Four isoforms of serum response factor that increase or inhibit smooth-muscle-specific promoter activity

Paul R. KEMP[1] and James C. METCALFE

Section of Cardiovascular Biology, Department of Biochemistry, University of Cambridge, The Downing Site, Tennis Court Road, Cambridge CB2 1QW, U.K.

Serum response factor (SRF) is a key transcriptional activator of the *c-fos* gene and of muscle-specific gene expression. We have identified four forms of the SRF coding sequence, SRF-L (the previously identified form), SRF-M, SRF-S and SRF-I, that are produced by alternative splicing. The new forms of SRF lack regions of the C-terminal transactivation domain by splicing out of exon 5 (SRF-M), exons 4 and 5 (SRF-S) and exons 3, 4 and 5 (SRF-I). SRF-M is expressed at similar levels to SRF-L in differentiated vascular smooth-muscle cells and skeletal-muscle cells, whereas SRF-L is the predominant form in many other tissues. SRF-S expression is restricted to vascular smooth muscle and SRF-I expression is restricted to the embryo. Transfection of SRF-L and SRF-M into C_2C_{12} cells showed that both forms are transactivators of the promoter of the smooth-muscle-specific gene SM22α, whereas SRF-I acted as a dominant negative form of SRF.

Key words: alternative splicing, dominant inhibitor, muscle-cell differentiation, SM22α gene promoter.

INTRODUCTION

The transcription factor serum response factor (SRF) was identified originally as an activator of the proto-oncogene *c-fos* [1,2]. Much attention has been given to the role of SRF in activation of this gene in the G0-to-G1 transition in the cell cycle and in many other cell-activation responses [3–8]. Recently, however, it has been found that SRF also plays a major role in the control of muscle gene expression and muscle differentiation. For example, the promoters that drive the expression of smooth-muscle myosin heavy chain, smooth-muscle α-actin and SM22α all contain multiple CArG [CC(A/Trich)6GG] boxes, the binding site for SRF [9–13]. These CArG boxes have been shown to be important in the control of gene expression in smooth-muscle cells *in vitro*. Furthermore, promoter analysis in transgenic mice has shown that the CArG boxes in the smooth-muscle α-actin and SM22α promoters are required for the expression of reporter genes in vascular smooth-muscle cells (VSMCs) in the adult mouse [14–16]. The promoters of several cardiac-muscle genes also contain CArG boxes, implying a role for SRF in the control of cardiac-specific gene expression [17–19]. Thus the CArG boxes present in the cardiac α-actin promoter are required for gene expression from this promoter in 10T1/2 fibroblasts [20]. Experiments using anti-SRF antibodies or antisense SRF mRNA in the skeletal-muscle myoblast cell line C_2C_{12} have also shown that SRF is required for the differentiation of these myoblasts into myotubes [21]. Targeted deletion of the SRF gene in mice has shown that the SRF is not required for the progression of the cell cycle as the cells of developing embryos divide normally [22]. However, the deletion is embryonic lethal due to a failure of gastrulation and mesoderm differentiation and the embryos die before E12.5.

The various roles of SRF in cell activation and in muscle-cell differentiation may be regulated by the large number of protein factors that interact with SRF to form functional transcription-factor complexes. For example, at the *c-fos* promoter SRF interacts with the ternary complex factors {TCFs, a subfamily of the *ets*-domain (or E26-specific domain) transcription factors [23,24]}. In contrast, at the smooth-muscle α-actin promoter the binding of SRF to the CArG boxes is enhanced by the protein Mhox. In cardiac muscle SRF has been shown to interact with the Nk homoeodomain protein Nkx 2.5, and the complex formed by these proteins activates transcription of the cardiac α-actin promoter [20,25] to a greater extent than either protein alone. At the promoters of skeletal-muscle-specific genes SRF has been shown to interact with members of the basic-helix-loop-helix (bHLH) family of transcription factors including MyoD and myogenin complexed with the ubiquitous bHLH E2A proteins [26]. One of the functions of these multiple protein complexes is probably to enhance binding of SRF to CArG boxes.

SRF is a member of the MADS (MCM-1, agamous and deficiens and SRF) box family of transcription factors. The DNA-binding domain of SRF is located in the N-terminal domain of the protein and includes the MADS domain. The transactivation domain is located in the C-terminal region of the protein, and mutational analysis has implicated amino acids 395–478 as playing a major role in transcriptional activation [27]. The activity of SRF is modified by phosphorylation at multiple sites throughout the protein. Phosphorylation at the N-terminus of SRF, in the DNA-binding domain of the proteins, occurs at serines 75, 79, 83, 85 and 103, probably via casein kinase II, which is activated by growth-factor stimulation [28–30]. Phosphorylation of these residues increases the binding of SRF to DNA and increases SRF-dependent transcription from target promoters. Phosphorylation of the C-terminal domain occurs at Ser-253 in a consensus site for casein kinase II [31], and at Ser-435 and Ser-446 mediated by DNA-activated protein kinase [27]. Mutation analysis has shown that phosphorylation of Ser-435

Abbreviations: bHLH, basic helix-loop-helix; CAT, chloramphenicol acetyltransferase; DMEM, Dulbecco's modified Eagle's medium; SRF, serum response factor; VSMC, vascular smooth-muscle cell; MADS, MCM-1, agamous and deficiens and SRF; RT-PCR, reverse-transcriptase PCR; CArG, CC(A/Trich)6GG.

[1] To whom correspondence should be addressed (e-mail pk@mole.bio.cam.ac.uk).

and Ser-446 is required for full activity of SRF but any role of phosphorylation of Ser-253 has yet to be defined.

In experiments to generate a full-length murine SRF clone we identified multiple isoforms of SRF mRNA in P19 embryonic carcinoma cells or VSMCs that are formed by alternative splicing. We examined the expression of the isoforms *in vivo* and analysed their ability to activate transcription from fragments of the promoter of the smooth-muscle-specific SM22α gene in the C_2C_{12} myoblast cell line.

EXPERIMENTAL

Reverse-transcriptase PCR (RT-PCR) and cloning

RNA was isolated from mouse tissue using a commercial kit (RNeasy, Qiagen). Cytoplasmic RNA was isolated from cells in culture as described previously [32]. RNA (1 μg) was reverse transcribed using avian myelomablastosis virus reverse transcriptase in a 20-μl reaction as described previously [12]. The cDNA produced by these reactions (2 μl) was amplified by PCR using *Taq* DNA polymerase as described previously [12] and the primers indicated in the text (see below; primer A, TGCCCCGATTCCTCGCTGACTTG, nucleotides 298–320; primer B, GTGACGGGCGGATCATTCACTC, reverse and complement of nucleotides 1874–1853; primer C, GAGGAAGACGGGCATCATGAAGAAG, nucleotides 824–848; primer D, GCTGCTCCCAGCTTGCTGCCCTATC, reverse and complement of nucleotides 1775–1799; primer E, CCAGCGCTGTCAGCAGTGCCAAC, nucleotides 1270–1292; primer F, GTCTGTGCTGCTGTCACGAGAG, reverse and complement of nucleotides 1448–1469; primer G, CATGTGCACCAGGCCCCACAGCAAG, nucleotides 1416–1440; all nucleotide numbers are relative to the sequence described by Belaguli et al. [33] and are shown in the $5' \rightarrow 3'$ orientation). Primers A and B were designed to amplify the full coding sequence for SRF, primer C was designed to amplify from the end of exon 1, thus avoiding the GC-rich region present in exon 1 which reduces the efficiency of the PCR. Primer D is functionally equivalent to primer B but has a higher melting temperature. Primers E and F were designed to amplify intron 3. Primer G was designed to confirm splicing of exon 5.

PCR fragments were cloned into pGEM-T easy (Promega) as directed by the manufacturer and clones were sequenced. Point mutations in individual clones were corrected by swapping segments of cDNA between clones to generate a correct cDNA for each form of SRF. For transfection the SRF cDNAs were released from pGEM-T easy with *Eco*RI and ligated into *Eco*RI-digested cDNA3. The orientation of the clones was determined by restriction digestion with *Pst*I and the sequence of clones in the correct orientation was confirmed. Northern-blot analysis was carried out using α-tropomyosin, SM22α and β-actin cDNA probes as described previously [12,34,35].

Southern blotting

A standard PCR reaction (5 μl) was loaded on to a 1.5 % agarose gel and electrophoresed. The DNA was denatured and blotted on to Hybond-N membrane as described in Maniatis et al. [36]. The membrane was prehybridized in hybridization buffer [3 × SSC (where 1 × SSC contains 150 mM NaCl and 15 mM sodium citrate), 5 % dextran sulphate, 10 × Denhardt's solution (where 1 × Denhardt's solution is 0.02 % Ficoll 400/0.02 % polyvinylpyrrolidine/0.02 % BSA), 250 μg/ml salmon sperm DNA and 0.1 % SDS] and then hybridized to a random-primed ^{32}P-labelled cDNA probe consisting of nucleotides 939–1115 of mouse SRF at 65 °C. The blot was washed twice for 30 min per wash at 65 °C in 0.1 × SSC/0.1 % SDS and exposed to a PhosphorImager screen overnight.

Cell culture, transfection and chloramphenicol acetyltransferase (CAT) assay

C_2C_{12} cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % foetal calf serum subcultured 1:8 every 2–3 days by trypsin treatment. To differentiate C_2C_{12} cells into myotubes, cells were grown to confluence and the medium was replaced with DMEM supplemented with 10 % dialysed horse serum.

Cells for transfection were seeded at 3×10^4 cells per ml into 12-well plates and grown overnight. The cells were washed with serum-free DMEM and then incubated with a mixture of 600 ng of DNA (made up from 300 ng of pCB80 or pCB81, 150 ng of pSVβgal, 75 ng of pCDSRFL, pCDSRFM, pCDSRFI or pCDNA3 and 75 ng of either pCDNA3 or pCDSRFI) with 4 μl of Lipofectamine in Opti-MEM prepared as described by the manufacturer (Gibco BRL). Cells were incubated with the lipid–DNA mixture for 5 h before the medium was replaced with DMEM supplemented with 10 % horse serum. Cells were harvested and extracts for β-galactosidase and CAT assay 48 h after transfection, as described in Manniatis et al. [36].

CAT assay was performed by incubation of the cell extract with [^{14}C]chloramphenicol and n-butyryl-CoA overnight at 37 °C. Butyrylated chloramphenicol was extracted from the reaction mix with xylene and the xylene phase was back-extracted twice with 100 μl of 250 mM Tris, pH 7.5. β-Galactosidase was assayed as described in [36]. CAT activities were normalized to the β-galactosidase activities for the same sample. Transfections were performed in triplicate and repeated at least twice.

Mouse P19 embryonal carcinoma cells were maintained in αMEM supplemented with 10 % foetal calf serum and subcultured 1:10 every 2 days by trypsin treatment. Adult rat aortic VSMCs and the clonal line of VSMCs were isolated and cultured as described previously [32,37].

Nuclear-extract preparation and electrophoretic mobility-shift assay (EMSA) analysis

Dishes (100 mm) of C_2C_{12} cells or P19 embryonal carcinoma cells were washed twice in ice-cold PBS, scraped in 0.4 ml of cell lysis buffer (10 mM Hepes, pH 7.9, 0.1 mM EGTA, 0.1 mM EDTA, 1 mM dithiothreitol, 10 mM KCl and proteinase inhibitors; Complete, Boehringer Mannheim) and transferred to a microcentrifuge tube. The cells were allowed to swell for 15 min before 25 μl of 10 % Nonidet P40 was added and the cells were vortexed for 10 s. Following centrifugation the pellet was resuspended in 50 μl of nuclear extraction buffer (20 mM Hepes, pH 7.9, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol and 400 mM NaCl) by pipetting and incubated on ice for 30 min. The samples were centrifuged for 5 min and the supernatants removed and frozen at −80 °C. Protein concentration in the nuclear extracts was determined using the Bio-Rad protein assay. Nuclear extract (10 μg) was incubated in 1 × binding buffer [2 × binding buffer is 20 % glycerol, 5 mM $MgCl_2$, 2.5 mM EDTA, 250 mM NaCl, 25 mM dithiothreitol, 50 mM Tris, pH 7.5, and 0.25 mg/ml poly(dI-dC):poly(dI-dC)] and 90000 c.p.m. of oligonucleotide probe in a total volume of 20 μl for 30 min at room temperature. The samples were then mixed with 2 μl of loading buffer (40 % glygerol and 0.2 % Bromophenol Blue) loaded on to a 0.5 × TBE/5 % acrylamide non-denaturing gel (where TBE is Tris/borate/EDTA, 1 × TBE = 45 mM Tris/borate/1 mM EDTA) and run at 10 V/cm. The gels were dried and exposed to a PhosphorImager screen. Oligonucleotides used were (shown in

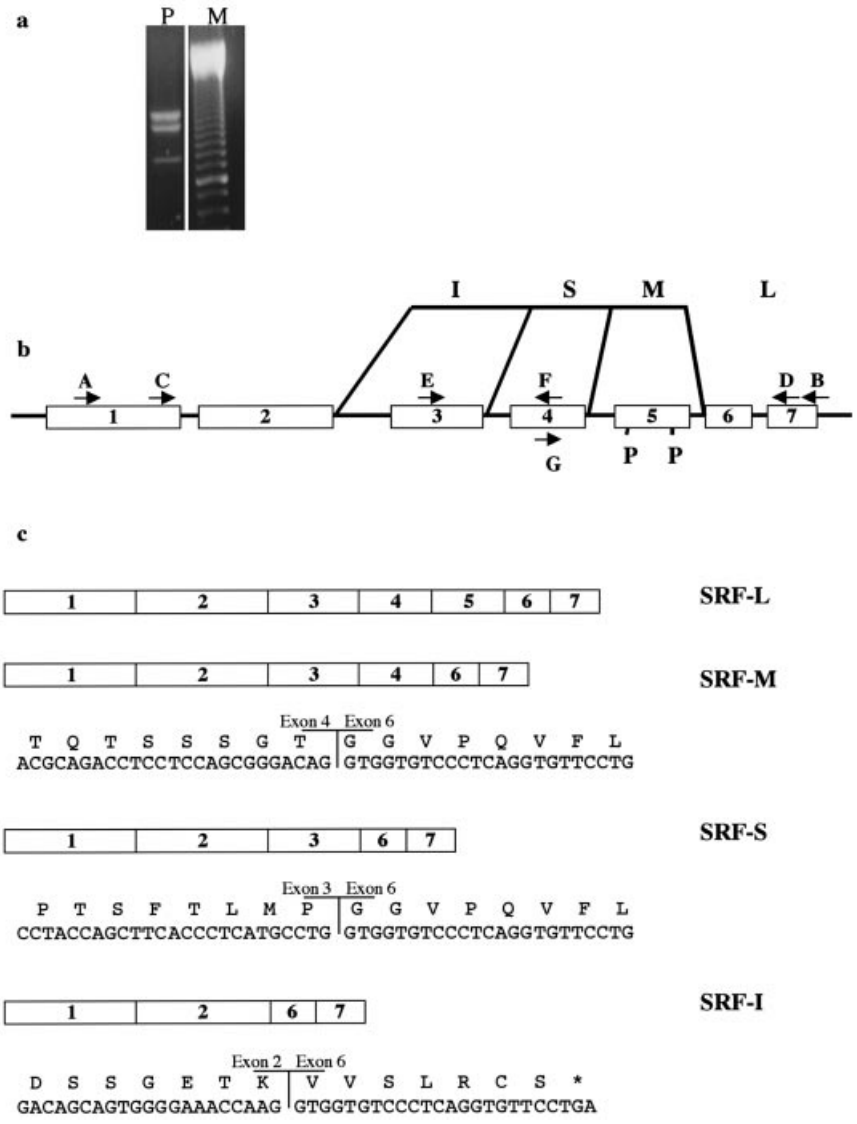

Figure 1 Alternative splicing of SRF

(**A**) Lane P, RT-PCR products of RNA isolated from P19 embryonic carcinoma cells amplified using primers A and B; lane M, 100-bp ladder. (**B**) Genomic structure of SRF. Lines indicate exons joined by alternative splicing. The DNA kinase-activated phosphorylation sites in exon 5 are marked (P). The positions of primers A–G used in this study are marked with arrows. (**C**) DNA and amino acid sequences of the alternatively spliced regions of the SRF isoforms.

the 5′ → 3′ orientation) CArG near (GTGTCTTTCCCCAAA-TATGGAGCCTGTGTG) and CArG far (TGGTCC TGCCC-ATAAAAGGTTTTTCCCGGC).

Western blotting

Nuclear extract (40 μg per lane) was run on an SDS/polyacrylamide (10 %) gel. The proteins were transferred on to a PVDF membrane at 0.8 mA/cm² of membrane for 1 h using a semi-dry blotter (LKB). The membrane was rinsed in PBS then blocked for 30 min in blocking buffer (1 × Tris-buffered saline/5 % dried milk powder/0.05 % Tween 20) at room temperature before being incubated with anti-SRF antibody (2 μg/ml; Santa Cruz) for 3 h in blocking buffer. The membrane was washed with four changes of Tris-buffered saline/0.05 % Tween 20 (5 min per wash) before being incubated with a 1 : 200 dilution of horseradish peroxidase-linked anti-rabbit IgG (Sigma) for 1 h in blocking buffer. Following washing as described above the proteins were visualized by chemiluminescence using the SuperSignal reagent (Pierce) as described by the manufacturer.

RESULTS

Identification of SRF-M and SRF-I isoforms

PCR amplification of cDNA from mouse P19 embryonic carcinoma cells, using primers designed to amplify the full coding sequence for mouse SRF (primers A and B; see the Experimental section), produced three products of 1.7, 1.5 and 1.0 kb (Figure 1a). Cloning and sequencing of the three products showed that they all had identical 5′ and 3′ ends (99.5 % identical to those

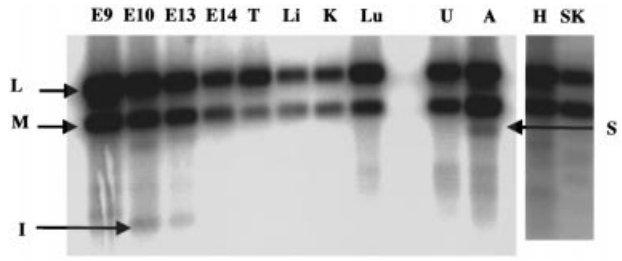

Figure 2 Tissue distribution of the alternatively spliced forms of SRF

RNA isolated from embryos after different periods of gestation, E9, E10, E13 and E14, and from different tissues of the adult mouse (T, testis; Li, liver; K, kidney; Lu, lung; U, uterus; A, aorta; H, heart; SK, skeletal muscle) were reverse transcribed and amplified as described in the text. The products were separated on a 1.5% agarose gel, Southern blotted and the blot was probed with a cDNA probe to SRF (see the Experimental section).

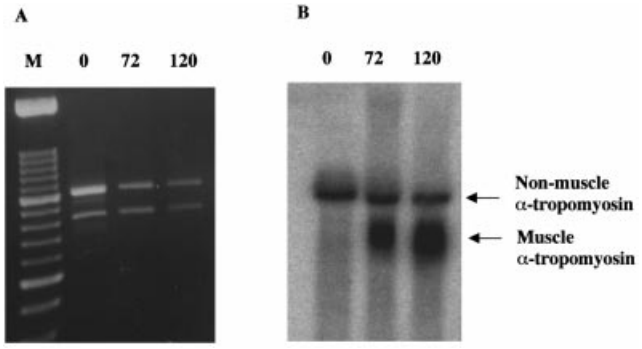

Figure 3 SRF isoforms in myoblast differentiation

(**A**) RT-PCR of RNA isolated from C_2C_{12} myoblasts maintained for 48 h in growth medium and then transferred to differentiation medium for 0, 72 and 120 h (lane M, 100-bp ladder). (**B**) Northern-blot analysis of the same RNA samples as in (**A**) for α-tropomyosin expression.

recently reported for the mouse SRF gene, nucleotides 348–700 and 1690–1862 of the sequence reported in [33] for the 5′ and 3′ ends respectively). Alignment of the sequences showed the longest sequence (termed SRF-L) was identical to the reported sequence, whereas the two shorter sequences lacked a 200-bp or a 700-bp internal sequence, suggesting that the mRNAs were alternatively spliced. To examine whether the sequences had been produced by alternative splicing or were a PCR artifact, the sequences were compared with the intron/exon structure of mouse SRF published recently by Belaguli et al. [33]. This comparison showed that the 1.5-kb sequence lacked exon 5 (termed SRF-M), whereas the 1.0-kb sequence lacked exons 3, 4 and 5 (termed SRF-I, Figures 1b and 1c). The splice junctions were therefore conserved in SRF-M and SRF-I, confirming that they had arisen by alternative splicing of the SRF pre-mRNA. Comparison of the SRF mRNA sequences with the EST database showed that there was a sequence present in the GenBank database (accession no. AA690609) for SRF-M that had been isolated from a myotube library.

Identification of SRF-S and tissue expression of the four isoforms

In experiments to examine whether SRF splicing occurred *in vivo* as well as *in vitro* and to determine the tissue distribution of the splice variants, RNA was isolated from adult mouse tissues and from mouse embryos after different periods of gestation. RT-PCR was performed using primers C and D. These reactions showed that all of the tissues examined expressed the longest form, SRF-L, and SRF-M, whereas SRF-I was only detected in the embryo (Figure 2). In addition to these three SRF forms a fourth band was observed in the aorta and embryo samples (Figure 2). Cloning and sequencing of this band from both samples showed that it was derived from the SRF gene and suggested that it resulted from splicing out of exons 4 and 5 (Figures 1b and c). However, the sequence obtained could not be explained by the exon 3–intron 3 splice-junction sequence reported by Belaguli et al. [33], because the sequence derived from exon 3 in this form of SRF was one base shorter than the reported exon 3 sequence. To determine whether this deletion was a PCR artifact or whether there was an error in the reported sequence, intron 3 was amplified by PCR from mouse genomic DNA using primers E and F and the 700-bp PCR product was sequenced from both ends. These data showed that G-1378 of the SRF cDNA was the first base of exon 4 and not the last base of exon 3 as previously reported [33]. This revised intron–exon boundary sequence was consistent with a fourth form of the SRF mRNA (termed SRF-S).

Expression of SRF mRNA isoforms in muscle

Although all of the samples analysed expressed both SRF-L and SRF-M, the proportions of the two forms expressed in each tissue type varied significantly (Figure 2). In most of the tissues analysed SRF-L was the predominant form of SRF mRNA present. However, in skeletal, cardiac and vascular smooth-muscle SRF-M and SRF-L mRNAs were present at similar levels, suggesting that SRF-M might be associated with muscle gene expression. To determine whether expression of SRF-M mRNA was correlated with the differentiation of muscle cells, RNA was isolated from C_2C_{12} myoblasts before and during differentiation into myotubes (Figure 3). RT-PCR using primers C and D showed that in undifferentiated C_2C_{12} myoblasts the predominant form of SRF was SRF-L. When the cells had been maintained in differentiation medium (DMEM + 10% horse serum) for 72 h they had started to differentiate, as marked by the expression of the skeletal-muscle-specific form of α-tropomyosin. At this stage, the expression of SRF-M had increased to a similar level to that of SRF-L. The amounts of the two forms of SRF remained similar when the cells had fused to form myotubes after 120 h in differentiation medium (Figure 3). As these data were derived from non-quantitative RT-PCR reactions they give an indication of the relative expression of the two isoforms within a sample but do not provide a reliable comparison of the amounts of SRF mRNA in different samples.

SRF-isoform expression was also compared in VSMCs at different levels of differentiation. RNA was isolated from fully differentiated VSMCs from intact adult rat aorta, from VSMCs from the same source that had de-differentiated after culture through nine passages and from a clonal line of neonatal rat aortic VSMCs. RT-PCR using primers D and G showed that the relative amount of SRF-M to SRF-L decreased with decreasing expression of SM22α in the three VSMC samples (Figure 4).

Expression of SRF proteins

To examine whether both the SRF-M and SRF-L mRNA species were translated into protein, nuclear extracts were made from

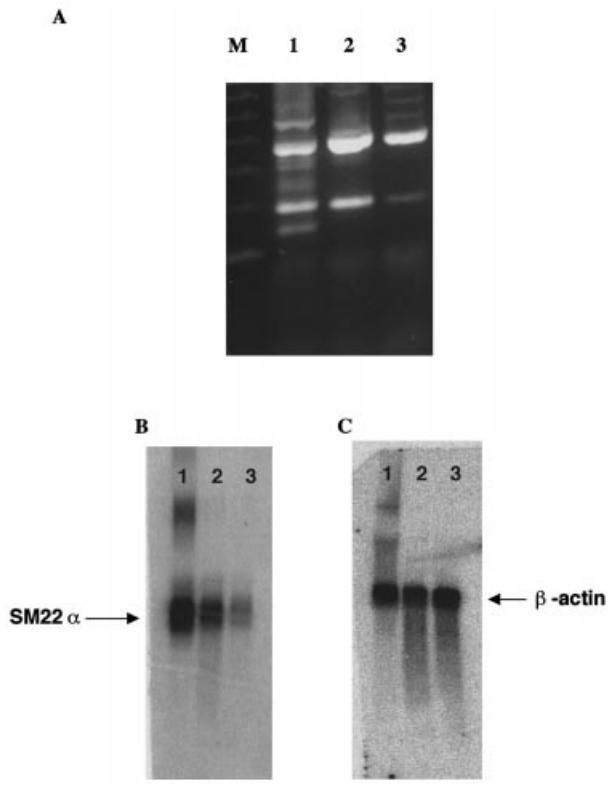

Figure 4 SRF isoforms in smooth-muscle-cell differentiation

(**A**) RT-PCR analysis using primers E and F of SRF isoforms in RNA isolated from intact adult rat aorta (lane 1), from adult rat aortic VSMCs subcultured for nine passages (lane 2) and from a clonal line of neonatal rat aortic VSMCs (lane 3). (**B**) SM22α and (**C**) β-actin expression were determined in the same RNA samples by Northern-blot analysis.

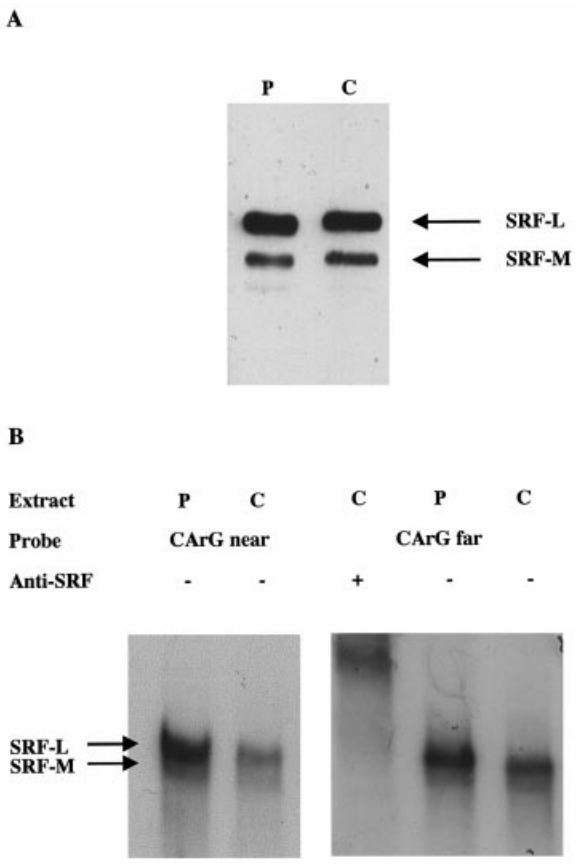

Figure 5 Translation of SRF-L and SRF-M mRNAs into proteins that bind to CArG elements

(**A**) Nuclear extracts were prepared from P19 embryonic carcinoma cells (P) and C_2C_{12} myoblasts (C) in growth medium. Each extract (40 μg) was separated by SDS/PAGE (10% gel) and Western blotted using an anti-SRF antibody. (**B**) The same nuclear extracts (10 μg) were incubated with oligonucleotide probes containing the CArG near and CArG far elements of the SM22α promoter. Both of the complexes formed were super-shifted with an anti-SRF antibody.

P19 cells and from C_2C_{12} myoblasts in growth medium. Western-blot analysis using an antibody against amino acids 486–505 of SRF, a C-terminal sequence present in both SRF-L and SRF-M, also identified two proteins, one of 67 kDa (SRF-L) and one of 60 kDa (the predicted size for SRF-M) in P19 and C_2C_{12} cells (Figure 5A).

Bandshift assays were performed using oligonucleotides containing the CArG boxes present in the SM22α promoter (see the Experimental section) to determine whether both species bound to DNA. These assays showed that in nuclear extracts from both the P19 cells and the C_2C_{12} myoblasts in normal growth medium two SRF species were detected (Figure 5B). Both of these species were super-shifted by an antibody against amino acids 486–505 of SRF, a C-terminal sequence present in both SRF-L and SRF-M. These data indicate that protein isoforms are translated from both the SRF-L and SRF-M mRNAs.

Effects of SRF isoforms on SM22α promoter activity

To determine the effects of the SRF isoforms on gene expression, cDNAs for SRF-L, SRF-M and SRF-I were cloned into pCDNA3 and the sequence of each construct was confirmed. The constructs were co-transfected into C_2C_{12} myoblasts with vectors pCB81 or pCB80 in which the *CAT* gene was under the control of bases $+65$ to -193 or $+65$ to -303 of the rat SM22α promoter respectively [12], and pSVβ-gal. Cell lysates were prepared 48 h after transfection and assayed for CAT and β-galactosidase activity. The basal activities of these two reporter constructs were markedly different with pCB81, having 5–10-fold less activity in the C_2C_{12} cells than pCB80 (results not shown). Co-transfections of the SRFs with pCB81 showed that SRF-L activated this promoter approximately 8-fold, whereas SRF-M activated the same construct approximately 5-fold (Figure 6). SRF-L and SRF-M also activated pCB80 in C_2C_{12} myoblasts with SRF-L causing a 4-fold increase in CAT expression and SRF-M causing a 2–3-fold increase in CAT expression (Figure 6). Co-transfection of SRF-I with either promoter construct did not affect CAT activity from either construct in C_2C_{12} myoblasts (Figure 6), suggesting that SRF-I does not affect basal activity of the SM22α promoter. To determine whether SRF-I acts as a dominant negative form of SRF, co-transfections were performed in which either pCB80 or pCB81 was co-transfected into C_2C_{12} myoblasts with SRF-I and SRF-L. In these experiments SRF-I inhibited the SRF-L-dependent increase in CAT expression from both promoters by approximately 50%, suggesting that it can act as a dominant negative form of SRF (Figure 6).

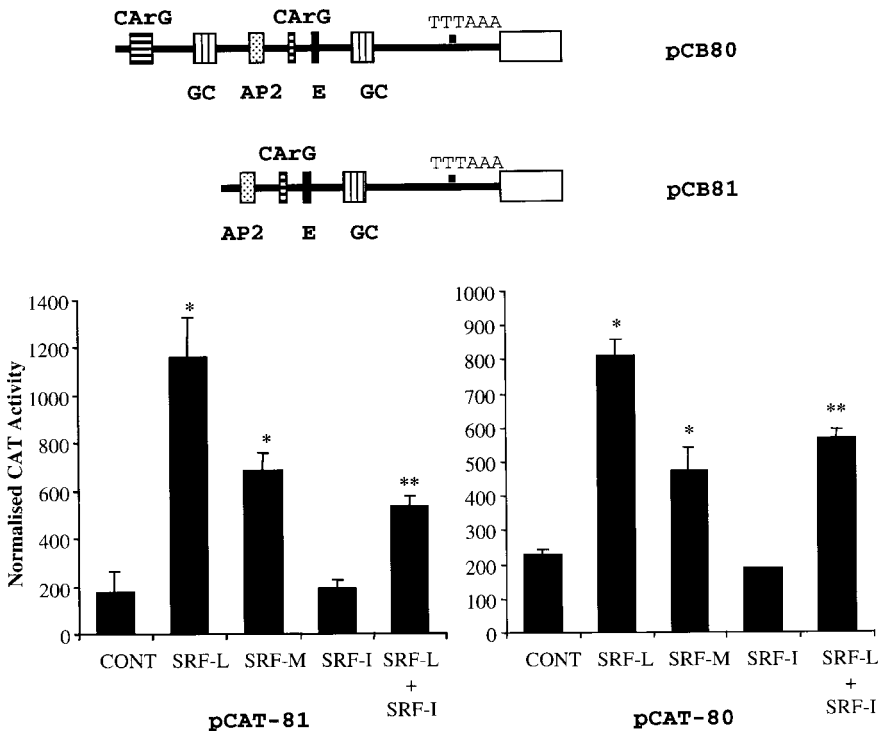

Figure 6 SRF-L and SRF-M activation of reporter-gene expression from the SM22α promoter in C_2C_{12} myoblasts

(Upper panel) Promoter elements present in the SM22α reporter constructs used. pCB81 only contained the CArG-near element, whereas pCB80 contained both the CArG-near and CArG-far elements. E, E box; GC, GC box; and AP2, AP2-binding site. (Lower panel) C_2C_{12} cells were transfected as described in the Experimental section with pCB81 or pCB80 and each of the SRF expression vectors. After transfection (48 h) the cells were harvested and CAT and β-galactosidase activities measured. CAT activity was normalized to the β-galactosidase from the same sample. Data presented are means $\pm$ S.E.M. Each transfection was carried out in triplicate and repeated at least twice. $^*P < 0.02$, sample versus control (CONT); $^{**}P < 0.01$, SRF-L versus SRF-L + SRF-I.

DISCUSSION

Multiple forms of SRF are expressed *in vivo* as a result of sequential deletion of exons (i.e. forms in which exon 5, exons 4 and 5 or exons 3, 4 and 5 were deleted). Previous analysis of SRF expression has shown that the length of the SRF message can also vary through the alternate use of polyadenylation sites in the 3′-untranslated region [33]. However, the functional significance of alternative use of the polyadenylation sites is not known and in contrast to the SRF isoforms identified here, polyadenylation does not affect the coding sequence. Alternative splicing in which multiple exons are removed is not unique to SRF. For example, forms of calretinin mRNA, a member of the troponin-C family of calcium-binding proteins, have been identified where exon 7 can be spliced to exon 8, exon 9 or exon 10. The exon deletions in SRF-M, SRF-S and SRF-I all affect the transactivation domain of the protein but leave the DNA-binding domain and the MADS box intact.

The data show that SRF-M mRNA is expressed in a wide range of tissues and is effectively translated into protein in C_2C_{12} myoblasts and P19 embryonic carcinoma cells. Comparison of SRF-M and SRF-L transfected into C_2C_{12} cells indicated that SRF-M had lower activity than SRF-L as a transactivator when co-transfected with either of the SM22α promoter constructs. The protein coded by the SRF-M message lacked amino acids Val-386 to Pro-449 that encode part of the transactivation domain. The deleted region contains two serine residues (Ser-433 and Ser-444) which are the equivalent residues to Ser-435 and Ser-446 in human SRF. These residues in human SRF are phosphorylated by DNA-activated protein kinase, which enhances the activity of the transactivation domain of human SRF and mutation of these sites has also been shown to reduce the transactivation efficiency of SRF [27]. Previous studies have also shown that deletion of amino acids 395–478 of human SRF in a fusion protein of the Gal4 DNA-binding domain with the C-terminus of human SRF reduced the ability of the C-terminus to transactivate reporter-gene expression [27]. However the transactivation activity of SRF is very dependent on co-activators and the activation of muscle-specific gene expression by SRF is enhanced by the activity of MyoD, MHox and Nkx 2.5 in skeletal, smooth and cardiac muscle respectively [20,26,38–40]. It is therefore possible that SRF-L and SRF-M each interact with different sets of co-activators that determine their activity in muscle cells. However, it should be noted that the residues required for interactions identified thus far between SRF and other transcription factors are located in the N-terminal domain of SRF, which is unaltered in all the forms of SRF described here.

SRF plays a significant role in tissue-restricted gene expression in the three major muscle-cell types and expression of SRF is highest in these tissues [33]. However, it is of interest that although SRF-L is the predominant form of SRF in most tissues, SRF-M is expressed at similar levels to SRF-L in all three types of muscle tissue in the adult mouse. This observation suggests that muscle-specific gene expression may be modulated by the relative proportions of SRF-M and SRF-L. The high levels of

SRF-M relative to SRF-L in differentiated C_2C_{12} cells and fully differentiated VSMCs compared with their less-differentiated counterparts are consistent with this hypothesis.

The smooth-muscle-specific SRF-S isoform was only detected in RNA from fully differentiated VSMCs in the intact adult rat aorta, suggesting that it may also have a role in maintaining the differentiated VSMC phenotype (Figure 4). However, SRF-S expression in mouse aorta was weak and any functional role remains to be determined. The SRF-S protein lacks amino acids Gly-347 to Pro-449 so that in addition to the sequence deleted to produce SRF-M, SRF-S lacks a further sequence containing seven serine residues and four threonine residues.

SRF-I is formed by splicing exon 2 to exon 6 which causes a frameshift relative to the normal reading frame for exon 6 and results in the formation of a seven-amino-acid region that is not present in the other forms of SRF. The resulting protein has a normal N-terminal DNA-binding domain and MADS box but no transactivation domain. Similar frameshift mutations occur in calretinin by splicing exon 7 to either exon 9 or 10. In contrast to SRF-L and SRF-M, SRF-I did not activate reporter-gene activity in C_2C_{12} cells and inhibited activation driven by SRF-L. These data and the predicted SRF-I protein sequence suggest that SRF-I protein is able to form heterodimers with the other forms of SRF, bind to DNA and act as a dominant negative form of the protein. Similar dominant negative activity has been demonstrated in primary chicken myoblasts for a form of SRF generated by mutagenesis in which the transactivation domain was deleted [41].

Detectable SRF-I expression is restricted to the embryo and raises the possibility that SRF-I acts as a temporally regulated inhibitor of SRF when this activity is not required. If SRF-I inhibitory activity in the embryo is highly localized, it may accentuate gradients of SRF activity across forming tissue boundaries, restricting the number of cells committed to a particular lineage. The balance of activating and inhibiting SRF isoforms would provide a mechanism for fine dynamic and spatial control of overall activity.

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