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Identification of Transcriptional Activation and Inhibitory Domains in Serum Response Factor (SRF) by Using GAL4-SRF Constructs

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The binding of serum response factor (SRF) to the c-*fos* serum response element has been shown to be essential for serum and growth factor activation of c-Fos. Since SRF is ubiquitously expressed, it has been difficult to measure the activity of SRF introduced into cells. To assay for functions of SRF in cells, we have changed its DNA binding specificity by fusing it to the DNA binding domain of GAL4. Transfection of GAL4-SRF constructs into cells has allowed us to identify SRF's transcriptional activation domain as well as domains which inhibit this activity. First, we found that the transcriptional activation domain maps to between amino acids 339 and 508 in HeLa cells and to between amino acids 414 and 508 in NIH 3T3 cells. Second, we show that in the context of GAL4-SRF constructs, there are two separate domains of SRF that can inhibit its activation domain. Although these domains overlap the DNA binding and dimerization domains of SRF, these functions were not required for inhibition. Finally, we show that one of the inhibitory domains is modular in that it can also inhibit activation when it is moved amino terminal to GAL4's DNA binding domain in an SRF-GAL4-SRF construct. The implications of these inhibitory domains for SRF regulation are discussed.

The serum response element (SRE) of the c-*fos* promoter is necessary and sufficient for the rapid and transient transcriptional activation of the gene in response to many different growth factors (reviewed in reference 38). Serum response factor (SRF) is a nuclear factor which binds specifically to the SRE (38). SRF appears to be required for growth factor activation through the SRE, since there is a strong correlation between mutations in the SRE that affect induction and SRF binding (38) and since microinjection of anti-SRF sera inhibits c-*fos* induction (6).

SRF is a 64-kDa protein and binds to the SRE as a dimer (26). It has been cloned and consists of 508 amino acids (aa) (26). The DNA binding and dimerization domains were mapped to aa 133 to 222 and 168 to 222, respectively (26). Phosphorylation of SRF by casein kinase II, predominantly at serine 83, increases its DNA-binding activity (15, 20–23); however, this phosphorylation does not appear to change in response to growth factors (22).

The mechanism of regulation of SRF and the SRE is still unclear. In most cell types, SRF's DNA-binding activity does not appear to change with growth factor treatment (4, 10, 34, 37). The exception is A431 cells, in which epidermal growth factor (EGF) induced SRF's DNA-binding activity (28). Biochemical characterization of SRF has also failed to detect covalent modifications or complexing proteins that change with induction (22). One interesting SRF-complexing protein, $p62^{TCF}$, has been identified. Binding of this factor depends upon contact with both SRF and sequences at the 5′ end of the SRE (32). Two genes, *SAP-1* and *elk-1*, with TCF-like properties have been cloned and are related to the *ets* proto-oncogene (3, 14). In addition, TCF from HeLa cells is immunologically related to Elk-1 (14). It has recently been found that phosphorylation of TCF by mitogen-activated protein kinase stimulates TCF binding to the SRF-SRE complex (7). It is still unclear, however, whether TCF is required for c-*fos* induction or whether phosphorylation of TCF controls c-*fos* activation. Mutations that abolish TCF binding without affecting SRF binding have been made in the SRE. The effects of these mutations in SRE reporter genes have been contradictory. They have either abolished serum induction (32), abolished phorbol ester (tetradecanoyl phorbol acetate [TPA]) but not serum induction (9), or had no effect on TPA or serum induction (16). We have also found a mutant of the SRE (in pFC53X [27]) that does not bind TCF in vitro but is still inducible by EGF in vivo (unpublished data).

Since biochemical characterization of SRF has not clearly elucidated its mechanism of regulation, it is necessary to develop other assays for regulation of the SRE. One approach is to assay for SRF function in cells. This would allow us to identify domains required for regulation and other critical activities. For instance, since the TCF binding domain in SRF has been mapped (25, 33), it should ultimately be possible to assay for the importance of TCF binding by mutation of this domain. It has been difficult, however, to assay for SRF function, since all cell types tested contain SRF (38; unpublished data). Overexpression of SRF has no effect on expression of an SRE reporter gene in HeLa cells, though it causes constitutive expression in NIH 3T3 cells by increasing the level of expression in uninduced cells (11; unpublished data).

As a first step toward assaying SRF function in cells, we have changed its DNA binding specificity to that of GAL4 by fusing the DNA binding domain of GAL4 to the amino terminus of SRF. We tested whether the GAL4-SRF chimera would activate transcription and cause a GAL4 site reporter gene to become growth factor responsive.

MATERIALS AND METHODS

Plasmid constructions. GAL4-SRF(1-508) was constructed by using a 1.6-kb *Xba*I-to-*Bam*HI fragment from pSRFXba, kindly provided by Michael Gilman and containing *Xba*I

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linker DNA 5′ to the SRF start codon followed by 1.6 kb of SRF cDNA sequence (26). This fragment was ligated into pGAL4-CREB (2) in which the CREB part was excised with *Xba*I and *Bam*HI. These constructs contain the cytomegalovirus promoter and are derivatives of the vector pCG (36). GAL4-SRF(204-508), GAL4-SRF(245-508), GAL4-SRF(266-508), GAL4-SRF(339-508), GAL4-SRF(414-508), and GAL4-SRF(433-508) were constructed by digesting pSRFXba with *Pst*I, *Bgl*II, *Nae*I, *Pvu*II, *Sph*I, and *Xmn*I, respectively, blunting with the Klenow fragment of *Escherichia coli* DNA polymerase where necessary, and ligating appropriate *Xba*I linker DNA such that the reading frame remained correct after fusion to the GAL4 DNA binding domain. The plasmids were then digested with *Xba*I and *Bam*HI and ligated into pGAL4-CREB as described above. GAL4-SRF(168-508) was constructed by digestion of pSRFXba with *Stu*I (at aa 171) followed by addition of adapter oligonucleotides to recreate sequence to aa 168 and an *Xba*I site. An *Xba*I-to-*Bam*HI fragment was then ligated into pGAL4-CREB as described above.

GAL4-SRF(pm1) was constructed by digesting pARSR-Fpm1 (29) with *Nde*I (at the start codon), blunting with Klenow fragment, ligating *Xba*I linker DNA, digesting with *Xba*I and *Bam*HI, and ligating the SRF fragment into pGAL4-CREB as described above. pGAL4-SRF(168-508BclX) was constructed by substituting the *Pst*I-to-*Bam*HI fragment of GAL4-SRF(168-508) with the *Pst*I-to-*Bam*HI fragment of pARSRFBclX (29). GAL4-SRF(204-465) and GAL4-SRF (204-433) were constructed by digesting GAL4-SRF(204-508) with *Bst*YI and *Xmn*I, respectively, blunting with Klenow fragment, and ligating an *Xba*I linker containing termination codons in all reading frames. The resulting *Xba*I fragment was ligated into *Xba*I-digested pGAL4-CREB which had its *Bam*HI site converted to an *Xba*I site by using the same termination linker. A construct expressing GAL4 aa 1 to 147 only [GAL4(1-147)], pCGT, was made from pGAL4-CREB as described above except for placing an *Xba*I termination linker between the *Xba*I and *Bam*HI sites without an insert.

GAL4-SRF(Δ171-203), GAL4-SRF(Δ141-203), GAL4-SRF (Δ93-203), and GAL4-SRF(Δ46-203) were constructed by converting the *Stu*I (aa 171), *Sma*I (aa 141), *Nar*I (aa 93), and *Sma*I (aa 46) sites to *Sal*I sites by using *Sal*I linker DNA and ligating the *Xba*I-to-*Sal*I fragment to *Xba*I- and *Sal*I-digested GAL4-SRF(1-508) in which the *Pst*I site at aa 204 had been converted to a *Sal*I site by using linker DNA. All of the N-terminal and internal deletion mutants were sequenced across the deletion junction to confirm the mutations.

SGS(1-45) and SGS(1-171) were constructed by ligating three fragments into *Xba*I- and *Bam*HI-digested pCG: (i) *Xba*I (at aa 1) to *Sma*I (aa 45) or *Stu*I (aa 171), respectively, from pSRFXba, in which the 3′ ends of the fragments were converted to *Xba*I sites with linker DNA; (ii) *Fok*I to *Hae*II of GAL4 (aa 5 to 92), in which these sites were converted to *Xba*I and *Bam*HI sites, respectively, with linker DNA; and (iii) *Bgl*II to *Bam*HI of pSRFXba (aa 245 to 508). SGS(1-45/172-266) was constructed by ligating an *Xba*I-to-*Sal*I fragment (aa 1 to 45) from GAL4-SRF(Δ46-203) with a *Stu*I-to-*Nae*I fragment (aa 172 to 266) from pSRFXba (in which these sites were converted to *Sal*I and *Xba*I sites, respectively) into *Xba*I-digested SGS(1-45).

SRF expression vector pCGNSRF(1-508) was as described previously (22). The C-terminal deletion mutant pCGNSRF(1-338) was constructed by digesting pCGNSRF (1-508) with *Pvu*II (at aa 338), ligating an *Xba*I termination linker, digesting with *Xba*I, and inserting the fragment into *Xba*I-digested pCGN (36).

Reporter plasmid pFC53G5 was constructed by inserting a cassette containing five GAL4 DNA binding sites from pGAL4$_5$E1bTATACAT (18) at the −53 position of pFC53 (5). The *Xba*I site in pGAL4$_5$E1bTATACAT was converted to *Bam*HI by using DNA linkers, and the GAL4 sites were excised with *Bam*HI and *Hin*dIII. This fragment was then ligated into pFC53 digested with *Hin*dIII and *Bgl*II. An internal control plasmid, phsp70CAT, that contains the human hsp70 promoter upstream of a chloramphenicol acetyltransferase (CAT) gene (same as p1170-CAT in reference 35) was used. Plasmid pFC53XGL, containing a high-affinity SRF binding site, was the same as pFC53X (27) except that it was in pUC19 rather than pUC18.

Transfection and RNA analysis. Reporter plasmids pFC53G5 (12 μg) and phsp70CAT (5 μg) (as an internal reference) were transfected with GAL4-SRF expression plasmids (3 μg) into HeLa or NIH 3T3 cells by the calcium phosphate precipitation method (31). Cells (set up at 10^6 per 10-cm-diameter plate and transfected 24 h later) were treated 48 h after transfection with or without 100 ng of EGF (Collaborative Research) per ml for 30 min, and total RNA was isolated by urea lysis (4). Specifically initiated *fos*-CAT transcripts were assayed by RNase protection as described previously (12). Transcript levels were quantitated by using a PhosphorImager and ImageQuant software analysis (Molecular Dynamics).

Immunoblotting and gel mobility shift assays. Cell lysates were prepared from transiently transfected HeLa cells (one 10-cm-diameter dish; approximately 6×10^6 cells) by resuspending the cells in 0.2 ml of 3× sodium dodecyl sulfate (SDS) sample buffer (6% SDS, 180 mM Tris-HCl [pH 6.8], 30% glycerol, 0.003% bromophenol blue). The amounts of the GAL4-SRF constructs transfected were as described above. The lysates (20 μl) were analyzed by immunoblotting using a 1:500 dilution of anti-GAL4 serum (kindly provided by Ivan Sadowski) and alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (Promega) as a secondary antibody.

Nuclear extracts were made from two 10-cm-diameter plates of transiently transfected HeLa cells (approximately 1.2×10^7 cells) (28) and analyzed by gel mobility shift assay for GAL4 site DNA-binding activity. A double-stranded 23-bp oligonucleotide, TCGAGCGGAGGAC(T/A)GTCCTC CGC, with *Xho*I ends and spanning a GAL4 DNA binding site (8) was used as a probe essentially as described previously (20) except that nuclear extract (5 μg) was used with poly(dI-dC) (instead of herring sperm DNA) at a final concentration of 75 mM KCl. GAL4-SRF proteins in nuclear extracts (about 30 μg) were also analyzed by immunoblotting with a 1:750 dilution of anti-SRF serum (22) or anti-GAL4 serum as described above.

RESULTS

Transfection of GAL4-SRF constructs. A GAL4-SRF fusion construct and variants with different parts of SRF's coding region (diagrammed in Fig. 1) were transfected into HeLa cells with a reporter gene (pFC53G5) with five GAL4 binding sites upstream of a minimal c-*fos* promoter (−53 to +42) and the bacterial CAT gene. Cells were treated with or without EGF for 30 min, and RNA was isolated. Specifically initiated *fos*-CAT transcripts were then analyzed by RNase protection assays. An internal control template with the hsp70 promoter (phsp70CAT) was also transfected to normalize for transfection efficiencies.

Surprisingly, we found that full-length SRF fused to GAL4

DNA Binding
CKII Site
Dimerization
GAL4 1-147 1 P 133 168 222 266 508
Basic region
206
PRGI

Construct	Activation
GAL4-SRF(1-508)	-
GAL4-SRFpm1	-
GAL4-SRF(168-508)	-
GAL4-SRF(168-508BclX)	-
GAL4-SRF(204-508)	+
GAL4-SRF(245-508)	+
GAL4-SRF(266-508)	+
GAL4-SRF(339-508)	+
GAL4-SRF(414-508)	- HeLa + NIH/3T3
GAL4-SRF(433-508)	-
GAL4-SRF(204-465)	-
GAL4-SRF(204-433)	-

FIG. 1. Diagram of GAL4-SRF constructs. The structures of constructs containing GAL4's DNA binding domain, aa 1 to 147, and various regions of SRF are indicated. The top diagram shows the position within SRF of known domains. GAL4-SRF(pm1) has three point mutations in the basic region that abolish DNA binding. GAL4-SRF(168-508BclX) has a linker insertion after aa 206 that abolishes dimerization. The ability of each construct to activate expression of a GAL4 site reporter gene (see Fig. 2 and 3) is summarized at the right.

[GAL4-SRF(1-508)] did not activate expression from pFC53G5 with or without EGF treatment of cells (Fig. 2). Expression was the same with GAL4-SRF(1-508) as with GAL4's DNA binding domain alone (aa 1 to 147 in construct CGT). To test whether the failure to activate could be due to the presence of two DNA binding domains in the fusion protein, we made a GAL4-SRF construct that had three point mutations in SRF that abolish DNA binding (29). This fusion protein, GAL4-SRF(pm1), also failed to activate (Fig. 2, lanes 5 and 6). Since SRF can activate transcription in vitro and since the carboxy-terminal region of SRF is required for optimal activation (29), we made deletions from the amino terminus to determine whether the carboxy-terminal part of the protein contains an activation domain that can function when fused to GAL4. GAL4-SRF(168-508) still did not activate transcription; however, deletion to aa 203, in GAL4-SRF(204-508), activated transcription independently of EGF treatment (lanes 7 to 10). These results suggest that SRF's transcriptional activation domain is between aa 204 and 508 and that when this domain is separated from the rest of SRF, its activity is constitutive. Thus, it

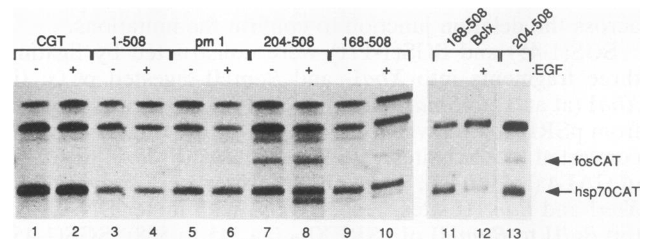

FIG. 2. Activation of expression by GAL4-SRF constructs. The indicated GAL4-SRF constructs were transfected into HeLa cells with a reporter gene, pFC53G5, containing five GAL4 sites upstream of a minimal c-*fos* promoter fused to the bacterial CAT gene. As an internal control, a reporter gene with the hsp70 promoter was also transfected. CGT is an expression construct with GAL4's DNA binding domain without SRF. Forty-eight hours after transfection, cells were treated with (+) or without (−) EGF for 30 min. RNA was then analyzed by RNase protection assays for specifically initiated transcripts. The positions of the *fos*-CAT and hsp70-CAT transcripts are indicated. The higher bands represent undigested probe and transcripts nonspecifically initiated upstream.

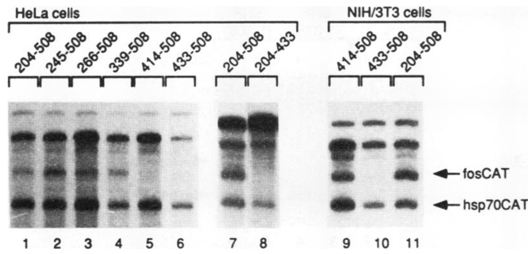

FIG. 3. Mapping of SRF's transcriptional activation domain. GAL4-SRF constructs containing the indicated regions of SRF were transfected into HeLa (lanes 1 to 8) or NIH 3T3 (lanes 9 to 11) cells. Transfection and RNase protection assays were as described for Fig. 2.

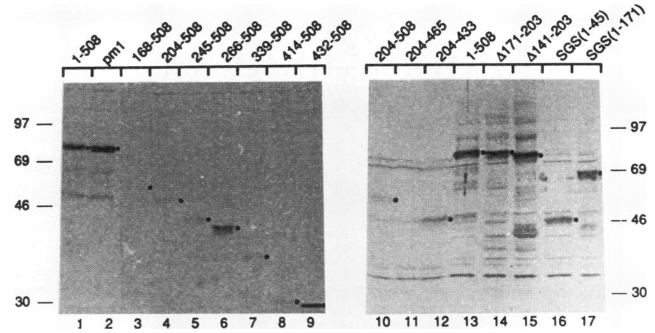

FIG. 4. Immunoblotting of transfected GAL4-SRF proteins. Nuclear extracts (lanes 1 to 9 and 13 to 17) or whole cell lysates (lanes 10 to 12) were probed with anti-SRF (lanes 1 to 9) or anti-GAL4 (lanes 10 to 17) serum. Bands with the expected size that were detected are indicated with a dot at the right of each band. Molecular weight markers are shown in kilodaltons on the side of each gel.

appears that the rest of SRF serves to repress the activation domain. The result, however, that GAL4-SRF(1-508) is not activated by EGF treatment implies that SRF must bind directly to DNA for proper regulation.

The dimerization domain of SRF is located between aa 168 and 222 (26) (Fig. 1) such that one obvious difference between GAL4-SRF(168-508) and GAL4-SRF(204-508) is that the former may be able to dimerize through SRF's dimerization domain. To test whether dimerization through SRF was responsible for the failure of GAL4-SRF(168-508) to activate, we mutated the dimerization domain with a linker insertion after aa 206, which we previously found to abolish SRF dimerization (29). This construct, GAL4-SRF(168-508BclX), was still unable to activate transcription, suggesting that dimerization through SRF is not the cause for lack of activity (Fig. 2, lanes 11 and 12).

The level of activation by SRF's activation domain in the GAL4-SRF constructs is similar to the level from an SRE in EGF-treated cells. We found that induction from a reporter gene with a high-affinity SRF binding site (pFC53XGL) in EGF-treated cells was similar to activation of the GAL4 site reporter gene by GAL4-SRF(204-508) (see Fig. 7B, lanes 1 to 4). This result suggests that activated endogenous SRF activates to a similar extent as SRF's activation domain fused to GAL4. Although this experiment was performed with one SRE site in pFC53XGL and five GAL4 sites in the GAL4 site reporter gene, activation by GAL4-SRF was relatively unchanged with one versus five GAL4 sites (data not shown). In contrast, activation by the herpesviral VP16 protein in GAL4 fusion constructs depended on the number of GAL4 sites in the reported gene. Thus, activation by SRF was approximately three times stronger than that by VP16 with one GAL4 site, but VP16 was about six times stronger with five sites (data not shown). Therefore, activation by multiple GAL4-VP16 factors binding simultaneously to the promoter appears to be much more synergistic than activation by SRF.

Mapping of SRF's transcriptional activation domain. To more precisely map SRF's transcriptional activation domain, we constructed further deletions from the amino terminus (diagrammed in Fig. 1). Truncations to aa 338 were equally able to activate (Fig. 3, lanes 1 to 4). Deletion to position 413 or 432, however, abolished activity (lanes 5 and 6). This places the amino-terminal border of the activation domain between aa 339 and 413 in HeLa cells. Surprisingly, in NIH 3T3 cells, we found that GAL4-SRF(414-508) was still able to activate transcription. Deletion to aa 433 was required to abolish activation in NIH 3T3 cells (lanes 9 to 11). Although activation by GAL4-SRF(414-508) in NIH 3T3 cells appears to be lower than that by GAL4-SRF(204-508) when normalized to the internal control, in several other experiments the levels were similar. Thus, a larger activation domain is required in HeLa cells than in NIH 3T3 cells.

Carboxy-terminal truncations in GAL4-SRF(204-508) were made to map the other end of the transcriptional activation domain. We found that deletion to aa 433 abolished activity (Fig. 3, lane 8). Similar results were obtained in NIH 3T3 cells (data not shown). This places the carboxy-terminal border of the activation domain between aa 433 and 508.

To ensure that the differences in transactivation potential of the various GAL4-SRF chimeras were not due to differences in expression, we made extracts from transfected cells and tested for expression by immunoblotting with anti-SRF (Fig. 4, lanes 1 to 9) or anti-GAL4 (lanes 10 to 17) serum. Endogenous SRF is difficult to detect in crude nuclear extracts with the anti-SRF serum. We found, however, that all of the proteins except one were expressed at detectable levels. The exception, GAL4-SRF(204-465), was not detectably expressed (lane 11); hence, we were not able to evaluate the effect of this smaller deletion on activation. Although the levels of expression of the other proteins varied, there was no correlation between the level of expression and the ability to activate. We also tested for DNA binding of the GAL4-SRF proteins to GAL4 sites to show that this critical activity was not affected by different SRF sequences. Using a gel mobility shift assay, we found that all of the proteins tested, including all those that failed to activate, bound a GAL4 site to comparable degrees (Fig. 5). The signal for GAL4-SRF(414-508) (lane 8) is not clear since it appears to comigrate with a background band. However, since this mutant was detected by immunoblotting (Fig. 4) and was active in NIH 3T3 cells (Fig. 3), its lack of activity in HeLa cells is unlikely to be due to lack of synthesis or stability. GAL4-SRF(168-508) migrated more rapidly than GAL4-SRF(204-508) in the gel mobility shift assay (Fig. 5, lanes 3 and 4). This difference is probably due to conformational or charge differences rather than size differences due to truncation because the proteins ran true to the expected sizes on SDS-polyacrylamide gels (Fig. 4, lanes 3 and 4). The expression of GAL4-SRF(168-508BclX) was somewhat low though detectable, perhaps as a result of instability of this mutant protein (Fig. 5, lane 10). It was also low but detectable in immunoblots (data not shown). We believe, however, that the level detected would be sufficient to activate expression,

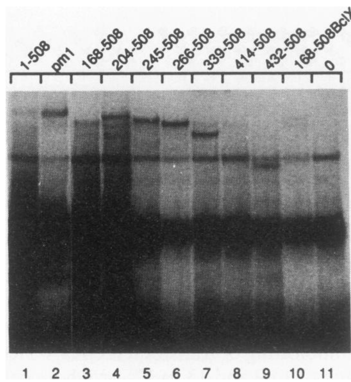

FIG. 5. DNA-binding activity of transfected GAL4-SRF proteins. Gel mobility shift assays were performed with nuclear extracts from transfected cells and with a ^{32}P-labeled oligonucleotide containing a GAL4 DNA binding site. HeLa cells were transfected with GAL4-SRF constructs, as indicated, or mock transfected (lane 11). Unbound oligonucleotide migrates just off the bottom of the gel. Nonspecific complexes are those seen with mock-transfected extracts (lane 11).

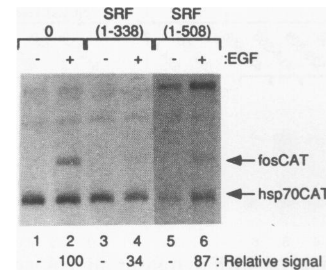

FIG. 6. Deletion of SRF's transactivation domain results in a dominant negative mutant. HeLa cells were transfected without (0) or with full-length (aa 1 to 508) or truncated (aa 1 to 338) SRF expression constructs and a reporter gene containing an SRE (pFC53E). In addition, phsp70CAT was transfected as an internal control. Transcripts were analyzed by RNase protection assays as described for Fig. 2. The relative levels of *fos*-CAT transcripts, normalized to that of the internal control, are indicated below the lanes.

since a fivefold-lower amount of GAL4-SRF(204-508) plasmid still activated transcription (data not shown).

To test whether the transcriptional activation domain identified with GAL4-SRF constructs is also required for SRF function on an SRE, we constructed an SRF mutant deleted of aa 339 to 508 [CGNSRF(1-338)]. If this region is required for activation, the mutant should function as a dominant negative inhibitor of activation. Overexpression of this mutant did, in fact, inhibit EGF activation of an SRE reporter gene by two-thirds compared with the level found in cells transfected with a vector control (Fig. 6, lanes 1 to 4). Overexpression of full-length SRF [CGNSRF(1-508)] had little effect on expression (lanes 5 and 6). The levels of expression were normalized to that of the internal control, and in several experiments full-length SRF did not significantly reduce the level of EGF-induced expression. In addition, the levels of truncated and full-length SRF proteins detected in immunoblots following transfection were similar (data not shown). These results suggest that the C terminus of SRF is required for normal activation in vivo in the context of SRF as well as GAL4-SRF.

Identification of inhibitory regions. The failure of GAL4-SRF(1-508) to activate transcription from a GAL4 site reporter gene suggests that the amino-terminal 203 aa contain a domain(s) that inhibits the transcriptional activation domain. As described above, we found that although this inhibitory region overlaps the DNA binding and dimerization domains of SRF, these functions were not necessary for inhibition. Specifically, DNA-binding mutations in GAL4-SRF(pm1) or dimerization mutations in GAL4-SRF(168-508BclX) did not affect the inhibition (Fig. 1 and 2).

Since GAL4-SRF(168-508) activated very weakly whereas GAL4-SRF(204-508) activated strongly, aa 168 to 203 are required for inhibition of SRF's activation domain in this context. To investigate whether deletion of these amino acids was sufficient to abolish inhibition in full-length GAL4-SRF, we made internal deletions spanning the region (diagrammed in Fig. 7A). Deletions removing more than aa 142 to 203 abolished inhibition, and the resulting fusion proteins strongly activated expression (Fig. 7B, lanes 5 to 10). However, activation was still inhibited in a mutant with only aa 171 to 203 deleted (lanes 11 and 12). The expression of GAL4-SRF(Δ172-203) and GAL4-SRF(Δ141-203) proteins was confirmed by immunoblotting of transfected cell nuclear extracts with anti-GAL4 serum (Fig. 4, lanes 14 and 15). These data suggest that there are two separable domains in SRF that can independently inhibit the C-terminal activation domain. One of these domains is located in the first 171 aa, as seen in GAL4-SRF(Δ172-203) compared with GAL4-SRF(204-508). The second is located C terminal of aa 168, as seen in GAL4-SRF(168-508) compared with GAL4-SRF(204-508) (summarized in Fig. 1 and 7A).

To test whether the inhibitory domains of SRF were modular and could also inhibit the activation domain when moved do a different position in the protein, we constructed GAL4 fusion constructs with the putative inhibitory domain amino terminal to the GAL4 DNA binding domain (Fig. 8A). The first inhibitory domain, in aa 1 to 171, inhibited activation in such a construct [SGS(1-171); Fig. 8B]. A similar construct, containing only the first 45 aa of SRF in front of GAL4 [SGS(1-45)], activated strongly. While weak EGF induction is apparent with SGS(1-171) (lanes 5 and 6), this induction was not reproducible. We confirmed that the SGS(1-171) protein was expressed well by immunoblotting (Fig. 4, lanes 16 and 17) and DNA binding assays (Fig. 8C) of transfected cell extracts. The second inhibitory domain, for which we used aa 171 to 266 of SRF, did not inhibit expression in this context [SGS(1-45/171-266); Fig. 8B], possibly because this inhibitory domain is sensitive to position in the protein. Alternatively, since we have not mapped the carboxy-terminal border of this domain, the fragment we used (aa 171 to 266) may not have spanned the entire inhibitory domain.

DISCUSSION

Using GAL4-SRF constructs, we have shown that SRF contains a transcriptional activation domain located near the carboxy terminus of the protein and two separable domains that can inhibit the activation domain.

Activation domain. The transcriptional activation domain was mapped to between aa 339 and 508 in HeLa cells. Surprisingly, in NIH 3T3 cells, a smaller region, aa 414 to 508, was sufficient for activation. The reason for this cell type difference is unclear. Two possibilities are that the smaller version of the activation domain adopts the correct conformation more easily in NIH 3T3 cells than in HeLa

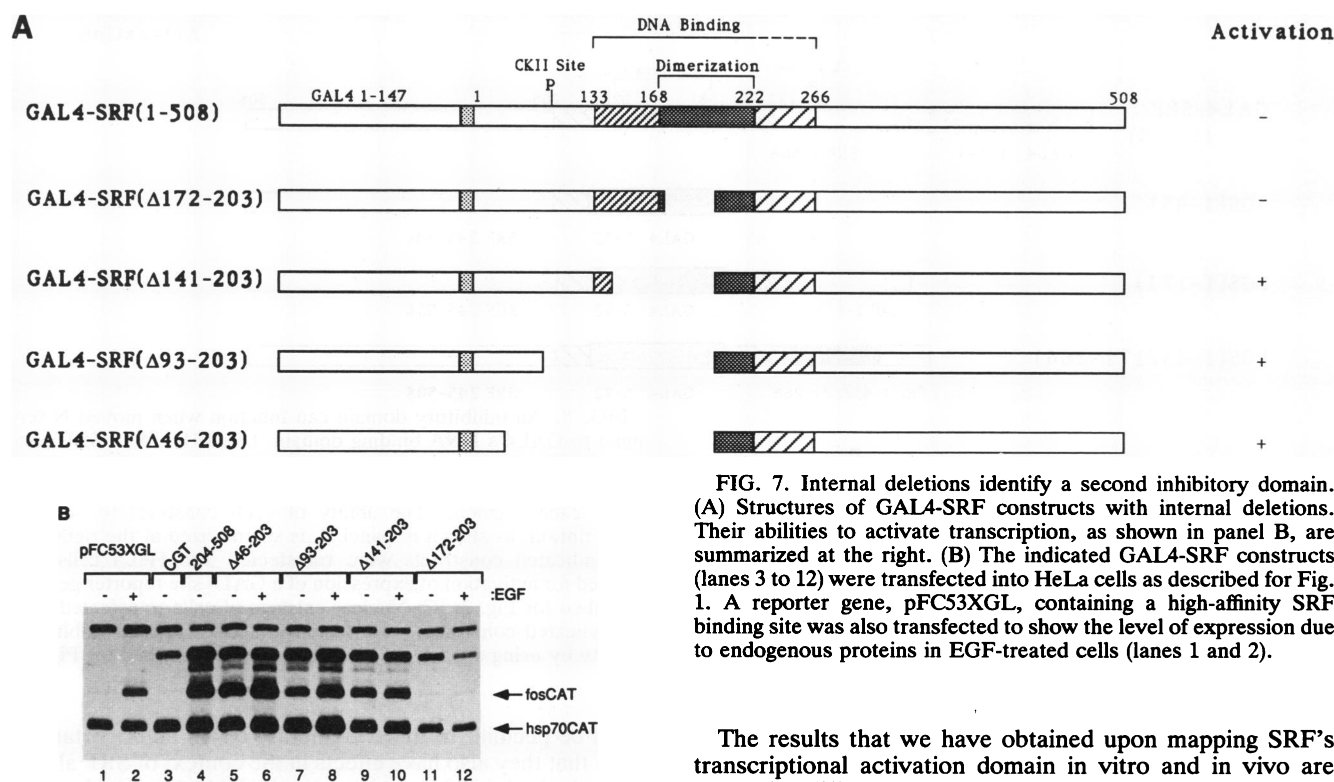

FIG. 7. Internal deletions identify a second inhibitory domain. (A) Structures of GAL4-SRF constructs with internal deletions. Their abilities to activate transcription, as shown in panel B, are summarized at the right. (B) The indicated GAL4-SRF constructs (lanes 3 to 12) were transfected into HeLa cells as described for Fig. 1. A reporter gene, pFC53XGL, containing a high-affinity SRF binding site was also transfected to show the level of expression due to endogenous proteins in EGF-treated cells (lanes 1 and 2).

cells or that there are multiple parts of the activation domain that are required for activation in HeLa but not NIH 3T3 cells. It should be noted that we used human SRF in these studies, so it is somewhat surprising that the minimal activation domain is smaller in mouse cells than in human cells.

We also found that the activation domain defined by using GAL4-SRF constructs was important in the context of SRF alone. Deletion of the activation domain in an SRF expression construct resulted in a dominant negative mutant for SRE activation. This finding further suggests that the activation domain is critical for SRF function and for EGF induction through the SRE. During the course of this study, Lee et al. (17) reported that SRF could activate expression in cultured myoblasts of the skeletal α-actin promoter (which contains an SRE). Consistent with our work, they found that deletion of SRF sequences C terminal of aa 245 abolished activation.

SRF's transcriptional activation domain does not fall into any standard class of activation domain (reviewed in reference 24). It is not rich in any particular amino acid, nor does it have a significant net charge. The mechanism by which SRF's activation domain activates transcription may involve its interaction with the general transcription machinery. It has been found recently that high levels of TFIIF are required for SRF activation of transcription in vitro and that SRF directly interacts in gel mobility shift assays with the RAP74 subunit of the general transcription factor TFIIF (39). A region of SRF spanning its activation domain, aa 245 to 508, was sufficient for this interaction. We also previously found by using in vitro transcription experiments that the order of addition of SRF and TFIID is critical for activation, such that SRF may also directly affect TFIID function (40). It will be interesting to determine more precisely whether the activation domain defined here binds specifically to TFIIF or TFIID.

The results that we have obtained upon mapping SRF's transcriptional activation domain in vitro and in vivo are somewhat different. In vitro, we found that deletion of aa 412 to 508 reduced activation by 50% but did not abolish it (29). We found here that similar deletions abolished activation by GAL4-SRF constructs. It appears that the in vitro conditions are more permissive for activation. Similar results have been found with GAL4; i.e., GAL4(1-147) activates in vitro but not in vivo (19). It is possible that deleted SRF(1-411) has an activity that contributes to activation in cells and can be detected in vitro but that is not sufficient for activation in vivo.

Inhibitory domains. We identified two regions of SRF that inhibited the activity of the transcriptional activation domain in GAL4-SRF constructs. The first domain mapped to aa 1 to 171 and overlaps the basic region of SRF's DNA binding domain. The second domain, C terminal to amino acid 171, overlaps the dimerization domain. These functions, however, do not account for the inhibitory activity, since point mutations or a linker insertion that abolish DNA binding or dimerization, respectively, had no effect on inhibition.

The importance of this inhibitory function is intriguing because it could be involved in regulation of SRF activity. SRF may need to be inhibited in cells when *fos* is not expressed. In untreated cells, *fos* transcription is low, is rapidly induced to a peak 15 min after growth factor treatment, and then is quickly repressed to uninduced levels by 60 min after induction (38). It appears that growth factor treatment does not change SRF's DNA binding activity (4, 10, 34, 37), and in vivo footprinting analysis suggests that SRF constitutively occupies the SRE (13). Thus, these results suggest that SRF is bound at the promoter before and after induction without activating expression. The observation that overexpression of SRF in NIH 3T3 cells activates expression in uninduced cells (11; unpublished data) is also consistent with negative regulation of SRF, since overexpression may serve to titrate out an inhibitory factor. Thus, the inhibitory domains could have a function in repressing SRF activity in uninduced cells. We have not, however,

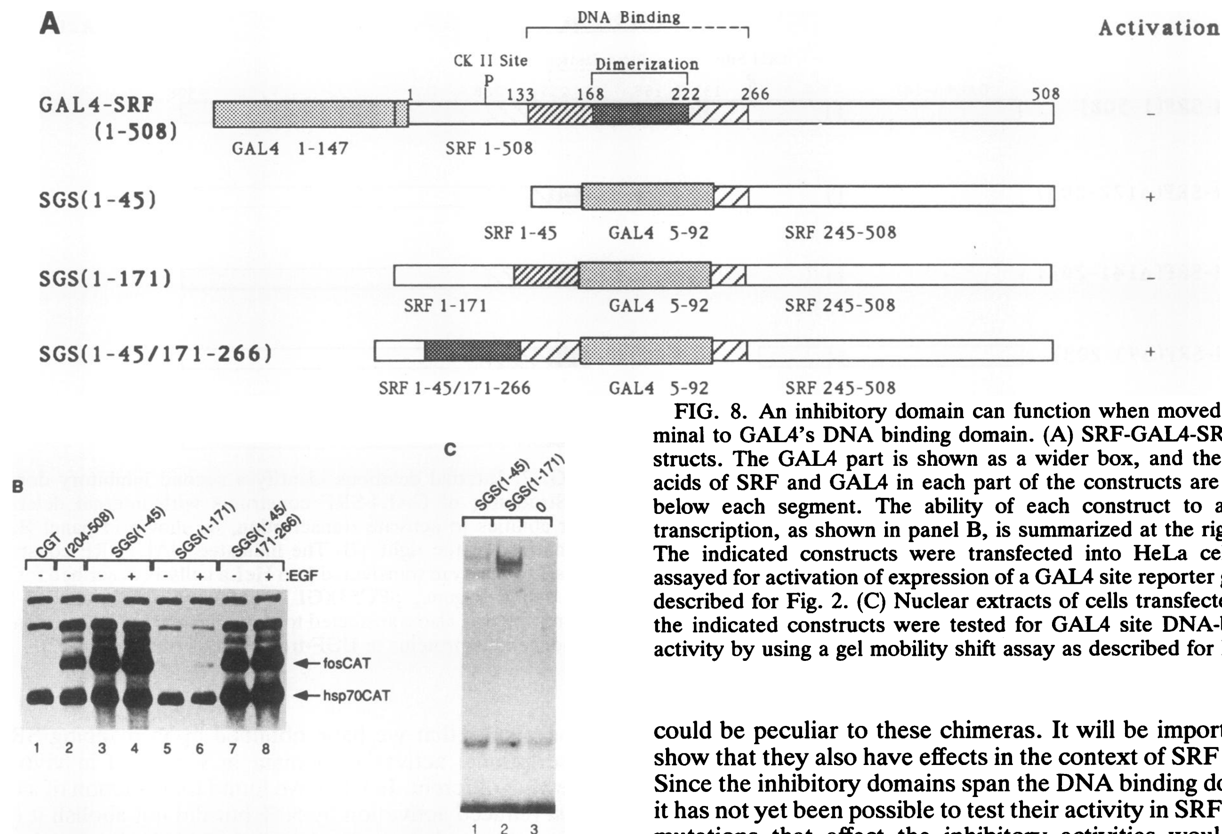

FIG. 8. An inhibitory domain can function when moved N terminal to GAL4's DNA binding domain. (A) SRF-GAL4-SRF constructs. The GAL4 part is shown as a wider box, and the amino acids of SRF and GAL4 in each part of the constructs are shown below each segment. The ability of each construct to activate transcription, as shown in panel B, is summarized at the right. (B) The indicated constructs were transfected into HeLa cells and assayed for activation of expression of a GAL4 site reporter gene as described for Fig. 2. (C) Nuclear extracts of cells transfected with the indicated constructs were tested for GAL4 site DNA-binding activity by using a gel mobility shift assay as described for Fig. 5.

observed growth factor regulation of the inhibitory activity in GAL4-SRF constructs; hence, the relief of inhibition may require SRF binding directly to DNA.

In GAL4-SRF constructs, the fusion protein is bound to DNA through GAL4 such that the SRF part may behave as if it is not bound to DNA. Thus, another possible role of the inhibitory domains is to repress the activation domains when SRF is not bound to DNA. This would stop SRF from inappropriately activating transcription complexes that it may contact without binding specifically to DNA. It may be important in general that transcriptional activators in the nucleus activate transcription only when they are bound to their specific sites.

The mechanism of inhibition could involve a direct effect of the inhibitory domains on the activation domain or could be mediated by the binding of an inhibitory factor(s) to these domains. We have not yet been able to distinguish these possibilities. If there is an inhibitory factor, it should be possible to titrate it out by overexpression of SRF and thereby increase expression of a reporter gene. This has been done with c-jun to identify a c-*jun* inhibitory factor (1). Using several SRF constructs, however, we have not been able to relieve the inhibition by overexpression of SRF (unpublished data). To test the specificity of SRF's inhibitory domain, we tried a chimeric construct of GAL4-SRF-VP16 that includes SRF's inhibitory domains and the transcriptional activation domain of the herpesviral VP16 protein. SRF's inhibitory domains could not inhibit VP16 activity such that inhibition of SRF's activation domain is at least somewhat specific (unpublished data).

Since the inhibitory effects that we have observed have all been detected in the context of GAL4-SRF constructs, they could be peculiar to these chimeras. It will be important to show that they also have effects in the context of SRF alone. Since the inhibitory domains span the DNA binding domain, it has not yet been possible to test their activity in SRF, since mutations that affect the inhibitory activities would also abolish DNA binding. Further mapping of the inhibitory domains, however, may identify mutations that affect this activity without affecting SRF DNA binding. There also has not been an in vivo assay for SRF regulation. Such an assay that reproduces SRF-dependent growth factor regulation of an SRE reporter gene will be critical for analyzing the importance of SRF's inhibitory domains and identifying other regions required for growth factor regulation.

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