Serum response factor (SRF) is a key regulator of many extracellular signal-regulated genes important for cell growth and differentiation. A form of the SRF gene with a double mutation (dmSRF) was generated. This mutation reduced the binding activity of SRF protein to the serum response element and reduced the capability of SRF to activate the atrial natriuretic factor promoter that contains the serum response element. Cardiac-specific overexpression of dmSRF attenuated the total SRF binding activity and resulted in remarkable morphologic changes in the heart of the transgenic mice. These mice had dilated atrial and ventricular chambers, and their ventricular wall thicknesses were only 1/2 to 1/3 the thickness of that of nontransgenic mice. Also these mice had smaller cardiac myocytes and had less myofibrils in their myocytes relative to nontransgenic mice. Altered gene expression and slight interstitial fibrosis were observed in the myocardium of the transgenic mice. All the transgenic mice died within the first 12 days after birth, because of the early onset of severe, dilated cardiomyopathy. These results indicate that dmSRF overexpression in the heart apparently alters cardiac gene expression and blocks normal postnatal cardiac growth and development.

Serum response factor (SRF) is a key regulator of many extracellular signal-regulated genes important for cell growth and differentiation (1–3). SRF was first identified as a critical transcription factor involved in mediating serum-induced transcriptional activation of the c-fos gene (4, 5). Attention has been given to the role of SRF in the activation of c-fos gene in the G1-to-G2 transition in the cell cycle and in many other cell activation responses (6, 7). The importance of SRF for growth factor-regulated transcription is further suggested by the identification of SRF-binding sites (serum response elements (SREs)) within the regulatory region of many other serum-inducible genes, including members of the immediate-early genes and muscle-specific genes (8–11).

SRF is a member of the MADS (MCM-1, Agamous and Deficiens, SRF) box family of transcription factors (5). The MADS box motif is a 56-amino acid region that comprises a highly conserved basic N-terminal region followed by a less well conserved, relatively hydrophobic C-terminal segment. The SRF-binding domain is located at the N terminus of the protein in which the MADS box (amino acids 142–198) forms the center of the binding domain that is important for DNA binding specificity, dimerization, and recruitment of p62TCF proteins (12–14). Mutations in this domain attenuate SRF binding specificity (12, 15).

Recently, several studies have suggested that SRF is directly involved in the regulation of expression of a number of muscle-specific genes, including skeletal α-actin (SKA), α-myosin heavy chain (α-MHC), β-MHC, and cardiac α-actin (16), and in the regulation of mesoderm formation (17, 18). SRF also plays an essential role in shaping the normal morphology and size of the embryonic body during embryonic cell differentiation and aggregation (19).

To define the role of SRF in the regulation of function and morphology of the postnatal heart and to test the hypothesis that functional SRF is required for postnatal cardiac growth and development, we overexpressed a double mutant form of SRF (termed dmSRF), which has impaired binding ability to SRE, in the mouse heart. Our results revealed that dmSRF overexpression in the heart elevated the levels of SRF mRNA and protein but attenuated the total SRF binding activity to the SRE. It resulted in remarkable morphological changes in the transgenic mice, such that all the transgenic mice died within 12 days after birth, because of the early onset of severe, dilated cardiomyopathy.

**EXPERIMENTAL PROCEDURES**

**SRF Mutant**—A functional double mutant form of human SRF gene, termed dmSRF, was generated by site-directed mutagenesis. It contains a double mutation at amino acid positions 159 and 163 of the SRF protein (Thr159→Ser159, Lys163→Glu163, based on the GeneBank™ sequence accession number J03161). When compared with wild type SRF, this mutant is severely compromised in its ability to bind to the c-fos SRE in *in vitro* binding assays.

**In Vitro Translation and Cotranslation of wtSRF and dmSRF Protein**—The DNA fragments corresponding to the full-length coding region of dmSRF and wild type SRF (wtSRF) were subcloned into plasmid pBluescript SK(-). The transcription of both the mutant and wild type SRF genes was under the control of T7 promoter. The dmSRF and wtSRF proteins were *in vitro* translated by using a TNT-coupled transcription/translation system (Promega). Equal amounts of the *in vitro*
Cardiac Changes in Mice Overexpressing Mutant SRF

translated dmSRF and wtSRF protein were used for electrophoretic mobility shift assays (EMSA). In the cotransfection experiment, 1 µl of in vitro translated wtSRF RNA was added to tubes containing 0, 1, 2, 4, or 6 µl of dmSRF RNA, respectively. The wtSRF and dmSRF proteins were synthesized in the same cotransfection reaction in the presence of[35S]methionine. The labeled probe to the ventricular tissue protein. DNA-protein complexes were resolved by electrophoresis through 4% native polyacrylamide gels containing 50 µM Tris, 45 mM boric acid, 0.5 mM EDTA. The gels were subsequently dried and exposed to Kodak X-Omat film. Gel supershift assays were performed as described above with the exception that subsequent to incubation of oligonucleotide probes with the whole cell extract, 1 µl of anti-SRF antibody (both from Santa Cruz Biotechnology) was added to the reaction mixture and incubated at room temperature for 30 min.

Measurement of SRF Protein Expression—50 µg of protein prepared as described in the electrophoretic mobility shift assays section above was separated by SDS-polyacrylamide gel electrophoresis on a 10% polyacrylamide gel and transferred to nitrocellulose. The membrane was blocked for 2 h at room temperature in 5% nonfat milk in TBS-T (20 mM Tris, 137 mM sodium chloride, 0.1% Tween 20, pH 7.6) and then incubated for 2 h at room temperature with SRF antibody followed by incubation for 1 h with horseradish peroxidase-conjugated secondary antibody (both from Santa Cruz Biotechnology). Immunoreactive bands were visualized by chemiluminescence (ECL, Amersham Pharmacia Biotech).

Northern Blot Analysis—Total RNA was isolated from mouse ventricular tissue using the ULTRASPEC RNA isolation reagent (Biotec Laboratories, Houston, TX). Approximately 10 µg of total RNA was then fractionated on a 1% formaldehyde-agarose gel and transferred to a Hybond nylon membrane (Amersham Pharmacia Biotech) by capillary transfer. The membrane was then washed in 0.2% SSC, 1× SSC, followed by 0.2× SSC, 0.1% SDS at 65 °C for 15 min, and then dried and exposed to Kodak X-Omat film. The sequences of the oligonucleotide probes were as follows: ANF, 5'-CGGGAGCTTCGGAGTACGAGGCTGTGGTTCAG-3', c-MHC, 5'-CGAACGTTT-CAGGGGGCTCAGAGGATTCCAAGAAGCACAATACGGTCATCCTAGCAGGCTTCAGACGAGGTTTCAGGAGGTTTACAA-3'; skeletal α-actin, 5'-TGCAGGACACAGAACGAGAAGAGAGAGAGGATAGAGG-3'.

Northern Blot Analysis—Total RNA was isolated from mouse ventricular tissue using the ULTRASPEC RNA isolation reagent (Biotec Laboratories, Houston, TX). Approximately 10 µg of total RNA was then fractionated on a 1% formaldehyde-agarose gel and transferred to a Hybond nylon membrane (Amersham Pharmacia Biotech) by capillary transfer. The membrane was then washed in 0.2% SSC, 1× SSC, followed by 0.2× SSC, 0.1% SDS at 65 °C for 15 min, and then dried and exposed to Kodak X-Omat film. The sequences of the oligonucleotide probes were as follows: ANF, 5'-CGGGAGCTTCGGAGTACGAGGCTGTGGTTCAG-3', c-MHC, 5'-CGAACGTTT-CAGGGGGCTCAGAGGATTCCAAGAAGCACAATACGGTCATCCTAGCAGGCTTCAGACGAGGTTTCAGGAGGTTTACAA-3'; skeletal α-actin, 5'-TGCAGGACACAGAACGAGAAGAGAGAGGATAGAGG-3'.

Morphologic Analysis—Fresh ventricular tissues were placed in 10% neutral buffered formalin for 24 h and processed for paraffin embedding. Sections (3–4 µm) were stained using standard hematoxylin and eosin or Masson trichrome staining protocols (Poly Scientific, Bayshore, NY). The sections were subjected to a dehydration series and embedded in paraffin. The sections were then fractionated on a 1% formaldehyde-agarose gel and transferred to a Hybond nylon membrane (Amersham Pharmacia Biotech) by capillary transfer. The membrane was then washed in 0.2% SSC, 1× SSC, followed by 0.2× SSC, 0.1% SDS at 65 °C for 15 min, and then dried and exposed to Kodak X-Omat film.

Electrophoretic Mobility Shift Assays—The in vitro translated protein of wtSRF and dmSRF and the whole cell lysate of mouse ventricular tissue were employed for EMAS. Whole cell extracts of ventricular tissue were prepared by a modification of the method described previously (20). Briefly, cells or tissue were washed with cold phosphate-buffered saline and then suspended in buffer containing 20 mM Heps (pH 8.0), 1.5 mM MgCl2, 25% (v/v) glycerol, 420 mM NaCl, 0.2 mM EDTA (pH 8.0), 1 mM diethiothreitol, and 1 × protease inhibitor mixture (Roche Molecular Biochemicals). They were homogenized and then incubated on ice for 30 min before centrifuging at 10,000 rpm for 15 min. The supernatant was then fractionated on a 1% formaldehyde-agarose gel and transferred to a Hybond nylon membrane (Amersham Pharmacia Biotech) by capillary transfer. The membrane was then washed in 0.2% SSC, 1× SSC, followed by 0.2× SSC, 0.1% SDS at 65 °C for 15 min, and then dried and exposed to Kodak X-Omat film.

Electron Microscopy—The tissue sections of the left ventricular free wall were processed immediately and then processed following standard procedures in the histology core facility of Beth Israel Deaconess Medical Center. The intracellular volume ratios (% of mitochondria, myofibrils, and remaining organelles) were measured by point counting using a line grid with 100 µm wide lines. The measurements were performed on electron micrographs from each specimen for each LV region in each group.
Cardiac Changes in Mice Overexpressing Mutant SRF

40035

SRE in vitro, wtSRF protein was cotranslated with dmSRF. EMSA using lysate from the cotranslation reactions revealed that increasing the amount of dmSRF RNA in the cotranslation reaction resulted in progressive reduction of SRF binding activity (Fig. 1B).

The effect of the double mutation on the function of SRF protein was further examined in ex vivo experiments. The plasmid containing the promoter of one of the cardiac marker genes, ANF, which contains an SRE site, was used to test this effect. NIH3T3 cells were cotransfected with plasmids containing the ANF promoter and with wtSRF and dmSRF plasmids. The results showed that wtSRF could significantly activate the ANF promoter activity relative to control (p < 0.01), whereas dmSRF did not significantly increase ANF promoter activity compared with control.

To elucidate the mechanism by which dmSRF inhibits SRF function, we sought to test whether dmSRF acts to inhibit the endogenous SRF or increase its activity. We cotransfected dmSRF with wtSRF and determined the ANF promoter activity. In the presence of the 2-, 4-, and 5-fold amounts of dmSRF relative to the amount of wtSRF, the ability of wtSRF to activate the ANF promoter was significantly and progressively attenuated (p < 0.01; Fig. 1C).

Generation of dmSRF Transgenic Mice—To examine the effect of the dmSRF protein on cardiac function in the postnatal period, the α-MHC promoter was employed to generate dmSRF transgenic mice (27). A plasmid construct containing dmSRF cDNA under the transcriptional control of the α-MHC promoter was injected into pronuclear stage zygotes using a standard microinjection procedure. Polymerase chain reaction was employed to identify the transgenic founder mice, and only two transgenic founder mice were obtained from a total of 159 mice that were screened (1.2% yield, which is much lower than the normal yield of between 20–30% at the transgenic facility of this institution). Southern blot analysis revealed that both founder mice A and B had only one single copy of the dmSRF transgene each (Table I). Furthermore, the transgenic mice were found to have elevated levels of SRF mRNA and protein in the heart. However, no overexpression of SRF was found in other muscle, such as skeletal muscle (see Fig. 3C).

Early Onset of Cardiomyopathy and Early Mortality in the dmSRF Transgenic Mice—The two male transgenic founder mice were bred with nontransgenic female mice, but the pregnancy rate among the nontransgenic female mice was low. Female mice that were mated with the transgenic founder mouse A gave birth to 8 litters of live birth progeny with litter sizes of 7–10. Only one or two neonates in each litter were found to be transgenic. The transgenic neonatal mice of line A apparently looked normal at birth and during the first few days gained body weight similar to the nontransgenic littermates. They were initially not distinguishable from the nontransgenic littermates. By ~7–8 days post-birth, however, their growth appeared to be blunted (Fig. 2A). All these transgenic mice died between the ages of 9 and 12 days (Fig. 2B). Autopsy revealed decreased body weight, increased heart weight, increased heart/body weight ratio, and four-chamber cardiac dilatation. The average body weight was 3.99 ± 0.15 g in transgenic mice,

RESULTS

Effect of the Double Mutation on SRF Protein Function—The amino acids Thr159 and Lys163 are located within an important domain of the SRF protein that is capable of dimerizing, binding to DNA, and recruiting p62TCF proteins (14, 15). To test the effect of the double mutation [Thr[ACG]159 → Ser[TCG]159 and Lys[AAG]163 → Glu[CAG]163] on the binding ability of SRF to its cognate binding site, in vitro translated dmSRF and wtSRF proteins were utilized in the gel shift assays. The results showed that the binding activity of the dmSRF protein was much lower than that of the wtSRF protein (Fig. 1A). To demonstrate that dmSRF can inhibit the binding of wtSRF to

Data Analysis—The values are expressed as the means ± S.D. The data were analyzed by two independent observers, blind to the transgenic status of the mice. Normality testing was performed on all data, and the nonparametric Mann Whitney U Test was used to determine the differences between the two groups. A p value of less than 0.05 was considered to be statistically significant.

Table I

The dmSRF founder mice and their lines

| Line | Sex | SRF transgene copy number | Transgenic progeny | Cardiomyopathy |
|------|-----|---------------------------|-------------------|---------------|
| A    | M   | 1                         | (+)               | (+)           |
| B    | M   | 1                         | (+)               | (+)           |

Two transgenic founder mice were identified among 159 mice that were screened for the presence of the transgene. Their live birth F1 progeny developed cardiomyopathy and suffered early mortality.

Fig. 1. Effect of double mutation on SRF function. A, the double mutation reduced the binding activity of SRF to SRE. [35S]-Labeled SRE probe and in vitro translated protein were employed for the gel shift assay. No protein was added in lane A. In vitro translated wtSRF protein (wt) was added in lane B, and in vitro translated dmSRF protein (dm) was added in lane C. B, competition for SRE probe between dmSRF and wtSRF proteins synthesized in vitro cotranslation in the presence of [35S]methionine. From lane 1 to lane 10, both equal amounts of wtSRF RNA (wt) and increasing amounts of dmSRF RNA (dm) were used in the cotranslation reaction. The lysate (1 μl in lanes 1–5 and 2.5 μl in lanes 6–10) of the cotranslation reaction and the SRE probe were employed in the gel shift assay. Note that SRF binding activity in the lysate of the cotranslation reaction decreased with increasing amounts of dmSRF RNA. C, double mutation reduced the capability of SRF to activate the ANF promoter activity. Transfection of wtSRF plasmid (200 ng) increased ANF promoter activity by 8.28-fold (*, p < 0.01), and transfection of dmSRF (200 ng) did not increase ANF promoter activity significantly. Note that 2-fold (400 ng), 4-fold (800 ng), and 5-fold (1000 ng) dmSRF plasmids significantly attenuate the ability of wtSRF (200 ng) to activate ANF promoter activity (*, p < 0.01).
Fig. 2. Early postnatal cardiac changes and premature death in dmSRF transgenic mice. A, early postnatal growth of the transgenic (Tg) and nontransgenic (NTg) mice. By ~7–8 days post-birth, growth of Tg mice was blunted. B, survival of the Tg mice. The F1 generation of Tg mice died between 9 and 12 days after birth. None of them survived. C, nontransgenic and transgenic mice at the age of 12 days. The body weight of the nontransgenic mouse was 5.11 g, and the body weight of the transgenic mouse was 3.94 g. The difference in body weight underlies an apparent difference in body size. D, hearts of the nontransgenic and transgenic mice at the age of 12 days. The heart weight was 28 mg in the nontransgenic mouse and 57 mg in the transgenic mouse. Note the severe dilatation of the transgenic heart. E, heart weight (mg) to body weight (g) ratio in nontransgenic and transgenic littermate mice. Note the increased heart weight/body weight ratio of transgenic compared with nontransgenic mice (15.75 ± 2.10 and 6.39 ± 0.81, respectively). The results are expressed as the means ± S.D. (n = 7, *, p < 0.001).
whereas it was 5.70 ± 0.34 g in age-matched nontransgenic littermate mice (p < 0.001, n = 7). The heart weight was 62.71 ± 6.62 mg in transgenic mice, whereas it was 36.71 ± 6.36 mg in nontransgenic littermates (p < 0.001, n = 7). The heart weight to body weight ratio was significantly increased in the transgenic (15.75 ± 2.10) relative to nontransgenic littermate mice (6.39 ± 0.81, p < 0.001, n = 7) (Fig. 2, C–E).

The transgenic founder mouse of line B was bred with 25 young adult female nontransgenic mice, but only six female mice became pregnant. They delivered 7–11 neonates/litter. However, only one of the F1 neonates was ever found to be transgenic. This transgenic neonatal mouse appeared to be extremely dyspneic and in respiratory distress shortly after birth. It became moribund within the first 24 h. Autopsy revealed that the body weight of this 1-day-old transgenic neonate was 1.36 g, which was similar to that of nontransgenic littermates (1.36 ± 0.051 g, n = 10), whereas the heart weight was 22.9 mg, which was 1.7-fold heavier than that of nontransgenic mice (13.39 ± 0.48 mg, n = 10). The heart weight to body weight ratio in the transgenic neonate was 16.8, which was higher than that of nontransgenic mice (9.74 ± 0.29, n = 10).

In an effort to better understand the low birth rate of the live birth transgenic offspring, three sets of mouse embryos at the embryonic development days 12–17 were obtained from line B to examine the presence of the dmSRF transgene. Only one of the 28 mouse embryos in these three litters was positive for the dmSRF transgene.

Elevation of dmSRF and Alteration of Gene Expression in the Heart—Northern blotting revealed that the total SRF mRNA level was increased by roughly 4-fold in the ventricle of the transgenic relative to nontransgenic animals. The total SRF protein in the ventricle of the transgenic mice was also higher than that in nontransgenic animals (Fig. 3, A and B).

To test whether overexpression of dmSRF could reduce the overall SRF binding activity to SRE in the heart, whole cell lysate from mouse ventricular tissue was employed in EMSAs. The whole cell lysate from transgenic and nontransgenic mice and a [γ-32P]ATP-labeled SRE oligonucleotide corresponding to the c-fos promoter region were used in the binding reactions. EMSAs revealed that the SRF binding activity was indeed decreased in the ventricle of the transgenic relative to nontransgenic mice (Fig. 3D).

Several cardiac genes that contain the SRF-binding site were examined in the dilated hearts of the transgenic mice. Elevated mRNA levels of ANF, SKA, β-MHC, and c-fos but decreased mRNA levels of α-MHC, MLC2v, cardiac α-actin, and SERCA2 were observed (Fig. 3E).

Because the α isoform of myosin heavy chain gene itself has a low level of expression in the ventricles during embryogenesis, the possibility that the α-MHC promoter drove dmSRF transgene expression during embryogenesis was examined. Northern blotting revealed elevated SRF mRNA level in the heart of the transgenic embryo at embryonic development day 17. In addition, the mRNA levels of β-MHC, MLC2v, and cardiac α-actin were notably increased in the transgenic compared with nontransgenic embryos. However, the SKA mRNA was hardly detectable in the transgenic embryos (Fig. 3E). The mRNA of the endogenous α-MHC isoform was not detected in the hearts of either nontransgenic or transgenic embryos (data not shown).

Morphologic Changes—Both atria and ventricles of the transgenic mice displayed substantial dilatation. Cross-sectional views of the ventricles revealed severe dilatation of both ventricular chambers in the transgenic relative to nontransgenic littermate mice (Fig. 4A). In addition, the ventricular free wall and septum of the transgenic mice were thinner than those of nontransgenic littermates. In the 12-day-old transgenic mouse, the left ventricle (LV) wall was 0.070 ± 0.0137 mm thick, which was 1/2 the thickness of that of nontransgenic littermates (0.102 ± 0.00619, n = 6, p < 0.001). The septum was 0.053 ± 0.0069 mm thick, which was 1/2 the thickness of that of nontransgenic littermates (0.103 ± 0.0096, n = 6, p < 0.001). The right ventricle (RV) wall was 0.0295 ± 0.0072 mm thick, which was 1/2 the thickness of that of nontransgenic littermates (0.0436 ± 0.0072, n = 6, p < 0.001) (Fig. 4B).
Cardiac Changes in Mice Overexpressing Mutant SRF

In the 1-day-old F1 neonate of the transgenic mouse from line B, the ventricular free wall and septum were much thinner than those of nontransgenic mice. The LV wall was 0.018 ± 0.0037 mm thick, which was ½ the thickness of that of nontransgenic littermates (0.0365 ± 0.0031, n = 6, p < 0.001). The septum was 0.0334 ± 0.00412 mm thick, which was ½ the thickness of that nontransgenic littermates (0.0540 ± 0.0028, n = 6, p < 0.001), and the RV wall was 0.0083 ± 0.0067 mm thick, which was only ½ the thickness of that of nontransgenic littermates (0.0235 ± 0.0067 mm, n = 6, p < 0.001). Part of the RV wall of the 1-day-old transgenic mouse appeared to be comprised of only a few layers of cardiac muscle cells and was only 0.002 mm thick, or only ½ of the average RV wall thickness of the nontransgenic littermate (0.0235 ± 0.0067 mm) (data not shown).

Mitotic cells were visible in the ventricular sections of 1-day-old mice. However, no significant difference of mitotic index was found between the transgenic (0.030 ± 0.018%) and nontransgenic (0.029 ± 0.005%) mice. No mitotic cells were found in the hearts of 12-day-old mice.

Immunohistochemistry confirmed elevated levels of SRF protein in the myocardium of the transgenic relative to nontransgenic mice (data not shown). Masson-Trichrome staining revealed minimal interstitial fibrosis in the myocardium of the transgenic mice at the age of 12 days (Fig. 4C), but no fibrosis was visible in the myocardium of the transgenic mouse at the age of 1 day (data not shown). No cardiac myocyte hypertrophy was observed in the ventricles of either of the transgenic lines. Instead, the myocytes in the transgenic mouse (24886 ± 8992, arbitrary units) were smaller than those in nontransgenic (33113 ± 8675, arbitrary units) mice (n = 100, p < 0.0001). The results are reported as the means ± S.D. Hematoxylin and eosin staining is shown (magnification of ×200). D, the cell size of the cardiac myocytes was determined in arbitrary units using Image-Pro Plus software. The myocytes in transgenic (24886 ± 8992, arbitrary units) were smaller than those in nontransgenic (33113 ± 8675, arbitrary units) mice (n = 100, p < 0.0001). The results are reported as the means ± S.D. Hematoxylin and eosin staining is shown (magnification of ×400). E, electron microscopic view of LV of nontransgenic mice and transgenic mice. There is myofibrillar degeneration and mitochondrial swelling and deterioration in the LV of transgenic mice. In addition, intracellular volumes of mitochondria and myofibrils are decreased in the LV of transgenic mice, and the intracellular volume of the remaining organellarae is increased in the LV of transgenic mice.

FIG. 4. Morphological changes in hearts of the transgenic (Tg) mice compared with nontransgenic (NTg) mice. A, cross-section of the ventricles of 12-day-old mice at the level of papillary muscle stained with hematoxylin and eosin. The hearts of transgenic mice were dilated in both RV and LV compared with that of nontransgenic mice. B, ventricular wall thickness. The LV wall of transgenic mice was 0.070 ± 0.0137 mm thick, which was ⅓ as thick as that of nontransgenic mice (0.102 ± 0.00619, n = 6, p < 0.001). The septum of transgenic mice was 0.053 ± 0.0069 mm thick, which was ⅓ the thickness of that of nontransgenic mice (0.103 ± 0.0096, n = 6, p < 0.001). The RV wall of transgenic mice was 0.0295 ± 0.0072 mm, which was ⅓ as thick as that of nontransgenic mice (0.0436 ± 0.0072, n = 6, p < 0.001). The data were obtained through direct measurement under the microscope, using an objective micrometer with 0.01-mm ruler markings. C, cross-section of the ventricles of 12-day-old mice stained with hematoxylin and eosin (magnification of ×400). D, the cell size of the cardiac myocytes was determined in arbitrary units using an electronic micrometer with 0.01-mm ruler markings. E, electron microscopic view of LV of nontransgenic mice and transgenic mice. There is myofibrillar degeneration and mitochondrial swelling and deterioration in the LV of transgenic mice. In addition, intracellular volumes of mitochondria and myofibrils are decreased in the LV of transgenic mice, and the intracellular volume of the remaining organellarae is increased in the LV of transgenic mice.

DISCUSSION

The complex of SRF and ternary complex factor is a downstream target of both the Ras/RhoA and mitogen-activated protein kinase pathways, which regulate cell growth and differentiation in response to a number of extracellular signals (23, 24). To test whether functional SRF is required for postnatal myocardial growth and development, a procedure such as targeted disruption of the SRF gene would be the preferred method. However, SRF null allelic (−/−) embryos die during embryonic development because of a gastrulation defect and the absence of mesodermal cells that are required for further embryonic development (17). In addition, in these SRF (−/−) embryos, expression levels of the SRF target genes, such as c-fos, egr-1, and the α-actin genes are either very low or not.
Cardiac Changes in Mice Overexpressing Mutant SRF

detectable by the sensitive reverse transcriptase-polymerase chain reaction measurement, suggesting that it would be extremely difficult if not impossible to study the function of SRF in the postnatal heart using conventional gene targeting techniques. We therefore chose to utilize a somewhat different approach, by overexpressing a mutant form of SRF driven by the α-MHC promoter to determine the role of SRF in the regulation of genes associated with postnatal development of cardiac structure and function.

The double mutation of amino acids 159 (Thr → Ser) and 163 (Lys → Glu) is located within the area of the SRF protein that is important for dimerizing, binding DNA, and recruiting other cofactors such as p62TCF (15). The mutation at these positions apparently reduced the binding ability of ΔmSRF protein to SRE and made it incapable of activating the ANF promoter, which contains the SRE site. In addition, 2-5-fold excess amount of ΔmSRF relative to the amount of wtSRF was able to significantly attenuate the ability of wtSRF to activate the ANF promoter activity. Furthermore, increasing the ΔmSRF/ wtSRF protein ratio in cotranslation experiments progressively attenuated the SRF binding activity. Overexpression of the ΔmSRF transgene elevated the levels of total SRF mRNA and protein but reduced the total SRF binding activity in the heart of the transgenic animals. These findings demonstrate that, as is the case in vitro, ΔmSRF was able to compete effectively with mouse endogenous SRF for SRE in vivo and thereby partially blocked the signals regulating cell growth and differentiation that are mediated through SRE via various signaling cascades, such as the Ras/RhoA and mitogen-activated protein kinase pathways (25, 26).

To study the effect of overexpression of ΔmSRF on postnatal cardiac function, the α-MHC promoter was selected for the transgenic procedure (27). We were able to obtain a few transgenic mice. However, the rate of live births to the transgenic founder mice was extremely low. Only two founder mice were identified of 159 mice that were screened, and each founder mouse had only one single copy of the transgene. Although a few live birth transgenic F1 progeny were eventually obtained, they all developed severe cardiomyopathy soon after birth and died during early postnatal development.

The F1 transgenic mice displayed severe dilatation of the atrial and ventricular chambers very early during the postnatal period, indicating that the heart of the ΔmSRF transgenic mouse was apparently unable to grow normally or maintain normal wall thicknesses in response to increased workload after birth. The blood volume in the circulation increases substantially around the perinatal period because of a rapid increase in body size (28–30). The normal perinatal heart is able to accommodate the increased workload by rapidly increasing cardiomyocyte size and myofibrillar protein content, thereby enhancing cardiac function (31, 32). The hearts of the ΔmSRF transgenic mice obviously failed to meet this challenge. The ventricular wall of the transgenic mice was much thinner than that of age-matched nontransgenic mice, and the cardiac myocytes in the transgenic mice were significantly smaller than those in nontransgenic mice. Furthermore, intracellular changes that could reduce the energy supply such as extensive mitochondrial swelling and deterioration were noted. Less myofibril volume and myofibril degeneration in the cardiac myocytes might have severely affected the cardiac performance of these animals. The morphological abnormalities in the heart of the transgenic mouse likely made it incapable of pumping blood with sufficient efficiency for survival.

It has been reported that SRF is required for a cell to establish proper cell-cell interactions and to maintain normal morphology. However, heterozygous SRF (−/+ ) embryonic stem cells apparently form embryonic bodies similar to wild type embryonic bodies. Only SRF (−/− ) null allelic embryonic stem cells form smaller embryonic bodies with 40–70% of the size of corresponding SRF (−/+ ) and SRF (++ ) embryonic bodies, and with abnormal morphology, which is presumably due to impaired interactions of SRF(−/− ) embryonic stem cells with each other during differentiation and aggregation (19). It is possible that ΔmSRF overexpression might make the heart more vulnerable to changes in cardiac morphology under increased workload during the early postnatal period, in part because of possibly altered cell-cell interactions.

The remarkable morphologic changes observed in the heart of the transgenic mice, especially in the 1-day-old transgenic mouse, could be a result of ΔmSRF overexpression before as well as after birth. During embryonic development, β-MHC is the predominant isoform of myosin heavy chain that is expressed in the heart, whereas the mRNA level of α-MHC is very low. The α-MHC promoter that was used in this study contains a small portion of the β-MHC gene and the entire portion of 5′-end of the α-MHC gene and is 5441 base pairs in length. Immediately behind the multiple cloning site of the promoter is a 622-base pair DNA fragment of human growth hormone intron (Hgh) sequence that could enhance the transgene expression (27, 33). This promoter is one of the most widely used promoters for transgenic mouse models of postnatal cardiac diseases. In the present study, the transcriptional unit α-MHC promoter-ΔmSRF transgene-Hgh was apparently activated at embryonic development day 17, as the mRNA level of the total SRF was moderately elevated in the heart of transgenic relative to nontransgenic embryos. Consequently, certain SRF-regulated genes, such as β-MHC, MLC2v, cardiac α-actin, and SKA were apparently dysregulated. These alterations could have changed the ratio and components of the cardiac structural proteins during embryogenesis, such as at day embryonic development day 17, and could have made the heart of the transgenic mouse more susceptible to the increased workload that occurred soon after birth.

The expression of the MLC2v, cardiac α-actin, and SKA genes at end stage cardiomyopathy (day 12) in the transgenic mouse was different from that of the ΔmSRF transgenic embryo at embryonic development day 17, whereas the expression of β-MHC remained the same at both periods. It is possible that different transcriptional regulators are active in the regulation of SRF target genes at these two periods. More work needs to be done to define the promoter region of the SRF target genes and to elucidate the potential role of other transcriptional modulators in the regulation of cardiac development and cardiac gene expression in response to hemodynamic stress.

Recently, Shioi et al. (34) reported that cardiac-specific overexpression of constitutively active PI3K results in transgenic mice with larger hearts and hypertrophied myocytes, whereas overexpressing dominant-negative PI3K results in mice with smaller hearts with comparable decreases in myocyte size. This suggests that the PI3K pathway is necessary and sufficient to promote organ growth in mammals (34). PI3K activates gene expression by transactivating SRF-dependent transcription independently of the Rho and ETS ternary complex factor pathways. Also, PI3K-stimulated cell cycle progression requires transactivation of SRF, and dominant-interfering SRF mutants could potentially attenuate mitogen-stimulated cell cycle progression (35). Therefore SRF might be one of the downstream regulators that mediates events or factors that contribute to the determination of cell size. We previously reported that overexpression of the wild type SRF transgene results in cardiac myocyte hypertrophy (16), and in the present study we found that overexpression of the ΔmSRF resulted in dilated
Cardiac Changes in Mice Overexpressing Mutant SRF

hearts with smaller myocytes. The lack of hypertrophy in the cardiac myocytes of these dmSRF mice raises the possibility that SRF may be important for mediating cardiomyocyte hypertrophy.

Taken together, the results of the present study indicate that overexpression of dmSRF in the heart could alter the mRNA levels of SRF-regulated genes and could thereby impair early postnatal cardiac growth and development. These changes result in the early onset of severe, dilated cardiomyopathy, and premature death. It appears that SRF may be important in the regulation of genes that mediate cardiac myocyte hypertrophy.

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