Organization and Myogenic Restricted Expression of the Murine Serum Response Factor Gene

A ROLE FOR AUTOREGULATION*

(Received for publication, May 6, 1997)

Narasimhaswamy S. Belaguli, Lisa A. Schildmeyer, and Robert J. Schwartz‡

From the Department of Cell Biology, Baylor College of Medicine, Houston, Texas 77030

Serum response factor (SRF), a member of an ancient family of DNA-binding proteins, is generally assumed to be a ubiquitous transcription factor involved in regulating growth factor-responsive genes. However, avian SRF was recently shown (Croissant, J. D., Kim, J.-H., Eichele, G., Goering, L., Lough, J., Prywes, R., and Schwartz, R. J. (1996) Dev. Biol. 177, 250–264) to be preferentially expressed in myogenic lineages and is required for regulating post-replicative muscle gene expression. Given the central importance of SRF for the muscle tissue-restricted expression of the striated a-actin gene family, we wanted to determine how SRF might contribute to this muscle-restricted expression. Here we have characterized the murine SRF genomic locus, which has seven exons interrupted by six introns, with the entire locus spanning 11 kilobases. Murine SRF transcripts were processed to two 3’-untranslated region polyadenylation signals, yielding 4.5- and 2.5-kilobase mRNA species. Murine SRF mRNA levels were the highest in adult skeletal and cardiac muscle, but barely detected in liver, lung, and spleen tissues. During early mouse development, in situ hybridization analysis revealed enrichment of SRF transcripts in the myotomal portion of somites, the myocardium of the heart, and the smooth muscle media of vessels of mouse embryos. Likewise, murine SRF promoter activity was tissue-restricted, being 80-fold greater in primary skeletal myoblasts than in liver-derived HepG2 cells. In addition, SRF promoter activity increased 6-fold when myoblasts withdrew from the cell cycle and fused into differentiated myotubes. A 310-base pair promoter fragment depended upon multiple intact serum response elements in combination with Sp1 sites for maximal myogenic restricted activity. Furthermore, cotransfected SRF expression vector stimulated SRF promoter transcription, whereas dominant-negative SRF mutants blocked SRF promoter activity, demonstrating a positive role for an SRF-dependent autoregulatory loop.

† To whom correspondence should be addressed: Dept. of Cell Biology, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030. Tel.: 713-798-6649; Fax: 713-798-7799.

‡ The abbreviations used are: SRF, serum response factor; mSRF, murine serum response factor; SRE, serum response element; bp, base pair(s); kb, kilobase pair(s); GST, glutathione S-transferase; PCR, polymerase chain reaction.

* This work was supported by National Institutes of Health Grants R01 HL50422 and P01 HL49953. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) U49759.

This paper is available on line at http://www.jbc.org
Thus, the increase in SRF mRNA appeared prior to the up-regulation of α-actin gene activity during myogenesis (28). Vandromme et al. (29) demonstrated that microinjection of SRF antibodies prevented the progression of myogenic differentiation, implying an early dependence on SRF. In addition, Croissant et al. (27) demonstrated that a dominant-negative SRF mutant, defective in DNA binding but capable of heterodimerization with other SRF monomers, inhibited the transcriptional activity of the skeletal α-actin gene promoter in myogenic cultures and also blocked terminal differentiation. Thus, SRF has a requisite role in α-actin gene transcription during terminal skeletal muscle differentiation.

Recently, Spencer and Misra (30) demonstrated that a 322-bp promoter region of the murine SRF gene was responsive to serum stimulation in NIH3T3 fibroblasts and that the SREs and Sp1-binding sites present within this promoter region were responsible. Since it is generally assumed that SRF is a ubiquitous transcription factor, no attempt has so far been made to understand the basis for the muscle tissue enriched expression of SRF. Given the central importance of SRF for the muscle tissue-restricted expression of the sarcomeric actin gene family, we wanted to determine how SRF might contribute to this muscle-restricted expression. Here we have characterized the SRF genomic locus. Murine SRF gene activity was reminiscent of the expression pattern of another MADS box-containing factor, MEF-2 (31), being primarily restricted to cell types derived from embryonic mesoderm such as skeletal, cardiac, and smooth muscles and, to a lesser extent, to cell types of neuroectodermal origins. Also, SRF was virtually absent in endoderm-derived tissues such as the liver, lung, and spleen. To understand the mechanisms responsible for the tissue-regulated expression of SRF, we also analyzed the cis-active elements in the SRF promoter region. Our results indicated the 310 bp of the SRF promoter region upstream of the cap site as the 5′-regulatory boundary required for muscle-restricted expression. Furthermore, dominant-negative SRF mutants blocked SRF promoter activity in muscle cells. SRF gene activity appeared to be under an autoregulatory loop, in which two high affinity SREs in the core promoter were required for the SRF expression in skeletal muscle cells.

MATERIALS AND METHODS

Cloning of Mouse SRF cDNA—A fragment of mouse SRF cDNA corresponding to nucleotides 834–1520 of human SRF (a gift from Dr. E. Olson) was used to screen a mouse heart cDNA library (Stratagene). One million plaques were initially screened with the above-mentioned mouse SRF cDNA fragment as the probe, and five clones were isolated. Restriction mapping and limited sequencing indicated that four of these clones were identical. The PstI fragment from the 3′-end of one of these clones corresponding to nucleotides 1747–1985 was used to rescreen the same library, and seven more clones were isolated. The remaining 3′-untranslated region sequences were isolated by screening 5 × 106 plaques with the NheI-BglII fragment of the mouse SRF genomic DNA, which corresponded to nucleotides 3354–4201 of human SRF cDNA. The mouse SRF cDNA restriction fragments were subcloned and sequenced using the Sequenase Version 2.0 sequencing system (U. S. Bioscience, Inc.).

Isolation of SRF Genomic Clones—The mouse strain 129 genomic library constructed in the EMBL2 vector was a gift from Dr. Philip Soriano. Three clones, each containing ~14 kbp of insert DNA, were isolated by screening one million plaques with the mSRF cDNA fragment corresponding to nucleotides 834–1520 of the human SRF cDNA. Restriction mapping and Southern analysis of these clones with various probes derived from the murine SRF cDNA indicated that clone 5 contained the complete SRF gene. Appropriate fragments from this clone were subcloned and sequenced. The exon/intron borders were assigned by aligning the cDNA and genomic DNA sequences.

RNA Isolation and Analysis—Total RNA was isolated from adult mouse tissues according to Chomczynski and Sacchi (32). Thirty μg of total RNA was resolved on formaldehyde-containing 1% agarose gel and then blotted onto GeneScreen membrane (NEN Life Science Products). The prehybridization, hybridization, and washings were according to the manufacturer’s recommendations. The preincubation (nucleotides 939–1747) and the post 1 poly(A) region probes were hybridized overnight in 50% formamide at 42 °C and washed at 65 °C. The 23-mer primer used for primer extension analysis was complementary to nucleotides 82–104 of mSRF cDNA. The primer was extended with T4 polynucleotide kinase and γ-32PATP, and 100,000 cpm of the labeled primer was hybridized overnight at 30 °C with 50 μg of skeletal muscle total RNA in 80% formamide, 100 mM sodium citrate, pH 6.4, 300 mM sodium acetate, pH 6.4, and 1 mM EDTA. The primer-RNA hybrid was precipitated and extended with 200 units of SuperScript II reverse transcriptase (Life Technologies, Inc.) in 50 mM Tris-HCl, pH 8.3, 50 mM KCi, 3 mM MgCl2, 0.01 mM dithiothreitol, and 0.5 mM dNTPs for 1 h at 45 °C. The reaction was treated with 1 μg of RNase A; extracted once with phenol/chloroform; and analyzed on an 8% urea, 6% denaturing polyacrylamide gel. A dideoxy sequencing ladder generated using the same primer was used as size markers.

In Situ Hybridization of SRF in Mouse Embryos—In situ hybridization was performed on 7-mm sections of day 11.5 mouse embryos as described by Croissant et al. (27). A murine SRF probe, corresponding to the 3′-untranslated region subcloned into pBluescript, was linearized with the appropriate restriction enzymes to produce antisense 32P-labeled copy riboprobes. Sections were hybridized overnight at 58 °C and washed at 64 °C. Sections were processed for emulsion autoradiography, post-stained with Hoechst 33258, and visualized by epifluorescence and dark-field microscopy.

Recombinant DNA Constructions—The wild-type p–456 plasmid was constructed by ligating the PCR-amplified 479-bp fragment containing 456-bp 5′- and 23-bp 3′-sequences relative to the transcriptional start site of the SRF gene and inserted between the NheI and XbaI sites of the luciferase reporter plasmid pGLO2. To construct plasmids p–310, p–292, and p–227, p–456 was digested with PspI and briefly digested with Bal31 exonuclease. After inactivation of Bal31 exonuclease, the 5′-overhangs were removed by T4 DNA polymerase digestion. The remaining DNA was trimmed with EcoRI, which cuts once within the luciferase vector. The SRF promoter region and part of the luciferase vector were gel-purified and ligated into the Smal/EcoRI-digested fragment of pGLO2. Plasmids p–136, p–57, and p–37 were derived from intermediate constructs containing a BglII site introduced by site-directed mutagenesis into plasmid p–456. The smaller BglII fragments from these intermediate plasmids containing 136, 57, and 37 bp of the SRF promoter were cloned at the BglII site of pGLO2-basic to construct p–136, p–57, and p–37, respectively.

Site-directed Mutagenesis of SRF Promoter Elements—Site-directed mutagenesis procedures were introduced into the p–456 backbone by PCR. Polymerase chain reactions were performed in a 50-μl volume of Pfu polymerase buffer (100 pmol each of the primer pairs, 0.2 mM dNTPs, and 5 μl of Pfu polymerase (Stratagene)). The upstream and downstream wild-type primers were designed with NheI and XbaI sites, respectively, at their 5′-ends. Mutagenic primers contained either BglII (for SRF mutation) or EcoRI (for Sp1 site mutation). The conditions for PCR were as follows: initial denaturation at 95 °C for 10 min and an additional 30 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 2 min. The final extension was at 72 °C for 20 min. The products of the secondary PCR containing the desired mutations were digested with NheI and XbaI and ligated at the NheI site of pGLO2. All mutations were confirmed by sequencing.

Tissue Culture and Plasmid DNA Transfections—Chicken embryo primary skeletal myoblasts were isolated from day 11 embryonic breast muscle tissue as described previously (33). After transfection, cells were placed in minimum Eagle’s medium containing 10% horse serum and 2% chicken embryo extract. CV1 and HepG2 cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum both before and after transfection. Cells were transfected with 1 μg of the indicated plasmid DNA along with 0.2 μg of pCMV-β-galactosidase vector by LipofectAMINE (Life Technologies, Inc.) according to the manufacturer’s recommendations. Transactivation assays were performed with SRF promoter-reporter constructs (1 μg cotransfected with 150 ng of SRF expression vector or empty vector). SRF promoter inhibition assays were performed with 150 ng of a SRF dominant-negative mutant (pCNSHIFpm1 or pCNSHIFAC). Cells were harvested 48 h post-transfection, and β-galactosidase activity was measured using standard methods in a luminometer. Luciferase activity was normalized for transfection efficiency using the β-galactosidase values. For the promoter transactivation experiments, luciferase activity was normalized to the total protein.

Preparation of Nuclear Extracts and DNA Binding Assays—Nuclear extracts were prepared according to Bohinski et al. (34). The protein
concentration of extracts was estimated by the Bio-Rad protein assay reagent. Electrophoretic mobility shift assays used 5–10 nmoles of poly(dG-dC) in binding buffer (50 mM NaCl, 20 mM HEPES-KOH, pH 7.5, 0.1 mM EDTA, 0.5 mM dithiothreitol, and 10% glycerol). Specific and nonspecific double-stranded oligonucleotides and antibodies were included in the reaction for competition and supershift assays, respectively. Subsequently, 0.01 pmol of the indicated end-labeled probe was added and incubated for a further 10 min. DNA-protein complexes were resolved on a 5% polyacrylamide gel cast and run in 0.5 Tris/glycine buffer. The gel was dried and autoradiographed.

**DNase I Footprinting of the SRF Gene Promoter**—The SRF promoter fragment from −253 to +23, relative to the transcriptional start site, was end-labeled at +23 and used for DNase I footprinting. One n mole of poly(dG-dC) was incubated at room temperature for an additional 10 min with increasing amounts of GST-SRF in 20 mM Tris binding buffer. The reaction was incubated at room temperature for an additional 10 min with increasing amounts of GST-SRF in 20 mM Tris binding buffer.

**Fig. 1.** Genomic organization of the murine serum response factor gene. A, a simple restriction enzyme map of the SRF gene locus is shown with the following abbreviations: B, BamHI; Bg, BglII; E, EcoRI; X, XbaI, and Xh, XhoI. B, regions comprising coding exons are shown as boxes. Closed and open boxes indicate the coding region and the 5′- and 3′-untranslated regions, respectively. The numbers below each box indicate the last nucleotide of the exon. The locations of the translational start codon (ATG), the translation termination codon (TGA), and the first and second polyadenylation signals (Poly(A)) are indicated. Introns and the flanking sequences are shown as thin lines. C, shown is the nucleotide sequence of the murine SRF gene starting at the 5′-cap site (+1) through the 5′-untranslated region, each of the coding exons, and the entire 3′-untranslated region. Only partial sequences were obtained to confirm exon/intron borders. Intron sequences were omitted from this figure.
after adding 5 \times 10^5 \text{ cpm} of probe. The MgCl_2 and CaCl_2 concentrations were adjusted to 5 and 1 \text{ mM}, respectively, and the probe was digested with 0.004 units of pancreatic DNase I at room temperature for 90 s. The reaction was stopped by the addition of 100 \mu\text{L} of loading buffer (100 \text{ mM} \text{ NaCl}, 10 \text{ mM} \text{ Tris-HCl}, pH 7.6, 15 \text{ mM} \text{ EDTA}, 0.375\% \text{ SDS}, 50 \text{ mg/mL} \text{ sonicated salmon sperm DNA, and 100 mg/mL proteinase K}) and incubated at 37 °C for 20 min. After phenol/chloroform extraction and ethanol precipitation, the samples were dissolved in loading buffer. Approximately 20,000 \text{ cpm} of the denatured samples was resolved on urea-6% polyacrylamide gel, dried, and autoradiographed.

**RESULTS**

Organization and Sequence Conservation of the Murine SRF Gene—

A mouse 129 genomic library was screened with the human SRF cDNA (35) corresponding to nucleotides 834–1520, overlapping the conserved MADS box region. Three overlapping clones, each containing ~14 kbp of mouse genomic DNA, were isolated, and one of these clones (\text{ASRF} 5) contained the complete SRF locus. The SRF structural gene extends over 10 kbp and consists of seven exons (Fig. 1A), encoding the SRF protein of 504 amino acids (Figs. 1B and 2). The sequence at the exon/intron borders conformed to the GT-AG consensus sequence as shown in Table I (36). The size of the exons ranged from 77 to 848 bp. However, based on which of the two polyadenylation signal sequences are used, the last exon was either 833 or 2337 bp in size. The methionine start codon (ATG) was located in the first exon, 347 bp downstream of the major cap site. The first exon contained 347 bp of GC-rich 5'-untranslated region and 501 bp of the coding region. The conserved MADS box region was split by the first intron and bordered by the second intron. The transcription activation domain was spread over exons 4–7. The stop codon (TGA) was located in the seventh and last exon.

Comparison of the mouse SRF amino acid sequence with the human, chicken, and X. laevis sequences revealed a very high degree of sequence conservation during evolution (Fig. 2). The 90-amino acid MADS box domain (Fig. 2, boldface) was identical in all three vertebrate SRF species. Mouse SRF is more closely related to human SRF than to X. laevis SRF. The coding region of mSRF is 94 and 95% similar to the nucleic acid and amino acid sequences of human SRF, respectively. The length of the coding region was also conserved for these two mammalian species. However, in comparison with more ancient relatives, such as the D. melanogaster SRF pruned, homologous sequences were limited only to the MADS box. Examination of the N-terminal domain revealed the presence of a conserved 36-amino acid insert in the amino-terminal region in human and mouse SRFs, which was absent in the X. laevis SRF. A higher degree of sequence divergence between mammalian and amphibian SRF species was observed in the N-terminal domain compared with the carboxyl-terminal domain, which is involved in transcriptional activation.

Characterization of the Murine SRF Promoter—

The sizes of exons in bp and of introns in kbp are shown in the second and fourth columns, respectively. The nucleotide sequence at the splice junctions is in upper-case letters for exons and in lower-case letters for introns. The codon interrupted by the splice junction is shown in the last column.

| Exon/Intron | Exon size | Splice junction sequence | Intron size | Protein interruption |
|-------------|-----------|--------------------------|-------------|---------------------|
| E1/1        | 848       | TAAGAAAG/gtacggga         | 1.6         | Lys-167/Ala-168     |
| E1/E2       | 273       | ctcceag/GCCATGA           | 1.8         | Lys-256/Asp-257     |
| E2/E3       | 263       | gtgtagat/GCAGCAGT         | 0.52        | Gly-344             |
| E3/E4       | 119       | ttttagat/CTGGGGCG         | 0.75        | Val-384             |
| E4/E5       | 192       | gatgtagat/GTGGTGTC        | 0.45        | Gly-448             |
| E5/E6       | 77        | ccctacag/ATGGCTGG         | 0.14        | Gln-474/Met-475     |
Potential binding sites for NF-Y, GATA factors, and TEF-1 are identified. Overlapping the Egr-1 sites are Sp1-binding sites. Immediate-early gene products (AP-1, Egr-1, and Ets-1) are cated at

There are two consensus SREs located at −42 and −62. In addition, there are two divergent SREs located at −142 and −222. Potential binding sites for other immediate-early gene products (AP-1, Egr-1, and Ets-1) are identified. Overlapping the Egr-1 sites are Sp1-binding sites. Potential binding sites for NF-Y, GATA factors, and TEF-1 are also present within the SRF promoter (Fig. 3B).

Two SRF mRNA Species Are Enriched in Cardiac and Skeletal Muscle Tissues—We asked, how does SRF fulfill its role in regulating striated α-actin genes, and how might SRF contrib-
tioned day 11.5 mouse embryos, SRF transcripts were seen in the neuroectoderm of the brain and the neural tube, but were absent in the underlying notochord (Fig. 5A). Transverse sections revealed high levels of SRF expression in the bulbus cordis and the right atrial portions of the myocardium, as shown in Fig. 5B. SRF was also detected at high levels in the myotomal portion of somites and in the emerging smooth muscle cells surrounding the second branchial arch artery (Fig. 5A). Lower levels of SRF was also detected in the sympathetic trunk (Fig. 5B) and in the cardinal vein. SRF was barely detected in the lung bud and liver (Fig. 5B). Sense probes in all cases showed background levels of hybridization in all tissues (data not shown). These in situ hybridization experiments demonstrated that SRF gene expression was developmentally regulated and largely restricted to the cardiac and skeletal muscle cell lineages, consistent with the early specific expression of the α-actin genes in the embryo.

Tissue-regulated Expression of SRF—To investigate the molecular basis for the muscle tissue enriched expression and to map the essential cis-acting elements, we transiently transfected chicken primary myotubes and the human liver cancer cell line HepG with SRF promoter constructs. The p–456 plasmid was 80-fold more active in myotubes than in HepG2 cells (Fig. 6). A further deletion to –310 uncovered the presence of a negative-acting element(s). This deletion of 146 bp resulted in a nearly 2.2-fold increase in the promoter activity in myotubes.

The promoter activity of the p–310 construct was 96-fold more active in myotubes than in HepG2 liver cells. A deletion of 18 bp to –292 decreased the promoter activity only slightly in myotubes and by 56% in HepG2 cells. A further deletion to –227 decreased the pro-

![Image: Fig. 5. Serum response factor expressed in murine embryonic neuroectoderm- and cardiac, somitic, and smooth muscle mesoderm-derived tissues. In situ hybridization localization (A and B) of SRF transcripts in sectioned day 11.5 mouse embryos was performed with a 35S-labeled cRNA probe as described under “Experimental Procedures.” Sections were processed for emulsion autoradiography, post-stained with Hoechst 33258, and visualized by epifluorescence, which labels cell nuclei as white spots against a blue background, and by dark-field microscopy, which shows autoradiographic grains as red spots. A shows an enlargement of the labeled neural tube. The notochord and the first and second branchial arches did not display SRF transcripts, except around the second branchial arch artery. B shows SRF transcripts in the outside wall of the bulbus cordis and right atria of the heart and the somitic myotomes. The sympathetic trunk and lung bud were lightly labeled, whereas the liver did not display in situ SRF labeling. The following abbreviations mark embryonic tissues as shown: A, atria; BA1, first branchial arch; BA2, second branchial arch; BART, branchial arch artery; BC, bulbus cordis; CV, cardinal vein; LG, lung; L, liver; M, myocardium; NT, neural tube; N, notochord; P, pharynx; ST, sympathetic trunk; S, somite.

![Image: Fig. 6. Preferential murine SRF promoter activity in cultured primary skeletal muscle cells versus HepG2 liver cells. Shown is a comparison of mSRF promoter activity in transfected primary chicken embryo myotubes and HepG2 cells. A schematic diagram of the murine SRF promoter and 5’-flanking sequences is shown to include the potential binding sites for several transcription factors. Serial 5’-deletion SRF promoter mutants were named according to the position of the 5’-deleted border, as shown on the left. The activity of each SRF promoter-luciferase (LUC) construct is expressed relative to that of the control SV40 promoter-enhancer-luciferase vector pSV2Luc. Data shown are the relative mean ± S.E. from three separate sets of transfection experiments of each plasmid construction done in duplicate.

![Image: Fig. 7. Bacterially expressed SRF protects SRE1, SRE2, and the TATA box of the mouse SRF promoter in DNase I footprints. The probe was the SRF promoter fragment from –253 to +23 end-labeled at +23. The probe was incubated with increasing amounts of purified GST-SRF and digested with DNase I. Lanes 1 and 7 did not contain any protein. The DNase I-protected region of the SRF promoter is demarcated by the open box. The closed box indicates the partial protection over the TATA box. At the bottom, regions of DNase I protection (SRE1, SRE2, and the TATA box) are underlined.
moter activity by −60% in myotubes, but not in HepG2 cells. Additional muscle-specific positive-acting element(s) were revealed by deletion to 2136, which resulted in a 4-fold decrease in the promoter activity in myotubes and a slight decrease in HepG2 cells. Two non-consensus SREs are present within this deleted 91-bp region. A further deletion of 79 bp to 257, which removed one of the two consensus SREs (SRE2) and overlapping Egr-1- and Sp1-binding sites, reduced the promoter activity by 12-fold in myotubes. Plasmid p257, which contains a single consensus SRE (SRE1) and the TATA box region, was 25-fold more active than the promoterless control plasmid pGL2 in myotubes. The p456 construct was not active in HepG2 cells, which express only low levels of endogenous SRF.

FIG. 8. The SRF promoter requires two intact SREs and an Sp1 site for transcriptional activity in muscle cells. A and B show binding of SRF and Sp1 from myotube nuclear extracts to cognate probes. End-labeled double-stranded SRE1 and SRE2 (A) and distal Sp1 (B) oligonucleotides were incubated with 5 µg of myotube nuclear extract in 1 × binding buffer. Where indicated, unlabeled competitor DNA was added at a 50-fold molar excess before adding the probe. Polyclonal antibodies for SRF, YY1, Sp1, and Egr-1 were used in supershift assays. Oligonucleotides to SRE1 (−53 to −30 bp, GCCTCGCCCTAAAGGAACATTTG), the SRE1 mutant (−55 to −25 bp, GGGCCCTCGCCATAGTACCGTTACG), SRE2 (−74 to −51 bp, GGCTGGCCCTATAAAGAGCCG), the SRE2 mutant (−75 to −46 bp, GGCCCTGGCCATAGTACCGTTACG), Sp1.1 (−94 to −71 bp, CCAATGGGGCGGGGGCTGGGGCC), the Sp1.1 mutant (−97 to −66 bp, GGACCAATGGGGCGGAATTCCGGTCTGGC), Sp1.2 (−263 to −244 bp, CAACCCAGGGGGCGGAAACT), and the Sp1.2 mutant (−266 to −236 bp, CTCAAAACCAGGGGGCGAATTCCGGTCTGGC) were used as duplexed DNA probes in band shift assays. Complexes were resolved by electrophoresis on 5% nondenaturing polyacrylamide gel cast and run in 0.5 × Tris borate/EDTA. SRF and YY1 complexes are identified in A and B. SRF promoter activity was regulated by both SRF and Sp1. Site-specific mutations at SRE1 and SRE2 (C) and proximal and distal Sp1 sites (D) were introduced into p−456 by PCR-directed mutagenesis using the mutated sequences shown above. The wild-type and mutant plasmids were transfected into primary embryonic myoblasts. Cells were cultured for 2 days in differentiation medium before harvesting. Luciferase activity was normalized to β-galactosidase activity. The promoter activity of mutated SRE and Sp1 site constructs was compared with wild-type p−456 activity, which was set at 100%. Data are presented as the mean ± S.E. of three experiments done in duplicate.

FIG. 9. SRF promoter activity elevated during myogenesis is stimulated by SRF in fibroblasts and blocked by SRF dominant-negative mutants. A, the p−456 SRF promoter-luciferase reporter gene was cotransfected with pCMV-β-galactosidase into primary chicken embryo myoblast cultures. Assays of luciferase light units normalized to β-galactosidase activity were carried out at 24-h intervals during myogenesis in culture. B, the p−456 SRF promoter-luciferase reporter gene or the SV40 promoter-enhancer-driven reporter was cotransfected in primary myoblasts with pCGNSRFpm1, pCGNSRFDAC, or the pCGN empty vector. Cells were harvested after 2 days of incubation, and luciferase activity was normalized to total protein. Normalized luciferase activity of pSV2Luc and p−456Luc cotransfected in primary myoblasts with the empty vector pCGN was set at 100. C, SRF promoter-luciferase reporter gene was cotransfected with pCGNSRF or the pCGN control vector in CV1 cells. Data are presented as the mean ± S.E. from three experiments done in duplicate.
A deletion of 20 bp to −37, which eliminated SRE1, reduced the promoter activity to background levels in myotubes, suggesting that the SREs were required for SRF promoter activity.

**Multiple SRF-binding Sites in the SRF Promoter**—Results of promoter deletion analysis suggested that the SREs present within the SRF promoter were required for transcriptional activity. DNase I protection assays performed with bacterially expressed purified SRF were used to ascertain if the two consensus SREs present in the SRF promoter bind SRF. Both SRE1 and SRE2 were well protected at the lowest levels of GST-SRF. In addition, the TATA box region was also protected, but at considerably higher inputs of GST-SRF (Fig. 7). No protection was observed over the two potential, but non-consensus SREs located at positions −142 and −222.

SREs may also serve as binding targets for YY1, NFIL-6, SRE-ZBP, SRE-BP, and several other uncharacterized factors. Many of these factors are also expressed in skeletal muscle tissue (Refs. 7 and 38; reviewed in Ref. 11). Having demonstrated specific binding of bacterially expressed SRF, we investigated the interaction of proteins from myotube nuclear extracts with SREs from the SRF promoter. Double-stranded oligonucleotide probes corresponding to SRE1 and SRE2 were incubated with the nuclear extract prepared from chicken embryo myotubes. A doublet of slowly migrating complexes (complexes I and II) and a fast migrating complex (complex III) were identified as a YY1-containing complex based on several criteria. These complexes contain Sp1 and not Egr-1. Even though the proximal Sp1 site contains an overlapping consensus Egr-1 site, binding of only Sp1 was evident under our electrophoretic mobility shift assay conditions (data not shown).

Spencer and Misra (30) showed by mutational analysis that the two Sp1 sites present in the murine SRF promoter were essential for serum induction of the promoter in 3T3 fibroblasts, but mutated Sp1 sites did not affect the basal promoter activity. The role of these Sp1 sites in the muscle enriched expression of the SRF promoter was also examined by mutagenesis. Sp1 site-directed mutations abolished the binding of Sp1 from myotube nuclear extracts (Fig. 8B). Mutagenesis of the proximal Sp1 site actually increased the promoter activity by 2-fold (Fig. 8D), whereas a mutation over the distal Sp1 site reduced the promoter activity by 60%.

**Myogenic Up-regulation of SRF Promoter Activity**—Recently, avian SRF RNA and protein and SRF DNA binding activity were shown to increase when cultured primary myoblasts were allowed to withdraw from the cell cycle and fuse to form multinucleated myotubes (7, 27). We wanted to determine if the up-regulation of SRF gene activity was under transcriptional control by examining the activity of transfected SRF promoter-reporter constructs in primary chicken embryo myogenic cultures. The SRF promoter fragment from −456 to +23 linked to a luciferase reporter gene displayed low activity in replicating pre-fusion myoblasts, but was up-regulated −6-fold in late-stage myotubes (Fig. 9A). Thus, cis-acting sequences required for the up-regulation of SRF gene activity are contained within the −456-bp promoter region.

The up-regulation of SRF during myogenesis, the presence of multiple SREs in the SRF promoter, and the high affinity binding of SRF to these SREs suggested the possibility that SRF autoregulates itself. Thus, we compared luciferase reporter activities of the p−456 SRF promoter construct cotransfected with or without SRF expression vectors in non-myogenic CV1 fibroblasts. Coexpression of an exogenous SRF resulted in up to a 5-fold increase in SRF promoter activity (Fig. 9C). We then asked if dominant-negative SRF mutants would block SRF promoter function in transfected chicken embryo myotubes. The SRFpm1 mutant (39) dimerizes with other SRF monomers and interferes with wild-type SRF by forming DNA binding-defective heterodimers. In addition, another dominant-negative SRF mutant, SRFAC, in which the C-terminal transcription activation domain (amino acids 266–504) was deleted, acts as a de facto repressor by occupying SREs through specific DNA binding, but is incapable of activating SRE-dependent transcription. Cotransfection of either SRFpm1 or SRFAC with p−456 resulted in 40 and 95% decreases in SRF promoter activity, respectively, thus suggesting that the myogenic up-regulation of SRF promoter activity was mediated by
SRF (Fig. 9B). Inhibition by SRFpm1 and SRFΔC was specific to the SRF promoter because SV40 promoter activity was not significantly affected by these dominant-negative SRF mutants. These results indicate that SRF autoregulates its own promoter and that this autoregulation is primarily mediated through SRE1 and SRE2.

**DISCUSSION**

The hypothesis that introns and RNA splicing facilitated the evolution of ancient genes in the progenote organism was recently reviewed (40). The function of introns in the evolution of genes can be explained by the proposal that either introns appeared late in evolution and could not participate in the construction of primordial genes or that RNA splicing and introns existed in the earliest organisms, but were lost during the evolution of the modern prokaryotes. Blake (41) suggested that evidence for intron-facilitated evolution of a gene might be found by comparing the borders of functional protein domains with the placement of introns. The recent elucidation of the x-ray crystal structure of the SRF MADS box demonstrated a novel DNA-binding motif, a coiled-coil, and a stratified structural subdomain involved in dimerization (2). We showed here (Fig. 1 and Table I) that the murine SRF gene consists of seven introns closely circumventing the dimerization subdomain involved in dimerization (2). Two showed introns of SRF were present between the conserved between the exons interrupted by six introns. Exon/intron borders are well conserved between the Xenopus SRF (42) and murine SRF genes. The first intron was found to sever the N-terminal extension, which makes specific base contacts within the murine gene of an SRF half-site, from the dimerization subdomain encoded in exon II. In comparison with the genomic organization of MEF-2B (43), which was conserved in Drosophila d-mef-2, other MEF-2 relatives, and the plant AGL3 gene (44), the first intron also bisected the unstructured N-terminal extension, whereas the second intron was close to the C-terminal border of MADS boxes found in animal and plant SRFs and the MADS/MEF-2 boxes in all MEF-2 genes. Thus, in all MADS box-containing genes yet examined, introns closely circumscribed the dimerization subdomain. Based on conservation of primary sequence of the MADS box region and gene organization analysis, introns might have participated in the construction of the earliest MADS box-containing genes, prior to the diversification of plants and animals that occurred at least one billion years ago.

Despite these similarities, MADS box proteins also have evolved to perform diverse functions such as specification of mating type in yeast, homeotic activities in plants, and pulmonary system development in Drosophila and elaboration of mesodermal structures in vertebrates. Interestingly, the overall structural divergence of SRF proteins among evolutionarily distant species of animals appears to be related to differences in the spatial expression pattern. For example, of the variety of animal species examined, pruned, the SRF homolog of Drosophila SRF (45), was the most divergent, in which sequence conservation was limited only to the MADS box domain. The localization of Drosophila SRF expression was different from that of Xenopus, avian, and murine SRFs, which are more uniform in structure and tissue expression. Drosophila SRF was localized to the insect tracheal system (46); whereas vertebrate SRFs, like several of their MEF-2 counterparts (reviewed in Ref. 5), were localized to avian and murine skeletal and cardiac muscle and neuroectoderm-derived tissues (Figs. 4 and 5) (27).

How does SRF play a central role in regulating muscle-specific genes that are expressed under cell differentiation-promoting conditions? We have shown that mSRF has a distinct striated muscle tissue enriched expression pattern and further identified the SRE as a mediator of SRF promoter regulation. Although it is generally assumed that SRF serves a role as a constitutive factor during its association with accessory factors, we have shown that SRF binding activity actually increased dramatically following the ending of cell replication primarily due to change in the cellular content of SRF in primary myoblasts (7). Surveys of early avian (27) and murine (Fig. 5) embryos also indicated tissue-restricted expression of SRF transcripts, which substantially increased the cellular mass of SRF in the myotomal portion of somites, cardiac myocytes, and vascular smooth muscle cells. The expression of SRF continues in these tissues at high levels through adulthood (Fig. 4). During primary myogenesis in culture, SRF promoter activity, RNA, and protein mass increase significantly preceding fusion of myoblasts and the appearance of muscle-specific structural genes (33). We showed that mutations of both SRE1 and SRE2 were required to down-regulate SRF promoter activity, suggesting that SRE1 and SRE2 were functionally redundant (Fig. 8C). Overexpression of SRF from a plasmid vector substantially increased SRF promoter activity in transfected fibroblasts (Fig. 9C). In comparison, the dominant-negative mutants of SRF, SRFpm1 and SRFΔC, inhibited the muscle enriched expression of SRF and other muscle-specific genes. Thus, SRF has a primary role in directing muscle differentiation.

Tissue-restricted expression of SRF was also evident from comparison of its promoter activity, in which the SRF promoter activity was at least 2 orders of magnitude greater in primary cultured myotubes than in liver HepG2 cells. Low SRF promoter activity cannot be attributed to the presence of strong liver tissue-specific silencer elements in the SRF promoter because serial deletions in the promoter did not activate the promoter. Although less likely, it is possible that a silencer element located within the 37-bp cap upstream region strongly repressed the SRF activity in liver cells. Another possibility for the lack of SRF promoter activity in liver cells could be the absence of SRF promoter-specific trans-acting factors from this cell type. Endoderm-derived liver tissue did not express endogenous SRF (Fig. 4). Furthermore, a minimal SRF promoter containing a single SRE, which was otherwise active in myotubes, was not active in HepG2 cells, indicating that the SRF promoter activity was SRF-dependent. The SREs were required for the higher basal level of expression in myotubes (Fig. 8C).

The basal activity of the SRF promoter in NIH3T3 cells was shown to be dependent on the more ubiquitous CAAT box-binding factor, whereas the SREs and Sp1 sites were dispensable (30). In contrast, SRE1 and Sp1 sites were required for both serum-induced promoter activity (30) and muscle tissue-restricted activity. SRF promoter deletion analysis demonstrated that SRF was necessary but not totally sufficient for driving the SRF promoter. Furthermore, autoregulation of the SRF promoter by SRF alone cannot account for the muscle tissue enriched expression of the SRF gene. The tissue enriched expression of SRF might be accomplished by the concerted action of SREs with other cis-acting elements. Accordingly, deletion of sequences 5′ to SRE1 and SRE2 decreased the promoter activity, suggesting that the interaction of these deleted sequences with SRE1 and SRE2 may be required for the complete activity of the promoter. Of the two Sp1-binding sites present in the SRF promoter, only the distal Sp1 site appeared to be important for SRF expression. Sartorelli et al. (47) have shown that a functional interaction among SRF, the ubiquitous transcription factor Sp1, and the cell type-restricted myogenic factor MyoD is required for human cardiac α-actin gene expression. One mechanism by which SRF might promote muscle-spe-
cific gene expression would be by interfering with the activity of negatively regulated factors of muscle differentiation. YY1, a ubiquitously expressed C2H2 zinc-finger protein (48, 49) that binds the consensus sequence AANATGGNG, has been shown to bind several SREs (7, 50). We observed that in proliferating myoblasts, the skeletal α-actin gene was repressed by mutually exclusive binding of YY1 over SRE1 (7). Interestingly, gel mobility shift assays with SRE2 from the SRF promoter also uncovered an overlapping YY1 site (Fig. 8A). Furthermore, comparison of nuclear extracts prepared from proliferating myoblasts with myotubes revealed mutually exclusive binding of SRF and YY1 over SRE2 (data not shown). Consistent with the repressor role of YY1, mutation of SRE2 resulted in a 2.2-fold increase in SRF promoter activity (Fig. 8). Displacement of YY1 by increased SRF binding activity that the SRF promoter activity might also be modulated by YY1. Displacement of YY1 by increased SRF binding activity during myogenesis may facilitate the SRF autoregulatory loop. YY1 and allow for saturation of the multiple SREs with positive-acting YY1. Functional interaction of SRF and MyoD or myogenin bound to muscle tissue specificity to SRF is not limited to physical interaction between SRF and a tissue-restricted transfactor. Functional interaction of SRF and MyoD or myogenin bound to different sites on the promoter can confer muscle tissue specificity to SRF (47). A similar interaction of SRF on the interleukin-2 receptor α-chain gene promoter with the Rel homology protein NF-κB confers T-cell specificity to SRF (55, 56). Thus, additional interactions of SRF with different cell type-restricted coactivators may also determine the response of different tissues to SRF.

Acknowledgments—We thank Lisa Goering and Dr. Ruxandra Dragha for assistance in preparing the in situ hybridization figure.

REFERENCES
1. Schwartz-Sommer, Z., Huijser, P., Nacken, W., Saedler, H., and Sommer, H. (1990) Science 290, 931–936
2. Pellegrini, L., Tans, S., and Richmond, T. J. (1995) Nature 376, 490–498
3. Shore, P., and Sharrocks, A. D. (1985) Eur. J. Biochem. 239, 1–13
4. Treisman, R. (1994) Curr. Opin. Genet. Dev. 4, 96–103
5. Olson, E. N., Perry, M., and Schultz, R. A. (1995) Dev. Biol. 172, 2–14
6. Lee, T.-C., Shi, Y., and Schwartz, R. J. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 9814–9818
7. Johansen, F. E., and Prywe, R. (1993) Mol. Cell. Biol. 13, 4640–4647
8. Treisman, R. (1985) Cell 41, 75–84
9. Greenberg, M. E., Siegfried, Z., and Ziff, E. B. (1987) Mol. Cell. Biol. 7, 1217–1225
10. Christy, B., and Nathans, D. (1989) Mol. Cell. Biol. 9, 4889–4895
11. Treisman, R. (1990) Cancer Biol. 1, 47–58
12. Mueller, C. G., and Norheim, A. (1991) EMBO J. 10, 4219–4229
13. Shaw, P. E. (1992) EMBO J. 11, 3011–3019
14. Rao, V. N., Huebner, K., Kiehart, D. P., Kars-Bushbi, A., Crote, C. M., and Reddy, E. S. P. (1989) Science 244, 66–70
15. Dalton, S., and Treisman, R. (1992) Cell 71, 395–406
16. Shore, P., and Sharrocks, A. D. (1994) Mol. Cell. Biol. 14, 3283–3291
17. Chen, C. Y., Croissant, J., Toupasis, S., Majesky, M., Frankovsky, M., and Walsh, K. (1992) Mol. Cell. Biol. 12, 4209–4214
18. Walsh, K. (1989) Mol. Cell. Biol. 9, 2191–2200
19. Croissant, J. D., Kim, J.-H., Helle, G. W., Goering, L., Jongsma, H. A., and Schwartz, R. J. (1986) Dev. Biol. 117, 250–264
20. Hayward, L. J., and Schwartz, R. J. (1986) J. Cell Biol. 102, 1445–1453
21. Vandermonde, M., Gauthier-Rouviere, C., Carne, G., Lamb, N., and Fernandez, A. (1992) J. Cell Biol. 118, 1489–1500
22. Sperger, J. A., and Misra, R. P. (1996) J. Biol. Chem. 271, 16535–16543
23. Yu, Y.-T., Breithart, R. E., Smoot, L. B., Lee, Y., Mahdavi, V., and Nadal-Ginard, B. (1992) Genes Dev. 6, 1783–1798
24. Chomczynski, P., and Sacchi, N. (1987) Anal. Biochem. 162, 156–159
25. Lee, T.-C., Zhou, Y., and Schwartz, R. J. (1994) Oncogene 9, 1047–1052
26. Bohinski, R. J., DiLauro, R., and Wissit, J. A. (1994) Mol. Cell. Biol. 14, 5671–5681
27. Norman, C., Runswick, M., Pollock, R., and Treisman, R. (1988) Cell 55, 899–903
28. Senapathy, P., Shapiro, M. B., and Harris, N. L. (1990) Methods Enzymol. 183, 255–278
29. Paradis, P., MacLellan, W. R., Belagudi, N. S., Schwartz, R. J., and Schneider, M. D. (1996) J. Biol. Chem. 271, 10827–10833
30. Catala, F., Wanner, R., Barton, P., Cohen, A., Wright, W., and Buckingham, M. (1996) Mol. Cell. Biol. 16, 5485–5496
31. Johansen, F. E., and Prywe, R. (1995) Biochem. Biophys. Acta 1242, 1–10
32. Stone, E. M., and Schwartz, R. J. (1990) Intervening Sequences in Evolution and Development, pp. 61–91, Oxford University Press, New York
33. Blakes, C. F. (1983) Nature 305, 537–537
34. Mahn, T. J., Chambers, A. E., Towers, N., and Taylor, M. V. (1991) EMBO J. 10, 933–940
35. Molkenstijn, J. D., Firulli, A. B., Black, B. L., Martin, J. F., Huntst, C. M., Copeland, N., Jenkins, N., Lyons, G., and Olson, E. N. (1996) Mol. Cell. Biol. 16, 3814–3824
36. Huang, H., Tudor, M., Weiss, C. A., Hu, Y., and Ma, H. (1995) Plant Mol. Biol. 28, 549–567
37. Guillemine, K., Groppe, J., Ucker, K., Treisman, R., Hafen, E., Affolter, M., and Kramon, M. A. (1996) Development (Camb.) 122, 1353–1362
38. Affolter, M., Montague, J., Walldorf, U., Groppe, J., Kloter, U., LaRosa, M., and Gehring, W. J. (1994) Development (Camb.) 120, 743–753
39. Sartorelli, V., Webster, K. A., and Kedes, L. (1990) Genes Dev. 4, 1811–1822
40. Shi, Y., Seto, E., Chang, L.-S., and Shenk, T. (1991) Cell 67, 377–388
41. Park, K., and Atibson, M. L. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 9894–9898
42. MacLellan, W. R., Lee, T.-C., Schwartz, R. J., and Schneider, M. D. (1994) J. Biol. Chem. 269, 16754–16760
43. Treisman, R. (1992) Transcriptional Regulation 881–905
44. Chen, C. Y., Croissant, J., Toupsias, S., Majesky, M., Frankovsky, M., McQuinn, T., and Schwartz, R. J. (1992) Development (Camb) 119, 119–130
45. Groisman, R., Masutani, H., Leibovitch, M.-P., Robin, P., Soudant, I., Trouche, D., and Harel-Bellan, A. (1996) J. Biol. Chem. 271, 5258–5264
46. Dehsehe, C. A. E., Wei, Q., Elderidge, J., Gannon-Zuki, L., Millasseau, P., Bouruelsenet, L., Caterina, D., and Paterson, B. M. (1994) Mol. Cell. Biol. 14, 5474–5486
47. Kuang, A. A., Novak, D. K., Kang, S.-M., Bruhn, K., and Lenardo, M. J. (1993) EMBO J. 12, 2536–2545
48. Pierce, J. W., Jamieson, C. A., Ross, J. L., and Sen, R. (1995) J. Immunol. 155, 1972–1980