Increased expression of alternatively spliced dominant-negative isoform of SRF in human failing hearts

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1Department of Surgery (Cardiac and Thoracic), University of Chicago, Chicago 60637; 2The Heart Institute for Children, Hope Children’s Hospital, OakLawn 60453; and Departments of 3Physiology and Biophysics and 4Medicine (Cardiology), University of Illinois at Chicago, Chicago, Illinois 60612

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Davis, Francesca J., Madhu Gupta, Steven M. Pogwizd, Emile Bacha, Valluan Jeevanandam, and Mahesh P. Gupta. Increased expression of alternatively spliced dominant-negative isoform of SRF in human failing hearts. Am J Physiol Heart Circ Physiol 282: H1521–H1533, 2002.—Serum response factor (SRF) has been shown to play a key role in cardiac cell growth and muscle gene regulation. To understand the role of SRF in heart failure, we compared its expression pattern between control and failing human heart samples. Western blot analysis of control samples showed expression of four different isoforms of SRF, with ∼67-kDa full-length SRF being the predominant isoform. Interestingly, in failing hearts we found robust expression of a low-molecular-mass (∼52 kDa) SRF isoform, accompanied by decreased expression of full-length SRF. By RT-PCR and Southern blot analyses, we characterized this ∼52-kDa SRF isoform as being encoded by an alternatively spliced form of SRF lacking exons 4 and 5 of the SRF primary RNA transcript (SRF-Δ4,5 isoform). We cloned SRF-Δ4,5 cDNA and showed that overexpression of this isoform into cells inhibits SRF-dependent activation of cardiac muscle genes. These results suggest that expression of SRF-Δ4,5 in failing hearts may in part contribute to impaired cardiac gene expression and consequently to the pathogenesis of heart failure.

serum response factor; cardiac hypertrophy; cardiac gene regulation; alternative gene splicing

The primary response of the heart to an increased workload is expansion of cell size and increase in sarcomeric proteins, resulting in increased ventricular mass. This is also accompanied by phenotypic changes of cardiac muscle cells caused by differential gene activation. These changes give rise to myocytes equipped with a new set of contractile proteins and ion channels that allows hearts to meet increased demands (adaptive hypertrophy). However, as overload progresses, contractile function of the heart diminishes and heart failure usually ensues (9, 21, 34). The mechanism by which cardiac hypertrophy is initiated and how this condition eventually progresses to heart failure remain poorly understood.

One of the conserved features of cardiac hypertrophy is the induction of fetal genes that have been repressed or shut off during development and downregulation of genes encoding corresponding adult isoforms. The most thoroughly studied examples of fetal genes that are activated during hypertrophy are skeletal α-actin, atrial natriuretic peptide, and β-myosin heavy chain (MHC) genes. The adult isoforms that are downregulated include α-MHC, cardiac α-actin, and sarcoplasmic reticulum Ca2+-ATPase genes (6, 9, 27, 34). This reprogramming of cardiac muscle gene expression was initially thought to be an adaptive one; however, it was recently proposed that such changes at the myocyte level may ultimately contribute to contractile failure commonly occurring in an overloaded heart (21, 27, 34). Therefore, delineation of the mechanisms behind this gene reprogramming process is important to the understanding of pathways responsible for cardiac growth and clinically relevant to therapeutic strategies aimed to protect the heart from progressing into failure.

Tissue-specific transcription of many myocardial genes is dependent on a cis element, CC(A/T)6GG element, also known as CArG box or serum response element (SRE), which is recognized by serum response factor (SRF) (36). Several reports have indicated that SRF and SRF-containing complexes binding to CArG box may play an important role in conversion of growth stimuli to cellular responses as reflected by reprogramming of the gene expression (18, 24, 28). SRF is a ∼67-kDa phosphoprotein that belongs to the MADS box family of transcription factors, named after four proteins identified with common DNA binding and dimerization domains (MCM1, Agamous, Deficiens, and SRF). SRF has been shown to be a versatile transcription factor capable of participating in many cellular processes, including cell growth, differentiation, apoptosis, and cell-specific gene regulation both in proliferative and in postreplicative cells (5, 7, 12, 35, 37).
Targeted destruction of SRF gene in mice has been found to be embryonic lethal because of defects in mesoderm differentiation during gastrulation (11). The various roles of SRF in cell activation and in muscle cell differentiation have been shown to be controlled by its different modes of actions. 1) SRF has the ability to interact physically with a large number of factors to form functionally active transcription complexes. For example, in immediate-early genes such as c-fos gene, SRF interacts with ternary complex factors of the Ets family (36). In contrast, in skeletal muscle cells SRF interacts with the myogenic basic helix-loop-helix protein MyoD-E12 complex (or myogenin-E-12) (13). In cardiac myocytes, SRF has been shown to interact with other cardiac myogenic transcription factors such as GATA-4, TEF-1, Nkx2.5, and a recently identified cardiac-restricted factor, myocardin (3, 11, 15, 38). These multiple-protein complexes are likely to change the affinity of SRF to SRE, which in turn would lead to cell-specific gene regulation. 2) Phosphorylation of SRF is another mechanism that regulates the activity of SRF complexes. SRF contains multiple phosphorylation sites and is a target of multiple signaling kinases (18, 24, 28). In C2C12 cells, RhoA-dependent activation of SRF has been shown to promote muscle cell differentiation and muscle gene expression (40). 3) SRF activity is also controlled by regulated nuclear localization of the factor. In tracheal smooth muscle cells, extranuclear redistribution of SRF in serum-deprived cells was shown to be responsible for its reduced transcriptional activity (10). 4) Yet another mechanism that regulates SRF activity involves the ability of SRF to generate different alternative spliced isoforms. Recently, three different truncated isoforms have been identified that are generated by alternative splicing of the same SRF pre-mRNA. These newly identified isoforms are SRF-Δ5 (SRF-M; lacking exon 5), SRF-Δ4,5 (SRF-S; lacking exons 4 and 5) and SRF-Δ3,4,5 (SRF-I; lacking exons 3, 4, and 5) (4, 22). These SRF isoforms lack different portions of the COOH-terminal activation domain of SRF; however, they possess the DNA binding and dimerization domain of the NH2-terminal region of the protein. Previous reports showed that SRF isoform mRNAs are expressed in a tissue-specific manner and at different stages of development (22). Recently, Yang et al. (42) reported that stretch-induced myogenic differentiation of smooth muscle cells occurs by reducing the activity of SRF-Δ5 isoform, which functionally antagonizes the activity of SRF and inhibits its myogenic potential.

Here we report that in hearts of both humans and animals all four SRF isoforms are expressed and in failing hearts the level of expression of one particular isoform, SRF-Δ4,5 (SRF-S), is markedly increased. The increased expression of SRF-Δ4,5 correlates with the repression of a key contractile gene that is SRF dependent. We have cloned the cDNA of SRF-Δ4,5 and have shown that increased expression of this isoform in myocytes exerts a strong negative effect on SRF-dependent gene expression. These new findings underscore the importance of SRF-dependent reprogramming of myocardial gene regulation during overloading of hearts and provide further insights into the pathogenesis of heart failure.

METHODS

Procurement of heart samples. Human heart samples were provided by the University of Chicago Cardiac Transplant Program. All procedures involving human tissue use were approved by the University of Chicago Institutional Review Board. Left ventricular (LV) samples from five nonfailing and seven end-stage heart failure patients were analyzed. Control myocardial specimens, \( 5 \times 7 \text{ mm} \times 1 \text{ mm}^2 \), were obtained intraoperatively from the posteroMedial ventricular wall from nonfailing patients undergoing valvuloplasty. Failing LV specimens (\( \sim 2 \text{ cm}^3 \)) were obtained from diseased hearts that were removed during orthotopic heart transplant. The samples were immediately frozen in liquid nitrogen and stored at \(-80^\circ\text{C}\) until being analyzed.

LV specimens from adult rabbits (New Zealand White) of either sex were obtained from the Department of Medicine, University of Illinois at Chicago. Heart failure in rabbits was produced by combined volume and pressure overloads from aortic insufficiency and aortic constriction as previously described (31). Age- and weight-matched rabbits served as controls (\( n = 5 \)). By echocardiographic examination, heart failure rabbits (\( n = 4 \)) demonstrated increased LV end-diastolic dimension (1.46 ± 0.05 to 2.23 ± 0.13 cm; \( P < 0.01 \)) and end-systolic dimension (0.9 ± 0.05 to 1.58 ± 0.11 cm; \( P < 0.01 \)) and decreased percent fractional shortening (38 ± 3 to 29 ± 2.0%; \( P < 0.01 \)). The heart weight-to-body weight ratio increased 92\% in heart failure rabbits compared with controls. In this model of heart failure, rabbits exhibit severe depression of LV function, nonsustained ventricular tachycardia/arrhythmia, sudden death in 10\% of animals, and pathological changes including marked cellular and interstitial fibrosis (31). These features closely resemble those of human patients with end-stage idiopathic dilated cardiomyopathy.

Preparation of nuclear extract and Western blot analysis. Unless otherwise specified, all common salts and reagents were obtained from Sigma (St. Louis, MO). Nuclear extracts were prepared from rabbit or human LV as described previously, with some modifications (16). The entire isolation procedure was done at 4°C. Briefly, frozen tissue was allowed to thaw in a buffer containing (in mM) 50 Tris-HCl (pH 7.4), 1.5 EDTA, 5 dithiothreitol (DTT), and 150 NaCl. Tissues were trimmed of adherent connective tissues and washed twice in the same buffer. Tissues were then washed twice in a hypotonic buffer containing (in mM) 50 Tris-HCI (pH 7.4), 1.5 EDTA, 5 dithiothreitol (DTT), and 150 NaCl. Tissues were trimmed of adherent connective tissues and washed twice in the same buffer. Tissues were then washed twice in a hypotonic buffer containing (in mM) 50 Tris-HCI (pH 7.4), 1.5 EDTA, and 5 DTT; a 33\% tissue homogenate was prepared in the same buffer with a polytron device (Tissuemizer; Tekmar, Cincinnati, OH). The sample was centrifuged at 3,000 \( g \) for 30 min, and the pellet obtained was washed three times and resuspended in the same buffer, followed by centrifugation at 3,000 \( g \) for 10 min. The pellet was resuspended in an extraction buffer, \( \sim 1 \text{ ml/g ventricular tissue, of the following composition: } 20 \text{ mM HEPES (pH 7.9), 25% glycerol, 0.55 M NaCl, 1.5 mM MgCl}_2, 0.2 \text{ mM EDTA, 0.5 mM DTT, 0.5 M phenylmethylsulfonyl fluoride (PMSF), 1 \mu M antipain, 1 \mu g/ml chymostatin, 1 \mu g/ml leupeptin, and } 1 \mu m\text{leupetan (Boehringer Mannheim, Indianapolis, IN). The nuclear pellet was homogenized with piston A in a Dounce homogenizer, using 20–30 strokes, transferred to precooled microfuge tubes, and centrifuged at 14,000 rpm for 30 min. The supernatant was dialyzed in 2 liters of dialysis buffer containing (in mM) 40 KCl, 15 HEPES (pH 7.9), 1 EDTA, 0.5
PMFS, and 0.5 DTT with 20% glycerol for 2–3 h. The dialyzed nuclear extract was snap frozen and stored at −80°C until used. Protein content of the extract was measured by using the Bio-Rad protein assay dye reagent (Bio-Rad Laboratories, Hercules, CA). For Western blot analysis, samples were suspended in Laemmli’s buffer (40 μg protein), denatured by boiling, and subjected to SDS-PAGE on a 10% gel. Proteins were transferred to a polyvinylidene difluoride membrane (Amersham Pharmacia Biotech, Piscataway, NJ) in a tank transfer system in buffer (25 mM Tris-HCl, pH 8.3, 0.192 M glycine, 20% methanol), at 4°C overnight. Membranes were blocked in 10% nonfat milk in PBS-0.5% Tween 20. Anti-SRF antibody directed against amino acids 486–505 (Santa Cruz Biotechnology, Santa Cruz, CA) was used (1:500 dilution) as the primary antibody, and rabbit IgG (1:2,000) coupled to horseradish peroxidase was used as the secondary antibody. An enhanced chemiluminescence kit (Amersham) was used for protein detection. Protein bands were quantitated with a computer program, Scion Image for Windows (release beta 4.0.2), based on NIH Image for Macintosh by Wayne Rasband (National Institutes of Health, Bethesda, MD).

RNA extraction and Northern blot analysis. Total RNA was extracted from control and failing human and rabbit LV with Trizol reagent (Life Technologies) according to the method provided by the manufacturer. Northern blot analysis was performed with synthetic oligonucleotide probes complementary to the unique 3′-untranslated sequences of the rabbit α- and β-MHC mRNA (17). Sequences of the probes are rabbit α-MHC: 5′CAGCGACTCGTGGTTATTGCGGGTTATAACAGACGGGTTTC 3′ and rabbit β-MHC: 5′GGAGATCTCAAGGCCTACAGGCTTCTATTCCTCATTCACTG 3′.

RT-PCR and Southern blot analysis. To analyze SRF mRNA, total cellular RNA (5 μg) was denatured and reverse-transcribed with thermoscript reverse transcriptase (RT; Life Technologies) in a RT-PCR buffer containing deoxyribonucleotide triphosphates and gene-specific primers. Nega
tive controls were carried out without RT in the reaction mix. After 1 h of incubation at 65°C the RT reaction was heat inactivated. PCR was performed in a separate tube with 6–8 μl of cDNA as a template. The 50-μl PCR reaction mix contained standard PCR buffer with 1.85 mM MgCl2, 0.2 mM deoxyribonucleotide triphosphates, and sense and antisense primers and platinum Taq polymerase (Life Technologies) (each 0.4 μM). The cycling conditions included an initial 3-min 95°C denaturation, followed by 50 cycles of 15 s of 95°C denaturation, 55 s of 50°C annealing, and 3-min extension at 72°C. A 5-μl aliquot of reaction mix was fractionated on a 1.5% agarose gel, stained with ethidium bromide, and photographed. For Southern blot analysis, the PCR products were run on a 1.5% agarose gel and transferred under alkaline conditions to Hybond-N+ membrane (Amersham). DNA was immobilized on the membrane by baking in a vacuum oven at 80°C for 2 h. Under standard hybridization conditions, the membrane was hybridized with end-labeled oligonucleotide probes specific to exon 4, exon 5, or MADS box regions of the SRF cDNA. Membranes were washed under standard conditions, exposed to X-ray film overnight, and autoradiographed. Sequences of primers and probes are as follows: SRF primers: sense 5′ CTACCGAGCTGTCGGA-GTCTGA 3′, antisense 5′ CCAGATGATGCTGTAAGACGA 3′; β-actin primers: sense 5′ GCTCGTGCTACACAGCGCT 3′, antisense 5′ CAAACTGTATGCTGGGTTATCTTCCTC 3′; probe sequences complementary to different regions of SRF: MADS box 5′ CCACGAAACAGCACCCTGTGCCCTTGTAGGGAGCATGCATTAGG 3′, exon 5 5′ TCTAGGGGTATATACCTGTTTCTGTTTTTTGAATTCGATTTGCCC-CTAGGACATGGTGCCTTTACATCATCATGTGGCCACCCACAGTTGTG 3′, exon 4 5′ TGTCCTGGGAGATGCTGTCGGA-GTCTGA 3′, exon 3 5′ GTTCCTGGTTCGTCGCGATCTGCTGCTGTCGTCACGCATGTGGCCACCCACAGTTGTG 3′, exon 2 5′ TCTAGGGGTATATACCTGTTTCTGTTTTTTGAATTCGATTTGCCCCTAGGACATGGTGCCTTTACATCATCATGTGGCCACCCACAGTTGTG 3′, exon 1 5′ GTTCCTGGTTCGTCGCGATCTGCTGCTGTCGTCACGCATGTGGCCACCCACAGTTGTG 3′.

Plasmid construction. Luciferase reporter constructs for skeletal α-actin and expression vectors pCDN and pCGNSRF were provided by Dr. R. Schwartz, Baylor College of Medicine, Houston, TX (11). The reporter construct with five SREs (5’sSRE-Luc) was described previously (10, 15). Expression vectors pCDNSRF-M (lacking exon 5) and pCDNSRF-I (lacking exon 3, 4, and 5) were obtained from Dr. P. Kemp, Cambridge University, Cambridge, UK. The α-MHC-Luc reporter construct was generated by cloning −612/+420 bp EcoRI/HindIII fragment of α-MHC gene into the pX-2-luc vector (provided by Dr. P. Bunn, Harvard Medical School, Boston, MA). To clone SRF−Δ4,5, exon 4 sequences of SRF were deleted from the pCDNSRF-M construct with a two-step PCR procedure. In the first step, PCR was performed with two sets of primers. In the first set, the forward (FP) primer contained the 298–317 bp of SRF (which is upstream of 1st codon ATG) with an EcoRI site (5′-TTTTTGAATTCGATTTGCCCCTAGGACATGGTGCTATTTCTG) and the reverse internal (IR) primer flanking the end sequences of exon 3 (1362–1379 bp) and beginning sequences of exon 6 (1690–1704 bp) of SRF gene (CCTGAGGAGCACCACCCACGGCATGGGTTG). The second set of primers comprised a forward primer that is complementary to IR primer and the reverse (RP) primer complementary to 1846–1865 bp (which includes last codon TGA) of SRF and an XbaI cloning site (TTTTTGAATTCGATTTGCCCCTAGGACATGGTGCTATTTCTG). All nucleotide numbers are relative to the mouse SRF sequences described by Belaguli et al. (2). PCR products of the two reactions were gel purified, annealed, and used as a template in the second-step PCR, in which FP and RP primers were utilized to reamplify SRF cDNA that lacks exon 4 and 5 sequences. The final PCR product was digested with EcoRI and XbaI enzymes and subcloned into the EcoRI and XbaI sites of pBluescript (Stratagene, La Jolla, CA). Deletion of exon 4 was confirmed by DNA sequencing and by translation of the SRF cDNA. Subsequently, SRF−Δ4,5 fragment was subcloned into EcorI/XbaI sites of an eukaryotic expression vector, pCDNA3 (Invitrogen).

Cell culture and transfection. Primary myocytes were cultured from 18-day-old fetal rat hearts. After differential plating to eliminate nonmuscle cells, myocytes were plated at a density of 2 × 106 cells/100-mm culture dish (Falcon; Becton Dickinson Labware) precoated with 0.1% gelatin in Ham’s F-12 medium (GIBCO-BRL) with 5% calf serum. More than 90% of the cells began to contract spontaneously within 24 h after plating. Cos7 cells were grown in growth medium containing Dulbecco’s modified Eagle’s medium (GIBCO-BRL) with 5% calf serum. More than 90% of the cells began to contract spontaneously within 24 h after plating. Cos7 cells were grown in growth medium containing Dulbecco’s modified Eagle’s medium (GIBCO-BRL) with 5% fetal bovine serum in an atmosphere of 5% CO2. All culture media contained penicillin (5 mg/ml), streptomycin (5 mg/ml), and neomycin (100 mg/ml). Primary cultures of cardiac myocytes were transfected after 48 h in culture, and Cos7 cells were transfected once they became confluent. Typically, 5 μg of DNA/100-mm plate was transfected by use of a Lipofectamine reagent (GIBCO-BRL). All transfections contained 1 μg of the pcMV-β-gal reference plasmid. The next morning (~18 h after transfection), the medium was changed. After an additional 48 h, cells were harvested. Cell lysates were prepared and assayed for luciferase and β-galactosidase activities and protein content. The luciferase activity for each construct was corrected for the protein content of each extract and normalized to the activity of β-galactosidase in the same cell extract.
RESULTS

Cardiac expression of SRF isoforms. Previous reports indicated that the primary transcript of SRF generates four different alternatively spliced isoforms, which are tissue specific (22). To examine expression of SRF isoforms in cardiac myocytes and to evaluate their role in heart failure, we analyzed LV specimens from human subjects by Western blot analysis with an anti-SRF antibody. The SRF antibody is against the COOH-terminal region of SRF and recognizes all four isoforms of the protein. In the hearts of patients with normal ventricular function (control patients), we found expression of all four isoforms of SRF, with the predominant isoform being the ~67-kDa full-length SRF (SRF-FL). Interestingly, in the ventricles of end-stage failing hearts we found robust expression of a ~52-kDa isoform that, based on molecular mass, corresponds to a previously documented SRF-Δ4,5 (SRF-S) isoform (Fig. 1B). Increased expression of SRF-Δ4,5 in the failing hearts was accompanied by proportionate decrease in levels of expression of SRF-FL (~67 kDa) as well as SRF-Δ5 (~57 kDa). However, there was no apparent difference in expression of SRF-Δ3,4,5 (~40 kDa) between the control and failing heart samples (Fig. 1B). These findings were repeated from LV samples of five control patients and seven heart failure patients, whose clinical characteristics are presented in Table 1. From each human sample the expression level of SRF proteins was quantitated with the Scion Image system computer program. As presented in Table 1, an average 56% of SRF-FL and 14% of SRF-Δ4,5 was found expressed in control LV samples; whereas in the failing heart samples SRF-FL was 20% and SRF-Δ4,5 level elevated to 40% of the total SRF protein.

Because the type and degree of cardiac abnormality were not the same in human subjects and the patients had received multiple pharmacological treatments that might have influenced expression of individual SRF isoforms, we also examined expression of SRF proteins in a rabbit model with experimentally induced heart failure (31). As shown in Fig. 1B, the LV of control rabbits showed a similar differential expression of the four SRF isoforms. In rabbits with failing hearts, a differential SRF isoform switch similar to that in humans was observed; however, the overall expression of SRF-Δ4,5 protein appeared to be considerably higher and was accompanied by almost complete disappearance of SRF-FL and SRF-Δ5 bands (Fig. 1B). These data thus demonstrate a marked increase of an ~52-kDa SRF isoform (SRF-Δ4,5) in the failing myocardium of both humans and rabbits. The difference in the levels of expression of SRF in human and rabbit failing heart samples could be from species variation or might reflect the experimental model (double overloads) of heart failure that we used in this study.

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Fig. 1. Serum response factor (SRF) is differentially expressed in the normal and failing myocardium. A: diagrammatic representation of four different isoforms of SRF: full-length SRF (SRF-FL), SRF lacking exon 5 (SRF-Δ5), SRF lacking exons 4 and 5 (SRF-Δ4,5), and SRF lacking exons 3, 4, and 5 (SRF-Δ3,4,5). B: Western blot analysis of cell lysates obtained from the left ventricle of human and rabbit hearts. C, control hearts; F, failing hearts. Right, in vitro translated SRF-Δ5 was used as a marker to determine electrophoretic mobility of different SRF isoforms. C: expression of SRF isoforms in different failing human heart (F-2–F-7) samples.

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ROLE OF SPLICED VARIANT OF SRF IN HEART FAILURE

Table 1. Clinical characteristics of patients and their corresponding levels of cardiac SRF isoforms

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SRF, serum response factor; SRF-FL, full-length SRF; SRF-Δ4,5, SRF lacking exons 4 and 5; NF, nonfailing; F, failing; MVD, mitral valve defect; A Myx, atrial myxoma; VSD, ventricular septal defect; IDC, idiopathic dilated cardiomyopathy; FDC, familial dilated cardiomyopathy; CAD, coronary artery disease; FS, fractional shortening; Dgn, digoxin; Dbt, dobutamine; β-Blk, β-adrenergic blocker; ACEI, angiotensin-converting enzyme inhibitor; Dutx, diuretics; Ca-Blx, calcium channel blockers; NA, not available.

To determine the contribution of other cell types in detection of SRF in these samples, we also examined expression of SRF isoforms in primary cultures of rat cardiac fibroblasts and myocytes. As shown in Fig. 2, the SRF-FL and SRF-Δ5 isoforms were expressed in both cardiac myocytes and fibroblasts. However, the SRF-Δ4,5 isoform was detected only in cardiac myocytes and not in fibroblasts even after longer exposure of the membrane. From these studies we conclude that the SRF-Δ4,5 isoform detected in our heart samples originated from cardiac myocytes and not from fibroblasts.

Decreased cardiac α-MHC gene expression in failing LV. We were interested in determining whether the biochemical profile of failing hearts in the two species used in this study is the same or different. Hence, we examined the expression pattern of α- and β-MHC genes, which are known to be significantly altered in failing hearts (29). To measure expression levels of human cardiac MHC mRNAs, we used a RT-PCR-based procedure previously described by Nakao et al. (29). According to this procedure, equal amounts of total RNA from control or failing heart samples were reverse-transcribed with common c primers. The cDNA so obtained was amplified by PCR to produce a 217-bp product that reflects the total MHC mRNA level. From control and failing heart samples, total MHC RT product was amplified with equal efficiency (Fig. 3B). Subsequently, the 217-bp PCR product obtained in the exponential phase of amplification (35 PCR cycles) was digested with PstI and SacI restriction enzymes. The PstI enzyme cleaves the β-MHC transcript at two sites (positions 5677 and 5697 bp) but not the α-MHC transcript; hence, any fraction of the 217-bp product resistant to PstI digestion reflects the amplified α-MHC mRNA. The SacI enzyme, on the other hand, cleaves both subtypes of MHC (at position 5631 bp of β-MHC and 5551 bp of α-MHC genes); hence, any undigested fragment remaining after this digestion corresponds to the background. In control hearts, as shown in Fig. 3B, a measurable amount of PCR product was left undigested after PstI digestion, reflecting the amount of α-MHC mRNA expression; however, in the failing heart samples it nearly disappeared. Further increasing the enzyme concentration had no effect, indicating complete digestion of PCR product in the assay conditions applied here. Because we did not perform a quantitative RT-PCR reaction, it is hard to derive relative levels of expression of α- and β-MHC mRNAs between the control and failing hearts. Nevertheless, the data presented in Fig. 3 demonstrate that nonfailing (control) human cardiac samples contained an appreciable amount (~20% of total MHC mRNA) of α-MHC mRNA, which was markedly reduced in failing heart samples, consistent with previous reports (29).
In rabbit hearts, expression of α- and β-MHC mRNAs was measured by Northern blot analysis with gene-specific oligo probes as described elsewhere (17). As shown in Fig. 4, the expression of α-MHC mRNA was reduced to almost undetectable levels in failing hearts compared with controls; however, there was no apparent difference in levels of expression of β-MHC mRNA in the two groups of hearts. These data corroborate previously reported hemodynamic data from the same model of heart failure in rabbits (31) and support the concept that this model, by combining α-MHC repression and mechanical dysfunction with arrhythmogenesis, closely simulates human heart failure.

Characterization of SRF isoforms. We next performed experiments to demonstrate that the SRF bands observed in our Western blot analyses were indeed generated as truncated isoforms of the full-length SRF protein. First, we blocked the SRF antibody with a blocking peptide containing COOH-terminal region of SRF (Santa Cruz Biotechnology). In subsequent Western blot analyses in which parallel blots were probed with the blocked or unblocked antibodies, the four SRF bands that are seen with SRF primary antibody were not detected once the antibody was blocked (results not shown), thus confirming that these bands are indeed specific for SRF.

Second, we analyzed the effect of dephosphorylation on the apparent mobility of the four SRF bands observed in our Western blot analyses. SRF, a phosphoprotein, is a known substrate for various kinase-mediated phosphorylation events involved in cell signaling. Therefore, it was likely that a change in the phosphor-
ylation state of SRF could have affected the mobility of SRF bands. To exclude this possibility, we immunoprecipitated SRF from cellular lysates from both human and rabbit hearts and treated the immunoprecipitated SRF with an alkaline phosphatase that nonspecifically dephosphorylates phosphoserine, phosphothreonine, and phosphotyrosine residues. The phosphatase activity of the enzyme was confirmed by its ability to reverse the protein kinase-induced phosphorylation of a target protein, and that protein served as a positive control. Subsequently, dephosphorylated ventricular lysate was analyzed by Western blot analysis, and the results revealed no apparent change in the gel mobilities of four SRF immunogenic bands (data not shown). These results confirm that the immunogenic bands observed in our Western blot analyses are indeed distinct isoforms of SRF and not the result of posttranscriptional phosphorylation of any one isoform with subsequent changes in electrophoretic mobility.

Finally, to demonstrate that the four SRF bands were in fact generated by alternative splicing of the SRF primary transcript, we analyzed human cardiac mRNA by RT-PCR analysis with gene-specific primers. For PCR amplification of SRF mRNAs, we used primers that amplify a minimum sequence common to SRF-FL, SRF-Δ5, SRF-Δ4,5, and SRF-Δ3,4,5 transcripts, i.e., the sense primer is within exon 2 (1099–1119 bp) and the antisense primer is within exon 6 (1728–1749 bp) (Fig. 5). These primers amplified four products of 651, 459, 340, and 78 bp, which are the predicted sizes for amplification of SRF-FL, SRF-Δ5, SRF-Δ4,5, and SRF-Δ3,4,5 transcripts, respectively. Importantly, the relative proportions of the four SRF transcripts expressed were significantly different in the failing vs. control heart samples. In the control hearts the predominant isoforms of SRF mRNA were SRF-Δ5 and SRF-FL, whereas SRF-Δ4,5 was minimally expressed. In contrast, SRF-Δ4,5 transcript was the predominant isoform in the failing hearts, with reduced expression of SRF-FL transcript (Fig. 5B). Expression of SRF-Δ3,4,5 transcript was minimal in both control and failing heart samples. To ensure that these bands were not the result of a PCR artifact or genomic contamination of samples, two negative controls were included in this experiment.
First, exclusion of RT during cDNA synthesis resulted in no PCR product. Second, under identical PCR conditions, same-intensity bands of \textit{H9252}\textsuperscript{-}actin transcript were amplified from control and failing heart samples (Fig. 5C), thus suggesting that the observed difference in intensity of SRF bands between heart samples is indeed due to different levels of expression of SRF isoform mRNA transcripts.

To confirm that the 459-bp and 340-bp bands amplified in our PCR analysis in fact originated from SRF-\textit{H9004}\textsuperscript{5} and SRF-\textit{H9004}\textsuperscript{4,5} transcripts, respectively, we performed Southern blot analysis. The PCR-amplified individual bands of 459 and 340 bp were gel purified and resolved on a 1% agarose gel. As positive controls, PCR products obtained from amplification of either pCGN-SRF-FL (predicted size 651 bp) or pCGN-SRF\textsuperscript{4,5} (predicted size 459 bp) were resolved on the same gel and blotted simultaneously on a nylon membrane. Each membrane was then probed with radiolabeled oligonucleotide probes specific to MADS box, exon 5, or exon 4 sequences of SRF. As shown in Fig. 6A, the probe specific for the MADS box hybridized with both 459- and 340-bp bands amplified from cardiac mRNA as well as with the positive controls amplified from pCGN-SRF-FL and pCGN-SRF\textsuperscript{4,5}. The probe specific for exon 5 hybridized with the PCR product obtained from pCGN-SRF-FL but not with the pCGN-SRF\textsuperscript{4,5} product or the 459- and 340-bp products obtained from sample mRNAs, suggesting a lack of exon 5 sequences in these products, as expected (Fig. 6B). The probe specific for exon 4 hybridized with the 459-bp but not the 340-bp band, indicating that the 459-bp product contains exon 4 sequences whereas the 340-bp product does not. Both positive controls hybridized to the exon 4-specific probe, as expected (Fig. 6D). In another set of experiments, by using the same set of primers, we were able to identify only the SRF-FL transcript from rat liver and HeLa cells (results not shown), demonstrating that expression of the alternatively spliced transcripts of SRF is not ubiquitous. From these results, we conclude that the preferential expression of SRF isoform in the failing human myocardium is SRF-\textit{H9004}\textsuperscript{4,5} mRNA, which is in agreement with the observation of increased expression of this truncated isoform at the protein level.

Functional role of SRF-\textit{H9004}\textsuperscript{4,5} in regulation of SRF-dependent muscle gene promoters. To determine the effect of SRF-\textit{H9004}\textsuperscript{4,5} on gene expression, cDNA for SRF-\textit{H9004}\textsuperscript{4,5} was cloned and confirmed by DNA sequencing and translation of the encoded protein. Amino acids at the junction of exons 3 and 6 in the SRF-\textit{H9004}\textsuperscript{4,5} cDNA clone and the molecular mass of its translated protein, in relation to other SRF isoforms, are shown in Fig. 7. For functional analysis, we evaluated the role of SRF-\textit{H9004}\textsuperscript{5} on three different gene promoters: cardiac \textit{α}-MHC, skeletal \textit{α}-actin, and an artificial promoter/reporter gene with multiple SRF-binding sites (SRE). As reported before, the promoter activity of these constructs is highly dependent on binding of SRF to SREs. For comparison purposes, we also evaluated the gene regulation ability of SRF-\textit{H9004}\textsuperscript{5} isoform in this assay. Plasmids encoding the wild-type SRF (pCGN-SRF-FL), SRF-\textit{H9004}\textsuperscript{5} (pCGN-SRF\textsuperscript{5}), SRF-\textit{H9004}\textsuperscript{4,5} (pCGN-SRF\textsuperscript{4,5}), and/or the empty vector (pCGN) were cotransfected with a promoter-reporter plasmid into Cos7 cells or in primary culture of cardiac myocytes, and the reporter activity was assayed in cell-lysates 48 h after transfection.
tion. To determine the levels of expression of different SRF isoforms in transfected cells, the lysate of cells was also subjected to Western blot analysis with SRF antibody. As shown in Fig. 8D, all three expression constructs synthesized a significant amount of their respective SRF proteins. Furthermore, forced expression of SRF-Δ5 as well as SRF-Δ4,5 completely abolished expression of the endogenous SRF-FL protein, suggesting an inhibitory effect of these isoforms on the synthesis of native SRF (Fig. 8D). The effect of the three SRF isoforms on promoter activity of skeletal α-actin gene is shown in Fig. 8A. SRF-FL activated skeletal α-actin gene promoter up to 30-fold in a concentration-dependent manner, whereas a lesser degree of activation (~10-fold) of this promoter was also observed with SRF-Δ5 isoform, suggesting that SRF-Δ5 isoform retains gene activation potential, consistent with a previous report (22). In contrast, SRF-Δ4,5 inhibited even the basal activity of the skeletal α-actin gene promoter at every concentration we examined. In cotransfection experiments, SRF-Δ4,5 repressed (>90%) the SRF-FL-stimulated activity of skeletal α-actin gene as well as cardiac α-MHC gene, indicating a dominant-negative role of SRF-Δ4,5 in the regulation of gene expression (Fig. 8, A and C). Furthermore, by analyzing expression of a promoter-reporter construct with multiple SREs (5xSRE-luc) we found SRF-Δ4,5 to be at least fivefold more potent than SRF-Δ5 in repressing the SRF-dependent gene activation. Thus these data collectively demonstrate SRF-Δ4,5 to be a highly potent repressor acting as a dominant-negative form of SRF and suggest that increased expression of this isoform in cardiac myocytes will have a detrimental effect on myocardial gene expression during cardiac overload.

**DISCUSSION**

Alternate splicing of the primary transcript is a commonly used posttranscription mechanism for creating a functionally diverse pool of gene products (25). Several proteins including members of the MADS box family, such as MEF2 proteins, have been shown to synthesize different tissue-specific isoforms (30). Both activator and repressor isoforms can be derived from the same gene by alternative splicing strategies (25). In this paper, we demonstrate that adult human and rabbit hearts express four different isoforms of SRF (SRF-FL, SRF-Δ5, SRF-Δ4,5, and SRF-Δ3,4,5), with SRF-FL being the predominant isoform. Interestingly, in the failing hearts of both humans and rabbits we found SRF-Δ4,5 to be the predominant isoform, accompanied by significant decrease in the expression levels of SRF-FL as well as SRF-Δ5. The expression level of SRF-Δ3,4,5 isoform, however, remained unchanged between the control and the failing hearts. On the basis of several criteria such as predicted molecular mass, comigration with exogenously expressed SRF isoforms, immune specificity, and dephosphorylation of bands, we demonstrated that the four bands observed in the heart samples are indeed SRF isoforms generated by alternative splicing of SRF primary RNA transcript.

We are not aware of any previous report in which the expression of SRF-Δ4,5 and SRF-Δ3,4,5, at the protein level has been shown in other cell types. Previously, Kemp and Metcalfe (22) reported expression of SRF-Δ4,5 (SRF-S) and SRF-Δ3,4,5 (SRF-I) mRNAs in the mouse aorta and embryo, respectively, and considered these to be tissue- and developmental stage specific. However, our data show that both isoforms are also
expressed in normal hearts; moreover, expression of SRF-Δ4,5 is significantly increased both at the mRNA and protein levels in the failing myocardium. It could be argued that the SRF isoforms detected in this study possibly originated from other cell types found in the LV, such as fibroblasts. However, given the high percentage of total cell volume and nuclear mass attributed to cardiac myocytes in the whole myocardium, we believe that the observed SRF isoforms are those expressed in myocytes. To further support this point, we also examined expression of SRF isoforms in primary cultures of cardiac myocytes and fibroblasts and noted that SRF-Δ4,5 was detected only in cardiac myocytes and not in fibroblast cultures.
The SRF isoforms SRF-Δ5, SRF-Δ4.5, and SRF-Δ3.4.5 are generated by sequential deletion of the COOH-terminal region of SRF, which comprises the activation domain of the protein. Thus different SRF isoforms contain the same NH2-terminal DNA-binding domain and MADS box, but with different lengths of activation domain. The activation domain of SRF was previously mapped to different COOH-terminal regions in different cell types: amino acids 339–508 in HeLa cells, 414–508 in NIH3T3 cells, and 406–475 in HuT-12 cells (19, 24). The SRF-Δ5 isoform lacks 389–449 amino acids, whereas SRF-Δ4.5 results from deletion of 347–449 amino acids in the activation domain of SRF. This additional segment deleted in SRF-Δ4.5 isoform contains seven serine and four threonine residues, which are substrates to phosphorylation by many signaling kinases (Ref. 24 and unpublished data). On the basis of these deletion sequences, it is conceivable that SRF-Δ5 will have higher activation ability than SRF-Δ4.5, as observed in our transfection experiments for the skeletal α-actin gene promoter activity.

Conflicting reports have previously appeared regarding the gene activation potential of SRF-Δ5 isoform. Kemp and Metcalfe (22) showed that SRF-Δ5 (SRF-S) isoform activates SM22α gene expression in C2C12 cells. Belaguli et al. (4) observed only a dominant-negative effect of this isoform in CV1 cells in the expression of different muscle gene promoters, including SM22 and skeletal α-actin gene promoters. A dominant-negative effect of SRF-Δ5 on the expression of smooth muscle marker genes in progenitors of coronary smooth muscle cells and lung embryonic mesenchymal cells has also been reported (23, 42). Because the gene activation potential of SRF is highly sensitive to the amount of SRF expressed in the cell, the reported differences could be explained by differences in the amounts of SRF-Δ5 plasmid utilized in these studies. We observed the activation effect of SRF-Δ5 to be only at low (50 ng) concentrations; however, at higher concentrations, similar to those used by Belaguli et al. (4), we observed either no effect or a negative regulatory effect. In addition, SRF isoforms may have cell type- and/or promoter-dependent effects, which could be explained by differences in the ability of the activation domain of SRF to interact with components of basal transcription machinery such as TFIIID, RAP74 subunit of TFIIF, ATF6, or cell-type-specific SRF coactivators (3, 11, 20, 43, 44). It is important to note that although SRF-FL is the predominant isoform in most tissues, SRF-Δ5 is expressed at levels comparable to those of SRF-FL in smooth, skeletal, and cardiac muscles of rodents (4, 42). This observation suggests that SRF-Δ5 may have a physiological role in muscle-specific gene expression, which may be modulated by relative proportions of SRF-Δ5 and SRF-FL in the cell. However, the same may not be true for SRF-Δ4.5 isoform because its levels are never found equal to SRF-FL in any of the normal tissues analyzed (M. P. Gupta, unpublished data).

The molecular and cellular mechanisms involved in the development of heart failure remain largely unknown. Among the different transcription factors studied, it appears that SRF might play a central role in the pathophysiology of heart failure. This is based on documented evidence that 1) SRF has an obligatory role in mesoderm formation and cardiac development (1); 2) the levels of expression of SRF in embryonic and adult cardiac myocytes are at least two orders of magnitude greater than those detected in cells of endodermal origin (2); 3) SRF has the ability to synergistically cooperate with many other known cardiac myogenic factors such as GATA-4, Nkx2.5, TEF-1, and myocardin (3, 11, 15, 38); 4) functionally relevant SREs in the promoter regions of several, if not all, cardiac contractile, Ca2+-transporting, and metabolic protein genes have been identified, indicative of a direct role for SRF in their transcriptional regulation (12, 32); and 5) forced expression of SRF together with Nkx2.5 has been found sufficient to induce endogenous expression of cardiac-specific proteins in pluripotent 10T1/2 fibroblasts (11). These studies strongly suggest an obligatory role for SRF in the induction and maintenance of the cardiac myogenic program; however, the mechanism for the defects in SRF-mediated cardiac gene expression during heart failure is not yet understood.

Data presented in this study implicate generation of the dominant-negative isoform of SRF by alternate slicing of mRNA as one such mechanism contributing to dysregulation of cardiac muscle gene expression and, consequently, to heart pump function. Although the precise mechanism by which alternative splicing of pre-mRNA of a gene is regulated is not known, a coupling between induction of intracellular signaling and alternative splicing of the target pre-mRNA transcript was demonstrated recently. Activation of PKC and Ras-Raf-mitogen-activated protein kinase kinase signaling pathways has been shown to regulate alternative splicing of CD44 pre-mRNA in lymphocytes (26, 39). Stress hormones and steroid receptors have been shown to control alternative splicing of potassium channel and dopamine D2 receptor mRNAs, respectively (14, 41). On the basis of these reports, it seems reasonable to speculate that during cardiac hypertrophy SRF pre-mRNA could be a downstream target of activated intracellular signaling mechanisms, leading to synthesis of antagonistic isoforms of the SRF protein. Studies are currently underway to delineate the signaling cascade contributing to synthesis of SRF-Δ4.5 transcript during cardiac hypertrophy and its role in endogenous expression of SRF-dependent cardiac muscle genes and contractile function of cardiac myocytes.

How do SRF isoforms modulate SRF-dependent muscle gene regulation? SRF-dependent cardiac muscle gene transcription could potentially be mediated by increased levels of SRF, as has been demonstrated during embryogenesis (8) and during myogenic differentiation (2, 12). Although we have not observed a quantitative difference in the expression of total SRF protein between control and failing hearts, the levels of individual isoform were significantly altered. Previously, Spencer and Misra (33) demonstrated that SRF...
by itself is a major regulator of its own promoter activation. We therefore speculate that one of the potential pathophysiological roles of the SRF-Δ4,5 isoform is to regulate expression of SRF-FL in myocardial cells in an autocrine manner, resulting in its decreased expression. This would be similar to the dominant-negative effect of SRF-Δ5 in suppressing the activity of SRF gene expression as reported previously (4). In fact, both in the failing myocardium and in transfected Cos7 cells as well as cardiac myocytes (not shown), we have observed a significant reduction in SRF-FL expression, in parallel with increased levels of SRF-Δ4,5 isoform. Thus the decreased activity of muscle gene promoters by SRF-Δ4,5 could be a consequence of the reduced levels of SRF-FL in the cell. Recently, reduced levels of SRF-FL were shown to prevent normal bronchial smooth muscle development (42). Besides direct suppression of SRF-FL expression, truncated isoforms of SRF can generate homo-/heterodimers, which interfere with the binding of SRF to SRE of muscle gene promoters and/or interact with other cardiac myogenic factors, resulting in repression of the target gene transcription activity. A negative role for SRF-Δ5 in the repression of SRE-dependent gene promoters was demonstrated to be a result of SRF-FL-SRF-Δ5 heterodimers and SRF-Δ5 homodimers at the SRE (4). SRF contains some important phosphorylation sites within exon 4 and 5 sequences; therefore, it is also likely that loss of these sites terminates the responsiveness of SRF to intracellular signaling mechanisms, leading to repression of SRF-dependent gene transcription. Together, it appears that alternatively spliced variants of SRF allow for a more diverse pool of isoforms whose combinatorial as well as inherent activity/repressive properties could result in varying SRF-dependent gene transcription in a cell type- and/or stimulus-dependent manner.

In summary, the major finding of this study is that a truncated isoform of SRF lacking exons 4 and 5, SRF-Δ4,5, is markedly increased in the failing hearts of both humans and animals. This isoform acts as a highly potent repressor of myocardial gene expression. Because elevated levels of SRF-Δ4,5 are not found in cardiac hypertrophy where myocardial function is still preserved, we believe that this isoform plays an important role in the pathogenesis of heart failure (32). Further studies aimed at understanding the molecular mechanisms of its synthesis, and how it represses gene expression, should provide valuable information on how SRF-mediated gene expression becomes dysregulated in the failing myocardium and how this can be modified. It has the potential for a new therapeutic strategy for protecting the heart from progressing into failure.

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