive cells among injected cells in each experiment. Eighty to 100 cells were injected for each experimental condition. Control represents the percentage of MyoD-positive cells after microinjection of only inert marker IgG.

binding or into an MEF2 consensus site *MyoD*-csMEF2 with enhanced MEF2 binding and no SRF binding, as shown Figure 2C. Each construct, called 17.11wt, 17.11csCARG, and 17.11csMEF2, was stably transfected into C2 cells and enhancer activity was tested in growing myoblasts at confluence and at different times of differentiation (Figure 5A).

As shown previously (Tapscott *et al.*, 1992), the *MyoD*-DRR construct (wtSMB) showed increased activity from proliferation to differentiation (Figure 5A, triangles). A mutation reverting *MyoD*-wtSMB to the consensus csCARG resulted in an increased enhancer activity in proliferation, likely related to increased SRF binding (Figure 2B). However, as C2 myoblasts reach confluence and stop proliferating, this activity decreased, and it became very weak after 2 d of differentiation into myotubes (Figure 5A, squares). These data confirm the microinjection data showing a lack of effect of SRF-binding oligonucleotides on endogenous *MyoD* expression in myotubes. In addition, the opposite mutation that created a MEF2 binding sequence that no longer binds SRF in *MyoD*-DRR showed weak enhancer activity in proliferating myoblasts, increasing at confluence and in differentiated myotubes to levels greater than DRR containing wt-SMB sequence (Figure 5A, circles). Together, these data show that although *MyoD*-DRR activity required SRF binding on its SMB-box in proliferative myoblasts, it needed MEF2 binding in differentiated myotubes. These results were qualitatively confirmed by in situ analysis of the expression of a similar transgene where the β -galactosidase

reporter was replaced with MyoD-EGFP fusion protein (see *Materials and Methods*) together with myosin heavy chain (MHC), in differentiated C2 cells stably transfected with either SRFcs- (Figure 5B), wt-SMB- (Figure 5C), or MEF2cs-containing constructs (Figure 5D). Whereas SRFcs-containing MyoD construct is only expressed in undifferentiated MHC-negative cells (Figure 5B, arrow), SMB- and MEF2cs-containing constructs are both expressed in differentiated MHC-positive myotubes (Figure 5, C and D), and the wt-SMB construct is also expressed in a MHC-negative myoblast (Figure 5C, arrow). Together, these data show that the hybrid SMB element in *MyoD* DRR is the site for a molecular relay from SRF-driven to MEF2-driven transcription of *MyoD* when progressing from proliferative myoblasts to differentiated myotubes.

DISCUSSION

We previously showed that *MyoD*-DRR enhancer contains a divergent SRE/CARg element that binds SRF with low affinity in proliferative myoblasts (L'honore *et al.*, 2003). This element is functionally active and required during myoblasts growth and toxin-induced muscle regeneration (Figure 1). Examining the role of this divergent CARg during differentiation revealed that it represents a new hybrid element that can bind MEF2 in addition to SRF. This SMB element forms a new complex in differentiation by binding MEF2 proteins. ChIP experiments reveal that whereas only

Figure 5. Point mutations in *MyoD*-SMB element modulate *MyoD*-PRR-DRR construct activity in the course of differentiation. C2 cells were stably transfected with constructs containing either wild-type CArG (SMB) (17.11wt), consensus CArG (17.11csCArG), consensus MEF2 site (17.11csMEF2), or mutated CArG (SMB) (17.11mut) as shown in Figures 1A and 2C. DRR enhancer activity was then examined by β -Gal activity measurements in proliferative cells, at confluence or 1 and 2 d after switch in differentiation medium. Values represent the average calculated from at least two different experiments on stably transfected C2 cell lines for each point. (B–D) C2 cells were stably transfected with a transgene where β -gal reporter was replaced with *MyoD*-EGFP fusion protein containing either the wild-type *MyoD*-SMB box (C), the SRF consensus mutated box (B), or the MEF2 consensus mutated box (D). Cells were fixed after 2 days of differentiation.

SRF is bound to this element in myoblasts, mostly MEF2 and with only little SRF are bound in myotubes and compete for the *MyoD*-SMB element during differentiation. Analyzing the short-term (within 2- to 3-h) direct effect of oligonucleotides microinjection on *MyoD* expression shows that it requires SRF in myoblasts and only MEF2 but not SRF in differentiated myotubes. Using SMB-mutated DRR-containing reporter constructs, we show that this new dual-specificity divergent CArG/SMB element in *MyoD*-DRR enables a relay from SRF-driven to MEF2-driven transcription of *MyoD* when progressing from proliferative to differentiating stages of myogenesis.

MyoD-SMB Element Is a Unique Hybrid Sequence Capable of Binding Either SRF or MEF2 Protein Complexes

We have identified that a single base divergence from G to A in the core sequence from CC(A/T)₆GG to CC(A/T)₆AG in *MyoD*-CArG element resulted in hybrid SRF/MEF2 binding sequence. Interestingly, a divergent CArG sequence in *MLC1A* gene, described as functional (Catala *et al.*, 1995), contains the same 3' G-to-A divergence as the *MyoD*-SMB element. In addition, the decanucleotide core sequences are identical in both cases, with the exception of one nucleotide divergence (A to T) in the A/T tract of *MLC1A* CArG. However, the *MLC1A*-CArG does not bind MEF2-containing complexes from differentiated myotubes, showing that the first A in the A/T tract is critical for MEF2 binding. This identifies the following consensus decanucleotide for an SRF and MEF2-binding SMB sequence: CCA(A/T)₄TAG. A search for this consensus in other regulatory regions of *MyoD*, particularly in the core enhancer (–20 kb) (Goldhamer *et al.*, 1992), revealed no other potential SMB elements.

ChIP analysis shows that in differentiated C2, SRF, or MEF2 can be bound to this new hybrid element. However, analysis of differentiated cultures after separating the population of undifferentiated reserve cells from differentiated myotubes reveals a much higher recruitment of MEF2 factors in the myotube fraction with only SRF being recruited to

the SMB element in reserve cells. In addition several lines of evidence indicate that the two factors cannot bind to the SMB together. First, the two proteins have overlapping binding sequences in *MyoD*-SMB. Second, no interaction is possible between two different members of the MADS-box family (for review, see Shore and Sharrocks, 1995), and we found no evidence of interaction (by coimmunoprecipitation) in differentiated myotube extracts (our unpublished observations). Third, SRF and MEF2 proteins compete for binding to *MyoD*-SMB element (Figure 4A). This mutual exclusion of SRF and MEF2 on *MyoD*-DRR SMB and the appearance of MEF2-containing complex specifically in differentiation led us to hypothesize that SRF and MEF2 have distinct and independent roles on this element to regulate *MyoD* expression in proliferation and differentiation.

Requirement for SRF and MEF2 Proteins in *MyoD*-DRR Activity during Myoblast Growth and Differentiation

The initial requirement for SRF in the induction of *MyoD* expression in proliferating myoblasts (L'honore *et al.*, 2003) fits with the classical function of SRF in the G0 to G1 induction of gene expression. Indeed, *MyoD* was shown to be induced within 4–6 h after entry of quiescent C2 myoblasts into the cell cycle (Kitzmann *et al.*, 1998) and upon activation of satellite cells in vivo (Beauchamp *et al.*, 2000) or reserve cells ex vivo (Rochat *et al.*, 2004). The SMB element, being divergent from the consensus, binds SRF with a low affinity that is consistent with the low level of *MyoD* expression in proliferative myoblasts. During toxin-induced regeneration, SRF levels remain constant (see figure 6B in L'honore *et al.*, 2003), whereas MEF2 protein induction and increase in transcriptional activity take place after 3 d (Akkila *et al.*, 1997), a time that corresponds to the transition from activation-proliferation of satellite cells to differentiation. Analyzing β -galactosidase and *MyoD*-GFP reporter activities of different SMB-mutated DRR constructs stably transfected in C2 myoblasts showed that whereas *MyoD*-DRR enhancer activity needs SRF binding to SMB to induce *MyoD* when cells proliferate, it needs MEF2 binding and no longer SRF when

cells stop proliferating and enter differentiation, corresponding to increased levels of MyoD.

Recent reports examined the role of SRF *in vivo* during postnatal myogenesis by using conditional knockout of SRF. These studies inducing the cAMP response element recombinase from either the myogenin and MCK promoters (Li *et al.*, 2005) or the skeletal α -actin promoter (Charvet *et al.*, 2006) found either no effect on MyoD mRNA level (Li *et al.*, 2005) or even increased MyoD expression (Charvet *et al.*, 2006). The knockout of SRF was induced in both case in differentiating (myogenin promoter) or differentiated (skeletal actin) myofibers. Their results are thus consistent with our conclusion that SRF, although required for MyoD expression in dividing myoblasts, is no longer necessary for MyoD expression in differentiated myotubes. The increased expression observed in the second study is interesting in view of the competition for binding the MyoD-DRR SMB element we found between SRF and MEF2 in differentiation where the possible binding of SRF may reduce the level of MyoD activation by MEF2.

Cross-Regulation between MEF2 and Myogenic bHLH Expression

Several studies have revealed a role for MEF2 factors in the regulation of myogenic bHLH genes. The *myogenin* promoter, for example, contains a MEF2 site required for high level transcription *ex vivo* in cultured muscle cells (Edmondson *et al.*, 1992; Buchberger *et al.*, 1994) and *in vivo* in the limb buds and somites of transgenic mice (Cheng *et al.*, 1993; Yee and Rigby, 1993). MEF2 is also crucial to *MRF4* gene expression. Indeed, the *MRF4* promoter contains an MEF2 binding site that positively regulates its activity, because mutations in this site reduce *MRF4* promoter activity in both C2 myotubes and primary chicken muscle cultures (Naidu *et al.*, 1995; Black *et al.*, 1995). Alternatively, forced expression of myogenic bHLH proteins in nonmuscle cells is sufficient to up-regulate MEF2 expression (Cserjesi and Olson, 1991; Lassar *et al.*, 1991). During developmental and postnatal muscle differentiation, MyoD and myogenin are expressed before, and are activators of, MEF2 transcription, suggesting a model in which MEF2 is induced by MRF transactivation, whereas amplification and maintenance are regulated by cooperative activation within MEF2 and MRFs families (Wang *et al.*, 2001). During regeneration, MyoD induction also precedes MEF2 expression. Thus, MyoD expression during regeneration may involve an autoregulatory and amplification loop with MyoD gene induction by SRF during satellite cells activation into proliferation, MEF2 induction by MyoD and myogenin at the onset of cell cycle exit, and MEF2 requirement for increase and maintenance of MyoD expression during differentiation. MEF2 transcription factor family includes four genes, MEF2A to MEF2D known to be sequentially expressed during muscle development and in cultured myoblasts: MEF2B and MEF2D are already expressed in myoblasts (Breitbart *et al.*, 1993; Molkentin *et al.*, 1996), whereas MEF2A and MEF2C accumulate during differentiation with MEF2A occurring early in differentiation and MEF2C latter in myotubes and mature muscle fibers (Martin *et al.*, 1993; McDermott *et al.*, 1993). We found no recruitment of MEF2 to MyoD-SMB in myoblasts supporting that the isoforms of MEF2 assembled on the SMB element in differentiation could be either MEF2A or MEF2C or both. Because MEF2A is induced early during differentiation and antibodies specifically directed against the MEF2C isoform failed to immunoprecipitate any SMB-bound MEF2 (our unpublished observations), MEF2A is the likely isoform to be involved in the complex assembled on the SMB element

during differentiation, consistent with an early role of MEF2 in a feedback loop to increase MyoD expression at the onset of differentiation.

Why would MyoD expression be under the control of such a switch between SRF and MEF2 compared with others MRFs? First, MyoD is the earliest MRF to be induced during regeneration and the only MRF to be expressed both in proliferating and differentiated myogenic cells. Second, whereas in proliferation, MyoD regulation is cell cycle dependent (Kitzmann *et al.*, 1998; 2001), it increases and becomes constant during early differentiation. Therefore, signaling pathways regulating MyoD gene are different, depending on the stage (proliferative or differentiated) of the skeletal muscle cells. In this respect, it is likely that SRF and MEF2 binding to SMB may depend the activity of these signaling pathways. Although in quiescent myoblasts only SRF binds MyoD-SMB (cf. Figure 3B) as an inactive dimmer (as assessed by EMSA and gel filtration; L'honore and Fernandez, unpublished observation) during mitogenic activation promoted by insulin and insulin-like growth factor-1, Rho GTPase signaling is activated (Sordella *et al.*, 2003), and as an upstream activator of actin dynamics and SRF (Kuwahara *et al.*, 2005), this activates MyoD transcription at the DRR where SRF binds to MyoD-SMB element as high-MW complexes containing SRF coactivators (L'honore *et al.*, 2003). In contrast, during differentiation, calcium signaling pathways are up-regulated (Delling *et al.*, 2000; Friday *et al.*, 2000, 2003; Olson and Williams, 2000a,b), and activation of the calcium-dependent phosphatase calcineurin may promote MEF2-dependent transcriptional activation at the MyoD-SMB element where we have identified the presence of MEF2-containing high-molecular-weight complexes (our unpublished observations). Our data show that the essential role of SRF in MyoD induction during activation and proliferation of satellite cells is relayed at the onset of differentiation by MEF2 proteins. Therefore, the dual binding specificity of MyoD-SMB element makes it a key molecular switch in the regulation of MyoD expression during postnatal myogenesis.

To our knowledge, these findings represent the first report of a regulatory DNA element with a dual binding activity that depends on proliferative or differentiated stage of the cells. Because SRF and MEF2 are expressed and involved in tissues other than skeletal muscle in particular cardiac, the identification of this element may shed new light on regulatory pathways controlling the balance between cell renewal and postmitotic differentiation. In this respect, it is interesting to note that the SRF-binding site recently described as conserved and essential in the upstream enhancer of the microRNAs miR-1-1 and miR-1-2 indeed corresponds to the SMB consensus (CCA(A/T)⁴TAG) (figure 2 in Zhao *et al.*, 2005). miR-1-1 and -2 are specifically expressed in cardiac and skeletal muscle and have been shown by Zhao *et al.* (2005) to regulate Hand2, a transcription factor critically involved in the transition between proliferation and differentiation, because it promotes cardiomyocyte expansion.

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