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Two Pathways for Serum Regulation of the c-*fos* Serum Response Element Require Specific Sequence Elements and a Minimal Domain of Serum Response Factor

FINN-EIRIK JOHANSEN AND RON PRYWES*

Department of Biological Sciences, Columbia University, New York, New York 10027

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The c-*fos* serum response element (SRE) is necessary and sufficient for induction of the c-*fos* gene in response to serum and growth factors. This activation is dependent upon serum response factor (SRF), a transcriptional activator which binds the SRE. A factor, $p62^{TCF}$, which binds in conjunction with SRF to the SRE and which is activated by mitogen-activated protein kinase, has also been implicated in c-*fos* regulation. By using a reporter gene system with weak SRE mutations that is dependent upon overexpression of SRF for serum induction, we have found that there are at least two pathways for serum induction that converge on the SRE. Loss of TCF binding by mutations in SRF and the SRE did not reduce serum induction of the reporter genes. We have found a pathway for serum induction that is sensitive to mutations in the A/T-containing central sequence of the SRE and which is independent of TCF. When this pathway was mutated, activation was dependent upon TCF binding, demonstrating that TCF can also function in serum induction. Both of the signalling pathways required a minimal domain of SRF. This domain, spanning SRF's DNA binding domain, was sufficient for serum induction when fused to a heterologous transcriptional activation domain.

We have investigated how serum regulates c-*fos* transcription through the c-*fos* serum response element (SRE). The SRE has been shown to be necessary and sufficient for the rapid induction of c-*fos* gene transcription by serum, growth factors, and phorbol esters (3, 6, 9, 41). Serum response factor (SRF) is a ubiquitously expressed transcription factor that binds to the c-*fos* SRE (7, 32, 42). SRF activity is required for serum induction of the c-*fos* gene since injection of anti-SRF antibodies into rat fibroblasts strongly inhibited serum induction of fos expression (4).

SRF is a 508-amino-acid-long protein with a central core (amino acids 133 to 222) that contains the DNA binding and dimerization domains (29). These domains span a region, termed a MADS box, which is similar in a group of DNA-binding proteins from various species (36). Other domains in SRF include a C-terminal transcriptional activation domain and an N-terminal domain that is phosphorylated by casein kinase II and ribosomal S6 kinase (18, 20, 23, 33). SRF can also form a ternary complex with DNA and an accesory factor, $p62^{TCF}$. TCF forms a ternary complex by making protein-protein interactions with SRF in a domain within SRF's dimerization domain and protein-DNA interactions with a purine-rich sequence (AGGA) at the 5′ end of the SRE (28, 37, 38). The genes *SAP-1* and *elk-1* encode Ets-related proteins with TCF-like properties (2, 14).

The requirement for TCF binding to the SRE for serum, growth factor, or phorbol ester induction has been the focal point of several studies and may vary with the cell type and experimental conditions used. On the basis of mutational analysis of the SRE, different laboratories have found that TCF is required for all induction through the SRE, is required only for phorbol ester induction, or is not required at all for transcriptional activation of the SRE (8, 19, 37). More recent studies on the nature of the DNA contacts made by TCF have shown that both the orientation and the exact distance of TCF's DNA binding site from that of SRF is flexible up to at least 25 bases (44). This finding raises the question of whether all SRE mutants used in the studies cited above did in fact abolish TCF binding. It has also been found recently that TCF (Elk-1) is phosphorylated by mitogen-activated protein kinase in response to serum and that this modification activates the transcriptional activation function of Elk-1 (5, 15, 25). Furthermore, Hill et al. (13), using altered specificity SRE and SRF variants, found that both TCF binding to the SRE (with SRF) and phosphorylation of TCF were required for serum induction of a reporter gene. These results suggested that modification of TCF by a MAP kinase pathway is the main mechanism for serum induction of the SRE. We have used similar methods, described here, and have found that TCF binding is not required for serum induction although it is one mechanism for activating the SRE. We find a second pathway which is dependent upon the A/T-rich core sequence of the SRE and a minimal domain of SRF but which is independent of TCF.

MATERIALS AND METHODS

Plasmid construction. The reporter genes pFSS-Fluc, pMSS-Fluc, pSRE.M-Fluc, pSRE.LM-Fluc, and pG1-Fluc were derived from pFos-lcf (27). They contain positions −53 to +45 of the c-*fos* promoter upstream of the luciferase gene with the respective oligonucleotides inserted at the −53 position. The sequences of the top strands of the five oligonucleotides are as follows:

FSS,	CTCGAGGATGTCCCTATTAGGTAATTAAGATCT
MSS,	CTCGTGTATGTCCCTATTAGGTAATTAAGATCT
SRE.M,	CTCGAGGATGTCCCAATCGGGACATCTAGATCT
SRE.LM,	CTCGTGTATGTCCCAATCGGGACATCTAGATCT
G1,	TCGAGCGGAGGACTGTCCTCCGC

G1 contains a single GAL4 binding site. Oligonucleotide XGL was as described previously (31).

* Corresponding author. Mailing address: Department of Biological Sciences, Columbia University, New York, NY 10027. Phone: (212) 854-8281. Fax: (212) 865-8246. Electronic mail address: Prywes@cubsps.bio.columbia.edu.

All expression vectors were derived from pCGN (39) and pCGNSRF (18), in which expression is driven by the cytomegalovirus promoter and the expressed proteins contain the influenza virus hemagglutinin antigen at the amino terminus. pCGNSMS has the basic region of SRF's DNA binding domain (amino acids 137 to 171) substituted with the homologous region of MCM-1 (amino acids 10 to 46) (designated SMS). The sequences of SRF (amino acids 137 to 171) and MCM1 (amino acids 10 to 46) with the differences indicated are as follows:

SRF, GKKTRGRVKIKMEFIDNKLRRYTTFSKRKTGIMKK
MCM1, NGQQKE-R--EIK--E--T--HV--F---H-----

pCGNSMAS has most of the TCF interaction domain (amino acids 172 to 205 in SRF) substituted with the homologous region of ARG80 (amino acids 109 to 142). The sequences of SRF (amino acids 172 to 205) and ARG80 (amino acids 109 to 142) are as follows:

SRF, AYELSTLTGTQVLLLVASETGHVYTFATRKLQPM
ARG80, -----V---ANI---ILANS-L-Y--T-P--EPV

MCM1-VP16 contains amino acids 1 to 98 of MCM1 fused to the transcriptional activation domain of VP16 (amino acids 412 to 490). Since these constructs were made by PCR, the DNA sequences of pCGNSMS and pCGNSMAS were confirmed by sequencing across the amplified regions. Details of these and all other constructs, which were made by using standard molecular biology techniques, will be provided upon request.

Transfections and luciferase assays. HeLa cells were grown in Dulbecco modified Eagle medium containing 8% newborn calf serum. One 10-cm plate was transfected by standard calcium phosphate methods (34) with 10 μg of reporter gene, 4 to 6 μg of pRSV-βgal or pCMV-βgal to normalize for transfection efficiency, and 0.1 to 5.0 μg of pCGN expression vector containing cDNA for the protein indicated. The expression level of the different proteins varied such that the amount of each expression construct was titrated, and amounts that yielded similar levels of expression were chosen as determined by immunoblotting with the antiepitope serum (monoclonal antibody 12CA5 [39]). pUC19 or pCGN plasmid DNA was added to the transfections as needed to achieve a final concentration of 20 μg of DNA per ml. Approximately 24 h after transfection, the cells were split onto three 6-cm plates and allowed to grow for an additional 24 h before being serum starved overnight in 0.2% newborn calf serum. Two plates were starved, and the third plate was used to prepare cell extracts for gel mobility shift assays or immunoblots. After overnight starvation, the cells were either treated with 20% bovine calf serum for 4 h or left untreated. Extracts and luciferase assays using the Promega luciferase assay system were prepared according to the manufacturer's protocol and counted for 1 min in the tritium channel in an Intertechnique SL 30 liquid scintillation spectrometer with the coincidence circuit disconnected. β-Galactosidase assays were performed with 100 μl of the same extracts, using 150 μl of 4-mg/ml *o*-nitrophenyl-β-D-galactopyranoside (ONPG) and 600 μl of Z buffer (100 mM Tris [pH 8.0], 10 mM KCl, 1 mM $MgSO_4$, 0.05 mM 2-mercaptoethanol). When a faint yellow color appeared, the reactions were stopped by the addition of 350 μl of 1 M Na_2CO_3, measured in a spectrophotometer at an optical density of 420 nm, and used to normalize the luciferase activity to transfection efficiency.

Gel mobility shift assays and expression of recombinant proteins. For gel mobility shift assays with TCF, 10 μg of pCGNSMS(1-266) or 20 μg pCGNSMAS(1-266) was used to transfect one 10-cm plate of HeLa cells. Whole cell extracts (40) were prepared 48 h after transfection and used in the gel mobility shift assays as described previously (18) except that 50 mM NaCl was used instead of KCl. The exogenous proteins were highly overexpressed such that the band shift due to endogenous SRF was very weak. The probes were made by digesting plasmid pFSS-Fluc or pMSS-Fluc with *Bam*HI and *Bgl*II and labeling with [γ-^{32}P]ATP, using T4 polynucleotide kinase. This produces a fragment with approximately 35 bases upstream and 10 bases downstream of the SRF binding site.

A TCF-containing fraction was purified from HeLa cell nuclear extracts on the basis of its ability to cause recombinant SRF to migrate more slowly to the position of the ternary complex in a gel mobility shift assay. This fraction exhibited no SRE binding activity alone (unpublished data). TCF initially fractionated in the phosphocellulose flowthrough and DEAE-cellulose flowthrough fractions (see reference 46 for a description of columns). The DEAE-cellulose flowthrough fraction was then loaded onto a Biorex 70 column in BC100 (100 mM KCl, 20% glycerol, 20 mM Tris-HCl [pH 7.9], 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol), and TCF was step eluted with BC350 (same as BC100 but with 350 mM KCl). This fraction was dialyzed in BC100 and loaded onto a double-stranded DNA-Sepharose column. TCF activity eluted in the flowthrough of this column, and this fraction was used for the gel mobility shift assays described above.

Gel mobility shift assays with full-length SMS were performed with extracts from cells transfected with 0.5 μg of pCGNSMS. Gel mobility shift assays were then performed as described above except that we also included monoclonal antibody 12CA5, which recognizes the influenza virus hemagglutinin epitope (39). We incubated the cocktails for 2 h before loading the gel to allow for antibody binding. The probe was made by labeling the double-stranded FSS oligonucleotide with T4 polynucleotide kinase, using [γ-^{32}P]ATP.

RESULTS

Mechanisms of regulation of SRF and the SRE have been difficult to elucidate biochemically. We therefore sought to develop a system for identifying domains of SRF responsible for serum regulation in vivo. Since endogenous SRF levels are sufficient to render an SRE-containing promoter construct serum inducible, it has been difficult to assay for the function of transfected SRF in vivo. In addition, GAL4-SRF chimeras were not able to render a promoter containing a GAL4 binding site serum inducible, suggesting that SRF may need to bind to DNA through its own DNA binding domain to support serum induction (18). Since SRF is ubiquitously expressed and overexpression of SRF in HeLa cells has no effect on the expression of a c-*fos* promoter construct (reference 10 and unpublished data), we wanted to set up a system with a reporter gene that would be sensitive to transfected SRF but not affected by endogenous protein.

To achieve this, we have used a reporter gene with a mutant SRE that has reduced affinity for SRF and which is only weakly serum responsive. Serum induction could then be reconstituted by overexpression of SRF. We used the observation that the yeast MCM1 protein, which is homologous to SRF in its DNA binding domain, binds SRE-like elements with five instead of six A/T core base pairs (11, 17, 30, 45). SRF has been reported to bind poorly to such an element (21). We therefore constructed a reporter gene with a mutated version of the SRE (termed FSS) designed to bind SRF poorly but bind the yeast MCM1 protein well. This site is composed of the 5′ flanking

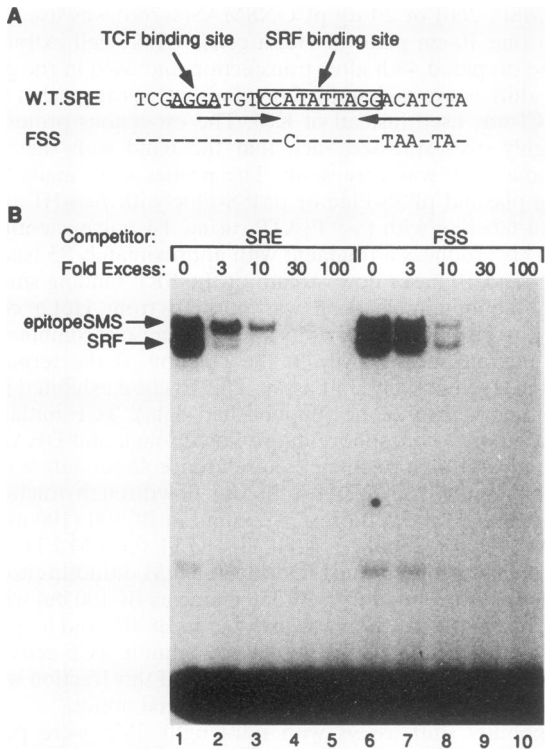

FIG. 1. Binding of SRF and SMS to the mutated SRE site. (A) Comparison of the wild-type c-*fos* SRE (W.T.SRE) with the mutant FSS site used in these studies. The minimal SRF binding site, the site of TCF's DNA contacts, and the inverted repeat of the SRE are indicated. (B) Gel mobility shift assay using extract from cells transfected with an epitope-tagged expression vector for SMS, using 1 ng of the ^{32}P-labeled FSS site as the probe. A monoclonal antibody to the epitope tag was included to separate transfected SMS from endogenous SRF. The positions of migration of antibody-bound, epitope-tagged SMS and endogenous SRF complexes are indicated. Increasing amounts of unlabeled SRE and FSS oligonucleotides were added as indicated.

sequence of the c-*fos* SRE and the central and 3′ flanking sequence from the yeast *STE6* gene, a natural target site for MCM1 (Fig. 1A). In addition, we made a mutant SRF with altered DNA binding specificity by exchanging the basic region of SRF's DNA binding domain with the homologous domain of MCM1 to create a chimeric protein with MCM1-like DNA binding specificity, termed SMS (Fig. 2A). Our prediction was that SRF endogenous to HeLa cells would bind poorly to the FSS site and that the chimeric SMS protein would bind well and cause the site to be serum responsive.

To test the DNA binding properties of SRF and SMS, HeLa cells were transiently transfected with an SMS expression construct (pCGNSMS) in which SMS was epitope tagged at the amino terminus. Extracts from the transfected cells were used in a gel mobility shift assay using the ^{32}P-labeled FSS site (Fig. 1B). Since the proteins produced by the transfected expression vector would comigrate with endogenous SRF, we included a monoclonal antibody to the epitope tag in the gel mobility shift reactions to further retard the transfected SMS complexes. Competitions with increasing amounts of c-*fos* SRE (lanes 2 to 5) and FSS (lanes 7 to 10) oligonucleotides showed that SRF has about a four-times-higher affinity for SRE than for FSS (compare the SRF signals in lanes 1 to 5 versus lanes 6 to 10). The affinity of SMS for FSS is about the same as for the SRE (compare the SMS signals in lanes 1 to 10). The antiepitope serum completely shifted up the SMS protein since an oligonucleotide (XGL) that bound SRF, but not SMS, competed for binding of the SRF band but had no effect on the SMS band (data not shown). Similarly, an oligonucleotide (SRE.M) that bound SMS but not SRF competed for binding of SMS but not SRF (see Fig. 5B).

The FSS oligonucleotide was inserted upstream of a minimal c-*fos* promoter fused to the firefly luciferase gene to create the reporter gene pFSS-Fluc. This reporter gene was only weakly serum inducible in HeLa cells compared with an identical reporter gene with a wild-type SRE (pSRE-Fluc) (Fig. 2B, columns 1 and 2 compared with columns 13 and 14). However, transfection of an expression vector for SRF (columns 3 and 4) or SMS (columns 5 and 6) partially restored serum induction of the reporter gene. Since SRF can still bind to the FSS site, albeit more weakly than to the c-*fos* SRE (Fig. 1B), it appears that overexpression of SRF is sufficient to cause occupation of the FSS site and serum regulation of the promoter. Representative transfections are shown in Fig. 2B and subsequent figures. Similar results were obtained in each case in at least three separate experiments. These results were also confirmed by using a similar reporter gene in which the chloramphenicol acetyltransferase gene was substituted for the luciferase gene, and RNase protection assays were used to measure the amounts of properly initiated transcripts in serum-starved and serum-treated cells (data not shown).

Mutation of SRF's TCF binding domain. To map domains of SRF important for serum activation, we made further mutations in the SMS protein. p62^{TCF} is a protein which forms a complex with SRF and the SRE (37). It contacts both a purine-rich sequence (AGGA) at the 5′ end of the SRE and a region in SRF. The TCF binding domain in SRF is located in the dimerization domain within the conserved MADS box region (28, 38). TCF can bind DNA with SRF and MCM1 but not with another homologous yeast protein, ARG80 (38). To make a mutant SMS protein that does not interact with TCF, we made a mutant similar to one previously described (56A/Q203E [38]) by exchanging part of SRF's dimerization domain with the homologous part of ARG80 to create a new mutant termed SMAS (Fig. 2A).

We confirmed that the mutation in SMAS abolished TCF binding by using a gel mobility shift assay. It has been shown that SRF's core DNA binding domain (amino acids 133 to 222) can efficiently form a ternary complex and that the use of a shorter SRF derivative increases the resolution of the binary complex (DNA-SRF) from the ternary complex (DNA-SRF-TCF) (35). We therefore used extracts from HeLa cells transfected with expression vectors for SMS(1-266) and SMAS(1-266) and titrated in a partially purified fraction from HeLa cells containing TCF (Fig. 3). With SMS(1-266), a ternary complex was detected with the lowest amount of TCF used, and increasing amounts of TCF gave an increasing amount of ternary complex (lanes 2 to 4). With SMAS(1-266), addition of TCF did not result in detectable levels of ternary complexes (lanes 6 to 8), although in separate experiments we have detected low levels of ternary complexes with high amounts of TCF (data not shown). We estimate that SMAS binding to TCF is at least 20-fold reduced compared with SMS binding. We then tested SMAS for activation of the pFSS-Fluc reporter gene and found that the strong reduction in the ability to complex with TCF did not affect the mutant's ability to activate the reporter gene (Fig. 2B, columns 7 and 8).

Requirement for SRF's transcriptional activation domain. We previously found using GAL4-SRF constructs in HeLa cells that SRF's transcriptional activation domain is located

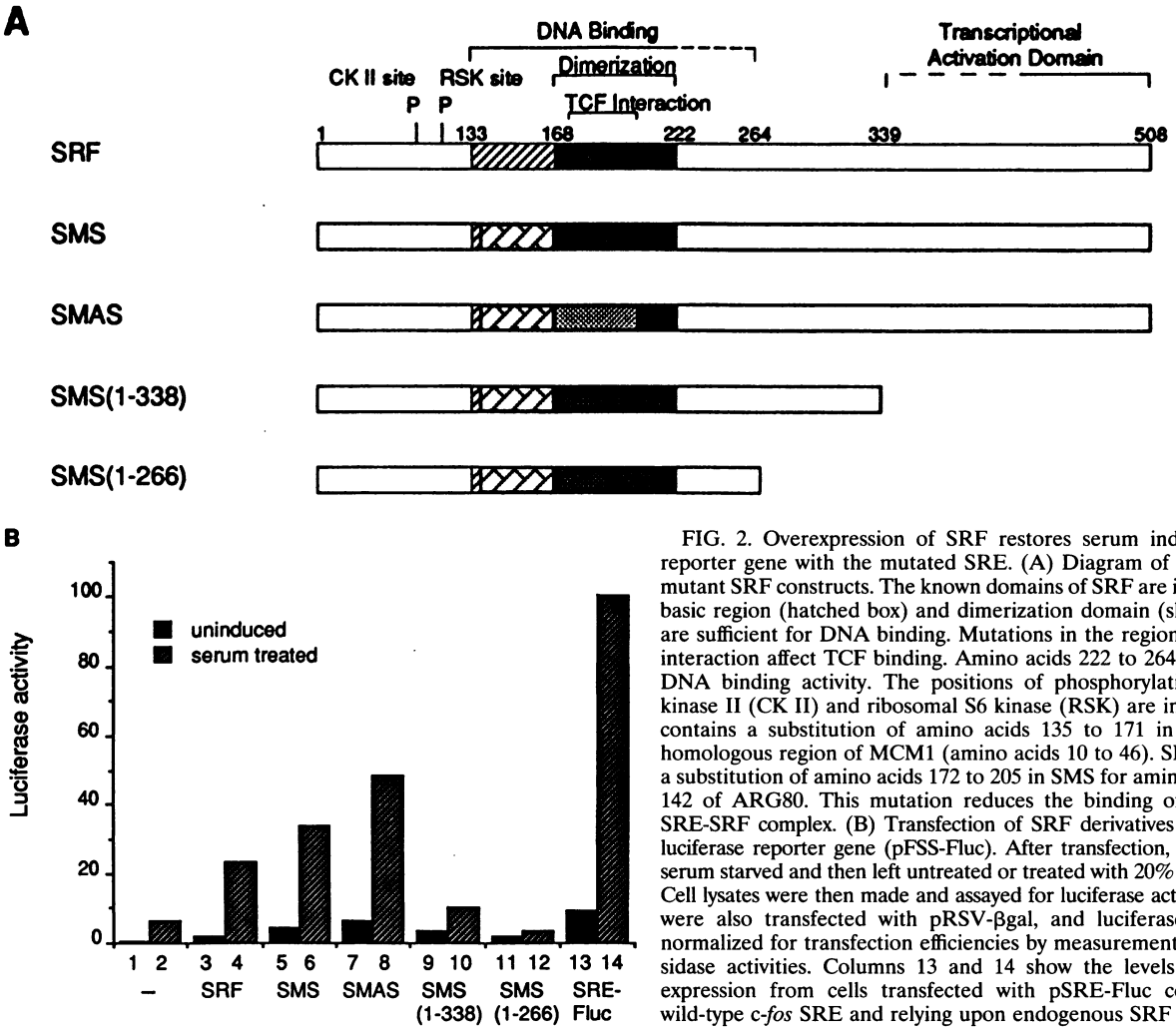

FIG. 2. Overexpression of SRF restores serum inducibility to a reporter gene with the mutated SRE. (A) Diagram of wild-type and mutant SRF constructs. The known domains of SRF are indicated. The basic region (hatched box) and dimerization domain (shaded darkly) are sufficient for DNA binding. Mutations in the region labeled TCF interaction affect TCF binding. Amino acids 222 to 264 increases the DNA binding activity. The positions of phosphorylation by casein kinase II (CK II) and ribosomal S6 kinase (RSK) are indicated. SMS contains a substitution of amino acids 135 to 171 in SRF for the homologous region of MCM1 (amino acids 10 to 46). SMAS contains a substitution of amino acids 172 to 205 in SMS for amino acids 109 to 142 of ARG80. This mutation reduces the binding of TCF to the SRE-SRF complex. (B) Transfection of SRF derivatives and the FSS-luciferase reporter gene (pFSS-Fluc). After transfection, the cells were serum starved and then left untreated or treated with 20% serum for 4 h. Cell lysates were then made and assayed for luciferase activity. The cells were also transfected with pRSV-βgal, and luciferase levels were normalized for transfection efficiencies by measurement of β-galactosidase activities. Columns 13 and 14 show the levels of luciferase expression from cells transfected with pSRE-Fluc containing the wild-type c-*fos* SRE and relying upon endogenous SRF for induction.

between amino acids 338 and 508 (18). To investigate whether SRF's activation domain is required for serum induction, we made C-terminal deletions to amino acid 338 and 266 in SMS (Fig. 2A). We found that both of these truncated proteins failed to activate transcription of the reporter gene in response to serum (Fig. 2B, columns 9 through 12). We confirmed that these truncated proteins were stably expressed by immunoblots of transfected cells by using an antibody against the epitope tag at the amino terminus of the expressed proteins. SMS(1-338) and SMS(1-266) were expressed at similar or higher levels than full-length SMS (data not shown). These results indicate that SRF's activation domain is required for serum induction through the SRE. We have also confirmed by immunoblotting that all of the SRF derivatives used in this study were expressed at similar levels (data not shown).

The level of reporter gene expression seen with SMS(1-338), although low, was consistently higher than the level of luciferase expression seen when the reporter gene was cotransfected with SMS(1-266), suggesting that SMS(1-338) may have some residual ability to activate transcription. Although the level seen with SMS(1-338) was only marginally higher than the level in cells cotransfected with the empty expression vector, we believe that it is significant for the following reason. In cells not transfected with expression constructs, the FSS site may be occupied by endogenous SRF at some low frequency causing a low but significant level of luciferase expression. However, in cells cotransfected with SMS(1-338), this exogenous protein is much more abundant than endogenous SRF. Therefore, most FSS sites will be occupied by SMS(1-338) rather than endogenous SRF, and the small activation seen is due to the transfected protein. This would also explain why SMS(1-266), completely lacking a transcriptional activation domain, gave a lower level of luciferase expression than did no exogenous protein (Fig. 2B, columns 1, 2, 11, and 12).

We also note that serum activation of the reporter gene is unlikely to be accounted for by heterodimerization of transfected SRF variants with endogenous SRF. The transfected SRF levels were much higher than enodogenous SRF levels. This can be seen, for instance, in Fig. 3, where the gel mobility shift assay shows strong binding by SMS(1-266) and undetectable binding by endogenous SRF (which would migrate higher on the gel). We have also not been able to detect any heterodimer of intermediate mobility of endogenous SRF with transfected SMS(1-266), which specifically binds to the wild-type SRE or FSS sites (data not shown).

Identification of a minimal domain of SRF required for serum regulation. We next wanted to determine whether serum activation specifically requires SRF's transcriptional

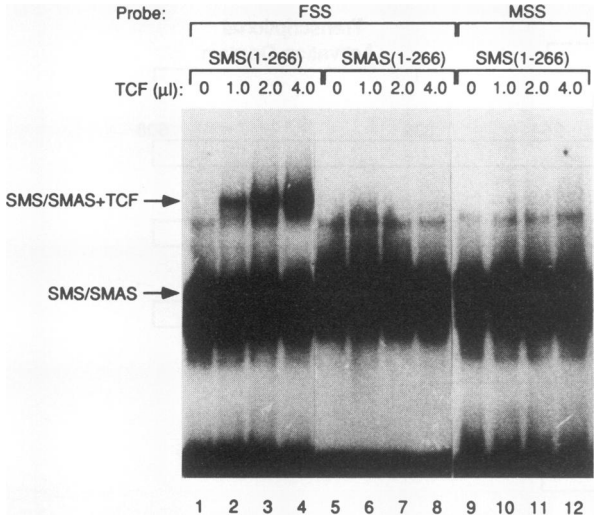

FIG. 3. Mutation of TCF binding. Extracts from cells transfected with either SMS(1-266) or SMAS(1-266) were used in a gel mobility shift assay with ^{32}P-labeled fragments containing the FSS or MSS site. To test for ternary complex formation, a partially purified preparation of HeLa cell TCF was added as indicated. The positions of ternary complexes as well as SMS(1-266) or SMAS(1-266) are indicated at the left.

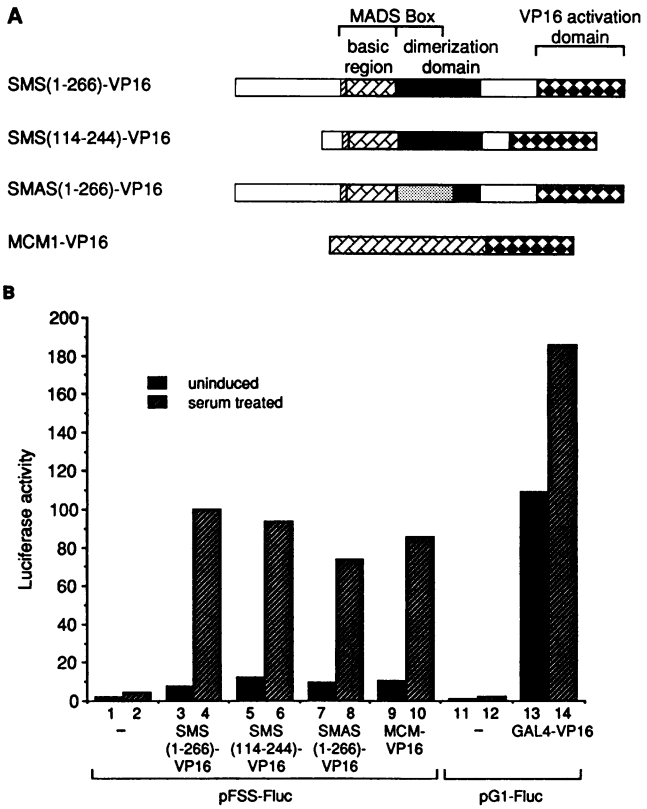

FIG. 4. SRF's DNA binding domain is sufficient for serum regulation when fused to the transcriptional activation domain of the acidic activator VP16. (A) Diagram of the SRF-VP16 derivatives used. The conserved MADS box, containing a basic region and dimerization domain, and VP16's transcriptional activation domain (amino acids 412 to 490) are indicated. The regions derived from MCM1 or ARG80 are as described for Fig. 2A. MCM1-VP16 contains amino acids 1 to 98 of MCM1 fused to the activation domain of VP16. (B) Luciferase expression was measured from cells transfected with pFSS-Fluc (columns 1 to 10) or pG1-Fluc (lanes 11 to 14) cotransfected with the indicated expression constructs. pG1-Fluc contains a single GAL4 binding site. As in Fig. 2B, the cells were serum starved and either left untreated or treated with 20% serum for 4 h.

activation domain or whether a heterologous activation domain could substitute. We therefore fused the activation domain of the herpesvirus VP16 protein to the carboxy-terminal end of SMS(1-266) to create SMS(1-266)–VP16 (Fig. 4A). When cotransfected with pFSS-Fluc, this construct was able to restore serum activation (Fig. 4B, columns 1 to 4). A similar construct, SMAS(1-266)–VP16 (Fig. 4A), containing the region of ARG80 that reduces TCF binding, also caused serum regulation of the reporter gene (Fig. 4B, columns 7 and 8). These constructs show that serum activation does not specifically require SRF's transcriptional activation domain but that a heterologous activation domain can suffice. Activation by SMS(1-266)–VP16 in serum-induced cells was about three times as strong as with SMS (data not shown), probably because of the relative strength of the SRF and VP16 activation domains.

We made further deletions in SMS(1-266)–VP16 to determine the minimal region of SRF required for serum regulation. We found that a construct essentially containing only SRF's DNA binding and dimerization domains, SMS(114-244)–VP16 (Fig. 4A), was sufficient to give serum induction of pFSS-Fluc (Fig. 4B, columns 5 and 6). This region of SRF does not contain potential regulatory phosphorylation sites in SRF for casein kinase II (amino acid 83), ribosomal S6 kinase (amino acid 103), or DNA-dependent protein kinase (amino acids 435 and 446) (16, 22, 24, 26, 33). Thus, the only domain of SRF that was required for pFSS-Fluc to be serum activated was the DNA binding and dimerization domain, containing the conserved MADS box sequence. We tested whether another MADS domain protein was also capable of supporting serum induction by constructing a chimeric protein containing the MCM-1 MADS domain fused to the activation domain of VP16 (Fig. 4A). Surprisingly, we found that MCM1-VP16 also caused serum induction of pFSS-Fluc (Fig. 4B, columns 9 and 10). This finding suggests that determinants in SRF required for regulation are conserved in MCM1.

Since all of the SMS-VP16 derivatives were positive for serum induction, we wanted to ensure that induction occurred specifically through the SRE site and not through flanking sequences in the reporter gene. We considered the possibility that an activator was required to raise the level of expression but that serum regulation was controlled by other DNA sequence elements. We therefore constructed a reporter gene, pG1-Fluc, containing a single GAL4 DNA binding site in place of the FSS site. This reporter gene gave low expression when transfected into HeLa cells (Fig. 4B, columns 11 and 12) but was activated by transfection of a GAL4-VP16 expression vector (columns 13 and 14). GAL4-VP16 caused a high level of expression of the reporter gene in serum-starved cells that was increased by two-thirds upon serum induction (columns 13 and 14). In contrast, SMS-VP16 caused a low uninduced level of expression that was typically induced 10- to 15-fold by serum.

Role of TCF binding for serum induction. Our results with the SMAS mutant (see above) suggested that TCF is not required for serum induction of the SRE. To investigate this possibility further, we mutated the TCF binding site in the FSS oligonucleotide. This mutation, in oligonucleotide MSS (Fig. 5A), has been reported to abolish TCF binding (13). We

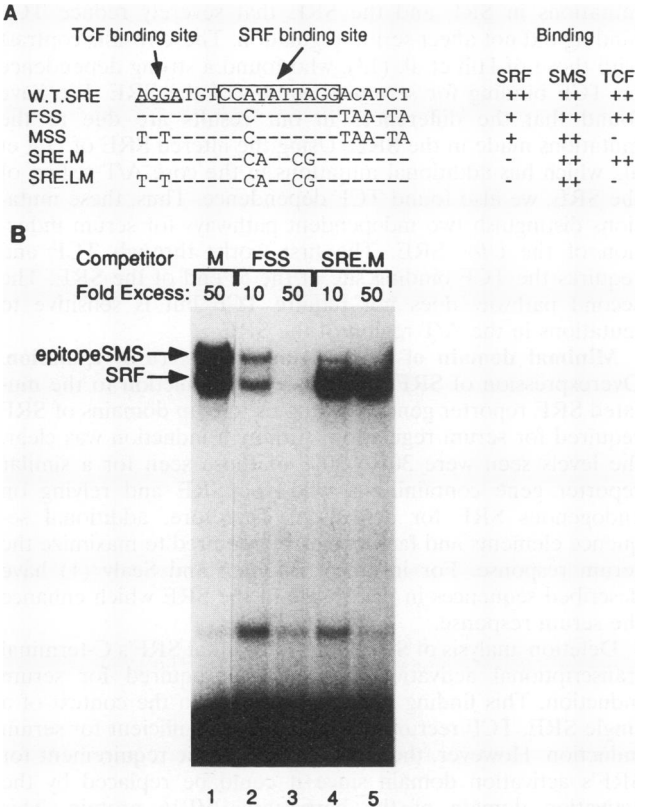

FIG. 5. Binding of SRF and derivatives to the mutated SRE sites. (A) Diagram of the four mutant SREs and summary of their binding properties. The top line shows the wild-type c-*fos* SRE (W.T. SRE) for comparison. (B) Gel mobility shift assay of SRF and SMS binding to the mutated SREs. Extracts from cells transfected with an epitope-tagged SMS expression vector were used with the ^{32}P-labeled FSS site as the probe. A monoclonal antibody to the epitope tag was included to separate transfected SMS from endogenous SRF as in Fig. 1B. Increasing amounts of unlabeled FSS, SRE.M, or XGLM (M) oligonucleotides were included as competitors as indicated. XGLM was used as a control and contains mutations in an SRF binding site that abolish binding (23).

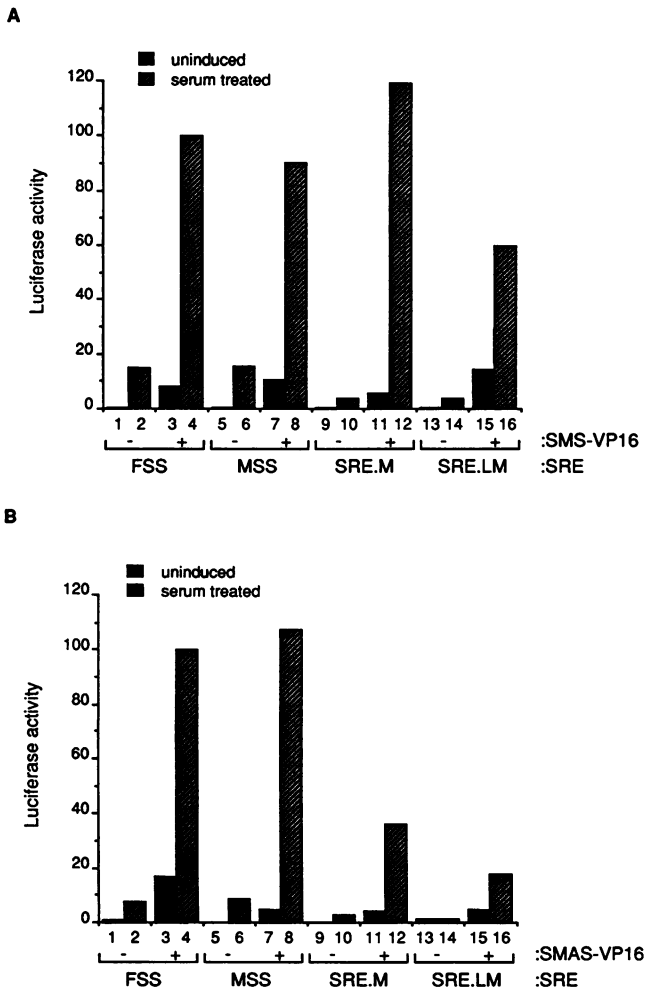

FIG. 6. Differences in serum induction through the mutated SRE sites. HeLa cells were transfected with the reporter genes containing the four different SRE sites with or without the expression vectors, as indicated. Expression vectors for SMS(1-266)–VP16 (A) and SMAS(1-266)–VP16 (B) were used. SMS-VP16 cotransfected with FSS or SRE.M should allow for optimal recruitment of TCF. Reduction of TCF binding is caused by the mutations in SMAS, MSS, and SRE.LM. SMAS-VP16 cotransfected with MSS or SRE.LM should cause the greatest reduction in TCF recruitment.

confirmed the loss of TCF binding to the MSS site compared with the FSS site by using a gel mobility shift assay (Fig. 3). The MSS site was then tested in our reporter construct for serum induction by SMS-VP16. The MSS and FSS constructs were similarly induced by serum (Fig. 6A, columns 1 to 8), suggesting again that serum induction of the SRE does not require TCF.

It has been reported that TCF binding to the SRE is flexible; i.e., that when the TCF binding site is mutated, it can bind through sequences further upstream of the SRE (44). Thus, TCF may still be binding weakly through other sequences in the plasmid. To further reduce the possibility of residual TCF involvement, we tested for serum induction when the TCF binding sites in both the SRE and SRF were mutated. We therefore assayed for induction by using SMAS-VP16 and found no effect of mutating the TCF binding site in the SRE (Fig. 6B, columns 1 to 8).

Differences with use of altered SREs. By using similar methods, it was previously found that serum induction through the SRE requires the participation of p62^{TCF} (13). Since this finding contradicted our results, we investigated the differences in experimental design. Hill et al. (13) also used an altered SRE, termed SRE.M, which could not bind endogenous SRF but could bind an altered SRF molecule similar to our SMS protein. Two SRE.M sites were required for efficient serum induction (13), while we have used a single FSS site. There is also a significant difference in the mutations in the SRE.M and FSS sites, with the SRE.M site having more changes in the core A/T region of the SRE (Fig. 5A). We compared the properties of single SRE.M and FSS sites in our system to determine whether the sequence differences were important.

We tested whether SMS bound the SRE.M site by using a gel mobility shift assay with extracts from HeLa cells transfected with the SMS expression vector (Fig. 5B). Excess SRE.M oligonucleotide was able to efficiently compete for SMS but not SRF binding (lanes 1, 4, and 5). SRE.M, in fact, competed slightly better for SMS binding than FSS (lanes 2 and 3). We have similarly found that SMAS binds efficiently to the SRE.M site (data not shown). We further tested a site described by Hill et al. (13), SRE.LM, that does not bind TCF

(Fig. 5A). They found that this mutation completely abolished serum induction in their system. SRE.LM has the same mutations that we used in MSS to reduce TCF binding. We found that SMS and SMAS bound SRE.LM well in a gel mobility shift assay and that TCF could not bind with SMS to the SRE.LM site (data not shown).

We transfected HeLa cells with the different reporter genes with or without SMS-VP16 (Fig. 6A) and performed luciferase assays as described above. We found that SMS-VP16 could support serum induction through the SRE.M site and that the TCF mutation in SRE.LM reduced induction by about 50% (columns 9 to 16). In contrast, the FSS and MSS sites showed no significant effect of the TCF binding mutation in several experiments (columns 1 to 8 and data not shown).

Since there could be residual TCF binding to upstream sequences in the SRE.LM construct, as discussed above, we tested for induction of the reporter genes with SMAS-VP16. We found that SMAS-VP16 activated SRE.M more poorly than FSS (Fig. 6B, columns 1 to 4 and 9 to 12). In contrast, SMS-VP16 activated the two sites similarly (Fig. 6A). The TCF binding mutation in SRE.LM further reduced induction by SMAS-VP16 (columns 13 to 16). Thus, while the combined mutations in SRE and SRF that affect TCF binding (SRE.LM with SMAS-VP16) dramatically reduced induction through the SRE.M site, there was little effect through the FSS site (MSS with SMAS-VP16). The effect of the combined mutations on induction with the SRE.M site suggests that these mutations are effective in reducing TCF binding in vivo and that induction through the FSS site is in fact TCF independent. The low level of induction observed with the SRE.LM and SMAS mutations (columns 15 and 16) could be due to residual activity of either the TCF-dependent or TCF-independent mechanisms or, alternatively, due to activation by yet another mechanism.

These results indicate that induction through the SRE.M site is primarily through a TCF pathway, while induction through the FSS site is independent of TCF. Therefore, the SRE.M mutations strongly reduce a TCF-independent mechanism for activation of the SRE. Note that the differences between the FSS and SRE.M sites are in the core A/T region and 3′ flanking sequence of the SRE (Fig. 5A). The differences in the 3′ flanking sequences are most probably not important since the FSS sequences are derived from the yeast STE6 site and are not present in the c-*fos* SRE, whereas the SRE.M flanking sequence is wild type and should therefore not be defective.

We found that MCM1-VP16 could also function through the two pathways. It activated through the SRE.M site, and this activation was reduced by the TCF mutations in SRE.LM. In contrast, with the FSS site, there was no effect of mutating the TCF binding site (data not shown). Similar results were obtained with the minimal SRF mutant SMS(114-244)–VP16 (data not shown). An additional variant, SMAS(114-266)–VP16, containing mutations in SRF's TCF binding domain, gave results similar to those for the longer SMAS(1-266)–VP16 variant (Fig. 4A). It could not activate through the SRE.LM site but activated well through the MSS site (data not shown). These results indicate that SRF's core DNA binding domain is sufficient for both signalling pathways.

DISCUSSION

We have used a reporter system based on mutated SREs to identify a minimal domain of SRF required for serum induction. This domain spans SRF's DNA binding and dimerization domains as well as its binding domain for $p62^{TCF}$. However, mutations in SRF and the SRE that severely reduce TCF binding did not affect serum regulation. These results contrast with those of Hill et al. (13), who found a strong dependence on TCF binding for serum activation of the SRE. We have found that the differences in our results are due to the mutations made in the SRE. Using the altered SRE of Hill et al., which has additional mutations in the core A/T region of the SRE, we also found TCF dependence. Thus, these mutations distinguish two independent pathways for serum induction of the c-*fos* SRE. The first works through TCF and requires the TCF binding site at the 5′ end of the SRE. The second pathway does not require TCF but is sensitive to mutations in the A/T region of the SRE.

Minimal domain of SRF required for serum regulation. Overexpression of SRF restored serum induction to the mutated SRE reporter gene, allowing us to map domains of SRF required for serum regulation. Although induction was clear, the levels seen were 30 to 50% of those seen for a similar reporter gene containing a wild-type SRE and relying on endogenous SRF for activation. Therefore, additional sequence elements and factors may be required to maximize the serum response. For instance, Boulden and Sealy (1) have described sequences in the 3′ side of the SRE which enhance the serum response.

Deletion analysis of SRF first showed that SRF's C-terminal transcriptional activation domain was required for serum induction. This finding demonstrates that in the context of a single SRE, TCF recruitment alone is not sufficient for serum induction. However, there was not a specific requirement for SRF's activation domain since it could be replaced by the activation domain of the herpesvirus VP16 protein. This finding is consistent with our previous result that a GAL4-SRF construct containing SRF's activation domain constitutively activated a GAL4 site reporter gene, further suggesting that regulation does not function directly through SRF's transcriptional activation domain (18).

Further deletions in SRF revealed that a region spanning its DNA binding and dimerization domains, amino acids 114 to 244, when fused to the VP16 activation domain was sufficient for serum regulation. These deletions remove potential regulatory phosphorylation sites for casein kinase II and ribosomal S6 kinase (23, 33). The core SRF domain also includes the binding site for TCF located within SRF's dimerization domain. Mutation of the TCF binding domain, however, did not affect serum induction by the SRF-VP16 derivative. To further reduce the possibility of the involvement of residual TCF binding, we tested together the function of derivatives of SRF and the SRE which had both been mutated to abolish TCF binding. Again, there was no effect of these double mutations on serum induction of the reporter gene.

SRF's DNA binding domain is homologous to those of a number of other DNA-binding proteins defining a conserved domain termed the MADS box (36). We tested the MADS box-containing yeast gene *MCM1*, which binds to SRE elements as well as the mutated SRE element (FSS) that we have used here. Surprisingly, an MCM1-VP16 fusion construct also supported serum induction of the reporter gene. Similar to induction with the SMS-VP16 protein, induction with MCM1-VP16 was not affected by reduction of TCF binding. Thus, it appears that the determinants for serum induction within the DNA binding domain are conserved between SRF and MCM1. It is quite plausible that regulatory protein interaction sites in SRF and MCM1 could be conserved since both have previously been found to interact with mammalian TCF (28).

An alternative possibility is that SRF binding to the SRE is regulated either directly by control of SRF's DNA binding

activity or by the antagonistic binding of another SRE-binding protein. Several lines of evidence, however, suggest that SRF is constitutively bound to the SRE in vivo. First, in vivo footprinting of the SRE shows identical footprints before and after growth factor treatment which are very similar to footprints with purified SRF in vitro (12). Second, SRF's DNA binding activity is unchanged in extracts from several untreated or serum-induced cell lines (reviewed in reference 43). Third, a SAP-1–VP16 fusion protein strongly induced expression from the SRE in uninduced NIH 3T3 cells (2). Since SAP-1 is a form of TCF and binds the SRE only in conjunction with SRF, this finding suggests that endogenous SRF occupies the site in uninduced cells. Finally, as discussed below, the SRE.M mutant appears to abolish a pathway for activation of the SRE. If this mutation affected the binding of an antagonist for SRF binding, one would expect constitutive expression with this site in cells containing SMS-VP16. Since this was not the case, the mutations in SRE.M must be affecting regulation in other ways.

Two pathways for induction through the SRE. We found that mutation of the TCF binding sites in SRF and the SRE did not affect serum induction in our system using the FSS site. Using similar methods, but with a different altered SRE (SRE.M), Hill et al. (13) found that mutation of the TCF binding site in SRF reduced serum induction, while mutation of the TCF binding site in the SRE completely abolished induction. We have found that the differences between our systems are accounted for by the different SRE mutants that we have used. With the SRE.M site, we also found TCF dependence. The SRE.M site has additional mutations in the A/T core of the SRE compared with the FSS site, though SMS bound similarly to both sites. It has been reported that TCF and SRF bind DNA cooperatively (35), and one could therefore speculate that a weaker SRF binding site is more dependent on TCF. However, the difference in TCF dependence of the SRE.M/SRE.LM sites versus the FSS/MSS sites is not likely to be due to differential affinities for SMS since we found that SMS bound better to SRE.M/SRE.LM than to FSS/MSS (Fig. 5B and data not shown).

These results suggest that there is a second pathway for activation through the SRE which is abolished by the mutations in SRE.M. The data additionally show that the TCF mutations that we have used are sufficient to severely disable the TCF pathway such that the pathway used by the FSS site is, in fact, TCF independent. Induction can also occur through the TCF pathway since TCF-dependent induction was observed with the SRE.M site. Simultaneous mutation of both pathways (in SRE.LM with SMAS-VP16) severely reduced induction. Individually, each pathway gave similar levels of induction such that there is a functional redundancy in these pathways, and it is at present unclear what the relative contribution of each is to serum induction. The existence of two pathways is consistent with the work of Graham and Gilman (8), who found that mutation of the TCF binding site in the SRE abolished phorbol ester but not serum induction.

The mechanism of regulation of the TCF-independent pathway is unclear except that it is abolished by mutations in the A/T region of the SRE and that SRF's core DNA binding domain, when fused to VP16's activation domain, is sufficient for serum regulation via this pathway. Mutations in the A/T region of the SRE may abolish binding by a novel factor which controls SRF function. Alternatively, the mutations in the SRE could affect the conformation of bound SRF such that it is no longer responsive to particular signals.

An additional complication to understanding SRF regulation is the low level of activation by SRF in uninduced cells. In fact, activation by the SRF-VP16 derivatives was low in uninduced cells compared with activation by a GAL4-VP16 construct of a similar reporter gene containing a single GAL4 site. Thus, if SRF is constitutively occupying the SRE, as discussed above, SRF-VP16 is inhibited in uninduced cells. One possibility is that an inhibitor binds SRF's core DNA binding domain in uninduced cells and blocks activation by VP16's activation domain. This view is consistent with the observation that high overexpression of SRF in certain cell types leads to constitutive activation of an SRE reporter gene such that high SRF levels could be titrating out an inhibitory factor (reference 10 and unpublished data). This inhibitor would then be displaced or inactivated by induction of one of the two pathways described above. Further work will be required to identify these putative regulatory proteins which control SRF activity.

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