Serum Response Factor Mediates AP-1-dependent Induction of the Skeletal α-Actin Promoter in Ventricular Myocytes*

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"Fetal" gene transcription, including activation of the skeletal α-actin (SkA) promoter, is provoked in cardiac myocytes by mechanical stress and trophic ligands. Induction of the promoter by transforming growth factor β (TGF-β) or norepinephrine requires serum response factor (SRF) and TEF-1; expression is inhibited by YY1. We and others postulated that immediate-early transcription factors might couple trophic signals to this fetal program. However, multiple fos/jun proteins exist, and the exact relationship between control by fos/jun versus SRF, TEF-1, and YY1 is unexplained. We therefore coin-transfected ventricular myocytes with Fos, J un, or J unB, and SkA reporter genes. SkA transcription was augmented by J un, Fos/J un, Fos/J unB, and J un/J unB; Fos and J unB alone were neutral or inhibitory. Mutation of the SRF site, SRE1, impaired activation by J un; YY1, TEF-1, and Sp1 sites were dispensable. SRE1 conferred J un activation to a heterologous promoter, as did the c-fos SRE. Deletions of DNA binding, dimerization, or trans-activation domains of J un and SRF abolished activation by J un and synergy with SRF. Neither direct binding of Fos/J un to SREs, nor physical interaction between Fos/J un and SRF, was detected in mobility-shift assays. Thus, AP-1 factors activate a hypertrophy-associated gene via SRF, without detectable binding to the promoter or to SRF.

Mechanical load induces adaptive growth of cardiac muscle by cell enlargement, with little or no capacity for proliferative growth in terminally differentiated ventricular muscle cells (hypertrophy, not hyperplasia). Characteristically, this increase in myocyte mass is associated with qualitative and quantitative changes in cardiac-specific gene expression, including up-regulation of an ensemble of genes (including skeletal α-actin (SkA), β-myosin heavy chain, and atrial natriuretic factor (ANF)) that are highly expressed in embryonic but not normal adult ventricular myocardium, a phenomenon referred to as reinduction of a "fetal" phenotype (1–3). SkA expression has been associated with increased contractility in the rodent heart (4) and with impaired contractility in patients with heart failure (5). In addition to this set of genes, two others are induced, and to varying degrees have been implicated in this response to mechanical load. First, "immediate-early" transcription factors including Fos and J un potentially might couple trophic signals to long-term changes in growth and gene expression. Second, myocardial growth factors including angiotensin II and transforming growth factor β (TGF-β) evoke most aspects of the fetal/hypertrophic program in cultured cardiac muscle cells (1–3). Induction of TGF-β by load, passive stretch, α-adrenergic agonists, and angiotensin II suggests that TGF-β might participate in the onset, maintenance, or inhibition of cardiac hypertrophy, as an autocrine or paracrine factor (6). We recently demonstrated that induction of the SkA promoter by TGF-β requires the MADS box protein serum response factor (SRF) and the SV40 enhancer-binding protein, TEF-1 (6); both also mediate activation of this promoter by α-adrenergic agonists (7). Dominant-negative mutations of the type II and type I TGF-β receptor, which share related serine/threonine kinase domains, suffice to disrupt TGF-β-dependent signal transduction (8, 9); however, the molecular circuit that confers signal from the TGF-β receptor complex to SkA promoter-binding proteins is unknown.

Among the secondary or tertiary messengers that might be involved in this signaling cascade, it is noteworthy that activation of nuclear oncogene transcription factors Fos, J un, and J unB precedes growth and the up-regulation of "fetal" cardiac genes, in cultured myocytes (10, 11) and intact animals (12–14). Fos and J un proteins (Fos, FosB, Fra-1, Fra-2, J un, J unB, and J unD) each possess a basic domain for DNA binding and a leucine heptad repeat (leucine zipper) as an interface for homo- or heterodimerization (15). Each member of this AP-1 transcription factor family recognizes the 12-o-tetradecanoylphorbol-13-acetate response element (TRE: TGA(G/C)TCA), although noncanonical sites also are reported (16–18). Three lines of evidence support the inference that Fos/J un proteins might mediate TGF-β signal transduction: TGF-β up-regulates the expression of junB and c-fos in skeletal myocytes (19), cardiac myocytes,* and other cell types, and AP-1 sites mediate autoinduction of TGF-β1 itself (20). Moreover, in skeletal muscle, forced expression of either Fos or J un reproduces the suppressive effect of TGF-β on myogenic differentiation, although the issue of physical association between Fos/J un with myogenic helix-loop-helix proteins is unresolved (21–23).

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† The abbreviations used are: SkA, skeletal α-actin; ANF, atrial natriuretic factor; SRE, serum response element; SRF, serum response factor; TGF-β, type β transforming growth factor; TRE, 12-o-tetradecanoylphorbol-13-acetate response element; CMV, cytomegalovirus; DF, Dulbecco’s modified Eagle’s medium; Ham’s nutrient medium F-12.

‡ T. G. Parker, P. Paradis, and M. D. Schneider, unpublished results.
Related co-transfection studies likewise support the premise that Fos/Jun mediate the induction of fetal cardiac genes by TGF-β, as shown for ANF (24, 25), β myosin heavy chain,3 and SKA (18). In the latter study, forced expression of Jun or Fos plus Jun up-regulated transcription of the human SKA promoter in cardiac myocytes from neonatal rats and in P19 teratocarcinoma cells. Deletion analysis of the SKA promoter indicated that nucleotides –153 to –36 were required for maximal trans-activation by Fos/Jun. Although no consensus AP-1 site was found within this region, sequence-specific binding to a noncanonical motif was believed to occur. Despite this suggestive information, the conclusion that Fos/Jun proteins augment the transcription of SKA and other fetal cardiac genes would be premature. Dichotomous results, repression (24) as well as activation by Fos/Jun (25), have been reported for ANF.

Mechanical load, ischemia, and isoproterenol each highly induce J unB in myocardium in vivo (13, 26, 27), but few functional comparisons among J un proteins are known for cardiac myocytes (24). Although prior results pointed to direct binding of Fos/Jun near the first SRE of the human SkA promoter, it has not been demonstrated whether point mutations of this construct that abolish AP-1 binding remain susceptible, or not, to induction by AP-1 factors. Finally, given the emerging importance of SRF in concert with TEF-1, and given the displacement of SRF at the first SRE by a GLI-Krüppel protein, YY1, the relationship between control by these three factors and the induction by Fos/Jun merits study.

In the present report, we demonstrate that Jun, Fos plus Jun, Fos plus JunB, and Jun plus JunB all transactivate the SKA gene in cardiac myocytes, whereas JunB, like Fos, is ineffective individually. Notably, a SRE is necessary for AP-1 responsiveness of the SKA promoter, and suffices to confer induction by AP-1 to a heterologous promoter. Induction required full-length Jun protein, and did not involve measurable binding of AP-1 factors to the SKA SRE1, physical association of AP-1 and SRF, augmentation of SRF binding by AP-1, or potentiation of the SRF trans-activation domain, residues 266–508 (SRF(266–508)). Together, these results indicate that AP-1 factors can act through SRF to induce a hypertrophy associated gene, SKA, but trans-activate SRF reporter genes in the absence of direct binding to the promoter or DNA-bound SRF.

**Experimental Procedures**

**Plasmids—**Luciferase reporter vectors driven by mouse c-fos core promoter (nucleotides –56 to +109, 356Fos), SKE1 R356Fos, c-fos SRE-J356Fos, chicken SKA promoter (nucleotides –394 to +24), and linker-scanning BglII mutations of the promoter were detailed previously (6, 28). The BglII mutations disrupt, respectively, the SRF binding site in SRE1 (M –94/–89), the overlapping YY1 site (M –81/–79), TEF-1 binding (M –70/–65), Sp1 binding (M –52/–47), and the TATA box (M –28/–23); nomenclature indicates the substituted nucleotides. A TRE reporter gene was constructed by subcloning a HindIII-BglII fragment containing nucleotides –71 to –65 of the human collagenase gene fused to nucleotides –105 to +51 of the herpes simplex virus thymidine kinase promoter (a gift from M. Karin), into the firefly luciferase expression vector pXFP2 (29). Nucleotides –456 to –23 of the mouse SRF promoter were cloned as a SmaI/XhoI fragment into the luciferase vector pGL2 (Promega, Madison, WI). Details of the sequence and construction will be described elsewhere.6 pULASGAL4EpLuc3, containing four yeast Gal4 binding sites fused to nucleotides –72 to +7 of the rat elastase gene, was constructed by subcloning a HindIII-BamHI DNA fragment of G4ElpHGH (a gift from R. W. Moreadith) (30), into the luciferase vector pGL3 (Promega).

Rat c-fos cDNA, provided by T. Curran (31), was subcloned as an EcoRI-XhoI fragment into the SV40-driven eukaryotic expression vector, pSV-SPORT1 (Life Technologies, Inc.). Mouse c-Jun and JunB cDNAs, provided by E. Olson (19), were subcloned as EcoRI fragments into pSV-SPORT1. Mouse JunR and JunLZ, a gift from I. M. Verma (32), were subcloned as XhoI-SacI and PstI DNA fragments, respectively, into pSV-SPORT1; JunR and JunLZ are mutations of Jun in which the DNA binding and dimerization domains have been deleted, respectively (amino acids 251–276 and 281–313). TAM67 (a deletion mutant of Jun) lacking amino acids 3–122 in the trans-activation domain (donor) and a CMV reporter construct derived from pCMV-β-gal (Clontech, Palo Alto, CA) were kindly donated by M. J. Birrer (33). Human SRF plasmids were kindly provided by R. Prywes. SRF (residues 1–508, wild-type), SRF (residues 1–338), and SRFpm1 (34) were, respectively, subcloned as XbaI-BamHI, XbaI-PvuI, and XbaI-BamHI DNA fragments into pSV-SPORT1. SRF(1–239) constructs a deletion of the transactivation domain of SRF, and SRFpm1 contains three point mutations in the basic region that abolish DNA binding. GAL4-SRF(266–508) comprises the DNA-binding domain of yeast GAL4 (amino acids 1–147), fused to residues 266–508 in the SRF trans-activation domain (35), and was subcloned into the CMV-driven expression vector pCMON. Plasmid DNAs were purified using Qiagen Maxiprep columns (Chatsworth, CA).

**Cell Culture and Transfection—**Primary cultures of cardiac myocytes and fibroblasts were prepared from 1–2-day-old Sprague-Dawley rats with modifications to previously described methods (8, 9). Ventrices of ~100 hearts were digested four times, 15 min each, in 20 ml of phosphate-buffered saline without Ca2+ and Mg2+, containing 1% collagenase, 0.1% elastase, 10 mM HEPES, pH 7.3, adjusted to final densities of 1.082, 1.061, and 1.051 g/ml. The enriched myocytes (banding between the 1.082 and 1.061 g/ml layers) and fibroblasts (banding between the 1.061 and 1.051 layers) were washed with medium DF (Dulbecco’s modified Eagle’s medium;Ham’s nutrient medium F-12, 17 mM HEPES, pH 7.4, 3 mM NaHCO3, 2 mM L-glutamine, 50 μM gentamicin) containing 5% horse serum (Hyclone, Logan, UT). Venticles as myocytes were further purified by preincubating non-myocytes by differential adhesiveness, then were plated at a density of 1 × 10^6 cells/35-mm dish (Primaria, Falcot) and cultured 24 h in DF containing 5% horse serum. Fibroblasts were cultured for 1–2 days in DF supplemented with 5% horse serum, then were passaged once in the same medium using 0.5 × 10^6 cells/35-mm dish (Falcon).

Cells were transfected by a modified DEAE-dextran method. DNA (2.5 μg/ml reporter and 0–10 μg/ml expression vectors) was mixed with 150 μg/ml DEAE-dextran (average molecular weight, 500,000; Sigma) in DF supplemented with 2.5% Cosmic calf serum (Hyclone). For all comparisons, DNA and promoter content were kept constant using equivalent amounts of vector. Cells were washed once, were incubated with 100 μg/ml DEAE-dextran complex, rinsed with medium, and then shocked for 30 s with 10% dimethyl sulfoxide in DF. Cells were cultured overnight in DF supplemented with 5% horse serum, after which the medium was replaced by DF supplemented with 5 μg/ml transferrin, 1 mM NaSeO4, 1 mM LiCl, 25 μg/ml ascorbic acid, and 100 μg/ml bovine serum albumin (fatty acid free). Twenty-four hour later, cells were harvested and assayed for luciferase activity (6) and for protein content using the Bradford assay (Pharmacia). Luciferase activity for each promoter was corrected for protein content of each extract and was normalized to the activity of each promoter in parallel cultures of control, vector-transfected cells. Co-transfection with a constitutively active lucZ gene was omitted for three reasons. The brief half-life of luciferase compared to other reporter proteins can lead to misleading interpretations; reports on activity of transcriptional repression (36) are unaffected by Fos/Jun, such as the c-myc promoter, and were passaged once in the same medium using 0.5 × 10^6 cells/35-mm dish/Falcon.

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**Electrophoretic Mobility Shift Assays—**Jun, Fos, and SRF were pro-

3 T. G. Parker, personal communication.

4 N. S. Belaguli and R. J. Schwartz, unpublished data.

5 P. Paradis and M. D. Schneider, unpublished results.
AP-1 Factors Activate Skeletal α-Actin Transcription

RESULTS

AP-1 Factors Differentially Activate the Skeletal α-Actin Promoter in Ventricle Myocytes—In preliminary Northern blot analyses, we observed a marked increase in c-fos and junB in ventricular myocytes treated with TGF-β, with little or no change in c-jun, as shown previously in skeletal muscle (19). To compare the potential for these three immediate-early genes to trans-activate the SkA promoter, ventricular myocytes were co-transfected with the SkA luciferase reporter gene and Fos, Jun, or JunB expression vectors. Control cultures were transfected with 2.5 μg of reporter plasmid and 10 μg of the empty SV40-driven vector. For each reporter plasmid, luciferase activity is expressed relative to that in vector-transfected cells. Results are the mean ± S.E. for at least four transfections with two exceptions; n = 3 for the SKA reporter co-transfected with Fos plus JunB, and for the TRE reporter co-transfected with Jun plus JunB. *p < 0.05, versus vector-transfected control cells; tp < 0.05, versus parallel cultures co-transfected with 5 μg of Jun.  

Reduced in vitro using the SP6 Tnt coupled reticulocyte lysate system (Promega). Gel mobility shift assays were performed as described previously (40): 6 μl of the coupled transcription/translation reaction mixture was incubated with 20,000 cpm of 32P-end-labeled DNA probe in 20 μl of 20 mM HEPES, pH 7.9, 25 mM KCl, 0.1 mM EDTA, 10% glycerol, 10 mM MgCl2, 0.2 mM dithiothreitol, 0.02% Nonidet P-40, 50 μg/ml of poly(dG-dC) or poly(dI-dC), 50 μg/ml of bovine serum albumin. Poly(dG-dC) was used for assays involving the SKA SRE1-TATA probe and poly(dI-dC) for the TRE probe. DNA-protein complexes were then analyzed on a 4% low ionic strength polyacrylamide gel (acrylamide: bisacrylamide, 80:1) containing 45 mM Tris-borate, 1 mM EDTA, and 0.05% Nonidet P-40, and were electrophoresed in 45 mM Tris-borate, 1 mM EDTA at 4°C and 250 V for 2 h. The SkA SRE1-TATA probe, comprising SkA SRE1 (nucleotides –100 to –73) fused to mouse c-fos nucleotides –56 to –19, was generated by digesting the SKA SRE1–56Fos luciferase expression vector with HindIII and PvuI. To ensure high specific activity, the probe was double labeled with the Klenow fragment of DNA polymerase I using α-32PdCTP and with poly nucleotide kinase using γ-32PdATP. Competing double-stranded oligonucleotides encompassing the M –94/–89 and M –81/–79 mutations of the SKA SRE1, which abolish SRF and YY1 binding respectively, were detailed previously (6). A double-stranded oligonucleotide containing a consensus TRE was subcloned into the Sac/NheI sites of pGL3 (5′-AGCCTCGGGTTAGTCTCAGC GGGAAGACGTAG-3′); the sense strand is 73 mutation of the YY1 site we weakly versus 587 mutation of the YY1 site we weakly versus transcription via the SkA promoter in a dose dependent fashion, up to 3.4-fold (p < 0.01). No further increase in Jun independent transcription was seen with a 5-fold larger, 2-kilobase pair segment of the promoter. Although Fos and JunB singly had no effect, in tandem they transactivated the SkA promoter synergistically (2.2 ± 0.16; p < 0.01). More than additive induction likewise was seen using Fos plus Jun (3.1 ± 0.32; p < 0.01), but not Jun cotransfected with JunB. Thus, co-expression of Fos and JunB, AP-1 factors that are highly induced by TGF-β, can suffice to increase transcription of the SkA promoter.

To verify that induction of the SkA promoter by forced expression of Fos, Jun, and JunB is specific, a canonical AP-1 responsive element and a constitutive neutral promoter were examined, as positive and negative controls, respectively. Control of the SkA promoter by the various permutations of AP-1 factors accurately resembled activation of the human collagenase TRE1, although the TRE1 was more highly induced. By contrast, little or no activation was seen using the c-fos neutral core promoter, 56Fos, Fos, FosB, and JunB up-regulated the 56Fos reporter by no more than 40, 70, and 60%, respectively (p < 0.01). Indeed, slight inhibition was observed at high concentrations of Fos or JunB (p < 0.01).

Trans-activation of the αSKA by the AP-1 Factors Requires SRE1 in Concert with the TATA Box—To test the hypothesis that induction by Fos/Jun might map to one or both TGF-β response elements of the SkA promoter (i.e. SRE1 and a TEF-1 site), cardiac myocytes were co-transfected with 10 μg of the Jun expression vector or the empty vector control, together with a luciferase reporter gene driven by the wild-type SkA promoter versus linker-scanning mutations as summarized in Fig. 2. Detailed previously (6), mutation of the YY1 site weakly activates basal activity; mutations of the SRF, TEF-1, Sp1, and TATA motifs inhibit basal activity of the promoter by up to 90%. Of the five linker-scanning mutations tested, three had no significant effect on induction by Jun. The mutation specific for YY1 (M –81/–79) lies immediately 3′ to the proximal SRF binding site, and disrupts a potential TRE-like sequence (18). Mutation of the YY1 site did not alter trans-activation by Jun (5.09 ± 0.42, relative to activity of this promoter in vector-transfected cells), nor did a mutation that destroys the binding
site for TEF-1 (M−70/65: 4.97 ± 0.76). The mutation that blocks the binding of Sp1 (M−52/−47) also had no significant effect (3.88 ± 0.41). M−94/−89 is a substitution in the 5' arm of the SRE1 palindrome, which specifically disrupts SRF binding while sparing YY1: by contrast, to activation of the wild-type promoter and these three mutations by Jun, mutation of the SRF binding site reduced Jun un-dependent transcription of the promoter by one-half (2.34 ± 0.40; p < 0.01). A similar decrease in induction by Jun un resulted from mutation of the TATA box (M−28/−23: 2.00 ± 0.07; p < 0.01). Together, these mutations suggest that SRF, but not TEF-1 or Sp1, is necessary for full trans-activation of the SKA promoter by Jun. That a mutation of the TATA box also inhibited trans-activation by Jun un is intriguing, as both SRF (40, 41) and Jun un (42) have been proven to associate with basal transcription factors.

A SRF Confers Jun un-dependent Transcription to a Heterologous Promoter—Conversely, to establish whether the SKA SRE1 suffices for trans-activation by Jun, we co-transfected Jun un with the SKA SRE1 upstream of the neutral promoter, Δ56Fos (Fig. 3A). Luciferase expression driven by Δ56Fos was not significantly changed by Jun un (1.39 ± 0.09). By contrast, Jun trans-activated the SRE1-Δ56Fos construct 5.02 ± 0.48 fold (p < 0.01). To distinguish whether AP-1-dependent activation was specific to the SRE1, this element was exchanged for the c-fos SRE: Jun un induced the c-fos SRE-Δ56Fos reporter to the same extent (5.11 ± 0.47; p < 0.01). By contrast, Jun un decreased transcription of these isolated elements in parallel cultures of cardiac fibroblasts under the same transfection conditions; where indicated, cardiac fibroblasts were cultured in the presence of TGFB−1, to demonstrate that the isolated SREs are functional in this cell background (Fig. 3B). Together with mutagenesis of the full-length SKA promoter, these findings indicate that SRF is both necessary and sufficient for activation of the SKA promoter in ventricular muscle cells by AP-1 transcription factors.

To test what domains of Jun might be necessary or sufficient for these effects, we co-transfected cardiac myocytes with 10 μg of the Jun expression vector, SRE reporter genes, and 0–100 ng of an SRF expression vector (Fig. 3, A and C). This concentration of SRF by itself did not significantly activate any of the three reporter genes. However, exogenous SRF was synergistic with Jun un, up-regulating both the SKA SRE1- and c-fos SRE-Δ56Fos constructs, up to 14-fold, nearly three times the level of induction produced by Jun un alone. When wild-type SRF was co-transfected with Jun un mutants, no trans-activation was observed with a deletion of the DNA-binding domain (Jun unΔRK), deletion of the dimerization domain (Jun unΔLZ), or partial deletion of the trans-activation domain (TAM67). Each of the three Jun un mutants also failed to transactivate, by themselves, the full-length SKA reporter gene. Conversely, in cells cotransfected with Jun un, the synergistic effect of wild-type SRF was abolished with SRFpm1, a point mutation of SRF which cannot bind DNA, and was markedly impaired with a mutation that deletes a trans-activation domain, SRF (1–338); the remaining cooperative effect is consistent with the residual activation domain of this mutation (43). As the high levels of endogenous Jun un and SRF in myocardium (6, 44), together with the limited efficiency for transfection of ventricular myocytes, would confound efforts to compare the levels of each protein in vivo, we synthesized each Jun un and SRF mutation in vitro in the presence of [35S]methionine and verified that the translational efficiency and inherent stability of each protein were equivalent to that of wild-type Jun un and SRF. Thus, functional DNA-binding, dimerization, and trans-activation domains were each necessary for up-regulation of the SKA promoter by Jun un. The necessity for full-length Jun un contrasts with effects shown for the isolated Jun un trans-activation domain in repression of myogenin activity (22).

Activation of SRF Transcription by Jun un—A requirement for full-length Jun un would be anticipated by either of two contrasting models: activation through direct binding of Jun un, suggested previously (18), or indirect activation by a Jun un-induced protein. Given the requirement for SRF binding to SRE1 (Fig. 2), Jun un might thus up-regulate the SKA promoter indirectly, by augmenting SRF expression or activity. Although the small proportion of cardiac myocytes that take up foreign genes during transient transfection precludes a direct test of whether Jun un induces SRF in this background, we co-transfected ventricular
muscle cells with J un together with a murine SRF luciferase reporter gene. Forced expression of J un increased transcription of the SRF promoter (2.29 ± 0.19; \( p < 0.01 \)). However, it is implausible that changes in SRF abundance alone could explain induction of the SkA promoter by FosJ un. First, the magnitude of SRF induction was modest. More importantly, overexpression of SRF at up to 100 ng/culture did not increase the magnitude of SRF induction was modest. More importantly, overexpression of SRF at up to 100 ng/culture did not increase transcription of the isolated SREs (Fig. 3), but causes squelch-overexpression of SRF at up to 100 ng/culture did not increase transcription of the isolated SREs (Fig. 3), but causes squelch-overexpression of SRF at up to 100 ng/culture did not increase transcription of the isolated SREs (Fig. 3), but causes squelch.

We conclude that AP-1 factors might modulate activity of SRF, or promote transcription via SREs, by other mechanisms.

To test whether J un could augment gene expression via the trans-activation domain of SRF, we employed a GAL4 fusion protein comprising the DNA-binding domain of yeast GAL4 fused to SRF amino acids 266–508, GAL4SRF (266–508). Five \( \mu \)g of GAL4SRF (266–508) were co-transfected with or without the J un expression vector, using a GAL4-dependent luciferase reporter (Fig. 4). In the absence of exogenous J un, the GAL4/SRF fusion protein increased transcription of the reporter B.39 ± 0.42 (\( p < 0.01 \)); transcription via the SRF activation domain was not augmented significantly by the addition of exogenous J un. Thus, J un did not increase transcription, when the SRF activation domain was tethered to DNA by a heterologous DNA-binding domain. This also argues against a generalized activation of transcription by J un.

To address two further possibilities, that J un might activate the SRE by physical association with native SRF, or facilitate DNA binding by SRF, gel mobility-shift assays were performed using SRF, Fos, and J un, produced by in vitro transcription and translation (Fig. 5A). A HindIII-PvuII DNA fragment of the SkA SRE 1–56Fos luciferase reporter gene was used as probe, encompassing the SkA SRE1 plus all sequences of the core c-fos promoter including the TATA box. A double-stranded oligonucleotide containing the consensus AP-1 binding site was used, for comparison. Recombinant SRF bound the SRE1–56 Fos probe, was displaced by the competitor that binds SRF but not YY1 (M–81/–79), and was displaced poorly by the reciprocal mutation, which disrupts SRF binding (M–94/–89). J un, Fos, and co-translated FosJ un showed no direct binding to the probe, did not form a higher order complex with DNA-bound SRF, and did not alter binding of SRF to DNA in any fashion. To ensure that the lack of protein-protein interaction was not due to inadequate expression of J un or Fos, parallel experiments were performed using the AP-1 consensus probe. J un and co-translated FosJ un bound the TRE, were displaced by excess unlabeled TRE, and were unaffected by the mutated TRE. Thus, both AP-1 factors were expressed as stable proteins with the expected DNA-binding and dimerization properties.

To exclude direct association between FosJ un proteins and SRF, we demonstrated no binding of recombinant SRF to J un homodimers or FosJ un heterodimers, and no effect of SRF on the binding of AP-1 factors to the TRE probe (Fig. 5B).

**DISCUSSION**

The present investigations show that transcription of SkA, a “fetal” cardiac gene associated with myocardial hypertrophy, can be augmented by AP-1 transcription factors (J un, Fos plus J un, Fos plus J unB, or J un plus J unB) in ventricular myocytes from neonatal rats. Despite marked differences in the constructs and procedures used, this corroborates and extends the report of Bishopric et al. (18), that forced expression of J un or Fos plus J un up-regulates the human SkA promoter in cardiac cells. Because dichotomous results, both induction and repression, were reported for the ANF gene (27, 28), consensus regarding the functional role of FosJ un factors in hypertrophy has been lacking. Although J unB, like Fos and J un, is highly induced by both mechanical load (13, 14) and trophic factors that up-regulate the endogenous SkA gene (TGF-β, cathecolamines, and angiotensin II) (18, 45), even less functional evidence has been available for J unB in cardiac muscle (24). Whereas Fos proteins do not form homodimers, J un and J unB form both homodimers and heterodimers with FosJ un proteins and more distantly related factors, via the leucine zipper. Our results demonstrate functional synergy between J unB and Fos in cardiac myocytes; either alone had no effect or was inhibitory, while Fos plus J unB activate the SkA promoter. Permutations of Fos, J un, and J unB that activate the SkA gene correspond to those that induced the human collagenase TRE, a canonical AP-1-responsive element.

Our results diverge from previous findings, however, on mechanisms to explain trans-activation of SkA by AP-1. Here, SRE1 was both necessary and sufficient. A molecular basis for activation of the SkA promoter by SRF in concert with TEF-1 has been proposed (6, 7), with virtually identical conclusions for the avian and rat promoters. Cooperation of SRF and TEF-1 is also implicated in two distinct transduction pathways for hypertrophy, TGF-β and α1-adrenergic agonists. By contrast, the TEF-1 site is dispensable for full augmentation of the SkA promoter by J un. In agreement, nested deletions of the human SkA promoter suggested that nucleotides encompassing the first SRE (−153 to −87) were required for maximal trans-activation by Fos plus J un, whereas more proximal sequences including the TEF-1 site at nucleotides −71 to −65 do not mediate AP-1 responsiveness (18). While direct binding of J un and FosJ un to a noncanonical site near SRE1 was reported for the human SkA promoter, our results point instead toward an indirect mechanism, mapped to SRE1 itself. First, J un can induce the avian SkA promoter in the absence of TEF-1, Sp1, or YY1 binding, yet the SRF binding site and TATA box are indispensable. This is intriguing, given that SRF contacts the RAP74 subunit of transcription factor IIF (39, 41), while Fos and J un contact other basal factors, transcription factor IIB and TATA box-binding protein (42, 46). Second, the isolated SkA SRE1 is sufficient for AP-1-dependent expression and is interchangeable, in this respect, with the c-fos SRE. Third, using recombinant Fos and J un produced in reticulocyte lysates, we detected no binding to the SkA SRE1. This discrepancy with direct binding of AP-1 factors reported for the human SkA promoter (18) may be explained by technical differences:

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6 W. R. MacLellan and M. D. Schneider, unpublished results.
in the earlier study, proteins were produced in E. coli, truncated Fos and Jun were used, and five times more protein was used for the SkA probe versus the authentic TRE. Alternatively, sequence dissimilarities may be germane. Among the characterized vertebrate SkA genes, only the human promoter matches the TGACTCA consensus TRE at five positions that include both cytosine residues.

Control of the SkA SRE1 by Jun and synergy with SRF both required full-length Jun protein with intact DNA binding, dimerization, and trans-activation domains. As no binding of AP-1 factors was detected to SRE1, mechanisms alternative to direct association must be considered. Conceivably, AP-1 factors might increase transcriptional activity of an SRF binding site through protein-protein interactions with SRF, increasing SRF abundance, or affecting transcriptional activity of SRF. Our results provide no support for a ternary or quaternary complex of Fos/Jun with DNA-bound SRF. No physical interaction was seen between SRF and AP-1 in gel mobility-shift assays, nor did AP-1 factors augment DNA binding by SRF. However, measurements of DNA-protein interaction might overlook low affinity binding or interactions that require a co-activator. Using GAL4 fusion proteins to overcome limitations of both gel retardation assays and endogenous SRF, we found that Jun could not potentiate the C-terminal activation domain of SRF. It is a formal possibility that Jun might interact (with affinity too low to be stable in vitro) only with native SRF or the native SRF-SRE complex. Increased SRF abundance is unlikely to explain AP-1-dependent SkA transcription, since SRF was not limiting in cardiac myocytes. Our findings are more consistent with the alternative, that Fos/Jun transcription factors increase, instead, the transcriptional activity of SRF. In principle, this could be contingent on altered expression of an autocrine or paracrine factor (23), a protein kinase modulating SRF activity (47), or a co-activator. Whereas SRF accessory proteins include most obviously the ternary complex factors Elk-1/TCF and SAP1-48, association and synergy with SRF both were demonstrated (49) for the cardiac-restricted homedomain protein, Nkd-2.5, vertebrate homologue of the Drosophila tinman gene (50, 51).

In summary, our studies reveal a novel AP-1-dependent pathway for gene induction in cardiac myocytes, via indirect activation of SREs. Hence, AP-1 factors might plausibly be involved in the up-regulation of genes containing SREs, including skeletal and smooth muscle α-actin and the immediately early gene c-fos, in the setting of cardiac hypertrophy. It is unknown whether the greater induction of “fetal” α-actin transcripts relative to cardiac α-actin reflects inherent differences among SREs, contextual sequences, or elements elsewhere in the respective promoters. The SRE has been implicated in numerous settings as a pivotal regulatory element for cardiac gene expression during hypertrophy, for activation of the SkA promoter by TGF-β (6), basic FGF (52), and α1-adrenergic agonists (7), up-regulation of ANF by α1-adrenergic signals (53), and induction of c-fos by load (54), passive stretch (55), or angiotensin II (56). Control of SRE-dependent transcription by AP-1 factors was selective, as the neutral core promoter was unaffected by Jun, contrasting with the global increase in transcription provoked by Ras under similar conditions (36). Conversely, TGF-β selectively up-regulates fetal cardiac genes, with little change in RNA or protein content (57). Hence, the overall increase in cell RNA and protein can be dissociated from the “fetal/hypertrophic” program. Analogously, rapamycin blocks the increase in total cell protein and p70 S6 kinase activity in angiotensin II-treated cardiac myocytes, while not affecting induction of c-fos or fetal cardiac genes (58). The latter report highlights other data pointing to the possibility, with which the present study and our Ras results concur (36), of distinguishable signaling cascades for these components of the hypertrophic phenotype, that the global increase of cell protein is mediated by p70 S6 kinase, while the fetal program might be mediated, at least in part, by mitogen-activated protein kinase induction of Fos, and Jun N-terminal kinase acting through Jun (58).

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