Expression of the Serum Response Factor Gene Is Regulated by Serum Response Factor Binding Sites*

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The serum response factor (SRF) is a ubiquitous transcription factor that plays a central role in the transcriptional response of mammalian cells to a variety of extracellular signals. Notably, SRF has been found to be a key regulator of members of a class of cellular response genes termed immediate-early genes (IEGs), many of which are believed to be involved in regulating cell growth and differentiation. The mechanism by which SRF activates transcription of IEGs in response to mitogenic agents has been extensively studied. Significantly less is known about how expression of the SRF gene itself is mediated. We and others have previously shown that the SRF gene is itself transiently induced by a variety of mitogenic agents and belongs to a class of "delayed" early response genes. We have cloned the SRF promoter and in the present study have analyzed the upstream regulatory sequences involved in mediating serum responsiveness of the SRF gene. Our analysis indicates that inducible SRF expression requires both SRF binding sites located within the first 63 nucleotides upstream from the start site of transcriptional initiation and an Sp1 site located 83 nucleotides upstream from the start site. Maximal transcriptional activity of the promoter also requires two CCAATT box sites located 90 and 123 nucleotides upstream of the start site.

SRF is a ubiquitous transcription factor that is a key regulator of many extracellular signal-regulated genes important for cell growth and differentiation. SRF was first identified as a critical factor involved in mediating serum and growth factor-induced transcriptional activation of the c-fos proto-oncogene (reviewed in Ref. 1). The importance of SRF for growth factor-regulated transcription is suggested by the identification of SRF binding sites (serum response elements) within the regulatory region of many other transiently expressed serum-inducible genes. These genes, which can be induced in the absence of new protein synthesis, have been termed cellular immediate-early genes (IEGs). They include new protein synthesis, have been termed cellular immediate-early genes. These genes, which can be induced in the absence of new protein synthesis, have been termed cellular immediate-early genes (IEGs) (2). They include krox-20/egr-2 (3), egr-1/zif-268/NGFI-A (4, 5, 6), cyr61 (7), pip92 (8), and members of the actin gene family (2). SRF has also been implicated in mediating IEG transcription in response to a variety of other agents, including agents that elevate intracellular calcium levels (9); viral activator proteins, such as the human T-cell lymphotropic virus type-1 activator protein Tax-1 (10, 11) and the hepatitis B virus activator protein pX (12); activated oncogenes including v-src (13, 14), v-fps (15), v-ras (16, 17, 18), and the activated proto-oncogene c-raf (19, 20) as well as extracellular stimuli such as antioxidants (21), UV light (22), and microgravity (23).

In addition to its role in mediating activation of genes expressed at early times after stimulation, some studies also suggest that SRF is involved in regulating later events, such as differentiation and cell cycle progression, presumably by regulating expression of key late response genes. Microinjection of anti-SRF antibodies blocks progression of stimulated fibroblasts from G1 to S phase (24), suggesting that SRF or an SRF-related factor is important for controlling cell cycle progression. This function of SRF may be conserved through evolution, since genetic analysis has revealed that the yeast SRF homolog, MCM1, is involved in cell cycle progression (25). The observation that in yeast, MCM1 binding sites are found in the promoters of the cyclin genes cn3 and dn2, and the gene for a cyclin interacting factor FAR1 (26), raises the possibility that SRF or a SRF-related factor may perform a similar function in mammalian cells.

Other microinjection studies suggest that SRF is also important for differentiation in two myoblast lines, mouse C2 and rat L6. These studies show that SRF antibodies lead to down-regulation of myogenin expression and block differentiation of myoblasts to myotubes (27), suggesting that SRF or SRF-related factors directly or indirectly regulate muscle-specific transcription factors important for conferring the myogenic phenotype. Additional support for SRF playing a role in development of the myogenic phenotype comes from numerous studies that have identified SRF binding sites in the promoters of a number of muscle-specific genes. These include the cardiac and skeletal muscle actin (28–30), dystrophin (31), myosin light chain (32), atrial natriuretic factor (33), and creatine kinase M promoters (34). In the case of the skeletal and cardiac actin and the dystrophin genes, SRF binding sites have been found to act as positive tissue-specific promoter elements.

While the role of SRF in tissue-specific gene expression is unclear, it has been suggested that SRF may interact with other transcription factors to confer tissue-specific expression. One model for how SRF can mediate disparate phenotypic consequences suggests that SRF interacts with different classes of cell type-specific accessory proteins to confer distinct phenotypic responses (35). Consistent with this hypothesis, SRF has been shown to interact with different classes of factors including the homeodomain protein Phox-1 (35) and a class of transcriptional activator proteins known as ternary complex factors (TCFs) that are members of the Elk-1 subfamily of the
ETS family of oncoproteins (reviewed in Ref. 36). It has also recently been reported that SRF and SRF-related proteins can interact through their conserved DNA binding/dimerization domain with myogenic basic helix-loop-helix proteins (37, 38).

In the case of SRF-mediated activation of IEGs, extensive studies of c-fos gene expression in fibroblasts indicate that in response to serum stimulation, SRF mediates gene activation by at least two distinct mechanisms (39). In one case, activation of the p21^{ras} signaling pathway leads to modification and subsequent activation of the TCF family of SRF-associated factors, thereby activating transcription. In a second, less well characterized SRF-dependent pathway, stimulation of cells can activate expression by a pathway that is dependent on members of the Rho subfamily of Ras proteins. This second pathway occurs in a TCF-independent manner. In both the TCF-dependent and -independent pathways, activation can occur in the absence of new protein synthesis and therefore relies on preexisting SRF protein.

While much is known about how SRF activates expression of IEGs such as c-fos, little is known about how SRF regulates genes involved in later responses. One possibility is that newly expressed SRF protein may be involved. To begin to address how SRF may be involved in regulating late responses, we have studied expression of the SRF gene and protein. In previous studies (40) we and others (41) found that the SRF gene is itself an IEG since its transcription can be induced in the absence of new protein synthesis. In response to serum and purified growth factors, peak expression of SRF mRNA occurs at 90–120 min after stimulation. The expression of SRF protein closely follows RNA expression. Unlike many IEG protein products SRF protein is relatively stable, having an in vivo half-life of 12–16 h (40). The stability of the SRF protein accounts for the apparent paradox that SRF protein is present prior to induction of the gene. In addition, the newly synthesized protein is extensively post-translationally modified by phosphorylation throughout the course of the cell cycle, raising the possibility that these modifications may be involved in regulating SRF’s ability to control expression of late acting genes (40).

The time of appearance of peak SRF mRNA levels suggests that the SRF gene belongs to a class of IEGs whose expression is delayed relative to other well characterized early IEGs such as c-fos, little is known about how SRF regulates genes involved in later responses. One possibility is that newly expressed SRF protein may be involved. To begin to address how SRF may be involved in regulating late responses, we have studied expression of the SRF gene and protein. In previous studies (40) we and others (41) found that the SRF gene is itself an IEG since its transcription can be induced in the absence of new protein synthesis. In response to serum and purified growth factors, peak expression of SRF mRNA occurs at 90–120 min after stimulation. The expression of SRF protein closely follows RNA expression. Unlike many IEG protein products SRF protein is relatively stable, having an in vivo half-life of 12–16 h (40). The stability of the SRF protein accounts for the apparent paradox that SRF protein is present prior to induction of the gene. In addition, the newly synthesized protein is extensively post-translationally modified by phosphorylation throughout the course of the cell cycle, raising the possibility that these modifications may be involved in regulating SRF’s ability to control expression of late acting genes (40).

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reactions using nuclear extracts, 6 μg of protein was used and incubation times were doubled. Conditions for the antibody supershift experiments were identical to the shiftsof nuclear extract with the exception that a 1:50 dilution of anti-SRF antibody R1122 (described in Ref. 40) was added to the shift reactions 10 min prior to electrophoresis.

Mutagenesis—Site-directed mutations were introduced in context in the SRF promoter by the technique of Deng and Nickoloff (48). The template for mutagenesis reactions was region 232 to 1229 of the SRF gene in a pUC19 background. The specific base changes were chosen based on their ability to disrupt factor binding in vitro as documented in the following references: CArG box 1, CCATAAAAGG to CCATAAAATT (this work); CArG box 2, CCATATAAGG to CCATATAATT (this work); SP1/254, GGGCGGG to GGTCTGG (49); SP1/83, GGGCGGGGGCCG to GGGGCTTTGGCG (49); CCAATCCAAT to AAAAT (50).

Combinations of mutations were generated either by performing mutagenesis reactions on a template already containing a mutation or by subcloning DNA fragments containing the appropriate mutation.

RESULTS

Structure of the SRF Promoter—To isolate sequences corresponding to the mouse SRF gene we used a 305-base pair restriction fragment, derived from the human SRF cDNA clone pTR7ΔATG (41), to screen a mouse genomic library. This probe contained sequences derived from the 5' region of a human SRF cDNA clone. Restriction analysis of the products of this screen identified three λ clones that contained overlapping sequences (data not shown). One clone containing a 5-kb fragment of murine genomic DNA was picked for further analysis. To verify that this clone contained the gene encoding the SRF protein and not a family member, a portion was sequenced and compared with the sequence of a partial mouse SRF cDNA clone. The mouse SRF cDNA clone was previously shown to encode a functional SRF protein and to have high homology to human SRF cDNA. This comparison revealed the λ clone to be 100% homologous to the mouse cDNA over the region corresponding to SRF sequences coding for the first 228 amino acids (data not shown). In addition, the presence of an intron (>1 kb) between the codons for amino acids 167 and 168 was noted.

To determine whether the clone we had isolated contained SRF promoter regulatory sequences, the region of the clone 5' to the protein-coding sequence was sequenced and compared with a full-length human cDNA in which the start site of transcription had been previously mapped (51). As shown in Fig. 1, there was 93% identity at the nucleotide level between the human cDNA and the mouse genomic clone over this region and 97% identity over the 100 nucleotides immediately 3' to the start site of transcription of the human gene. Based on this analysis, the start site of transcriptional initiation in the mu-

FIG. 1. Nucleotide sequence of the mouse SRF promoter and 5'-untranslated regions. Boldface and underlined sequences include two CArG boxes, two CCAAT boxes, two SP1 sites, and one high affinity Ets binding site. The lower sequence corresponds to the 5'-untranslated region of the human SRF cDNA (51). The vertical lines indicate sequence identity, and the dots correspond to gaps inserted in the sequence for optimal alignment.
rine clone was assigned, and the 5-kb genomic fragment was determined to contain 1 kb of SRF coding sequence and 4 kb of sequence 5' to the start site of transcriptional initiation. To measure serum responsiveness of the SRF promoter, a 2.7-kb restriction fragment spanning 2500 to 1229 relative to the start site of transcriptional initiation was then isolated from the 5-kb fragment and inserted into a luciferase reporter construct.

Serum-inducible Expression Is Mediated through the SRF Promoter—Previously we have shown that the SRF gene is transiently induced when serum-starved NIH3T3 cells are treated with serum (40) or purified growth factors.3 In unstimulated serum-starved NIH3T3 cells SRF mRNA levels are virtually undetectable. SRF mRNA reaches a maximum by 90–120 min after stimulation of cells with 20% fetal calf serum and then returns to nearly basal levels by 6 h after stimulation. To determine the regulatory elements required for transcriptional activation of the SRF gene, progressive 5' promoter deletion constructs containing different amounts of the SRF promoter and 229 nucleotides of the SRF 5'-untranslated region were fused to a luciferase reporter gene. These constructs, schematically depicted in Fig. 2A, were transiently transfected into NIH3T3 cells, and luciferase activity was measured after serum starvation or 2 h after serum stimulation of starved cells. To normalize for transfection efficiency, each construct was co-transfected with a constitutively expressed Rous sarcoma virus-β-galactosidase reporter. The results of one typical set of experiments are shown in Fig. 2B. Upon serum stimulation of cells containing a reporter with 2500 nucleotides of upstream sequence, there is an approximately 5-fold increase in luciferase activity relative to the unstimulated cells. Roughly the same 5-fold stimulation is observed for constructs in which all but 111 nucleotides of upstream sequence have been deleted. In contrast, a construct with 35 nucleotides of upstream sequence, containing only the SRF TATA element, is stimulated 1.3-fold. These results indicate that the major sequence determinants of serum responsiveness in the SRF promoter reside between 35 and 111 nucleotides upstream from the start site of transcription.

The results presented in Fig. 2 indicate that sequences required for maximal serum-stimulated expression of the SRF gene are present within the first 322 nucleotides upstream of the start site of transcription. Computer analysis of the sequence of this region identified a number of potential regulatory elements that are in boldface type and underlined in Fig. 1. Most notably, two CArG box elements are located within 60 nucleotides of the start site of transcription. In addition, there are potential Sp1 binding sites at −83 and −254, an Ets bind-

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3 J. A. Spencer and R. P. Misra, unpublished results.
ing motif at $-103$, and two CCAAT box elements located at $-90$ and $-123$.

A CARG Box and the $-83$ Sp1 Binding Site Are Major Determinants That Mediate Serum Induction of the SRF Promoter—To begin to determine which of the potential regulatory elements located in the $-35$ to $-322$ region mediate serum responsiveness of the SRF promoter, we mutated the Sp1, CCAAT box, or CARG box elements, either alone or in various combinations and then measured luciferase activity before and after serum stimulation. Point mutations of each putative regulatory element, which abolish factor binding, were introduced into the indicated elements in the context of the wild type $-322$ reporter construct (Fig. 3A), and their effects on expression were tested in transient transfection assays. As seen in Fig. 3B, the most dramatic effect on serum responsiveness occurred when both CARG boxes were simultaneously mutated or when the $-83$ Sp1 site was mutated.

Induction of the double CARG mutant was reduced from 5-fold for the wild type $-322$ construct to 1.3-fold for the mutant. This value was similar to the induction of the $-35$ TATA-only minimal construct. Mutation of either CARG box alone had either no effect (CARG box 1), or a modest 25% reduction in responsiveness (CARG box 2). Since as shown below, the responsiveness of the individual CARG boxes correlates with their relative affinity for SRF, these results suggest that they may serve redundant functions during serum stimulation of the SRF gene.

Similarly, mutation of the Sp1 site at $-83$ diminished serum responsiveness to a level comparable with that of the double CARG box mutant, reducing the induction to 1.5-fold. This effect appears to be dependent on the distance of an Sp1 site from the start site of transcription since mutation of the Sp1

**FIG. 3.** Functional analysis of SRF promoter elements. A, a schematic representation of reporter constructs used. In each case, site-specific mutations that disrupted the indicated binding sites (see "Experimental Procedures") were introduced in context into SRF-luciferase reporter constructs containing 322 nucleotides of sequence upstream of the transcriptional start site. B, luciferase assays were carried out on extracts from NIH3T3 cells transiently transfected with the indicated reporter constructs and either serum-starved (−) or serum-stimulated (+) for 2 h. Basal expression refers to the level of luciferase activity in unstimulated cells transfected with the indicated reporter construct. The -fold induction is determined for each construct by comparing the luciferase activity in the stimulated and unstimulated case. The basal expression of the $-322$ construct containing wild type elements is arbitrarily assigned a value of 100%. Luciferase activity is reported as relative light units (RLU). For each point, values were determined in triplicate and corrected for transfection efficiency. Results from at least three independent experiments are shown (means ± S.E.).
site located at −254 has significantly less effect on serum-stimulated expression of the −322 construct, reducing activation approximately 33% from 5- to 3.2-fold. In contrast to the effect of the CArG box or Sp1 site mutants, mutations in either one or both CCAAT boxes located at −90 and −123 have virtually no effect on the fold induction of the −322 construct. Together, these results suggest that the factors that bind the −83 Sp1 site and the CArG boxes are together responsible for mediating serum responsiveness of the SRF promoter.

Transcriptional Efficiency of the SRF Promoter Is Regulated by the −90 and −123 CCAAT Boxes—As seen in Fig. 2, while the fold induction of both the −322 and −111 constructs are similar upon serum stimulation, the overall transcription efficiency of the −111 construct is dramatically reduced. This effect is observed for both unstimulated and stimulated samples, suggesting that elements contained between −111 and −322 are important for basal transcription. A likely candidate for one element is the CCAAT box located at −123. Mutation of this site leads to a 60% reduction in the expression of the −322 construct in unstimulated cells. When the −90 CCAAT box is mutated there is a 30% reduction in expression. An even more dramatic effect is observed when both the −90 and −123 elements are mutated. Expression from the double mutant is reduced to 13% of wild type levels in unstimulated cells, comparable with expression of the −111 construct. These results suggest that two CCAAT boxes are required for maximal transcriptional efficiency of the SRF promoter.

CArG Box 1 and CArG Box 2 Bind SRF—It has previously been shown that CArG box-containing elements can mediate serum-stimulated gene expression by an SRF-dependent mechanism. In the case of the c-fos SRE, which is the most extensively studied CArG box-containing element, a CArG box is flanked on either side by regions of imperfect dyad symmetry (52). In fibroblasts, SRF appears to be the major c-fos CArG box binding factor, although at least eight other transcription factors have been shown to interact with the c-fos SRE in vitro (53, 54). Mutations in the c-fos CArG box that abolish SRF binding also abolish serum responsiveness of the c-fos promoter, and minimal SRF binding sites are capable of imparting serum responsiveness to promoter minimal reporter plasmids (46). In the case of the c-fos promoter, these studies indicate that serum responsiveness requires SRF. In the case of the SRF promoter, the results in Fig. 3 indicate that a reporter containing mutant CArG boxes, incapable of binding SRF, is severely impaired in its serum responsiveness. This suggests that SRF is responsible for mediating the serum response. Therefore, we wanted to determine whether SRF was a major SRF CArG box binding protein in NIH3T3 nuclear extracts. To do this, we performed electrophoretic mobility shift assays using nuclear extracts prepared from either serum-starved or serum-stimulated NIH3T3 cells, and a 32P-labeled DNA probe corresponding to −165 to −134 of the SRF gene. Reactions were electrophoresed on a nondenaturing polyacrylamide gel. The positions of the free probe, two specific complexes (I and II), and the SRF-DNA complex whose migration is further retarded by interaction with anti-SRF antibodies (Ab/SRF/DNA) or left unaffected by the addition of preimmune sera (PI), were detected by autoradiography. Complexes I and II were competed with the SRF promoter DNA fragment but not with a nonspecific DNA fragment (not shown).

CArG box 2, binding assays using in vitro translated SRF were performed. In one experiment, radiolabeled wild type probe was competed with increasing concentrations of nonlabeled DNA containing either CArG box mutant. As seen in Fig. 5 both CArG boxes compete effectively with the wild type SRF promoter fragment for SRF binding. Under the binding conditions used here, however, CArG box 2 has an approximately 2-fold greater affinity for SRF than CArG box 1. In a second experiment, the relative affinity of CArG box 1 and CArG box 2 for SRF was determined by measuring their ability to compete against each other for SRF binding. In Fig. 5B, it can be seen that CArG box 2 competes approximately 2-fold more efficiently for binding to a CArG box 1 labeled probe than CArG box 1 competes for a CArG box 2 labeled probe (e.g., compare lanes 4 and 8). In this experiment, to ensure that the specific activities of both probes were identical, oligonucleotide primers were labeled and used in separate polymerase chain reaction reactions using templates containing either a CArG box 1 or CArG box 2 mutation. Identical amounts of radioactivity from each probe synthesis reaction were then added to each shift reaction. The relative binding was determined by directly comparing the differing amounts of shifted probe and was quantified by Phosphorlmager analysis.

Binding of SRF Simultaneously to CArG Box 1 and CArG Box 2 Is Inefficient—Mutant reporter constructs that contain only one functional CArG box are capable of responding to serum nearly as well as a wild type promoter containing two intact CArG boxes (Fig. 3). This suggests that in the case of serum stimulation, CArG boxes 1 and 2 perform redundant functions. This raises the possibility that SRF binding to each CArG box may be mutually exclusive. To determine whether SRF was capable of simultaneously binding both CArG boxes 1 and 2, in vitro translated SRF was complexed with probes mutated in either CArG box 1 or box 2, and the mobility of
SLOW MOTION COMPLEX IS LIKELY TO REFLECT INEFFICIENT BINDING OF SRF TO BOTH SITES, SINCE IT IS NOT OBSERVED WHEN AUTORADIOGRAPHS ARE EXPOSED FOR SHORTER PERIODS OF TIME, OR WHEN LESS SRF PROTEIN IS USED IN THE SHIFT REACTIONS (FIG. 5A, LINES 1, 5, AND 9). IN ADDITION, INCREASING THE AMOUNT OF SRF ADDED TO THE SHIFT REACTIONS DOES NOT CHANGE THE RATIO OF THE SLOWER TO FASTER MOBILITY COMPLEX. TOGETHER WITH THE OBSERVATION THAT CARG BOX 1 AND 2 DIFFER IN THEIR AFFINITY FOR SRF BY ONLY 2-FOLD, THIS SUGGESTS THAT FORMATION OF THE SLOWER MOBILITY COMPLEX IS NOT DEPENDENT ON FIRST SATURATING A HIGH AFFINITY SITE. THE POOR EFFICIENCY OF FORMATION OF THE SLOWER COMPLEX SUGGESTS THAT IN VITRO, ON A SINGLE PROBE MOLECULE, OCCUPANCY OF BOTH CARG BOXES BY SRF IS LARGELY MUTUALLY EXCLUSIVE.

**DISCUSSION**

In the present study, we have examined the promoter regulatory elements involved in mediating serum induction of the SRF gene. Our results show that a 111-nucleotide sequence immediately upstream of the SRF start site of transcriptional initiation is sufficient to confer serum responsiveness to a heterologous reporter gene. This region contains two CARG boxes and an Sp1 binding site. While –111 constructs exhibit serum responsiveness, maximal expression also requires additional sequences located between –111 and –322. This region does not affect -fold stimulation, suggesting that elements contained in this region are not targets for regulation by serum-stimulated signaling pathways. However, this region does appear to affect the transcriptional efficiency of the SRF promoter as evidenced by the elevated levels of both induced and basal transcription observed from the –322 reporter constructs. Our analysis also indicates that transcriptional efficiency is affected by two CCAAT box elements located 90 and 123 nucleotides upstream from the start site of transcriptional initiation. Disruption of one or both of these elements decreases overall transcription. Previously, it was shown that the CCAAT box binding factor NF-Y can facilitate in vivo recruitment of upstream factors in the HLA-DRA promoter (50). It has been proposed that CCAAT box factors may function to enhance transcription by stabilizing binding of upstream factors. Our observations are consistent with this role of CCAAT box binding factors in the SRF promoter.

What protein factors are required for mediating serum responsiveness of the SRF promoter in vivo? Our analysis indicates that at least one of two CARG box sequences located between –35 and –111 nucleotides is required for mediating serum stimulation of a luciferase reporter. CARG box 2 is identical to a serum and growth factor-responsive CARG box found in the zif-268 promoter (5). CARG box 1 and 13 of 15 flanking nucleotides match the reverse sequence of a chick β-actin promoter element (55). In vitro both CARG boxes bind SRF with similar affinities, yet binding to SRF appears to be mutually exclusive. Previously, it has been shown in in vitro protection analyses that SRF protects 10 nucleotides flanking either side of the c-fos SRE CARG box (52). Since CARG boxes 1 and 2 in the SRF promoter are separated by 10 base pairs, this raises the possibility that the inability of SRF to efficiently bind both CARG boxes simultaneously may be due to steric hindrance. Electrophoretic mobility shift assays using nuclear extracts from either serum-starved or serum-stimulated NH3T3 extracts, performed in the presence or absence of antibodies specific for SRF, suggests that SRF also binds CARG box 1 or 2 in vivo. Serum responsiveness of reporter constructs containing mutant CARG boxes, which are deficient for SRF binding in vitro, placed in their natural context in the wild-type promoter is drastically reduced. Taken together, these results indicate that SRF is likely to be a major regulator of SRF promoter serum responsiveness.

Studies of the mechanism by which SRF mediates inducible

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**Fig. 5.** A, SRF binds CARG boxes 1 and 2 with similar affinity. DNA mobility shift assays were carried out using in vitro translated SRF, a 32P-labeled DNA probe corresponding to –165 to +14 of the SRF gene, and the indicated molar excess of competitor DNA consisting of unlabeled promoter fragment containing point mutants in CARG box 1, CARG box 2, or both. Lanes 1–8 were exposed longer than lanes 9–13 to reveal the additional complex. B, binding of SRF simultaneously to CARG box 1 and CARG box 2 is inefficient. DNA mobility shift assays were carried out using in vitro translated SRF and a 32P-labeled DNA probe corresponding to –165 to +14 of the SRF gene containing a fragment containing mutant CARG box 1 or mutant CARG box 2 sequences. Each reaction also contained the indicated molar excess of competitor DNA consisting of unlabeled promoter fragment containing a mutation in the reciprocal CARG box. Mutant CARG boxes are represented by X-filled boxes and wild type by open boxes. Reactions were electrophoresed on a nondenaturing polyacrylamide gel. The positions of the free probe and the SRF-DNA complex were detected by autoradiography and subsequently quantified by PhosphorImager analysis. Unprogrammed reticulocyte lysates gave no observable complex (not shown).
transcription, carried out mainly on the c-fos promoter, have revealed that SRF acts to activate transcription by interacting with other transcription factors. These include YY1 (56), Phox-1 (35), and members of the Elk-1 subfamily of the Ets family of transcription factors. SRF has also recently been shown to interact with myogenic basic helix-loop-helix regulatory factors (38); however, the significance of this interaction for serum-regulated gene expression is unclear.

In the SRF promoter, CArG boxes 1 and 2 contain overlapping consensus binding sites for the YY1 transcription factor. YY1 is a multifunctional transcription factor that has been reported to function in transcriptional activation and repression. Recent reports indicate that the ability of YY1 to bend DNA may account for some of these disparate functions (56, 57). It has been proposed that YY1 acts as a structural factor to organize DNA-protein complexes that form on a promoter. In the c-fos promoter YY1 either represses or enhances transcription, depending on which promoter binding site it occupies.

When YY1 binds to the c-fos SRE it bends DNA in a fashion that enhances SRF binding in the c-fos promoter, thereby potentiating transcription. In contrast, when YY1 binds to a site located between the c-fos cAMP response element and the TATA box it bends DNA in a fashion that represses transcription. Based on our mutational analysis, the role of YY1 in mediating serum responsiveness of the SRF promoter is unclear. It is likely that YY1 binding is not sufficient to mediate serum induction, since double CArG box mutants, in which YY1 binding sites are left intact, are unresponsive to serum stimulation. However, since it has been reported that YY1 can potentiate SRF binding and SRF-mediated transcription by transiently binding to SRF-occupied CArG boxes, it is possible that YY1 may be playing a role in potentiating the serum response of the SRF promoter. So far, however, under the shift conditions used here we have not been able to detect YY1 in CArG box complexes containing SRF. However, we are continuing to investigate a possible role for YY1 in SRF promoter activity.

Analysis of the c-fos promoter has also identified a family of SRF-associated factors involved in mediating SRF-dependent activation of the c-fos gene (reviewed in Ref. 36). These factors, which belong to the Ets-1 family of transcription factors, have been termed p62TcF's, based on their ability to interact with an SRF-DNA complex to form a ternary complex. In the c-fos promoter, p62TcF's bind to a site immediately adjacent to the c-fos SRE. Interaction of p62TcF's with SRF allows p62TcF to bind a CAGGAT binding site adjacent to the c-fos SRE. In the case of activation of the c-fos gene, various mitogenic agents including serum, phorbol ester tumor promoters, and purified growth factors can stimulate mitogen-activated protein kinase-dependent activation of p62TcF, thereby stimulating c-fos expression. In some cases, such as phorbol ester-mediated activation, SRF-dependent gene expression is p62TcF-dependent (58). In other cases, such as serum-mediated activation, SRF-dependent activation can also occur in a TcF-independent fashion, indicating that alternative SRF-mediated activation pathways exist (58).

Since p62TcF's have been demonstrated to play a role in serum-mediated gene expression, we also wanted to determine whether p62TcF may be involved in mediating serum-stimulated expression of the SRF promoter. We investigated whether in vitro translated p62k-1 was capable of forming a ternary complex with SRF on the SRF promoter. A consensus Ets motif (CA)IC/AGA(T) is important for promoting efficient ternary complex formation has been identified (59). In the first 322 nucleotides upstream of the start site of transcription, there are three potential consensus Ets binding sites located at −32 (overlapping CArG box 1), −103, and −195. We found that under conditions in which SRF and p62k-1 formed an efficient ternary complex with the c-fos SRE, we were unable to detect efficient ternary complex formation using a SRF promoter fragment spanning −165 to +14 (data not shown). We found similar results using either in vitro translated p62k-1 or nuclear extracts from NIH3T3 cells. Our observations suggest that serum-mediated activation of the SRF promoter is not mediated by ternary complex formation, although it is possible that in vivo ternary complex formation may be occurring, which we are unable to detect using our in vitro shift conditions. Alternatively, it is possible that ternary complex formation may be occurring preferentially using the −195 site.

In addition to SRF-dependent DNA binding, p62TcF's can also bind DNA autonomously through high affinity Ets binding sites (59). Thus, although ternary complex formation may not be important for serum responsiveness, TcF's may be playing a role in serum stimulation by autonomous binding to the SRF promoter. One such site, identical to an Ets binding site found in the Drosophila E74 gene, is located at −103 of the SRF promoter. DNA binding assays reveal that in vitro translated p62k-1 can bind efficiently to this site (not shown). This site, however, does not appear to be necessary for serum responsiveness of the −322 reporters, although basal expression may be affected (data not shown). We have also not been able to detect Elk-1 in complexes formed with NIH3T3 extracts using anti-Elk-1 antibodies. Together, these observations suggest that factor binding to the −103 Ets site is not necessary for serum responsiveness in vivo. This is consistent with the observation that serum responsiveness of the c-fos promoter can occur in a SRF-dependent yet TcF-independent manner (58).

However, a more careful analysis of the factors that bind this region of the SRF promoter in vivo and their effect on SRF promoter responsiveness is still required.

While our analysis suggests that YY1 and Ets factors are likely not to be necessary for serum stimulation of the SRF promoter, the results shown in Fig. 3B suggest that another transcriptional activator protein Sp1 may act together with SRF to activate serum-mediated expression. Disruption of either Sp1 binding site in the SRF promoter reduces the level of stimulation of the −322 reporter while leaving basal expression unaffected. In particular, disruption of the −83 Sp1 site has significantly more effect than disruption of the −254 site, reducing expression to levels comparable with the double CArG box mutant. Since Sp1 sites in the absence of intact CArG boxes are not sufficient to mediate serum stimulation of the SRF promoter, these results suggest that in the context of intact CArG boxes Sp1 may interact with CArG box factors to mediate serum stimulation. One possibility is that Sp1 is directly interacting with SRF to mediate serum responsiveness. This interpretation is supported by the observation that full-length SRF and Sp1 can interact in a yeast two-hybrid assay system. Consistent with the idea that SRF and Sp1 can interact to mediate gene expression, intact SRF and Sp1 binding sites have been shown to be important for regulating muscle-specific expression of the cardiac c-actin gene (60). While our results in Fig. 3B suggest that Sp1 is involved in mediating serum responsiveness of the SRF gene, the mutation used to disrupt Sp1 binding also disrupts a zif-268 consensus binding site. It is therefore possible that zif-268 or a related factor may be playing a role and not Sp1. We are currently investigating further the nature of Sp1 and SRF interactions and their role in SRF promoter serum responsiveness as well as the role of zif-268 in the function of the −83 element.

4 D. Krainc and R. Misra, unpublished observations.
The SRF gene belongs to a class of IEGs whose expression is delayed relative to other IEGs. The molecular basis for temporal control of expression of different classes of IEGs is not known. One possibility is that similar signaling pathways target distinct complexes of promoter regulatory factors to regulate temporality of expression. Our observations that, although the SRF promotes is regulated in an SRE-dependent manner, different combinations of interacting elements are required for maximal expression relative to other SRE-controlled early IEGs is consistent with this interpretation. One intriguing possibility is that signaling pathways that regulate fos expression at earlier times than SRE-dependent promoters, such as the SRF promoter, in which SRF interacts with other transcription factors. We are currently investigating this hypothesis.

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REFERENCES

1. Treisman, R., and Ammerer, G. (1992) Curr. Opin. Genet. & Dev. 2, 221–226
2. Treisman, R. (1990) Semin. Cancer Biol. 1, 47–58
3. Chavlier, P., Witten-Timmen, U., Matts, M.-G., Zerial, M., Bravo, R., and Charnay, P. (1989) Mol. Cell. Biol. 9, 787–797
4. Chambless, P. S., Fong, P., King, T. C., and Milbrandt, J. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 377–381
5. Christy, B., and Nathans, D. (1989) Mol. Cell. Biol. 9, 4889–4895
6. Tsai-Morris, C.-H., Cao, X., and Sukhatme, V. P. (1988) Nucleic Acids Res. 16, 8035–8046
7. Lattink, B., O’Brien, T., and Lau, L. (1991) Nucleic Acids Res. 19, 3261–3267
8. Lattink, B. V., and Lau, L. F. (1991) J. Biol. Chem. 266, 23163–23170
9. Misra, R., Bonni, A., Miranti, C. K., Rivera, V. M., Sheng, M., and Greenberg, M. E. (1994) J. Biol. Chem. 269, 25483–25493
10. Fujii, M., Tsuchiya, H., Chuhjo, T., Akizawa, T., and Seiki, M. (1992) Genes & Dev. 6, 2066–2067
11. Fujii, M., Tsuchiya, H., Chuhjo, T., Minamoto, T., Miyamoto, K., and Seiki, M. (1994) J. Viral. 68, 7275–7283
12. Avantaggiati, M. L., Natoli, G., Balsano, C., Chirillo, P., Artini, P., DeMarzio, E., Collepardo, D., and Levrero, M. (1996) Oncogene 8, 1575–1576
13. Fuji, M., Shalloway, D., and Verma, I. M. (1989) Mol. Cell. Biol. 9, 2493–2499
14. Qureshi, S. A., Cao, X., Sukhatme, V. P., and Foster, D. A. (1991) J. Biol. Chem. 266, 10802–10806
15. Alexandropoulos, K., Qureshi, S. A., Rim, M., Sukhatme, V. P., and Foster, D. A. (1992) Nucleic Acids Res. 20, 2355–2359
16. Fukumoto, Y., Kaibuchi, K., Oku, N., Hori, Y., and Takai, Y. (1990) J. Biol. Chem. 265, 774–780
17. Gauthier-Rouviere, C., Fernandez, A., and Lamb, N. J. C. (1990) EMBO J. 9, 171–180
18. Gutman, A., Wasylyk, C., and Wasylyk, B. (1991) Mol. Cell. Biol. 11, 5381–5387
19. Jaram, S., and Ziff, E. B. (1990) Nature 344, 463–466
20. Kaibuchi, K., Fukumoto, Y., Oku, N., Hori, Y., Yamamoto, T., Toyoshima, K., and Takai, Y. (1989) J. Biol. Chem. 264, 20835–20838