AKAP12α, an Atypical Serum Response Factor-dependent Target Gene

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We recently identified three AKAP12 isoforms that are differentially regulated by distinct promoters. During a screen to identify molecular determinants distinguishing the activities of these promoters, we found a potential binding site for the serum response factor (SRF) in the promoter of the ubiquitously expressed AKAP12α isoform. SRF is an evolutionarily conserved transcription factor that governs disparate programs of gene expression linked to cellular growth and differentiation. Using a combination of reporter assays and RNA interference, we demonstrate that SRF is required for AKAP12α expression. SRF regulates the activity of the AKAP12α promoter through two conserved CArG boxes that bind SRF with different affinities. Unlike other SRF-dependent genes, AKAP12α is not regulated by growth or differentiation stimuli. Molecular analysis of the AKAP12α SRF-binding sites, or CArG boxes, indicates that sequences flanking these sites are the determinants of sensitivity to SRF-activating signals. Specifically, the AKAP12α CArG boxes are shielded from growth stimulation by the absence of a binding site for Ets transcription factors. Similarly, sensitivity to the differentiation-associated co-factor, myocardin, was also determined by responsive flanking sequence; however, unlike growth stimuli, sensitivity to myocardin was found to also be dependent on a consensus CArG box. Collectively, our data demonstrate that AKAP12α belongs to a novel class of atypical SRF-dependent target genes. Furthermore, we provide new insight into the role of flanking sequences in determining sensitivity to SRF-myocardin activity.

Serum response factor (SRF)1 is a single copy gene encoding an evolutionarily conserved transcription factor that governs disparate programs of gene expression linked to cellular growth and differentiation (1, 2). SRF dimers bind a 1,216-fold degenerate cis-element known as a CArG box (consensus = CCWGG) found singly or in multiple copies around the proximal promoter region of over 60 target genes (1). The importance of SRF-dependent gene expression is underscored by the essential role of SRF in normal eukaryotic homeostasis. For example, mutation of the yeast ortholog of SRF, MCM1, elicits perturbations in mating and sporulation (3). SRF inactivation in Dictyostelium discoideum (4), Caenorhabditis elegans (5), and Drosophila melanogaster (6, 7) results in deficits in normal locomotion and cellular outgrowth. The latter defects are hypothesized to be linked to the role of SRF in regulating normal cytoarchitecture (6, 8). Targeted SRF inactivation in mice results in early embryonic lethality because of abnormal gastrulation and loss in key mesodermal markers (9). More recently, tissue-restricted inactivation of SRF resulted in severe defects in cardiovascular development (10, 11). Collectively, molecular inactivation studies of SRF have revealed a critical role for this transcription factor in cellular differentiation, migration, and cytoarchitecture.

Insight into how SRF regulates distinct gene sets linked to such disparate biological processes first emerged when it was discovered that SRF interacts with other transcription factors that bind to sites flanking the CArG box. For example, SRF regulation of the c-fos proto-oncogene was found to be dependent upon interaction with Ets family members, such as ELK-1 and SAP-1, which bind to cognate sites flanking the CArG box (12, 13). A number of other growth-related genes were soon found to be governed through similar cooperative SRF-Ets associations (2). In contrast to SRF-Ets complexes activating growth-related SRF target genes, several distinct SRF associated complexes exist that coordinate programs of muscle differentiation including SRF-myogenin (14), SRF-Nkx2.5/3.1 (15, 16), SRF-GATA4/6 (15, 17), and SRF-CSRP1 (18). Recently, myocardin (MYOCD) was cloned in an in silico screen for cardiac-restricted genes, was shown to physically interact with SRF, and greatly accentuated SRF-dependent gene activity (19). Our laboratory first proposed the paradigm of SRF-MYOCD coordinating a program of SMC differentiation (20), a concept subsequently confirmed by several independent groups (21–23). Direct evidence implicating MYOCD in the control of SMC differentiation was verified in mice null for MYOCD (24). Importantly, whether a target gene is responsive to one SRF complex over another will depend on both the CArG sequence itself as well as flanking sequences immediately adjacent to CArG (25–27).

We recently characterized the regulatory regions controlling three isoforms of AKAP12 (A kinase anchoring protein) (28). During further sequence analysis, we identified two evolutionarily conserved CArG boxes in the 5’ promoter of the ubiquitously expressed α isoform of AKAP12. Because AKAP12 has been implicated in both growth and cytoskeletal controls (29),
we sought to characterize the role of SRF in regulating AKAP12α as a means to further understand the function of this AKAP12 isoform. In the present study, we demonstrate that the AKAP12α promoter is responsive to SRF. Moreover, AKAP12α expression is highly dependent on SRF, because RNA interference knockdown of SRF severely attenuates endogenous AKAP12α expression. SRF binds with varying affinity to two CARG boxes in the AKAP12α promoter, and both CARG sites are required for full regulation by SRF. Unexpectedly, AKAP12α is refractory to growth- (serum) and differentiation-inducing (MYOCD) stimuli, suggesting the existence of a unique class of SRF target genes. Extensive analysis of each AKAP12α CARG element and its immediate flanking sequences reveals an inherent shielding of the AKAP12α promoter from both growth factor- and muscle/cytoskeletal-inducing cues. Collectively, our data demonstrate that AKAP12α belongs to a novel class of ubiquitously expressed SRF target genes whose basal expression is regulated by SRF. Furthermore, we provide new insight into the role of flanking sequences in determining sensitivity to SRF-MYOCD activity.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—NIH-3T3 (3T3) and A7r5 smooth muscle cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS), 100 units/ml penicillin, 100 µg/ml streptomycin, and 250 ng/ml amphotericin B. Rat-2 fibroblasts were maintained in DMEM supplemented with 10% FBS. All of the cells were grown at 37 °C in a humidified incubator.

**Plasmids**—The AKAP12α promoter-luciferase reporter plasmids were as recently described (28). Mutation of the two AKAP12α CARG boxes was performed using a QuikChange site-directed mutagenesis kit (Stratagene) as per the manufacturer’s directions, the −1034 AKAP12α luciferase construct followed the wild-type primers: CARG1mutFv, 5'-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT
oligonucleotides were radio end-labeled using T4 polynucleotide kinase (New England Biolabs). The gel shifts were then performed essentially as described previously (30).

RESULTS

AKAP12α Is a New SRF Target Gene—The AKAP12 gene encodes three transcriptionally distinct isoforms, designated α, β, and γ, to reflect both the timeline of their discovery and their position within the AKAP12 locus (28). These three isoforms have distinct subcellular distribution and expression profiles, with the latter property presumably caused by differences within the regulatory regions or promoters of the isoforms. Because identifying factors that regulate AKAP12 expression could provide a foundational step toward identifying unique roles for each isoform, we performed an in-depth survey of potential transcription factor-binding sites within the promoters of the major AKAP12 isoforms, AKAP12α and AKAP12β.

Consistent with our original foundational study of AKAP12 localization, we identified several GC box and E box elements in both the α and β promoters. Because we previously demonstrated that similar transcriptional factors, such as USF-1, can bind to elements found in both promoters (28), we chose to focus our search on sites found in only one of the promoters. One of the sites identified in this fashion was a CarG box, located at −502 from the start site of AKAP12α transcription (GenBank™ accession number AY695059), that could potentially serve as a binding site for SRF.

Both SRF and AKAP12α are ubiquitously expressed, suggesting that SRF may be involved in AKAP12α regulation. Because no CarG boxes were found in the AKAP12β promoter, we chose to analyze this site further in the hope of gaining new insight in the regulation of AKAP12α.

To determine whether AKAP12α is regulated by SRF, we tested the ability of a constitutively active form of SRF, SRF-VP16, to regulate activity of a large AKAP12α promoter construct encompassing this consensus CarG element. SRF-VP16 activates both a control serum response element (SRE) reporter derived from the c-fos promoter and the AKAP12α promoter (Fig. 1A) but has no effect on a control construct lacking a CarG box (data not shown). To confirm that SRF regulates AKAP12α in its native context, we generated an adenovirus, named shSRF, expressing a double-stranded hairpin targeted against SRF under the control of the U6 promoter, to knock down endogenous SRF expression. Transduction of A7r5 smooth muscle cells with shSRF reduces SRF protein levels by over 90% (data not shown). In contrast, a similarly constructed adenovirus, shGFP, expressing a hairpin targeting enhanced green fluorescent protein, does not affect SRF expression (data not shown).2

Knockdown of SRF expression plateaus at 3 days post-transduction.

Fig. 2. The AKAP12α promoter contains two conserved CarG boxes. A, 3T3 cells were transfected with the indicated AKAP12α-Luc reporters and either pEMSV (black bars) or pEMSV-SRF-VP16 (gray bars). Unlike in Fig. 1, these data are not normalized to the EMSV controls and instead show the relative activity of both the control and SRF-VP16 stimulated reporters. B, nucleotide alignment of the AKAP12α CarG boxes and their flanking sequence from human, dog, rat, and mouse shows few sequence differences between these four species. Sequence for the AKAP12α promoters from these species was obtained through the University of California, Santa Cruz genome browser (genome.ucsc.edu).
duction and persists for at least 4 more days (data not shown). We analyzed the expression of AKAP12α by semi-quantitative reverse transcription-PCR at two points after SRF expression is reduced. Knockdown of SRF results in a substantial reduction of AKAP12α expression at both times tested (Fig. 1B). This effect is specific, because expression of the SRF-independent tubulin gene, TUBA1, was not changed (Fig. 1B). Together with the promoter analysis, these data indicate that SRF is a key regulator of AKAP12α expression, thus making it a new SRF target gene.

A Second Nonconsensus CArG Box in the AKAP12α Promoter—To determine whether SRF regulates AKAP12α through the −502 CArG box, we used several promoter deletion constructs to identify the SRF-responsive region of the AKAP12α promoter. Consistent with our above data, SRF-VP16 increases activity of both a control SRE reporter and a large AKAP12α promoter construct (Fig. 2A). Surprisingly, however, a construct lacking the region between nucleotides −1034 and −174 that contains the consensus CArG box is only slightly less activated by SRF-VP16 (Fig. 2A). Further deletion of the AKAP12α promoter indicates that the region from nucleotides −71 to −31 is the main SRF-responsive region, because deletion of this region abolished induction by SRF-VP16 (see −30 LUC in Fig. 2A).

Although our initial scan of the AKAP12α promoter identified only one consensus CArG box, our deletion analysis indicates that a second SRF-responsive site exists in the AKAP12α promoter. When we re-examined the sequence of the SRF-responsive region spanning nucleotides −71 to −31, we identified a CArG-like motif at −32 that differs from the consensus at the 5′ nucleotide position (Fig. 2B). Based on their position relative to the transcriptional start site, we refer hereafter to the nonconsensus CArG as CArG1 and the consensus CArG as CArG2. The newly identified CArG1 is flanked at the 5′ end by a polythymidine tract and at the 3′ end by the transcriptional start site.

Both CArG Boxes Bind SRF with Varying Affinity—Both CArG boxes are well conserved across multiple species (Fig. 3B). These results are consistent with data from another study (30) and likely reflect the two CArGs differential binding affinities for SRF. Interestingly, these data contrast with our demonstration in Fig. 2, where CArG1 appeared to be more critical. This discrepancy likely reflects the effect of additional promoter sequence on the ability of CArG1 to mediate response to SRF. Despite the different contributions of the two AKAP12α CArG boxes, both appear to be necessary for full SRF regulation. The construct with both CArG boxes mutated has the largest reduction of basal activity, indicating that although CArG1 may only contribute modestly to SRF regulation of the AKAP12α promoter, it is still required for full regulation (Fig. 4B). Consistent with this finding, the dual mutant construct also was the least responsive to regulation by SRF-VP16 (Fig. 4C). The two AKAP12α CArG boxes are thus essential for SRF-dependent AKAP12α promoter activity.

**Fig. 3.** SRF can bind to both AKAP12α CArG boxes. Incubation of radiolabeled oligonucleotides encompassing either AKAP12α CArG1 (near) or CArG2 (far) with in vitro translated SRF results in the formation of a large nucleoprotein complex. The different binding capacities of the two CArG boxes are apparent in this exposure. Both SRF-CArG complexes are supershifted with SRF antibody, indicating that the complex contains a protein immunoreactive for SRF. Oligonucleotides containing CArG mutations (Mut Comp) used to examine the role of each CArG in AKAP12α expression (see Fig. 4A) are unable to bind SRF (at 100× molar concentration), as indicated by their inability to compete with the radiolabeled probe for SRF. Cold competitor probes, however (Comp), effectively reduce the nucleoprotein complexes.

**SRF Does Not Regulate AKAP12α Expression in Response to Growth Stimuli—**Because SRF is a central regulator of AKAP12α expression (Fig. 1B), we sought to determine what aspects of AKAP12α expression are regulated by SRF. AKAP12α has been shown to be regulated by many growth promoting stimuli, such as angiotensin (32) and serum stimulation (29). Because these signals also activate SRF-dependent gene expression (2), we postulated that SRF may mediate AKAP12α expression.
stimulation by these factors. As a first step toward addressing this notion, we needed to identify which AKAP12 isoform is regulated by serum stimulation. Counter to our expectation, expression of the SRF-regulated AKAP12α isoform is not increased by serum stimulation (Fig. 5A). Instead, expression of the AKAP12β isoform, which lacks any discernable CArG box, is increased following serum stimulation in a manner consistent with other early growth-related genes (Fig. 5A). AKAP12γ was not analyzed because it is solely expressed in cells of the spermatid lineage (28).

Our finding that AKAP12α is not regulated by SRF is surprising in light of the contributions of the two CArG boxes in its promoter. We thus sought to independently confirm that AKAP12α is not regulated by serum and rule out potential cell line artifacts. Because serum can nonspecifically activate the tk-RL plasmid that we use as an internal control, we used constitutively active (CA) MEK1 construct to mimic growth stimulation. CA-MEK1 activates MAPK signaling, which ultimately increases SRF activity. Activation of this pathway stimulates activity of the SRE control reporter (Fig. 5B). However, consistent with our Northern analysis, activity of the AKAP12α promoter remained unchanged in the presence of CA-MEK1 (Fig. 5B). Together, these data indicate that AKAP12α is not the AKAP12 isoform regulated by growth stimulation. Moreover, our data suggest that AKAP12α is not a member of the class of growth-related SRF target genes.

**AKAP12α Is Not Regulated by Cytoskeletal or Muscle-restricted SRF Activation**—Because AKAP12α does not appear to belong to the growth-related class of SRF-dependent genes, we postulated that it might belong to the muscle/cytoskeletal class of SRF target genes. Given the broad expression pattern of AKAP12α, this classification appeared unlikely to be validated because the majority of these genes have highly restricted expression profiles. However, as AKAP12 has been implicated in regulation of cytoskeletal architecture (29), a plausible fit within this set could be justified because SRF activity is regulated in part by actin dynamics (33). To determine whether actin cytoskeletal dynamics affect AKAP12α expression, we tested the effect of jasplakinolide (Jasp) on the activity of the AKAP12α promoter. Jasp promotes actin filament formation and depletes the G-actin stores of the cell, leading to downstream activation of SRF (33). Activation of SRF by G-actin depletion with Jasp results in elevated SM22α promoter (34) (Fig. 6A). In contrast, Jasp treatment had no affect on the AKAP12α promoter, suggesting that this promoter is not responsive to cytoskeletal dynamics (Fig. 6A).

As with our serum stimulation experiments, we sought to independently confirm that AKAP12α is not regulated as other cytoskeletal/muscle-restricted SRF genes. Signaling from the actin cytoskeleton to SRF is mediated in part by MRTF-A (also known as MKL1 or MAL) (35, 36). MRTF-A is one of three members of the MYOCD family of SRF co-factors that have recently been identified (37–39). Because the precise roles of
MYOC, MRTF-A, and MRTF-B are still under investigation, we tested the ability of each of these co-activators to stimulate the AKAP12α promoter. As with Jasp, each of these factors activated the SM22α promoter but had no effect on the AKAP12α promoter (Fig. 6B and data not shown). These experiments indicate that AKAP12α is not a member of the class of cytoskeletal/muscle-restricted SRF-dependent genes.

**Sequences Flanking the AKAP12α CArG Boxes Lack Specifying Information for Sensitivity to Growth Stimuli**—Our data indicate that AKAP12α does not belong to either of the two broad sets of SRF-dependent genes. Instead, our data demonstrate that AKAP12α is a founding member of a novel third set of ubiquitously expressed SRF-dependent genes whose basal expression is dependent on SRF regulation. To determine how SRF can distinguish the AKAP12α promoter from the other types of SRF-dependent promoters, we first examined whether the CArG boxes and flanking sequence were sufficient to confer specificity with respect to growth factor stimulation. For this, we cloned a minimal tk promoter in front of either Me2SO (black bars) or jasplakinolide (100 nM; gray bars) for 4 h to deplete G-actin stores. The activity of the each reporter construct was normalized to the average activity of the appropriate Me2SO control to determine fold induction. B, myocardin does not enhance AKAP12α promoter activity. 3T3 cells were co-transfected with the indicated reporters and either pCDNA3.1 (black bars) or pCDNA3.1-mouse Myocardin (gray bars). The activity of the each reporter construct was normalized to the average activity of the appropriate pCDNA3.1 control to determine fold induction.

Moreover, this activation was dependent on the Ets site because mutation of this site abolishes response to CA-MEK1 (Fig. 7C, F5M-C1). These results suggest that the 5′-flanking sequence of CArG1 in AKAP12α does not contain the appropriate sequence information necessary to mediate responses to growth stimuli.

To determine whether this situation also exists with CArG2, we constructed a similar series of chimeric reporters (Fig. 7D). As before, the sequence of the CArG box was not an important determinant of sensitivity to growth stimuli (Fig. 7E, C2-F). Replacement of the 5′-flanking sequence of CArG2 with the corresponding c-fos flanking sequence, however, results in sensitivity to CA-MEK1 (Fig. 7E, F5-C2). Again, the Ets-binding site was necessary for responsiveness to CA-MEK1 (Fig. 7E, F5M-C2). Taken together, these data indicate that the AKAP12α promoter is refractory to growth stimuli because of flanking sequences surrounding each of its two conserved CArG boxes.

**The Type of CArG and Flanking Sequence Determines Sensitivity to MYOC—**Because the flanking sequences around the AKAP12α CArGs appear to insulate this promoter from growth factor stimuli, we sought to determine whether these sequences also controlled the insensitivity of AKAP12 to MYOC activation. Although the precise mechanism by which MYOC selectively activates a subset of SRF-dependent genes has yet to be fully elucidated, Olson and colleagues (23) have proposed an intriguing hypothesis positing the necessity for multiple CArG boxes. To determine whether MYOC can activate through a single CArG box and whether the AKAP12α CArGs confer specificity, we tested the ability of MYOC to stimulate activity of our native and chimeric reporters as outlined above. MYOC activates a reporter containing CArG near the SM22α promoter (40) (Fig. 8A). This indicates that a single CArG box is sufficient to sustain strong activation by MYOC. In contrast, the CArG boxes of AKAP12α were only weakly responsive to MYOC (Fig. 8A), suggesting that the insensitivity of the AKAP12α promoter to MYOC is inherent within each AKAP12α CArG sequence. To further assess this notion, we constructed multiple reporters in a fashion similar to that used to test responsiveness to growth stimuli in Fig. 7 to determine the specifying sequence responsible for responsiveness to MYOC (Fig. 8B). Compared with SM22α CArG near, the AKAP12α CArG1 reporter was insensitive to MYOC (Fig. 8C, S versus C1). When we replaced the nonconsensus CArG1 with SM22α CArG near, the resulting reporter was strongly activated by MYOC, suggesting that SM22α CArG near contains inherent sequence determinants for MYOC responsiveness (Fig. 8C, C1–S). To determine whether this was specific to
SM22a CArG near or was simply the result of converting a nonconsensus CArG into a consensus CArG, we mutated CArG1 to a consensus CArG box. This construct was just as responsive to MYOCD as SM22a CArG near (data not shown), indicating that a consensus CArG is required for a high level of responsiveness to MYOCD activation.

Additional specifying information is also contained within sequences flanking each AKAP12a-CArG box. For example, exchange of the CArG1 flanking sequences with that from SM22a CArG near results in elevated MYOCD activation, despite the presence of a nonconsensus CArG box (Fig. 8C, S-C1). Interestingly, exchange of only the 5' flanking sequence resulted in a greater level of activation (Fig. 8C, S5-C1). Because the converse exchange of only the 3' flanking sequence did not affect sensitivity (Fig. 8C, S3-C1), these results suggest that both 5' flanking sequence of the SM22a CArG near and 3' flanking sequence of CArG1 are responsive to MYOCD. However, under native conditions, the 3' flanking sequence of CArG1 is insufficient for MYOCD activation of CArG1. This indicates that both the flanking sequence and the type of CArG box are important determinants of sensitivity to MYOCD.
To validate these findings with respect to CArG2 in AKAP12α, we performed a similar series of experiments (Fig. 8D). The consensus CArG2 reporter is not as responsive as the SM22α CArG near reporter, indicating that, as with the consensus CArG of c-fos, the CArG2 of AKAP12α does not have appropriate specifying sequence for responsiveness to MYOCD (Fig. 8E). Exchange of the CArG boxes from these two constructs did not influence outcome (Fig. 8E, C2-S), indicating that a high level responsiveness to MYOCD is not conferred solely by a consensus CArG; rather, surrounding sequences appear to play a critical accessory role in mediating the activation potential of MYOCD. Accordingly, when we exchange the flanking sequences, CArG2 becomes nearly as responsive to MYOCD as the SM22α reporter (Fig. 8E, S-C2). This responsiveness remains when we exchange only the 5′-flanking sequence (Fig. 8E, S5-C2) and is lost when we exchange only the 3′-flanking sequence (Fig. 8E, S3-C2). Taken in aggregate, the data are consistent with the notion that the lack of sensitivity of the promoter of AKAP12α to MYOCD is conferred by sequences flanking its two CArG boxes.
DISCUSSION

We recently characterized the AKAP12 gene to establish a foundation for future investigation of the regulation of AKAP12 expression (28). In continuation of this endeavor, we have here characterized the regulation of the ubiquitously expressed α isoform of AKAP12 by SRF. SRF regulates AKAP12α expression through the AKAP12α promoter and is required for normal AKAP12α expression in cultured cells. Two highly conserved CArG boxes were identified in the AKAP12α promoter, and both mediate SRF regulation despite clear differences in their affinity for SRF binding. Additionally, the sequences flanking these binding sites were found to be important determinants in specifying the response to SRF-regulating signals and co-activators. Our study demonstrates that AKAP12α is a novel SRF-dependent gene that is regulated in a manner distinct from all other SRF-dependent genes characterized to date. Specifically, it appears that AKAP12α is a prototypic member of a new class of ubiquitously expressed SRF-dependent genes that are shielded from known SRF-activating signals. We therefore propose that AKAP12α be categorized as an atypical SRF-dependent target gene. As of this writing, AKAP12α brings the total number of functionally validated SRF target genes to 62.3

The AKAP12 gene encodes three transcriptionally distinct isoforms (α, β, and γ) with different physical properties (28). This genetic regulatory mechanism affords independent regulation of the three isoforms, allowing them to be utilized in unique functions. In the present study, we have shown that AKAP12α is specifically regulated by SRF. The promoter of the other major AKAP12 isoform, AKAP12β, does not contain any discernable SRF binding sites and is not regulated by SRF-VP16 (data not shown), confirming that the differential expression of AKAP12 isoforms is indeed conferred by their respective promoters. Interestingly, AKAP12β, not AKAP12α, is the AKAP12 isoform selectively regulated by growth stimulation (Fig. 5A). Because these two AKAP12 isoforms have different subcellular distributions (28), the findings highlight the importance of recognizing the isoforms as distinct entities when attempting to elucidate functional roles for AKAP12.

The primary function of the AKAPs is to target PKA and other signaling enzymes to discrete subcellular compartments to facilitate correct spatial positioning between signaling components and their substrates (41). AKAP12α is localized to the endoplasmic reticulum via an N-terminal myristoylation motif (28). Despite much interest in the cellular function of AKAP12, no downstream targets have been identified to date. Thus it is unclear what role AKAP12α performs at this locale. In addition, SRF, to our knowledge, has not been previously associated with regulation of an ER-targeted gene. Although future work will be necessary to characterize AKAP12α function at the ER, it is likely that AKAP12α is involved in one of the many aspects of ER biology, such as protein synthesis and trafficking. Because proper functioning of the ER is essential to cell viability, defects in AKAP12α expression may in part underlie the lethal phenotypes of SRF knockout animals (9–11).

SRF directs disparate programs of gene expression through a toggle switch that involves its interaction with many associated co-factors. The composition of the SRF transcriptional complex of each gene set is defined by the inherent sequence of the CArG box itself as well as sequences adjacent to the CArG element. Although this mechanism has previously been recognized for SRF regulation of muscle-restricted genes such as SM22α (40, 42) and growth-related genes such as c-fos (12, 13), our data extend this phenomenon to an atypical SRF-dependent target gene. For example, although MYOCD was proposed to specifically activate genes with multiple CArG boxes, we were able to not only demonstrate that a single CArG could be robustly activated by MYOCD but could convert a MYOCD-insensitive CArG box into a responsive CArG box by exchanging sequences flanking the CArG. Because there are known contact sites for SRF binding immediately adjacent to the CArG box (43), sequence changes altering the ability of SRF to bind CArG may be expected to have a corresponding effect on the activation potential of MYOCD. In this context, recent studies on the SM22α promoter have revealed an Ets-binding site flanking a CArG box which, when mutated, resulted in heightened responsiveness to MYOCD (44).

It is unclear at this time how MYOCD and, by extension, the MRTFs recognize specific flanking sequences. Although it has been reported that MYOCD does not bind DNA (19), the ability of MYOCD and the MRTFs to bind DNA sequences has not yet been thoroughly examined at the atomic level. Aside from directly recruiting MYOCD via SRF binding, CArG flanking sequences may alternatively direct binding of other SRF co-activators that in turn may recruit or stabilize the association of MYOCD with the SRF scaffold. Although recent work suggests that this may indeed be the case for some SRF-dependent genes (45), the ability of MYOCD to associate with CArG-bound SRF in the absence of other co-factors (19) argues against this scenario. Another possible mechanism is that the flanking sequence of MYOCD-sensitive CArG boxes regulates the structural conformation of bound SRF in a manner that exposes the MYOCD-binding site on SRF. A similar conformational change has been proposed to occur when SAP-1 binds to the Ets site flanking the c-fos CArG (46). Given the phenominal diversity in CArG sequences (1,216-fold) and the likely greater variety of flanking sequences around all functional CArG elements, it will be a major challenge to fully elucidate rules governing how inherent sequences specify the responsiveness of any given CArG element to the array of signaling pathways known to regulate SRF-dependent gene expression. High throughput structural biological studies will certainly be an important approach to this daunting task.

Because the flanking sequence appears to dictate the nature of SRF-mediated regulation, further study will be necessary to examine its role in basal regulation of the AKAP12α gene and other newly identified SRF-dependent genes. More generally, it will be necessary to determine how flanking sequence regulates recruitment of SRF co-factors that ultimately determine the nature of SRF regulation. To that end, identification and definition of flanking sequence signatures will be needed to establish recognition of distinct SRF-dependent gene sets whose products define functional cellular systems. This will be particularly important in high throughput in silico and wet lab screens for SRF target genes. Indeed, we have recently identified and begun characterizing over 100 SRF target genes using a combination of these approaches.4 Identification and characterization of the entire CArGome will ultimately yield a rich source of insight into SRF function in both biology and disease.

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3 An updated SRF target gene list is available upon request.

4 J. W. Streb and J. M. Miano, manuscript in preparation.
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Jeffrey W. Streb and Joseph M. Miano

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