Serum Response Factor, an Enriched Cardiac Mesoderm Obligatory Factor, Is a Downstream Gene Target for Tbx Genes*

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We tested the idea that T-box factors direct serum response factor (SRF) gene activity early in development. Analysis of SRF-LacZ “knock-in” mice showed highly restricted expression in early embryonic cardiac and skeletal muscle mesoderm and neuroectoderm. Examination of the SRF gene for regulatory regions by linking the promoter and 5′-flanking sequences, up to 5.5 kb, failed to target LacZ transgene activity to the heart and the tail pre-somatic mesenchyme. However, linkage of a minimal SRF promoter with the SRF 3′-untranslated region (UTR), inactivated with multimeric T-box binding sites (TBEs), restored robust reporter gene activity to embryonic heart and tail. Finer dissection of the 3′-UTR to a small cluster of TBEs also stimulated transgene activity in the cardiac forming region and the tail, however, when the TBEs contained within these DNA sequences were mutated, preventing Tbx binding, transgene activity was lost. Tbx2, Tbx5, and the cardiogenic-enriched MYST family histone acetyltransferase TIP60, were observed to be mutual interactive cofactors through the TIP60 zinc finger and the T-box of Tbx factors. In SRF-null ES cells, TIP60, Tbx2, and Tbx5 were sufficient to stimulate co-transfected SRF gene activity, as shown by strict correlation with SRF-LacZ “knock-in” mice. Although this is a continuous developmental process, one transcription factor, serum response factor (SRF),1 may play a leading role in the commitment of pre-cardiac cells. SRF is a 67-kDa DNA-binding protein that was first cloned from a HeLa cDNA library and was generally presumed to be a ubiquitous transcription factor. SRF binds DNA as a dimer and symmetrically contacts various serum response elements with a consensus sequence CCAATGG. SRF is member of the “MADS” box transcription factor family (2, 3), and, despite their similarities, MADS box proteins have evolved to perform disparate important biological functions. Functions of MADS transcription factors include specification of mating type in yeast, homeotic activities in plants, pharyngeal muscle specification in Caeorhabditis elegans, pulmonary development in Drosophila, and elaboration of mesodermal structures in vertebrates (reviewed in Ref. 4). Recent homologous recombinant knock-out of the murine SRF gene locus supports the observation that SRF is absolutely required for the appearance of mesoderm during mouse gastrulation (5).

Analysis of SRF null mice not only revealed a severe block to the activation of SRF-regulated immediate early genes, but it also showed inhibited expression of the cardiac, skeletal, and smooth muscle α-actins. These results indicate that SRF is an essential regulator of muscle-specific gene activity. Further, the regulatory regions of a number of muscle-specific genes, such as skeletal, cardiac (6–8), and smooth muscle α-actin (9, 10), contain serum response elements, which are required for promoter activity and depend upon SRF (see a recent review, Ref. 11). Mutations that prevent SRF binding severely impair the expression of c-fos, as well as these muscle-restricted promoters (12). Taken together, these data further support a role for SRF in muscle formation, a subset of mesoderm-derived cell populations. We therefore examined the expression pattern of SRF in the developing embryos and show that SRF is not ubiquitously expressed but is rather restricted to tissues of mesoderm and neuroectoderm origins. We show that staining of embryos generated from β-galactosidase “knock-in” mouse lines closely mirror actual SRF gene activity, as shown by strict correlation with in situ hybridization analysis.

Although SRF is important for muscle formation in the developing embryo, the one or more mechanisms governing its expression remain elusive. The upstream promoter region of

1 The abbreviations used are: SRF, serum response factor; UTR, untranslated region; Tbx, T-box; UTR, untranslated region; EMSA, electrophoretic mobility shift assay; CMV, cytomegalovirus; GST, glutathione S-transferase; E, embryonic day; HAT, histone acetyltransferase; T, brachyury; ES cells, embryonic stem cells.
SRF contains binding sites for the cardiac transcription factors GATA, Nkx, and SRF (12). Because it has been shown for the cardiac α-actin promoter that these three factors can bind to DNA and synergistically activate gene transcription (8), we asked if sequences in the SRF promoter region were sufficient to up-regulate expression of the β-galactosidase reporter gene in the cardiac regions of transgenic mice. We found that the promoter sequences containing the GATA, Nkx, and SRF binding sites were insufficient to drive expression in the developing heart, although skeletal and smooth muscle populations expressed SRF in these mice. Where then are the SRF genetic sequences that allow for cardiac expression?

Tbx factors are potential candidates for directing SRF gene activity. One example comes from Eomesodermin, a T-box (Tbx) transcription factor expressed in the early mesoderm of Xenopus embryos (13). When the T-domain of this factor is fused to a transcription factor expressed in the early mesoderm of Xenopus embryos (13), when the T-domain of this factor is fused to an engrailed repressor domain, arrest of gastrulation results, similar to that of the SRF null mutant. Other examples show similar roles for Tbx factors in the formation of the heart. The cardiac Tbx factor Tbx5 is known to physically interact with the cardiac factor Nkx2.5 (14). Mutation or ablation of Tbx5 from the embryo causes Holt-Oram syndrome, a condition characterized by defects in limb and heart development (15–17). In addition, Tbx2, Tbx5, and SRF are co-expressed in overlapping patterns, indicative of mutual co-regulation. SRF transcripts were examined by in situ hybridization in Tbx5 “knock-out” mice, which display the Holt-Oram phenotype (18). SRF transcripts were reduced in the cardiac and tail regions of these mice, indicating that a loss of Tbx5 results in a reduction in cardiac SRF expression. Furthermore, the presence of a plethora of evolutionarily conserved consensus T-box DNA sequences (18), many of which are located in the 3′-UTR of the SRF gene, influence the notion that SRF may be a downstream gene target of the Tbx factors.

Our study demonstrates that SRF transgenes, containing a minimal promoter and multimeric Tbx-containing 3′-UTR sequences, are capable of inducing expression in heart and tail mesenchyme of the developing embryo. This inductive activity was lost when the T-box sites were mutated. In addition, it has recently been shown that Tip60, a founding member of the MYST family of histone acetyltransferases, appears in the embryonic chick myocardium and physically interacts with SRF (19). Although the function of Tip60 in the context of the early heart is unknown, we imagine that Tip60 may participate in the transcriptional regulation of early myocardial genes. Co-transfection assays done in SRF−/− murine embryonic stem cells indeed indicated that Tbx2, in combination with Tbx5, can up-regulate SRF reporter activity, but only in the presence of the SRF 3′-UTR sequences and Tip60. Inactivation of the HAT domain in a TIP60 mutant or ablation of its zinc finger domain abrogated up-regulation of SRF gene activity. Our study indicates that cardiac expression of SRF is achieved through the combination of Tbx factors and a MYST histone acetyltransferase via a T-box-rich enhancer region in the 3′-UTR of the gene.

MATERIALS AND METHODS

Targeting Strategy and Generation of NLS-β-galactosidase Recombination into the SRF Genomic Locus—SRF genomic DNA used for targeting was isolated from the isogenic strain 129A library (12). A cassette containing nuclear localization signal-β-galactosidase cloned upstream of neo selectable marker was inserted between the NotI site located upstream of the SRF ATG and the BglII site located within the second exon. The cassette was designed with the pGKneo reading frame in an opposite orientation to that of the endogenous SRF allele. Insertion of this cassette deleted both the DNA binding and dimerization domains of SRF. The targeting vector contained 2.4 kb and 6.7 kb of homologous sequences on 5′ and 3′ regions of the cassette, respectively. Sall-linearized targeting vector was electroporated into AB2.2 ES cells and selected in 200 μg/ml G418 for 10 days. Genomic DNA from 100 clones was analyzed by Southern blotting after cutting the DNA with BamHI (for the 3′-end external probe) and HindIII (for 5′-end internal probe). Two correctly targeted clones were used to generate chimeras.

Generation, Collection, and Identification of Transgenic Embryos—Constructs for the generation of LacZ transgenic mice were generated by restriction endonuclease digestion of vector sequences using the following enzymes: HindIII/NotI (5.5 kb), Scal/NotI (3.5 kb), XhoI/NotI (1.0 kb), EcoNI/NotI (0.541 kb), XhoI/Scal (1.0 kb plus UTR), EcoNI/Scal (0.541 kb plus UTR), XhoI/BSrGI (1.0 kb plus UTR2313), and EcoNI/BSrGI (0.541 kb plus UTR2313). Restriction endonuclease digestion was followed by size separation on agarose gels and purification with Qiagen beads (Qiagen, Chatsworth, CA). DNA fragments were then eluted with 0.1× Tris-EDTA and phenol/chloroform-purified. Transgenic embryos were produced by pro-nuclear injection of one-cell-stage embryos (20). Embryos were harvested 7.5–10.5 days post coitum following transfer and stained for β-galactosidase activity (21). Paraffin-embedded embryos were sectioned (10 μm, day 10) and counterstained with nuclear fast red. Transgenic embryos were identified by PCR analysis of placental or embryo DNA with a combination of LacZ SA primers: upstream, 5′-CTCAAACTGCGATGCGA-3′; downstream, 5′-CGTTGCGACCACATGAAACCC-3′. PCR reactions were performed using 1 μg of DNA isolated from the amnion or embryo (20) as template.

Transient Transfection Assays—SRF−/− murine embryonic stem cells, generated as described by Du et al. (22), were maintained in ES medium supplemented with leukemia inhibitory factor to prevent differentiation. Cells were plated at 1 million cells per well in a 6-well dish and transfected 24 h later with DNA mixtures containing a total of 1 μg of total DNA, which included 250 ng of luciferase reporter vector and a total of 750 ng of pCg-derivated vectors. Cells were transfected using Effectene reagent (Qiagen). Briefly, DNA solution levels were brought to a total volume of 150 μl with the Effector kit (EC buffer). Next, 8 μl of enhancer was added to each sample, and these solutions were incubated for 5 min at room temperature. 25 μl of Effectene was then added to each sample, and after mixing these were incubated an additional 10 min. Following washing with phosphate-buffered saline, and application of 1 ml of ES medium/well, 1 ml of ES medium was added to each DNA sample, and then cells were incubated for 48 h at 37 °C, 5% CO2. Medium was then changed, and cells were incubated for an additional 24 h. Cells were washed with phosphate-buffered saline and lysed with 200 μl of Reporter lysis buffer (Promega). Cell lysates were scraped from the dishes and centrifuged at 13,000 × g for 10 min. Supernatants were tested for luciferase activity using a Luminometer Monolight 2010 (Analytic Luminescence Laboratory). 20 μl of cell extract was added to 20 μl of luciferase substrate solution (20 mM Tris-HCl, pH 8.0; 4 mM MgSO4; 0.1 mM EDTA; 30 mM dithiothreitol; 0.5 mM ATP; 0.5 mM β-luciferin; 0.25 mM coenzyme A), and the emitted light automatically mixed with 100 μl of luciferase reagent. Bacterial proteins were prepared in Epicurian coli as template.

Electrophoretic Mobility Shift Assays—Double-stranded oligonucleotides, corresponding to nucleotides 11,038–11,097 and 11,091–11,150 of the SRF gene sequence, as well as a consensus Tbx binding sequence (5′-CGTGTGGTGGTCTACTAACC-3′) as described by Gosh et al. (17) were synthesized and used for electrophoretic mobility shift assays (EMSAs). Each reaction mixture (10 μl) contained 20 mM Tris (pH 7.6), 50 mM NaCl, 1 mM dithiothreitol, 5% glycerol, 1 mM sodium phosphate, 0.5 μg of poly(dI-dC), and purified bacterial proteins. Bacterial proteins were prepared in Epicurian coli (BL21-Gold (DE3) cells (Stratagene). End-labeled probes (0.02 pmol; 10,000–20,000 cpm) and proteins were incubated for 15 min at room temperature. For competition assays, cold competitors were incubated with the proteins for 5 min prior to the addition of probe. The DNA-protein complexes were fractionated on 5% polyacrylamide gels (acrylamide:bisacrylamide ratio, 29:1) in Tris-bo- rate-EDTA buffer as described by Belaguli et al. (12). The gels were visualized by autoradiography.

TIP60 Mutants—TIP60 was amplified from reverse-transcribed mouse testis total RNA using primers complementary to the first and last nucleotides of TIP60 cDNA; the forward primer was 5′-CGCCGAATTCTATGGGCCGGAAGTTG-3′ (note underlined EcoRI site), and the reverse primer was 5′-GGCGCCATCCACCTCGTGTGGTGAGTCG-3′ (note underlined BamHI site). Following directional ligation into the EcoRI/BamHI sites of pSF2FLAG-CMV-7.1, sequencing was performed.
**RESULTS**

Early Embryonic Expression of SRF Is Largely Restricted to Cardiac and Skeletal Muscle Tissues—*In situ* hybridization analysis of mouse embryos, using an SRF RNA probe, indicate that transcripts of SRF are somewhat diffuse early in development, with concentrated expression in the lateral plate mesoderm and primitive streak (Fig. 1A, E7.5). These areas are of significance to cardiac and skeletal muscle, because pre-cardiac mesoderm cells migrate through the streak to take up residence in the anterior lateral plate, whereas skeletal muscle originates in myotomes of the paraxial mesoderm. As development proceeds, SRF becomes robustly expressed in the cardiac crescent (Fig. 1B) and later in the heart tube and developing somites (Fig. 1, C and D, E8.5). These tissues continue to express high levels of SRF throughout development. Similar to chick embryos previously analyzed, SRF transcripts appear highly restricted to myogenic mesoderm and neuroectoderm (7, 8). Both in chick and mouse embryos, SRF is enriched in the cardiac crescent (and later the heart tube), somites, and the tail region. The concentration of SRF in muscle precursors early in development suggests that SRF plays a specific role in muscle specification and differentiation.

SRF β-galactosidase “Knock-in” Reporter Mice Faithfully Reproduce the Endogenous SRF Expression Pattern—We generated β-galactosidase reporter “knock-in” mice to analyze SRF gene activity during embryogenesis. The organization of the SRF gene and the knock-in cloning strategy used to disrupt the SRF genomic locus with a β-galactosidase-PGK neomycin cassette are shown schematically in Fig. 2A. Southern blots of mouse-tail DNA cut with HindIII and probed with the 5′-galactosidase reporter “knock-in” mice to analyze SRF gene activity during embryogenesis. The organization of the SRF gene and the knock-in cloning strategy used to disrupt the SRF genomic locus with a β-galactosidase-PGK neomycin cassette are shown schematically in Fig. 2A. Southern blots of mouse-tail DNA cut with HindIII and probed with the 5′-galactosidase reporter gene would faithfully reproduce the expression pattern of their younger counterparts, showing expression in the myocardium of the heart, the somites, and strong staining in the tail region (Fig. 2D, panel q). This pattern is consistent with the endogenous gene. E10.5 embryos (Fig. 2D) continue the expression pattern of their younger counterparts, showing expression in the myocardium of the heart, the somites, and strong staining in the tail region (Fig. 2D, panels s and t). β-Galactosidase expression also extended to the smooth muscle of vessels (Fig. 2D, panel p) and the ventral portion of the neural tube (Fig. 2D, panel q). Thus, SRF transcriptional activity, shown by LacZ
FIG. 2. β-Galactosidase reporter gene knock-in into the SRF genomic locus reveals restrictive expression in embryonic cardiac, skeletal, and smooth muscle tissues. A, a simple map of the SRF gene locus organization of the knock-in vector with regions comprising coding exons shown as boxes. Filled and open boxes indicate the coding region and the 5- and 3-untranslated regions, respectively. The numbers below each box indicate the last nucleotide of the exon. The locations of the translational start codon (ATG), the stop codon (TGA), and the first and second polyadenylation signals (Poly(A)) are indicated. Introns and the flanking sequences are shown as thin lines. The bacterial β-galactosidase gene was inserted into the translation start site of SRF (Approximately 2 kb of homologous sequence was linked to the PGK-neo cassette (phospho-glycerate kinase) promoter driving neo in the opposite orientation to lacZ). B, a Southern blot of tail DNA derived from two wild-type controls and a β-galactosidase knock-in mouse line. HindIII digestion probed with a DNA fragment overlapping the core promoter revealed two hybridized bands of 10.9 kb, representing the wild-type control, and the foreshortened 8.7-kb band, representing the β-gal-Pgk-neo knock-in control. Panel C, SRF lacZ expression in E7.5–E9.5 mouse embryos. a, negative control (whole mount view); b–e and i, SRF knock-in expression in whole mouse embryos; f–h, and j, transverse sections through SRF β-galactosidase embryos shown in a–e and i. In D, SRF β-galactosidase expression observed in E10.5 mouse embryos. k, whole mount view; l–t, transverse sections through the embryo shown in k. The following embryonic tissues are marked: nt, neural tube; h, heart; ht, heart tube; so, somites; a, allantois; hf, head fold; t, tail; lb, limb bud; e, endocardium; m, myocardium; en, endothelium; cc, cardiac crescent; ys, yolk sac; v, blood vessel.
FIG. 3. The SRF promoter and contiguous 5’-flanking sequences, up to 5.5 kb, are insufficient to direct SRF β-galactosidase reporter activity in the heart and tail. A, a schematic representation of the SRF promoter constructs analyzed in F0 day 7.5–10.5 embryos and a summary of the transgene expression. The number of transient transgenic embryos generated with each construct is expressed as the number...
expression, reinforced the notion of restricted developmental expression of SRF and demonstrates that transcriptional expression of the reporter is consistent with the appearance of the endogenous gene.

The Core Promoter and Contiguous 5' Flanking Sequences Are Insufficient to Direct Cardiac Expression of SRF—Because the SRF core promoter was shown to contain binding sites for the cardiac transcription factors GATA4, Nkx2–5, and SRF, we asked whether these sequences and additional 5'-flanking sequences were sufficient to drive β-galactosidase activity in the heart, using transient transgenics of Fo embryos. Fig. 3A shows a schematic representation of the constructs used to test SRF promoter and 5'-flanking sequences, as well as a summary of the β-galactosidase staining in six different embryonic tissue types. The expression pattern in mice containing 5.5 kb of the SRF promoter and contiguous 5'-flanking sequences included the ventral neural tube and somites, but no staining was observed in the heart tube (Fig. 3B). The 3.5-kb flanking sequences yielded staining in the ventral neural tube and in somites (Fig. 3, C and D). Mice transfected with up to 1.0 kb of SRF contiguous sequence showed staining in the neural crest cells, particularly those in the outflow tract of the heart and pharyngeal arches, but not in the distal tail and the heart (Fig. 3, E–J). No β-galactosidase staining was observed in the embryo when 0.541 kb of the promoter was linked with LacZ (Fig. 3K). These results indicate that the promoter sequences, containing well known cardiogenic transcription factor binding sites and extensive 5'-flanking sequences, are incapable of directing cardiac expression in the embryonic heart and tail. We then attempted to identify the SRF genetic sequences that drive SRF expression in the heart.

A Potential Candidate Role for Tbx Factors to Direct SRF Gene Activity—We reported on the appearance of Tbx2, Tbx3, and Tbx5 transcripts during early chick embryogenesis (23) and noticed that Tbx2 and Tbx5 proteins were expressed in regions of the developing heart that might be co-expressed with SRF. As shown in Fig. 4 (A–D), Tbx5 transcripts are expressed in the anterior lateral plate mesoderm (stage HH6), the cardiac crescent, and heart tube (stage HH8). Expression overlaps with that of SRF. Because Tbx5 co-expresses with SRF, SRF mRNA levels were evaluated by in situ hybridization in Tbx5 mutant mice. These mice have a Holt-Oram phenotype, showing limb and cardiac defects later in development. As can be seen in Fig. 4E, SRF transcripts evaluated by in situ hybridization are significantly reduced in the heart tube of the mutant embryo relative to the wild type, but not in the tail where Tbx6 resides.

We then asked if consensus T-box binding sequences, TG(C/G)CACACCTAGGTGTGAAATT, were present in the SRF genetic locus. A search revealed the presence of many combinations of full and half palindromic T-box sites throughout the transcribed portion of the SRF gene. T-box sites that were both highly conserved between mouse and human genomes and are located in the second poly(A) 3'-UTR region of the SRF gene are shown in Fig. 5A. To verify that some of these sequences were indeed functional T-box binding sites, double-stranded oligonucleotides, including the T-box consensus binding sequence (TbxSEQ1), a conserved pair of T-boxes (SEQ1), and another set of T-boxes containing intervening NKE binding sites (SEQ3) from the murine 3'-UTR were synthesized and used for electrophoretic mobility shift assays (EMSAs) using bacterially expressed Tbx5. Authentic T-box sites in SEQ1 provided two strong shifted DNA-protein complexes, whereas SRF SEQ2 and SEQ3 DNA showed two and three shifted species that were well resolved upon the addition of Tbx5 (Fig. 5B). The addition of unlabeled Tbx SEQ1 DNA competed well with shifted Tbx5 and SEQ3 complexes, indicating specific Tbx binding to a portion of the SRF 3'-UTR.

A Tbx-responsive Downstream Enhancer, Located in the 3'-UTR of the Gene, Directs Early Cardiac Expression of SRF—Given the expression pattern of Tbx factors and the presence of Tbx binding sites in the 3'-UTR of SRF, we tested several of the promoter constructs in transgenic mice again, but included the heavily enriched T-box-laden region. Fig. 6A shows a schematic representation of the constructs used to test SRF promoter and UTR sequences, as well as a summary of the results in six tissue types. Because there were conserved T-boxes also in the
FIG. 5. Highly conserved T-box binding sites are located in the 3′-untranslated portion of the murine SRF gene and bind Tbx5. A, a schematic drawing of a portion of the 3′-UTR of the murine SRF gene, showing the location of T-boxes. T-box sequences are highlighted in gray, whereas the central “guanine” (critical for Tbx binding) is shown in dark shading. The blue highlighted region indicates the 231-bp fragment used...

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**A**

| T-box Sequence | SRF Gene Location |
|----------------|-------------------|
| TTGAGG         | 11038-11097       |
| TTGAGG         | 11091-11150       |

**B**

| T-box Seq | SRF Seq 2 | SRF Seq 3 | Unlabeled T-box Seq 1 |
|-----------|-----------|-----------|-----------------------|
| 1 2 3     | 4 5 6     | 7 8 9     | 10 11 12 13 14 15 16|

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**NKE**

-231 bp fragment
first and second introns, an internal ribosomal entry site construct containing 3.5 kb of upstream sequence and the first intron was also investigated. Results show that 1.0-kb plus UTR transgenic mice have robust expression in the cardiac crescent as well as in the tail (Fig. 6, B and C). Later in development, cardiac expression becomes highly restricted, reminiscent of Tbx2 and Tbx5 expression. Analysis of the 0.5-kb plus UTR transgene in slightly older embryos also showed robust β-galactosidase staining in the heart tube and intense staining in the tail region (Fig. 6, F–K). Like the 1-kb plus UTR transgene, cardiac expression in these mouse embryos becomes restricted later in development. To begin fine mapping of the Tbx sites in the 3′-UTR, a transgene containing the 3′-UTR plus UTR transgene, cardiac expression in these mouse embryos was me may include or be required for cardiac expression. To demonstrate that expression of the LacZ transgene in these mice was truly controlled by Tbx factors, the T-boxes included in the 231-bp region of the 3′-UTR were mutated to prevent Tbx binding. T-box sequences were changed to GGATCC, thus eliminating the central “guanine” residue (dark shading in Fig. 5A), which has been shown to be critical for Tbx binding (25). These mice, shown in Fig. 6 (N and O), show complete absence of transgene activity, demonstrating that Tbx-protein binding is essential. Finally, the intron-containing construct (Fig. 6, D and E) showed staining only in the outflow tract of the heart, indicating normal crest expression, and in the somites. The SRF promoter region of 0.5 kb is included in Fig. 6M as a negative control for comparison.  

**Tbx2 and Tbx5, Along With the Cardiac MYST Family**  

**Tbx5, Along With the Cardiac MYST Family**  

**Histone Acetylase TIP60, Activates Transcription Through the SRF 3′-UTR—**TIP60 is a founding member of what is known as the “MYST” family, related by a central domain of ~174 amino acids; moreover, most MYSTs contain chromo, zinc finger-like, and histone acetyltransferase (HAT) domains (26). Although the function of TIP60 in the context of the early myocardium is unknown, as inferred from its chromodomain, zinc-finger-like, HAT, and LX domains (shown schematically in Fig. 7A), TIP60 was tested for role in SRF gene regulation. We observed that TIP60 physically interacts with SRF and Tbx5 in GST pull-down assays (Fig. 7, B and C). In addition, TIP60 forms specific contacts with Tbx5 via the zinc finger in the MYST domain (Fig. 7C). We asked whether Tbx factors and TIP60 could facilitate expression of a luciferase reporter, using a minimal SRF promoter linked with its 3′-UTR. Tbx2, Tbx3, or Tbx5 factors alone had minimal effects on SRF reporter activity in co-transfected SRF−/− ES cells (Fig. 8A, lanes 2–4). Combinations of Tbx2 with Tbx3 and or Tbx5 resulted in preferred co-activation of SRF promoter linked with the 3′-UTR about 6-fold above basal levels (Fig. 8A, lanes 7, 8, and 12) and to about 5-fold with the addition of SRF (Fig. 8A, lane 13). However, in the presence of paired Tbx2 and Tbx5 factors, the addition of TIP60 co-activated the SRF promoter plus 3′-UTR reporter activity 16- to 18-fold over control levels and about three to four times greater than the SRF promoter without the 3′-UTR (Fig. 8A, lanes 10 and 14). The inclusion of a TIP60 mutant in which the HAT domain was specifically mutated (TIP60mHAT) inhibited transcriptional activity ~50% in a dose-dependent manner (Fig. 8, C and D, lanes 6–13 and 12–15). In addition, TIP60mHAT failed to activate the reporter gene when paired with the Tbx factors in the absence of wild-type TIP60 (Fig. 8D, lane 10). This effect was only observed in situations where the 3′-UTR sequence was linked downstream of the promoter and reporter gene in the co-transfection assay and is consistent with the requirement of the 3′-UTR for cardiac expression in transgenic mice. Because TIP60 was found to interact with Tbx5 through its zinc finger domain, a TIP60 mutant in which the zinc finger was ablated (TIP60ΔZn) was tested to determine whether disruption of the interaction with Tbx5 would prevent activation through the 3′-UTR. Fig. 8D (lanes 11 and 16–19) shows that, like TIP60mHAT, TIP60ΔZn is incapable of activating the reporter with T-box factors in the absence of wild-type TIP60, and inhibits activation with TIP60 in a dose-dependent manner. These results suggest that the interaction between Tbx5 and TIP60 is essential for activation of the reporter gene, presumably due to the function of Tbx5 to bind and recruit TIP60 to the complex.  

**DISCUSSION**  

In contradistinction to skeletal muscle differentiation, in which a single “master gene” is responsible for the induction of the myogenic program, the process of cardiogenesis involves temporal layers of multiple signaling factors, which cooperate to drive naive mesenchymal cells progressively into mesodermal, muscular, and finally cardiac populations. It is a process that continues throughout development, because pre-cardiac cells migrate through the embryo, encountering new combinations of signaling factors that progressively narrow their pluripotency. Exogenous growth factor signals are translated in the cell by the up-regulation and action of transcription factors, which activate type-specific sets of genes. SRF is one such factor, which was first discovered in HeLa cells and was generally presumed to be a ubiquitous transcription factor (1). More recently, however, SRF has been shown to have specific importance for the development of all three types of muscle. High levels of SRF expression and increased SRF mass appear to coincide with the expression of cardiac, skeletal, and smooth muscle α-actins (7), noted as early markers for terminal striated muscle. The second Pol(A) of SRF is indicated in green, and NKE sites present are in italics. Sequence analysis of the SRF gene shows that the indicated T-boxes are conserved across species. B, double-stranded oligonucleotides were synthesized and used for EMSAs using bacterially expressed Tbx-5 as described under “Materials and Methods.” Binding targets used included the T-box consensus sequence (TbxSEQ1; lane 1), and two regions from the murine 3′-UTR (SRFSEQ2, lanes 3–7; SRFSEQ3, lanes 9–13). (Numbers above these sequences indicate the base pair location of these sequences within SRF.) T-box sites in SRF sequenced two strong shifted EMSA DNA-protein complexes. EMSA with Tbx5 and SQK2 and SQQ3 DNA showed two and three shifted species. The addition of TbxSEQ1 DNA competed the Tbx5 and SQK2 and -3 complexes (lanes 14–16), indicative of specific Tbx binding.
ated and smooth muscle differentiation (27). In addition, SRF protein levels have been shown to increase approximately two orders of magnitude during cardiogenesis (7).

Because of the central importance of SRF in the specification and differentiation of cardiac muscle, we examined the expression pattern and embryonic regulation of this factor in chick and mouse embryos. We have demonstrated that SRF is expressed in a highly restrictive, rather than ubiquitous pattern throughout development. This pattern of expression is consistent with SRF function in muscle formation, as transcripts were

**FIG. 6.** Early cardiac expression of SRF is directed by a downstream enhancer, located in the 3′-UTR of the SRF gene. A, schematic representation of the SRF promoter constructs analyzed in F0 day 7.5–10.5 embryos and a summary of the transgene expression. The number of transient transgenic embryos generated with each construct is expressed as the number of β-galactosidase positive relative to the number of PCR-positive embryos. A “+” or “−” symbol indicates whether β-galactosidase expression was detected. B and C, −1.0-kb-UTR expression pattern (arrow indicates expression in the cardiac crescent). D–K, −0.541-kb-UTR expression pattern; D and E, internal ribosomal entry site expression pattern; L, expression pattern in mice containing −0.541 + 231 bp of the UTR region. N and O, expression pattern of mice with the −1.0-kb-UTRm (231) transgene. Note the lack of cardiac staining. M, −0.541-kb (no UTR) embryo included for comparison with F. The following embryonic tissues are marked: nt, neural tube; h, heart; m, myocardium; e, endocardium; a, allantois.
enriched early in development in the cardiac crescent, the developing somites, and the tail. SRF transcripts were detected later in development in the smooth muscle of vessels, the somites, and the heart tube itself. This expression continues throughout all stages tested, indicating the continuity of the development of muscle tissue. To study the regulation of SRF, we constructed SRF-β-galactosidase knock-in mice. These mice were used as transient transgenics, incorporating different portions of the SRF sequence. Before we could test various SRF sequences in these mice, we first had to determine whether the knock-in would reproduce the complete endogenous SRF expression pattern. We have demonstrated that these mice show the same restricted expression pattern of SRF transcripts as the endogenous gene, as shown by in situ analysis.

The SRF promoter has previously been shown to contain binding sites for the cardiac transcription factors GATA, Nkx, and SRF (12). It has been shown, in the case of cardiac α-actin, that these three factors can bind to DNA and synergistically activate gene transcription (8). It seems reasonable, therefore, that the cardiac regulation of SRF is controlled by these three, overlapping transcription factors. To test this hypothesis, we constructed SRF promoter deletion constructs, linked to β-galactosidase, and tested LacZ expression in transgenic mice. These studies clearly show that, although expression of SRF is observed in several tissues (neural tube and somites), no SRF expression could be detected in developing cardiac tissue. This observation is important for two reasons; first, it illustrates that the mechanism of SRF regulation in cardiac muscle differs from SRF regulatory mechanisms in other cell types, implying the existence of a cardiac-specific enhancer for SRF. Second, it demonstrates that even though functional, accessible binding sites exist in these mice for GATA, Nkx2.5, and SRF, these factors cannot drive cardiac expression of SRF.

Because GATA, Nkx, and SRF cannot activate cardiac expression of SRF on the promoter, the regulatory sequences governing this expression must lie outside the promoter sequences and 5.5 kb of 5′-flanking sequence. In fact Nelson et al. (28) used 1500 bp of the 5′-flanking SRF sequences and failed to observe expression in the myocardium of developing hearts. They identified an E-box/Ets containing 270-bp cis-acting module in the SRF promoter that mediates expression in the epicardial organ in which derivatives give rise to the coronary vasculature but did not express in the epicardium. A search of the SRF gene for transcription factor binding sites revealed an area of the 3′-UTR that contains many sites for T-box proteins. Tbx factors have been well established to play an important role cardiac development. Mutant mice lacking Tbx5 have been shown to exhibit Holt-Oram syndrome (9). In addition, Tbx5 antagonism with a hormone-inducible dominant negative form of the protein blocks heart development altogether (29). It has also been shown that temporally persistent ventricular expression of Tbx5 inhibits normal chamber formation (30). Tbx2 has been shown to cooperate with Nkx2.5 (31), which in turn binds Tbx5 (14). Involvement of T-box genes in heart development and the discovery of T-boxes in the 3′-UTR of SRF suggest that Tbx proteins may regulate cardiac expression of SRF.

We first performed in situ hybridization analysis in chick embryos to determine whether Tbx factors are expressed temporally and spatially with SRF. We have demonstrated that indeed Tbx2 and -5, but not Tbx3, are co-expressed with SRF in the cardiac crescent. That SRF is expressed outside areas of the embryo where Tbx2 and -5 are co-expressed (outside the cardiac crescent) is consistent with the fact that the mechanism of cardiac regulation of SRF is unique. That transgenic mice lack the 3′-UTR, where SRF is expressed only in non-cardiac cells, supports this conclusion. Tbx3 is expressed in the tail and head-fold and is not involved in the cardiac component of the expression pattern. This is consistent with the fact that Tbx3 mutant mice result in Ulnar-Mammary syndrome, which shows no cardiac defects (32). It is interesting to consider, in light of the co-expression of Tbx5 and SRF, that cardiac defects observed in Holt-Oram embryos may be caused at least in part by the lack of SRF expression. To address this possibility, we performed in situ hybridization analysis for SRF on Tbx5-mutant mice. We have shown in this study that SRF expression, while not eliminated, is indeed reduced in these mice. Remaining SRF transcripts could be a result of redundancy of Tbx2 activity, or the fact that regulatory actions of Tbx factors on SRF expression in the heart are at least in part translational in nature. In addition, the embryonic expression of SRF-β-galactosidase in the tail mesenchyme is reminiscent of the embryonic pattern of expression of Tbx6 in somite precursor cells that is involved in the specification of paraxial mesoderm. Chapman and Papaioannou (33, 34) have shown that mutated Tbx6 blocks the differentiation of paraxial mesoderm and the formation of posterior somites, but allowed for differentiation.
The histone acetyltransferase activity of TIP60 and association with Tbx5 are essential for activation of SRF. A, activation of the SRF promoter in murine SRF\(^{-/-}\) ES cells requires both the presence of the T-box-containing SRF 3'-UTR sequences (1.5 kb, shown in Fig. 5), as well as TIP60. B, brachyury (T), which plays a crucial role in mesoderm specification, can replace Tbx2, but not Tbx5, in SRF 3'-UTR activation. This suggests that T and Tbx5 can hetero-dimerize on the 3'-UTR to activate SRF during mesoderm specification. C and D, further assays with mutant forms of TIP60, with a mutated HAT domain (TIP\(_{mHAT}\)) or ablated zinc finger domain (TIP60\(_{\Delta Zn}\)), demonstrate that TIP60 HAT activity and the interaction domain for Tbx5 are absolutely required for SRF promoter-3'-UTR-dependent activation.
along a neural pathway. Their study indicates that Tbx6 is needed for cells to choose between a mesodermal and a neuronal differentiation pathway during gastrulation. The Tbx5-null mouse embryo did not reveal reduced expression of SRF in the tail. Thus, it is highly likely that SRF may be a Tbx6 target that might be essential for the specification of posterior paraxial mesoderm.

To examine further Tbx regulation of SRF in the heart, we constructed transgenes as described above, and linked the Tbx 3′-UTR region to them. We show here that the promoter sequences, with the 3′-UTR DNA, can robustly direct cardiac expression of SRF in mouse embryos. Because the T-boxes in the UTR are arranged in two “clusters,” we asked if the first of these clusters (contained within the first 5′ 231 bp) was sufficient for this expression. Constructs containing 541 bp of the SRF promoter linked to the 231-bp first T-box cluster were indeed capable of driving SRF expression in the heart. Older embryos with these DNA constructs show a more restricted pattern of SRF expression in cardiac tissue, which is strikingly similar to Tbx2 expression. It is intriguing to postulate that, early in development, Tbx2/5 heterodimers drive SRF cardiac expression, but that later Tbx2 is primarily responsible. Because SRF has been shown to auto-regulate itself through the promoter (12) once initiated, it is more likely that Tbx2 initiates new areas of SRF expression later in development. Expression in older mice is also seen in the pharyngeal arches, which is interesting, because it suggests that SRF is expressed in neural crest cells, known to express Tbx1 (33). internal ribosomal entry site mice containing the first intron of SRF (where several more T-boxes were discovered) show expression in the out-flow tract of the heart, suggesting neural crest regulation also, but not in the heart itself. These data clearly suggest that regulation of SRF in the heart is controlled by the Tbx sequences in the 3′-UTR. To strengthen this point, a transgenic mouse was constructed using the same 231 bp of UTR sequence, with all T-box sites “knocked-out.” We have shown here that this segment of DNA fails to drive LacZ expression in transgenic mice, demonstrating that functional T-box sites are required for cardiac expression. A summary of all transgenic mouse findings, in the form of the location of each tissue-specific enhancer, is shown in Fig. 9.

To investigate the function of T-box proteins on the UTR enhancer, we designed transfection experiments using various combinations of Tbx and cardiogenic factors. Early experiments in CV1 cells showed a high degree of squelching of luciferase reporter activity due to the high levels of SRF expressed in these cells. Essentially, SRF activation by SRF through the promoter sequences was masking the role of the 3′-UTR enhancer. As an alternative approach, experiments were performed using SRF−/− ES cells, which lack expression of functional SRF protein. These experiments demonstrate that Tbx2, Tbx5, and a HAT protein (TIP60) can strongly induce SRF expression in these cells. All of these effects were seen only in the presence of the 3′-UTR sequences. Results from Habets et al. (31) appear in contradiction to these findings, because they demonstrate that Tbx2, along with Nkx2.5, represses expression of ANF. However, this is only an apparent contradiction as demonstrated by two findings. First, ANF activation/repression occurs by competition between Tbx2 and Tbx5 for a single half site in the promoter, working with Nkx2.5. Paxton et al. (35) have concluded that, although the “T-box” of Tbx2 represses expression in the presence of a single half site (consistent with Ref. 31), it activates promoter activity of a reporter gene containing five multimerized cis-acting elements. In the case of SRF, there are many cis-acting T-boxes contained within the UTR sequences, consistent with activation by Tbx2. Second, Paxton et al. (35) also show that Tbx2 contains 5′ and 3′ repressor domains. These data, along with the finding of Hiroi et al. (14) that physical interaction occurs between Tbx factors and Nkx2.5, suggest that Tbx2 can repress the activity of Nkx2.5, thus accounting for the repression activity of Tbx2 on ANF. Indeed, Hiroi et al. (14) show that Tbx5 (which lacks the repression domain) and Nkx2.5 activate Nppa in combination. Thus, although Tbx2/Nkx2.5 heterodimers may repress activity through Tbx repression of Nkx signaling, Tbx2/Tbx5 heterodimers, which may form on the 3′-UTR of the SRF gene, may be mutually positive regulators. Our data strongly suggest that TIP60 is an essential element in the activation of SRF in the heart as well, and the inclusion of this factor may also serve to negate the repressive activities of Tbx2. Expression of SRF through the 3′-UTR enhancer is located in the tail region as well as the heart. This area of expression suggests regulation of SRF during a much earlier developmental time point, perhaps during mesoderm specification. A prominent T-box factor that is expressed at this time in emerging mesoderm is brachyury (T). Thus, T was tested in transfection as well and was found to be able to substitute for Tbx2, but not Tbx5. Co-transfected cells with T and Tbx5 showed a 6-fold higher induction than cells with T and Tbx2, suggesting that Tbx2 may inhibit T, but not Tbx5 activation, or that Tbx2 has no synergy with T. The ability of Tbx5 and T to activate SRF through the 3′-UTR enhancer is developmentally relevant, because both of these T-box factors are expressed during mesoderm specification. In investigating the function of T-box proteins on the UTR further, we examined protein-protein and protein-DNA interaction. As shown in Fig. 7 (B and C), Tbx5 interacts strongly with Tbx2, but only weakly with Tbx3, and Tbx5 was found to bind to TIP60 through the zinc-finger-like domain. In addition, Fig. 5B demonstrates that Tbx5 can bind to several T-boxes located in the 3′-UTR. Our data strongly support the hypothesis that Tbx2, Tbx5, and TIP60 are the primary components driving cardiac expression of SRF, through interaction on the T-boxes in the 3′-UTR of the SRF gene.

Previous studies addressing the role of MYST family members in steroid receptor activated transcription have indicated that, although TIP60 may activate the function of steroid-responsive genes (36), the related human MYST protein HBO1 functions as a transcriptional repressor (37). It was
recently reported that TIP60 resides in a chromatin-remodeling complex involved in DNA repair and apoptosis (21). Most important, however, was the identification of the zebrafish mutant lieberskummer (lik), which inhibits an ATPase complex involved with cell autonomous proliferation of cardiomyocytes (38). lik is related to Reptin, a DNA helicase, found in all organisms as a part of the TIP60 complex (24). Our data strongly suggest that TIP60 is a primary component driving cardiac expression of SRF. To test this hypothesis further, we constructed mutant TIP60 proteins, one of which contained a mutation in its HAT domain (TIP60mHAT) and the other, which lacked the zinc finger domain (TIP60ΔZn; this was constructed to disrupt TIP-Tbx5 binding). Transfection experiments using these constructs (Fig. 8, C and D) show that not only are these mutants incapable of driving luciferase activity when substituted for TIP60WT (with Tbx proteins), but they also repress activity in a dose-dependent manner when co-transfected into these complexes with TIP60WT. The function of TIP60 in regulating SRF may involve chromatin remodeling, which may overlap with the entire SRF gene transcriptional locus and be cell type-dependent as a function of local Tbx gene activity; thus it plays a key role in activating SRF transcription during embryogenesis.

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Serum Response Factor, an Enriched Cardiac Mesoderm Obligatory Factor, Is a Downstream Gene Target for Tbx Genes
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