Conserved Enhancer in the Serum Response Factor Promoter Controls Expression During Early Coronary Vasculogenesis

Timothy J. Nelson, Stephen A. Duncan, Ravi P. Misra

Abstract—Serum response factor (SRF) is a transcription factor required for mesoderm formation in the developing mouse embryo that is important for myogenic differentiation, including notably, the differentiation of the proepicardial organ (PEO) into coronary vascular cells during early development. To identify regulatory sequences that control SRF expression during early mouse development, we used a novel transgenic approach to study the role of conserved noncoding DNA sequences (CNCS) in the SRF gene. Embryonic stem (ES) cells containing a targeted single-copy of putative SRF regulatory sequences were used to directly generate transgenic embryos by tetraploid aggregation. Because the ES cell–derived targeted embryos are genetically equivalent, except for the putative regulatory sequence of interest, differences in transgene expression can be attributed directly to these sequences. Using this approach, we identified an E-box/Ets containing 270-bp cis-acting module in the SRF promoter that mediates expression in the PEO. Reporter transgenes containing this module express in derivatives of the PEO that give rise to the coronary vasculature, but do not express in the PEO-derived epicardium. These results are the first reported in vivo analysis of SRF regulatory elements that control expression during early development. Using this reporter module and this approach, it should be possible to begin to elucidate molecular mechanisms involved in the differentiation of coronary vasculature progenitor cells, as well as identify additional SRF regulatory elements important during mammalian development. (Circ Res. 2004;94:1059-1066.)

Key Words: serum response factor • gene targeting • transcriptional regulation • proepicardial organ • coronary vascular development

Serum response factor (SRF), a member of the MADS (MCM1, Agamous and Deficiens, SRF) box family of transcription factors, is essential for regulation of early embryonic development. SRF is tissue-specifically enriched in cardiac, skeletal, and smooth muscle progenitor cells during embryogenesis, as well as in terminally differentiated adult muscle cells. SRF plays a key role in the differentiation of a variety of cell types including mesoderm cells formed during gastrulation, skeletal myoblasts, and smooth muscle progenitor cells (see review). Various lines of evidence indicate that the role of SRF in cellular differentiation and development is dependent on proper spatial and temporal expression of the SRF gene.

Notably, the induction of SRF is pivotal in regulating the development of the coronary vasculature from progenitor cells derived from the proepicardial organ (PEO). The PEO is a transient “grape-like” embryonic structure found at stage 16 to 17 in the chick and 9.5 days postcoitum (dpc) in the mouse that consists of mesothelial and mesenchymal cells overlying the septum transversum. Cells within the PEO make contact with the inferior surface of the developing myocardium, migrate over the surface of the heart forming the epicardium, and penetrate into the myocardium at the junction between the atrium and ventricle (AV junction) to give rise to subepicardial mesenchymal cells (SEMCs) (see reviews). SEMC are important for heart development and give rise to the entire coronary vasculature consisting of fibroblasts, endothelial, and smooth muscle cells. The PEO and epicardial-derived subepicardial mesenchymal cells first give rise to endothelial tubes within the myocardium that subsequently recruit additional SEMCs to differentiate into smooth muscle cells around the endothelial tubes. Landerholm et al demonstrated that induction of SRF in explanted PEO cells is required for the differentiation of smooth muscle cells, and that overexpression of SRF is sufficient to induce the expression of smooth muscle markers. SRF expression may contribute to the specification and differentiation of mesenchymal cells in vivo. SEMCs strongly express SRF as they form the coronary vasculature whereas the PEO-derived epicardium does not express SRF, suggesting that induction...
of SRF is important as PEO-derived epicardial cells differentiate to coronary vascular cells. To identify elements that control SRF gene expression, we examined the regulatory function of conserved noncoding sequences (CNCS) in developing mouse embryos. Because mutational analysis of CNCS can result in subtle but important changes in gene expression, it was critical to use a transgenic system that minimized spurious variations in expression. We therefore used a transgenic approach in which targeting of putative regulatory sequences to the hypoxanthine phosphoribosyl transferase (HPRT) locus of embryonic stem (ES) cells14,15 was coupled with tetraploid embryo aggregation to generate completely ES cell–derived embryos from online data supplement. Completely ES cell–derived embryos were efficiently produced, thus allowing direct comparison of equivalent, differing only in the CNCS being examined, are efficiently produced for each reporter construct. Embryos containing different reporter constructs that are genetically equivalent, differing only in the CNCS being examined, are also efficiently produced, thus allowing direct comparison of regulatory sequences and identification of subtle changes in expression.

The reproducibility and sensitivity of this approach allowed us to identify a previously unknown SRF regulatory module that confers expression of a β-galactosidase reporter gene to PEO cells at 9.5 dpc, cells within the AV junction by 10.5 dpc, and early endothelium of the coronary vasculature in the developing mouse embryo. Mutational analysis reveals that a single E-box/Ets protein-binding site is necessary, but not sufficient, for the function of this module. Identification of this PEO-specific regulatory module is a first step in understanding SRF-dependent signaling pathways as well as characterizing additional elements and molecular mechanisms responsible for de novo coronary vasculogenesis during mammalian development.

Materials and Methods

Plasmids

HPRT targeting vectors were generated from the parent plasmid, pMP8NEBlacZ.16 Sequences from −1500 to +200 bp or −322 to +200 bp of the SRF promoter were subcloned from previously reported luciferase vectors17 and an expression cassette from pnlacF20. The 270-bp CNCS module between positions −726 and −456 was PCR amplified and placed upstream of the 322 SRF construct. Truncations of the 270-bp CNCS module, termed distal 127, middle 199, and proximal 160 were cloned into the 322 SRF context by PCR amplified and placed upstream of the 322 SRF construct. Adjacent E-box and Ets protein-binding sites were located within 1500 bp upstream of the transcriptional start site of the SRF gene. We focused on this limited region in the proximal promoter based on the reasoning that these sequences were likely to contain regulatory functional significance. As illustrated in Figure 1A, the two regions labeled b and c were located within 1500 bp upstream of the transcriptional start site of the SRF gene. We focused our initial in vivo studies on these regions and generated two reporter constructs that either contained one or both of these CNCS labeled −322 SRF and −1500 SRF (Figure 1A).

Generation of Single-Copy Transgenic Mouse Embryos for In Vivo Promoter Analysis

To investigate the function of these CNCS during mammalian development, we generated genetically equivalent transgenic mouse embryos. LacZ reporters driven by putative SRF regulatory sequences were introduced as a single-copy to the X-linked HPRT locus, as originally described in Bronson et al.,14 using an HPRT-negative ES cell line described in Misra et al. Figure 1B shows the F3 HPRT-deficient ES cell line, the rescuing targeting vector used to introduce the SRF promoter constructs, and the recombinant F3 locus containing the functional HPRT gene with the transgene of interest. The production of recombinant ES cells is greatly simplified by the powerful selection criteria afforded by the newly acquired functional HPRT gene, which allows cells to become resistant to HAT media. These ES cells are then used to directly produce embryos by tetraploid embryo aggregation16 as summarized in Figure 1C. This results in completely ES cell–derived transgenic embryos in only 2 weeks and avoids the need for breeding. Because the transgenes are integrated processed using Photoshop 7.0. Stained embryos were paraffin-embedded and sectioned following standard protocols.

Immunofluorescence

Staged CD-1 mouse embryos were dissected in ice-cold PBS, fixed in 4% paraformaldehyde for 20 minutes at room, and frozen in Tissue-Tek OCT media. Cryosections were cut at 7 μm and stained with primary and secondary antibodies in following dilutions: 1:200 rabbit anti-SRF (from R. Prywes, Columbia University, New York), 1:300 Wilms’ tumor suppressor (Wt-1 or C-19, Santa Cruz), 1:500 MF-20 (Developmental Studies Hybridoma Bank, NICHD), 1:100 PECAM (Pharmagen), 1:500 goat anti-rabbit Alexa Fluor 488, goat anti-mouse Alexa Fluor 568, and goat anti-rat Alexa Fluor 568 secondary antibodies (Molecular Probes), 1:1000 Hoechst stain (Sigma).

In Situ Hybridization

SRF 3’ UTR sequences (9199 to 10217 nt of the mouse genomic SRF sequence, accession No. AB038376), were PCR-amplified, cloned into pGEM T-easy vector (Promega), and used to produce a 32P-labeled anti-sense RNA probe for in situ hybridization. CD-1 mouse embryos were harvested and stained for lacZ activity.

Results

Identification of Conserved Noncoding Sequences in the SRF Promoter

The AVID/VISTA online search engines19–21 identified putative regulatory sequences in the SRF gene that are conserved between mouse and human. Alignment between mouse and human highlighted multiple conserved sequences (see online data supplement), but only two CNCS in the SRF proximal promoter region. We focused on this limited region in the proximal promoter based on the reasoning that these sequences were likely to contain regulatory functional significance. As illustrated in Figure 1A, the two regions labeled b and c were located within 1500 bp upstream of the transcriptional start site of the SRF gene. We focused our initial in vivo studies on these regions and generated two reporter constructs that either contained one or both of these CNCS labeled −322 SRF and −1500 SRF (Figure 1A).
Conserved Sequences Between −1500 and −322 of the SRF Promoter Direct Tissue-Specific Expression in the Mouse

Transgenic embryos containing either −322 SRF or −1500 SRF promoter constructs reproducibly displayed unique expression patterns (Figure 2). Figure 2A illustrates the orientation of the embryonic outflow tract, ventricle, and atrium relative to the PEO from three different views. Embryos derived from nontargeted, parental F3 ES cells display no detectable lacZ activity even after 2 weeks in X-gal stain (data not shown). In contrast, the outflow tract (OFT) expresses the lacZ reporter from both the −322 SRF and −1500 SRF promoters at 9.5 and 10.5 dpc (Figures 2B and 2C). Although, the −322 reporter construct only expresses in the OFT, the −1500 SRF reporter construct is also expressed in cells adjacent to the caudal surface of the developing ventricle (Figures 2B and 2C). The lacZ-positive cells from the −1500 SRF construct at 10.5 dpc appear concentrated in the AV junction (Figure 2C). The apparent location of these lacZ-positive cells in the embryos containing the −1500 SRF construct is consistent with their localization in the PEO. The expression pattern was consistent for all embryos examined at 9.5 dpc (Table).

To more carefully characterize lacZ-positive expression from the −1500 SRF promoter, 9.5-, 10.5-, and 14.5-dpc embryos were stained as whole embryos and sectioned (Figure 3). Expression of lacZ at 9.5 dpc was detected in a small number of cells in the PEO as well as cells in the AV junction (Figures 3A and 3B). By 10.5 dpc, lacZ-positive cells were detected throughout the migrating PEO as it extended over the inferior surface of the ventricles (Figure 3D). At this stage, there was also an enrichment of lacZ-positive cells within the heart at the AV junction (Figure 3C). By 14.5 dpc, lacZ-positive cells were detected within the SEMC layer as well as nascent vessels that were forming in this layer (Figures 3E through 3G). Interestingly, lacZ expression was never found in the epicardium, the single cell layer overlaying the myocardium by 10.5 dpc.

Highly Conserved 270-bp E-box/Ets–Dependent Module in the SRF Gene Confers PEO-Specific Expression at 9.5 dpc

The expression pattern difference between the −322 SRF and −1500 SRF reporter constructs may be due to the presence of the conserved sequence in region b shown in Figure 1. To examine this possibility, the 270-bp region b was cloned into the −322 SRF construct to generate the −322 SRF reporter construct along with a series of region b truncations depicted in Figure 4. Specifically, the distal 127 bp, middle 199 bp, or the proximal 160 bp of the 270-bp fragment were placed upstream of the −322 SRF construct to generate the D127+322 SRF, M199+322 SRF, and P160+322 SRF reporter constructs, respectively. Examination of the transcription factor binding sites within region b revealed that all three species contained an E-box binding site. Thus a 270-bp...
module without the E-box, and containing nucleotides that confer no recognizable binding sites in place of the E-box, termed \(H9004\) E270 \(H11001\) 322 SRF reporter, was generated. As shown in Figure 5, the 270\(H11001\)322 SRF construct is expressed in the PEO and OFT similar to the larger \(H11002\)1500 SRF construct. Truncations of this 270-bp module were not able to direct PEO expression. This indicated that the 270-bp module was responsible for directing PEO expression. In addition, when the E-box/Ets binding site within the 270-bp PEO-specific module was ablated, within the context of an otherwise intact 270-bp module, no lacZ activity was observed in the PEO despite OFT expression. Because all constructs contained the \(-322\) SRF reporter, which is sufficient for OFT expression, the OFT expression pattern served as a convenient internal control to ensure proper staining of transgenic embryos. Taken together, this data indicates that PEO-specific expression requires both the E-box/Ets binding site, as well as additional conserved sequences within the 270-bp module for tissue-specific expression.

Endogenous SRF Is Expressed in the PEO but Is Absent in the Epicardium

Cells from the PEO give rise to the coronary vasculature as well as the epicardium. Studies using quail PEO cells demonstrate that SRF expression is necessary for the differentiation of PEO-derived coronary vasculature smooth muscle progenitor cells, but that SRF is not expressed in the epicardium.\(^9\) Cells within the epicardium, however, are able to undergo an epithelial-to-mesenchymal transformation (EMT) to generate SEMC that contribute to cell types of the coronary vasculature\(^22\)–\(^25\) that subsequently do express SRF. Together, these studies indicate that in avian systems SRF is induced as progenitor cells differentiate into the coronary vasculature from derivatives of the PEO. To more carefully characterize the expression of SRF during early mouse coronary vasculogenesis, we examined the location and embryonic stage of PEO-derived progenitors cells in 9.5- to 14.5-dpc mouse embryos for endogenous SRF expression. Figure 6 indicates that the endogenous SRF gene is transcribed in the 9.5-dpc mouse myocardium as previously described,\(^3\) but also shows that SRF is transcribed in the 9.5-dpc mouse myocardium but is barely detected in the PEO. However, a day later at 10.5 dpc, SRF protein is detected in the expanding PEO tissue. The conserved sequences identified in Figure 1 are not able to fully recapitulate expression of endogenous SRF. However, the conserved SRF sequences identified in this study direct lacZ expression in the PEO at a similar developmental stage as expression of the endogenous SRF gene. Interestingly, similar to the pattern seen for the \(-1500\) SRF lacZ reporter, endogenous SRF protein is not detected in the mouse epicardium at any stage of development, even though these cells are derived from the PEO.

Wt-1 Identifies Early PEO and PECAM Identifies Nascent Coronary Endothelium During Coronary Vasculogenesis

Lineage analysis of PEO-derived cells into the coronary vasculature has been extensively performed using chick:quail chimeric embryos. However, investigation of the PEO during mammalian vasculogenesis has been limited by the lack of markers that effectively identify PEO derivatives as they develop into the SEMC and nascent coronary vessels. Wilms’ tumor (Wt-1) expression has been used in mouse\(^26\) and
avian embryos. It has been shown that Wt-1 marks PEO cells, in addition to many other cells that involve an epithelial to mesenchymal transition, although it is not a persistent marker for PEO-derived vascular cells. This is exemplified in Figure 7, which shows the majority of cells within the mouse PEO at 9.5 dpc are Wt-1 positive. By 10.5 dpc, these cells expand and migrate to begin covering the myocardium as they form the primitive epicardium. However by 14.5 dpc, Wt-1-positive cells from the PEO are only detected in the single cell layer of the epicardium even though many PEO-derived cells have migrated into the myocardium to form the SEMC layer and have begun to form the coronary vasculature by this stage of development. Shown in Figure 7, PECAM expression in the nascent coronary vasculature is evident by 14.5 dpc within the myocardium. At 9.5 dpc of mouse development, however, PECAM expression is not present in the PEO but is strongly detected in other tissues such as the endocardium. There is significant cellular differentiation occurring during coronary vasculogenesis between the time of Wt-1 expression in PEO cells at 9.5 dpc and PECAM expression in nascent coronary vessels at 14.5 dpc. The expression of the −1500 SRF construct is notable within this timeframe of coronary vasculogenesis in that lacZ expression is detected in a small population of PEO cells at 9.5 dpc, in an enriched population of cells within the AV junction at 10.5 dpc, and in SEMC and nascent coronary vessels by 14.5 dpc, despite not being detected in the PEO-derived epicardium.

**Discussion**

Coronary smooth muscle differentiation has been extensively studied in chicken and quail model systems where progenitor cells originate from the PEO and differentiate into smooth muscle cells, fibroblasts, and endothelial cells (see reviews10,12). Using quail PEO cells, Landerholm et al9 have shown that SRF is induced before, and required for, the differentiation of PEO cells to smooth muscle cells. Using a transgenic approach coupling HPRT-targeted ES cells and tetraploid aggregation to analyze the role of CNCS in the SRF gene during early mouse development, we have identified a 270-bp, E-box/Ets-dependent module that confers PEO-specific expression to the SRF promoter. To our knowledge, this is the first study to identify and demonstrate in vivo function of regulatory DNA sequences from the SRF gene, and the first study to identify sequences of any kind that direct an expression pattern characteristic of coronary vasculogenesis. This study also shows for the first time that develop-
mental control of SRF gene expression is regulated by modular elements controlling expression in the OFT and coronary vasculature, similar to that seen for other genes such as Nkx-2.5 during heart development.28 Because endogenous SRF is expressed during embryogenesis more extensively than the reporter constructs, there must be additional regulatory sequences required to fully recapitulate the in vivo expression pattern. Consistent with this idea, the analysis presented in Figure 1 has identified additional conserved elements the in mouse and human gene that are likely to regulate further expression of the SRF gene. Further characterization of other regulatory sequences in transgenic embryos will likely provide significant insight into signaling mechanisms and the ability to identify novel, in vivo cell populations based on transcriptional networks.

Our data indicates that the 270-bp module that mediates PEO-specific expression of the transgene requires an E-box/Ets binding site, suggesting that helix-loop-helix (HLH) and the Ets family transcription factors are important for its action. Consistent with this possibility, it has recently been shown that Ets-1 and Ets-2 are necessary for the development of chicken SEMC and coronary vasculature.29 Moreover, bHLH proteins such as dHand and epicardin are expressed in the mouse PEO at 9.5 dpc30,31 and could be involved in regulating SRF expression in the differentiating PEO. Whether these transcription factors or other family members are responsible for regulation of PEO-specific expression during mouse coronary vasculogenesis remains to be investigated. Because the expression pattern of these potential regulatory transcription factors is more ubiquitous than the expression pattern of the SRF/LacZ constructs used in this study, other possibilities such as control by novel transcrip-

Figure 5. A 270-bp module mediates PEO-specific expression through an E-box/Ets binding site-dependent mechanism. The 270-bp module containing the highly conserved SRF sequence is sufficient to confer PEO-specific expression to the −322 SRF reporter while maintaining OFT expression. Truncation mutants D127, M199, and P160 of the 270-bp module placed in the context of −322 SRF, as well as point mutations in the E-box/Ets binding sites are unable to confer PEO expression (dashed line), whereas all constructs retained OFT expression.

Figure 6. Endogenous SRF is expressed in the myocardium and PEO but absent from the epicardium. A, 9.5-dpc embryo sagittally sectioned and H&E-stained shows the relationship between the ventricle (V), atrium (A), and PEO in the mouse embryo. B, Sagittal section of a 9.5-dpc mouse embryo after in situ hybridization using 35S-labeled antisense SRF RNA. SRF expression is enriched in the myocardium and PEO. C through E, Expression of endogenous SRF protein in the heart region of 9.5- to 14.5-dpc mouse embryos. Anti-SRF (green), myocardium marked with MF-20 (red), and counterstained with DAPI to mark nuclei (blue). C, 9.5-dpc mouse embryo shows the myocardium strongly expressing SRF protein with little SRF expression in the PEO. D, 10.5-dpc mouse embryo shows continued expression of SRF in the myocardium with significant increase in SRF expression in the PEO. E, 14.5-dpc mouse embryo shows continued expression of SRF in the myocardium but no expression detected in the epicardium (inset).
A significant issue raised by this study is whether expression of the 270-bp PEO-module containing lacZ reporters is confined to a specific cell population within the PEO. At this time, direct demonstration that lacZ-positive PEO cells are contributing to specific cell types has not been possible due to low levels of transgene expression in our current studies. However, the observation that this PEO-specific module mediates expression in a unique spatial and temporal pattern consistent with the differentiation of SEMC and nascent coronary vessels is consistent with the idea that this module is marking coronary vasculature progenitor cells in the mouse PEO. It has been previously shown that early SEMCs in the AV junction can arise from direct migration of PEO cells, and that epicardial-derived SEMCs congregate within the AV region. Based on these published observations, it is tempting to speculate that the subpopulation of cells at 9.5 dpc that are marked by the PEO-specific SRF module are recruited to the AV junction and contribute to SEMCs and subsequent nascent coronary vessels, rather than being destined to the epicardium like other PEO cells. Consistent with this possibility, Mikawa and coworkers have proposed a model in which the PEO consists of a chimeric population of cells destined to become distinct cell types of the coronary vasculature prior to migration out of the PEO. To resolve this issue it will be necessary to perform careful and definitive lineage studies that allow migration of PEO cells to be followed during early coronary vasculogenesis. This type of experiment may be possible using a chick:mouse chimeric model using genetically labeled mouse PEO cells in wild-type chick embryos as previously used to study migrating neural tissue.

In summary, this study has identified regulatory sequences in the SRF gene that direct a unique expression pattern during coronary vasculogenesis. By using a novel rapid and sensitive approach for in vivo analysis of regulatory DNA sequences in which target site integration is coupled with tetraploid aggregation, we have characterized CNCS in the proximal SRF promoter. This in vivo promoter analysis approach only requires 35 to 40 days to produce 9.5-dpc hemizygous transgenic mouse embryos containing a DNA sequence of interest. The 270-bp module identified in this study can confer expression to a reporter in PEO cells at 9.5 dpc, AV junction at 10.5 dpc, SEMCs at 14.5 dpc, and nascent coronary vessels at 14.5 dpc. Expression is limited to a subset of PEO cells, and is absent in the PEO-derived epicardium, supporting the possibility that this module marks cells destined for the coronary vasculature. This module requires an E-box/Ets binding site that likely functions in conjunction with other highly conserved sequences. This PEO-specific module should be useful in gaining further insight into the molecular mechanisms of gene expression underlying differentiation of the PEO and mammalian coronary vasculogenesis, and the novel strategy used here should facilitate identification of other regulatory elements important during early development.

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Methods:

Plasmids

The 270bp CNCS module with a Stu1 site (–726) at the 5’ end and Nhe1 site (–456) at the 3’ end was PCR amplified with primers starting at positions 1276 and 1622 in the SRF mouse sequence (Accession # AB038376). This module was placed in the forward orientation, 200bp upstream of the 322 SRF promoter to generate the 270+322 SRF targeting vector. Truncations of the 270bp CNCS module, termed Distal 127, Middle 199, and Proximal 160, were PCR amplified (primer sequences available on request) and cloned into the 322 SRF context by replacing the 270bp module. Adjacent E-box and Ets binding sites in the 270bp module as shown in bold GCCGGCAGCTGGAAGTCTT were mutated to GCCTAGCGCTGTAGCTCTT.

HPRT targeting of F3 ES cells

To prepare for targeting, F3 ES cells were split from a 70-80% confluent 100mm plate onto 5 plates that each contained 30%, 25%, 20%, 15%, and 10% of the total cells. The five plates were harvested together after 1-3 days when the first plate became 90% confluent and resuspended at 5x10^7 cells/ml in growth media. Linearized targeting vector (100µg) was mixed with 3.1ml of F3 ES cells and incubated at RT for 15min before electroporation. All cells were then collected in 50ml of ES media and plated onto five-100mm plates. HAT (Sigma) selection was started 2 days later and 10-20 colonies were picked within 10-16 days. Transgenic ES cell lines were genotyped by PCR amplification of the lacZ gene.
Results:

Vista Plot Alignment of human and mouse SRF locus oriented in the 5’ to 3’ direction.

Purple-exons 1-7
Pink-larger CNCS with the highest homology
White-smaller CNCS with moderate homology
Light blue-5’ and 3’ UTR

Proximal CNCS shown in pink in front of the 5’ UTR were included in this study were found to be sufficient to mediate OFT expression. The CNCS upstream from this proximal promoter, depicted in white, were also conserved in *Fugu* (not shown) and found in this study to direct PEO expression. Also shown in this alignment is significant homology between mouse and human sequences within the 2nd intron and the 3’ UTR suggesting these regions may contain additional regulatory sequences.