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Inhibition of the Cardiac α-Actin Gene in Embryonic Cardiac Myocytes by Dominant-Negative Serum Response Factor

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ABSTRACT

Serum response factor (SRF), a transcription factor ubiquitously involved in the processes of cellular proliferation and differentiation, has been implicated in cardiac and skeletal muscle development because of its strong expression in embryonic muscle lineages, and its necessity for the transcription of transiently transfected muscle genes that contain SRF binding sites. This study was designed to ascertain whether SRF is required for the expression of an endogenous SRF-dependent gene during differentiation of early embryonic cardiac myocytes by introducing a dominant-negative SRF construct via retroviral delivery. Although no effect on overt cellular differentiation was detected, semi-quantitative RT-PCR revealed that expression of the SRF-dependent gene cardiac α-actin was inhibited, whereas expression of the non-SRF-dependent genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and cardiac troponin-C was unaffected. No effect on myocyte proliferation was detected. Curiously, immunohistochemical localization of SRF protein suggested that whereas endogenous SRF was homogeneously dispersed throughout the cytoplasm and nucleus, the dominant-negative SRF protein was concentrated in the nucleus. These results extend previous findings using transiently transfected genes to the endogenous level, indicating that SRF is required for the full expression of muscle genes that contain SRF binding sites during cardiac myocyte differentiation. Anat Rec Part A 271A:315–321, 2003.

Key words: cardiac α-actin; glyceraldehyde-3-phosphate dehydrogenase (GAPDH); immunohistochemistry; quantitative RT/PCR; serum response factor; troponin C; Northern blotting

Studies have shown that endoderm cells in the heart-forming region of the embryo are necessary and sufficient to sustain terminal cardiogenesis in adjacent precardiac mesoderm (for review, see Lough and Sugi, 2000). The characterization of medium conditioned by explanted cultured endoderm has revealed the presence of carrier proteins and specific growth factors among the secretory products of these cells (Lough and Sugi, 2000). Work in this laboratory has shown that among the secreted growth factors, the fibroblast growth factors (FGFs)—alone in defined medium—can replace the cardiogenic efficacy of whole endoderm cells on precardiac mesoderm (Sugi and Lough, 1995; Zhu et al., 1996). Later, upon appearance of the definitive heart tube, the myocardium itself begins to

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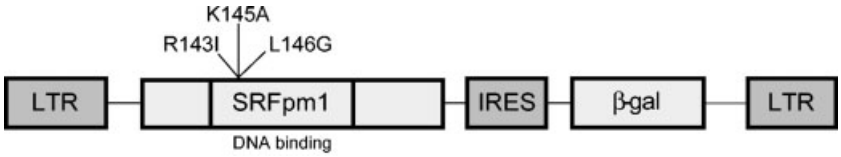

Fig. 1. Diagram of the pSRFpm1Z cassette that was inserted into plasmid pCXIZ for use in these experiments.

manufacture FGF (Parlow et al., 1991), which is used in autocrine fashion to regulate its continued differentiation and proliferation (Sugi et al., 1993; Mima et al., 1995).

Among molecules that may function in the myocardial signal transduction pathway initiated by FGF, serum response factor (SRF) (for reviews, see Shore and Sharrocks, 1995; Treisman, 1996) becomes strongly expressed in the avian myocardium at stage 10 (Croissant et al., 1996). SRF, which has been shown to mediate FGF signaling in other cells (Parker et al., 1992; Moss et al., 1994), is a 67 kDa transcription factor that activates or co-activates a variety of genes, such as c-fos (Treisman, 1985), in transient transfection assays, and muscle-specific genes, including SM22α (Li et al., 1997), atrial natruiretic factor (ANF) (Morin et al., 2001), and the muscle α-actin genes (Shore and Sharrocks, 1995). To activate transcription, SRFs must form dimers and bind to a promoter sequence [$CC(A/T)_6GG$ = CArG box] in SRF-dependent genes (termed the serum response element (SRE)).

The requirement for SRF to dimerize in order to activate transcription has been exploited in loss-of-function experiments utilizing a dominant-negative SRF construct (termed SRFpm1) that contains point mutations in SRF's DNA binding domain. SRF/SRFpm1 dimers cannot bind SREs in target promoters, thereby preventing transcription of SRF-dependent genes. For example, transfection of SRFpm1 into differentiating skeletal myotube cells blocks transcription of a cotransfected luciferase reporter gene that is regulated by the SRE-containing skeletal α-actin promoter (Croissant et al., 1996).

Because SRF, which is strongly expressed in the definitive heart, regulates transfected SRE-dependent promoters, it was reasonable to infer that endogenous SRE-dependent genes would be affected by disrupted SRF signaling during the cardiac myocyte development. To examine this possibility, we determined the effect of SRFpm1 (packaged in a bicistronic retroviral construct with a lacZ reporter gene) on the expression of endogenous cardiac α-actin, a gene that is increasingly and strongly expressed during early stages of cardiac myocyte development. Although cells infected with SRFpm1 exhibited no effects on expression of non-SRF-dependent genes (cardiac troponin-C and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)), the expression of cardiac α-actin was inhibited. These results extend previous findings using transiently transfected genes, indicating that SRF is required for the expression of an endogenous SRF-dependent gene in cardiac myocytes.

MATERIALS AND METHODS

Heart Cell Culture

Hearts were removed from stage 14–17 (Hamburger and Hamilton, 1951) chicken embryos by severing the inflow and outflow tracts. Myocytes were liberated from minced hearts by digestion with 0.05% trypsin/EDTA (Gibco, Invitrogen, Carlsbad, CA) at 37°C for 15 min, followed by resuspension in defined cell culture medium (HCCM-1; HyClone, Logan, UT) supplemented with 0.1% fetal bovine serum (FBS; HyClone) and 0.1% chick embryo extract (CEE) (Lough and Bischoff, 1977). Cells were diluted to 2×10^5 cells/ml, and 500 μl aliquots were placed in 15-mm-diameter fibronectin-coated wells of a 24-well culture plate (Costar, Corning Costar, Acton, MA).

Dominant-Negative SRF Mutant Construct

The dominant-negative SRFpm1 contains point mutations of three amino acids ($R \rightarrow I^{143}$, $K \rightarrow A^{145}$, and $L \rightarrow G^{146}$) in SRF's DNA-binding domain. Although these mutations allow dimer formation between wild-type and mutant SRFs, binding to the SRE of gene promoters is prevented (Johansen and Prywes, 1993). SRFpm1 cDNA was directionally subcloned between the 5′-LTR and the bacterial β-galactosidase (β-gal) reporter gene of pCXIZ, a retroviral vector plasmid derived from a replication-defective variant of spleen necrosis virus (Mikawa and Fischman, 1992) obtained from Dr. Takashi Mikawa of Cornell University. The presence of an internal ribosome entry sequence between the SRFpm1 and β-gal cDNAs enables their simultaneous expression and identification using X-gal histochemistry. The bicistronic construct, termed pSRFpmZ, is depicted in Figure 1.

Stable Transfection of Packaging Cells

The pSRFpmZ plasmid was cotransfected with a neomycin resistance plasmid (pSV-neo) into a canine packaging cell line (D17.2G) using calcium phosphate precipitation. Neomycin-resistant clones were selected with G418 (Gibco) through tertiary screening that was based on 1) the coexpression of β-galactosidase and SRFpm1, and 2) viral titers that exceeded $>10^5$ virions/ml. For controls, a D17.2G line transfected with plasmid pCXIZ containing only the β-galactosidase gene was obtained from Dr. Mikawa. Virus harvested from a conditioned medium of packaging cells was concentrated by ultracentrifugation. Titers were determined by adding serial dilutions of harvested virus to log-phase host cells (D17.2G). After 4 hr incubation the medium was replaced with fresh medium without virus. The cells were incubated for an additional 2 days, and were then evaluated for viral gene expression via X-gal histochemistry.

Infection of Cardiac Myocytes With Retrovirus

After an overnight culture of cardiac myocytes the medium was replaced with 500 μl DMEM/7% FBS medium containing 10^6 virions/ml; polybrene (10 μg/ml; Sigma, St.

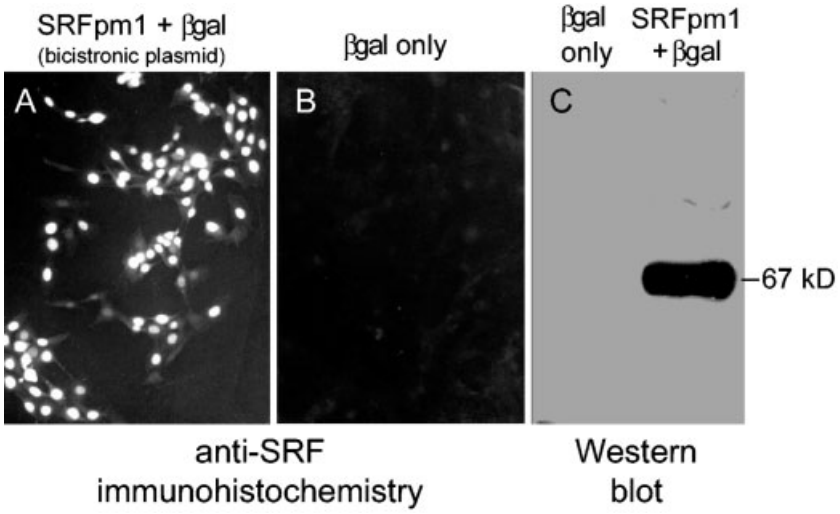

Fig. 2. Immunochemical assessment of SRFpm1 expression in stably transfected (D17.2G) packaging cells. Panels **A** and **B** depict cells that were stained with SRF antiserum after stable transfection with (A) bicistronic plasmid containing dominant-negative SRFpm1 and β-galactosidase cDNAs, or (B) control plasmid containing only β-galactosidase cDNA. **C:** Western blot revealing the presence of a single 67 kD band in cells transfected with the SRFpm1, but not the control (βgal only), plasmid.

Louis, MO) was included to facilitate infection. After 4 hr incubation the virus-containing medium was replaced with HCCM-1/0.1% FBS/ 0.1% CEE, and cell growth was continued for an additional 48 hr.

Immunostaining and Western Blotting

Immunostaining to detect sarcomeric α-actin (Sugi and Lough, 1992, 1994), SRF (Croissant et al., 1996), and BrdU (Sugi et al., 1993) was performed as previously described. Western blotting was performed as described in Sugi et al., 1995. Each antibody's specificity and efficacy is described in these works as well.

Quantitative PCR

Total RNA was purified from heart cell cultures using RNA Stat-60 (Tel-Test, Friendswood, TX), using 10 μg linear polyacrylamide as the carrier. Purified RNA was treated with DNAse I (Roche, Indianapolis, IN) to remove contaminating genomic DNA. Reverse transcription (RT) was performed using oligo-dT as the primer and M-MLV reverse transcriptase (Promega, Madison, WI). Aliquots consisting of one-tenth of the RT product were used as a template for each of the following PCR amplifications, which were performed in parallel. Amplification was catalyzed with Thermus aquaticus (Taq) DNA polymerase (Promega) by denaturing (94°C, 30 sec), annealing (55°C, 30 sec), and extending (72°C, 30 sec) the template in standard reaction buffer, which included 1.0 μCi ^{32}P-dCTP. Sequences of the forward (F) and reverse (R) primers (Operon, Alameda, CA) used to amplify each cDNA, and the size of each amplified product, were as follows: cardiac α-actin (Chang et al., 1985): F, 5′-CGTAAGGATCTGTATGC-3′; R, 5′-TGTCATCATCCTGAGTG-3′; 302 bp (in order to select only the cardiac isoform of α-actin, the reverse primer is complementary to a domain in the 3′-UTR); troponin C (Toyota et al., 1989): F, 5′-ATCTATAAGGCGGCGGTTGA -3′; R, 5′-CAGTGATCGTCTCTCCAGTT-3′; 379 bp); and GAPDH (Panabieres et al., 1984): F, 5′-ACGCCATCACTATCTTCCAG-3′; R, 5′-CAGCCTTCACTACCCTCTTG-3; 578 bp). All reactions were amplified for 25 cycles, during which accumulation of each PCR product remained linear while quantifiable amounts of troponin-C and cardiac α-actin were amplified without generating saturating amounts of GAPDH, which is relatively abundant. Ten microliters of each PCR product were separated on a 4.5% acrylamide gel, which was dried and visualized on a Storm 860 Optical Scanner (Amersham Biosciences, Piscataway, NJ). The relative amount of radioactive PCR product in each band of the dried gel was determined by Image-Quant analysis. This determination was performed six times. Statistical significance was determined by application of Student's *t*-test, with two samples paired for means analysis (two-tail).

RESULTS

Plasmid pSRFpmZ was used to stably transfect the virus packaging cell line D17.2. This bicistronic construct, which coexpresses SRFpm1 and β-galactosidase cDNAs, is schematically shown in Figure 1. Clones of D17.2 were selected for virus propagation based on the criteria described in Materials and Methods. All cells in each selected clone expressed SRFpm1 protein, which was detected only in the nucleus (Fig. 2A). Verification of the immunodetected protein as SRF, as well as indication of its robust expression, was performed by Western blotting (Fig. 2C). Because the packaging cell line does not express (endogenous) SRF, no immunostained protein was detected in cells that were transfected with the control plasmid, which does not contain the SRFpm1 cDNA (Fig. 2B). However, as expected, X-gal histochemistry revealed the presence of β-galactosidase activity in cells that were transfected with either plasmid (not shown).

Cultures of primary cardiac myocytes infected with freshly harvested retrovirus and immunostained 48 hr later to detect SRF protein are shown in Figure 3. Virtu-

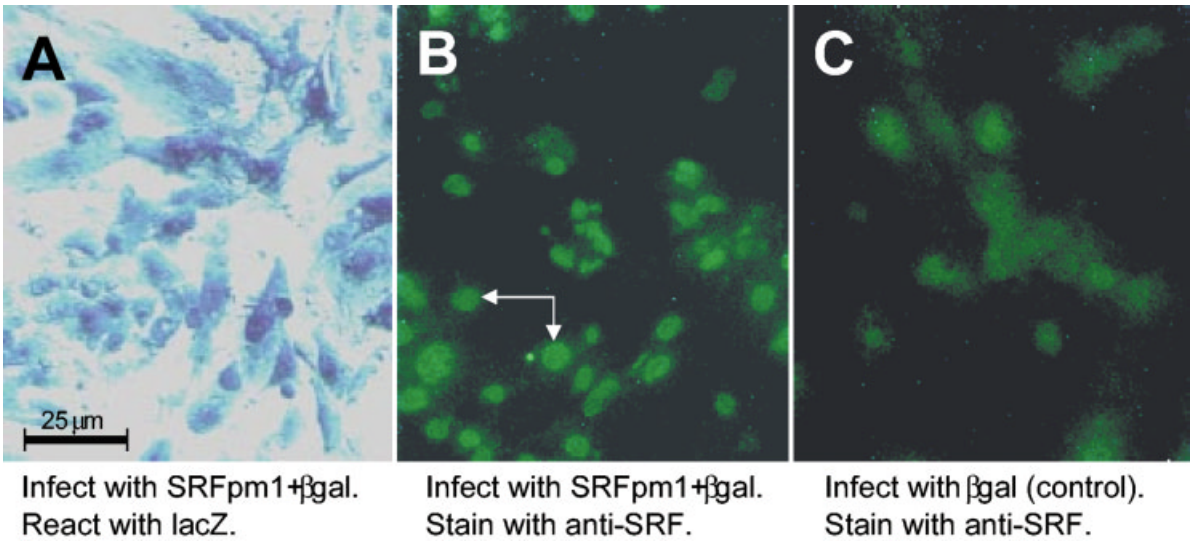

Fig. 3. Expression of SRFpm1 in embryonic cardiac myocytes. Cells from embryonic stage 14 hearts were infected with either (**A** and **B**) bicistronic (SRFpm1 + βgal) or (**C**) control (βgal only) retrovirus. Two days later, cultures were reacted to detect β-galactosidase activity (panel A) or were immunostained to detect SRF protein (panels B and C). Panel C depicts immunostained endogenous SRF, whereas panel B depicts retrovirus-encoded SRFpm1 over a background of endogenous SRF; white arrows in B point to nuclei.

ally all cells (which were essentially devoid of fibroblasts due to the early embryonic stage [Hamburger-Hamilton stage 14] at which the cells were harvested) exhibited evidence of infection based on detection of the blue X-gal reaction product of the β-galactosidase gene (Fig. 3A). Because cardiac myocytes, unlike the packaging cells shown in Figure 2, express endogenous SRF, it was necessary to evaluate the expression of infected SRFpm1 on this background. As revealed by immunostaining with anti-SRF antibody, myocytes that were infected with the control virus exhibited endogenous SRF signal that was homogeneously distributed throughout the cytoplasm and nucleus (Fig. 3C). By contrast, myocytes that were infected with virus containing SRFpm1 cDNA exhibited a preponderance of SRF signal in the nucleus (Fig. 3B), similar to the packaging cells (Fig. 2A). In determinations not shown here, similar effects were obtained using myocytes from stage 17 or 20 embryonic hearts. Moreover, evidence that SRFpm1 did not overtly affect myocyte cytodifferentiation was indicated by the absence of detectable differences in cardiac myocyte contractility: virtually all myocytes, whether infected with control or SRFpm1-containing retrovirus, displayed rhythmic contractions after 2 days. Also, extent of differentiation was estimated by immunostaining sarcomeric α-actin proteins, which revealed no detectable differences between control and SRFpm1-infected cells.

Because SRF has traditionally been considered to be associated with cell proliferation, it was of interest to ascertain whether SRFpm1 altered this process. Figure 4 shows that SRFpm1 did not affect numbers of cultured cardiac myocytes that were able to incorporate 5′-bromodeoxyuridine (BrdU), a parameter indicating numbers of cells in cell-cycle transit.

To assess whether SRFpm1 affected the expression of SRF-dependent genes, such as the cardiac and skeletal isoforms of α-actin, Northern hybridization was utilized as a first approach. As shown in Figure 5, these determinations detected no effect of SRFpm1 on gene expressions, assessed at the level of sensitivity and resolution provided by Northern hybridization. However, multiple assessments via semi-quantitative PCR (Fig. 6), initiated using identical quantities of the RT template for each primer pair and carried out during the phase of amplification when accumulation of all PCR products was linear, revealed that cells infected with SRFpm1 expressed subtly but significantly less amounts of cardiac α-actin than the non-SRF-dependent genes GAPDH and cardiac troponin C, which were relatively unaffected.

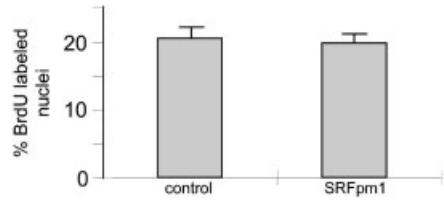

Fig. 4. Effect of SRFpm1 on cardiac myocyte proliferation. Stage 14 heart cells were cultured and infected with either the control β-galactosidase or the bicistronic retroviral vector containing SRFpm1. After 2 days, the cultures were treated with 50 μM BrdU for 2 hr, followed by immunostaining with anti-BrdU to detect the presence of cycling cells, and by counterstaining with propidium iodide to label all nuclei in the cultures. These data were compiled from duplicate cultures in two separate determinations. At least 500 nuclei were scored, and it was observed that SRFpm1 did not detectably affect cell proliferation. Error bars indicate ± standard error of the mean (SEM).

DISCUSSION

The semi-quantitative PCR analysis shown in Figure 6 revealed that retroviral-mediated expression of a dominant-negative SRF protein in cultured primary embryonic

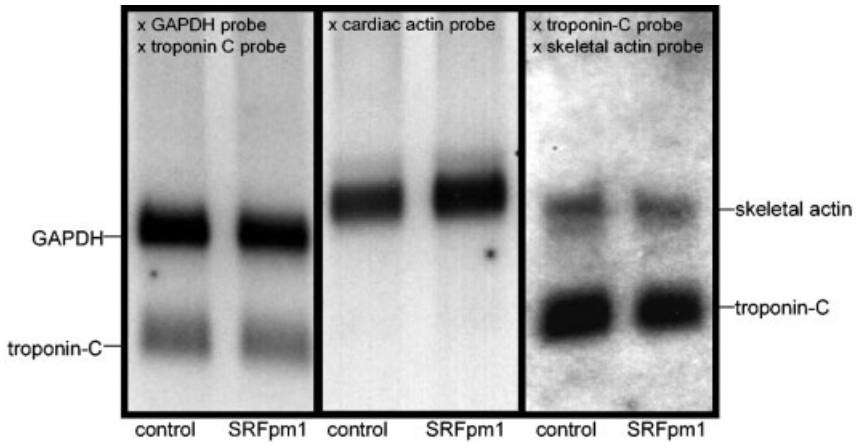

Fig. 5. Northern hybridization to detect mRNA transcript levels expressed by genes that are (cardiac and skeletal actin) and are not (GAPDH and troponin-C) SRF-dependent. Each lane in the gels used to create the blots shown in each panel contained exactly 10 μg total RNA purified from cardiac myocyte cultures. Blots were hybridized with the indicated (ribo)probes complementary to the coding regions of GAPDH and troponin C, and to the 3′-untranslated region of cardiac and skeletal α-actin. Blots shown in the left and right panels were sequentially hybridized.

heart cells inhibits expression of the cardiac α-actin gene by ~22.5%, without significantly affecting expression of GAPDH and cardiac troponin C. Inhibition of cardiac α-actin, which is expressed in both cardiac and skeletal muscle lineages, was an anticipated result, since all known vertebrate α-actin genes possess three SREs, and functional characterization of the skeletal α-actin promoter has revealed that binding of SRF to all three SREs is required for maximum activation (Lee et al., 1991). Moreover, it was previously shown that transfection of a plasmid containing SRFpm1 into cultured skeletal myotube cells inhibits binding of endogenous SRF and activation of a transfected skeletal α-actin gene containing a minimal promoter (Croissant et al., 1996). The current finding is consistent with that of Croissant et al. (1996), as well as that of Arsenian et al. (1998), who reported the absence of all α-actin gene expressions (cardiac, skeletal, and smooth) in embryos containing global deletion of the SRF gene. This indicates that the requirement for SRF to activate SRF-dependent promoters extends to the level of endogenous genes.

Although expression of cardiac α-actin was significantly inhibited in cells infected with SRFpm1, the extent of inhibition detected by quantitative PCR was only 20–30%, depending on the experiment. While this contrasts with the strong inhibition (>90%) observed for the skeletal α-actin promoter by Croissant et al. (1996), this disparity can be explained by the fact that the previous study assessed the activity of a transfected gene, while the current study assessed the activity of the endogenous cardiac α-actin gene. Moreover, because the level of endogenous SRF is very high in cardiac cells (Croissant et al., 1996), there may have been sufficient numbers of wild-type SRF/SRF dimers to limit the extent of inhibition caused by SRF/SRFpm1 heterodimers. Finally, it must be considered that since cardiac α-actin transcripts initially appear very early during cardiac myogenesis (Ruzicka and Schwartz, 1988), approximately coincident with the appearance of SRF at stage 8, dominant-negative SRFpm1 introduced to stage 14 heart cells would presumably have to displace wild-type SRF from established transcription complexes to inhibit endogenous promoters. The introduction of dominant-mutant SRFpm1 during embryogenesis prior to the activation of cardiac α-actin and other SRF-dependent cardiac genes may be required to achieve full transcriptional inhibition.

A similar rationale may explain the absence of an effect on cell proliferation. Although this finding was surprising considering the presumed role of SRF in proliferative processes (for review, see Herschman, 1991), it was consistent with observations that cell proliferation is normal in cells of SRF-null mice despite their early embryonic lethality (Arsenian et al., 1998). It was also noted in Croissant et al. (1996) that cell proliferation genes were not affected in transfected skeletal myogenic cell lines that were inhibited from differentiating by SRFpm1.

The observation that endogenous SRF is homogeneously dispersed in the nucleus and cytoplasm of cardiac myocytes, both in vivo (Croissant et al., 1996) and in vitro (current work), while SRFpm1 is apparently localized to the nucleus, is difficult to explain. The mechanism of SRF nuclear localization is complex and involves a poorly understood A-kinase regulatory step superimposed on an obligatory nuclear localization sequence (NLS) located at residues 95-103 of SRF (Gauthier-Rouviere et al., 1995). Recently, Ding et al. (2001) reported that although SRF nuclear localization is suppressed during cellular senescence and differentiation, this is subject to tissue-specific variability. While it could be conjectured that the apparently strong localization of mutant SRFpm1 protein to nuclei in D17.2G cells (Fig. 2) and embryonic myocytes (Fig. 3) is caused by the three point mutations in SRFpm1, these are situated between residues 143–146, a domain that is significantly downstream from the NLS domain. Perhaps the best explanation for this phenomenon may be that slow accumulation of SRF in SRF-rich tissues, such as the heart, results in homogeneous distribution throughout the cell, whereas the rapid accumulation that accompanies synthesis of exogenous SRFpm1 somehow promotes sequestration in the nucleus.

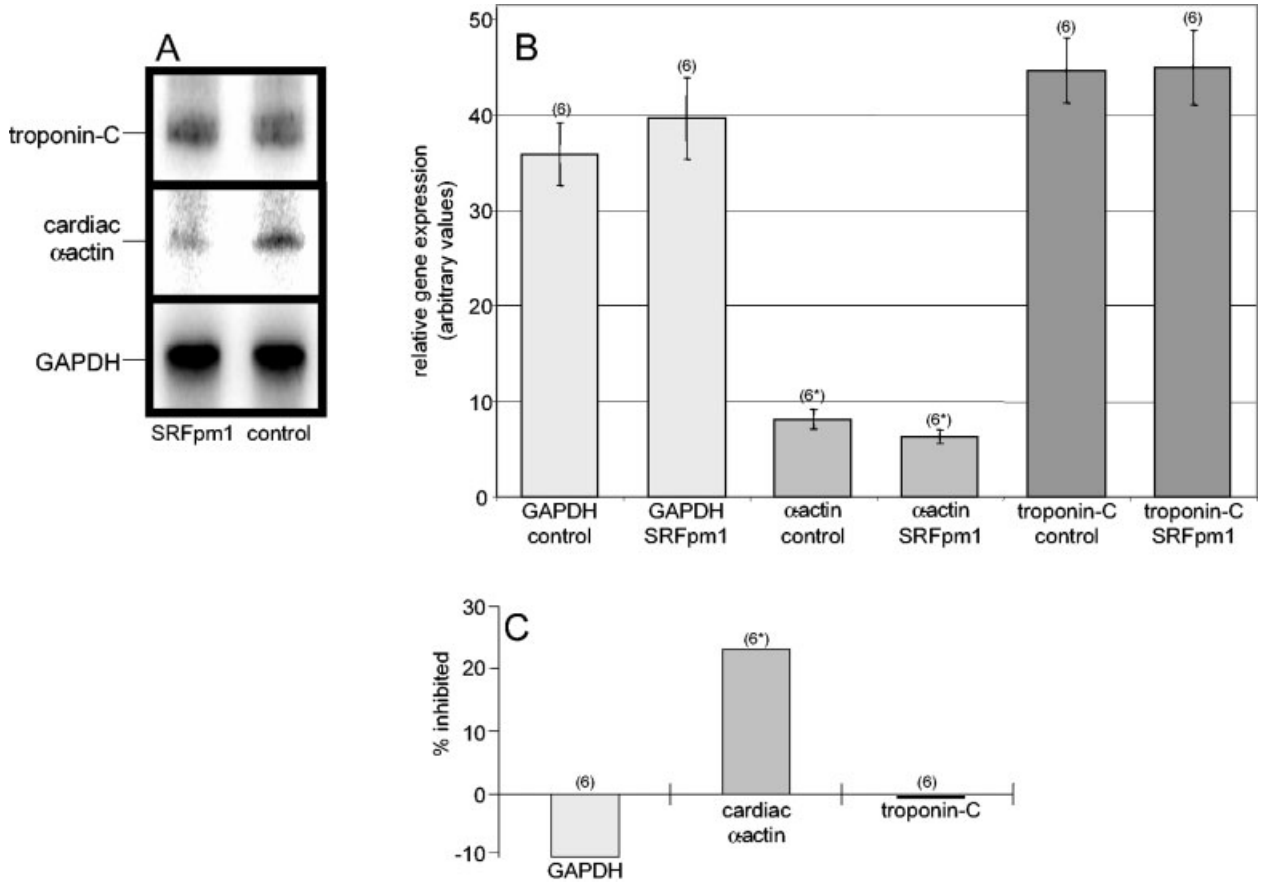

Fig. 6. Quantitative PCR assessment of gene expression in myocytes infected with SRFpm1. **A:** Representative Phosphorimager depiction of ^{32}P-labeled PCR products synthesized during the linear phase of PCR product accumulation. **B:** ImageQuant statistical analysis of digital images obtained from five repetitions of the determination shown in panel A. Vertical bars indicate the SEM; the asterisks (*) indicate significance between means as determined by Student's *t*-test ($P = 0.027$). Panel **C** expresses the data in panel B as a percentage of inhibition caused by SRFpm1.

In summary, these determinations provide additional evidence that SRF is required for α-actin gene transcription during myogenesis. The extent to which inhibition of SRF-dependent genes affects heart development remains to be determined by experiments in which the dn-SRFpm1 mutant is introduced to presumptive heart cells prior to transcriptional activation of endogenous SRF. Unfortunately, our attempts to infect cultured precardiac mesoderm cells explanted from the heart-forming region of stage 6 embryos with SRFpm1/βgal have not been successful. The inability to obtain satisfactory infection may be caused by these cells' propensity to assemble as a complex cellular multilayer that impedes virus penetration, combined with our inability to propagate and harvest the retrovirus virus at high ($>10^{10}$–10^{12}) titers. Because the clarification of SRF's role in heart development requires its tissue/stage-specific deletion during heart development in vivo, we are designing an approach to enable injection of SRFpm1 vector constructs into the heart-forming region of whole embryos grown in culture, between stage 3 (into the anterior primitive streak during gastrulation) and stage 5 (into precardiac mesoderm within the anterior lateral plate).

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