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Serum-regulated transcription by Serum Response Factor (SRF): a novel role for the DNA binding domain

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The transcription factors Serum Response Factor (SRF) and Ternary Complex Factor (TCF) form a ternary complex at the c-*fos* Serum Response Element (SRE). We show that in NIH3T3 cells TCF binding is required for regulated transcription in response to stimulation by phorbol myristate acetate (PMA), but not by whole serum. We constructed a novel transcriptionally inactive SRE variant whose serum-regulated activity can be partially restored by overexpression of SRF in the absence of bound TCF. Activation by SRF does not require the SRF N-terminal phosphorylation sites, but is potentiated 2- to 3-fold by the SRF C-terminal activation domain. Mutations in the SRF DNA binding domain, which do not affect the ability of SRF to bind DNA, abolish its ability to mediate TCF-independent serum-regulated activation and reduce activation by the SRF/TCF(Elk-1) ternary complex. Efficient activation requires that SRF be targeted to DNA via its own DNA binding domain.

Key words: c-*fos* gene/serum response element/serum response factor/ternary complex factor/transcription

Introduction

The Serum Response Element (SRE) is a promoter element required for the regulation of many cellular immediate-early genes by growth factors (Treisman, 1990). Studies of the activation of the prototypic c-*fos* SRE, both by diverse extracellular signals and by intracellular signalling molecules, indicate that its activity can be regulated by the conserved MAP kinase signalling pathway (for reviews, see Johnson and Vaillancourt, 1994; Marshall, 1994; McCormick, 1994). The SRE binds the ubiquitous transcription factor serum response factor (SRF), the DNA binding domain of which is 70% identical to that of MCM1, a yeast protein which binds SRE-related sequences which are also regulated by extracellular signals (Norman *et al.*, 1988). Mutational analysis indicates that SRF binding is required for SRE function (for a review, see Treisman, 1990). SRF contains a transactivation domain at its C-terminus (Norman *et al.*, 1988; Prywes *et al.*, 1988; Hipskind and Nordheim, 1991; Zhu *et al.*, 1991; Prywes and Zhu, 1992; Hill *et al.*, 1993; Johansen and Prywes, 1993; Liu *et al.*, 1993) and interacts with TFIIF (Zhu *et al.*, 1994). SRF is subject to a growth factor-regulated phosphorylation by pp90^{rsk} at serine 103 (Rivera *et al.*, 1993) and is constitutively phosphorylated at its N-terminus by casein kinase II (CKII) (Manak *et al.*, 1990; Janknecht *et al.*, 1992; Marais *et al.*, 1992). However, the regulatory significance of these modifications remains obscure since SRF alone does not potentiate the growth factor-regulated activity of a co-transfected SRE (Hill *et al.*, 1993; Johansen and Prywes, 1993; S.John and R.Treisman, unpublished).

SRF forms a ternary complex at the c-*fos* SRE with members of a family of Ets domain accessory proteins, the Ternary Complex Factors (TCFs), which bind to a conserved Ets motif (Shaw *et al.*, 1989; reviewed in Treisman, 1994). These proteins, which include SAP-1 (Dalton and Treisman, 1992), Elk-1 (Rao *et al.*, 1989; Hipskind *et al.*, 1991) and ERP-1/NET/SAP-2 (Giovane *et al.*, 1994; Lopez *et al.*, 1994; M.A.Price, A.Rogers, J.Wynne and R.Treisman, manuscript in preparation), contain two conserved N-terminal regions required for DNA binding and ternary complex formation with SRF (Dalton and Treisman, 1992; Janknecht and Nordheim, 1992; Rao and Reddy, 1992; Treisman *et al.*, 1992), and a conserved C-terminal domain containing potential MAP kinase consensus sites. Growth factor-stimulated activation of the MAP kinase pathway results in phosphorylation of the Elk-1 C-terminus, potentiating its ability to activate transcription (Gille *et al.*, 1992; Janknecht *et al.*, 1993; Marais *et al.*, 1993; Zinck *et al.*, 1993; Janknecht *et al.*, 1994; Kortenjann *et al.*, 1994; M.A.Price, A.Rogers, J.Wynne and R.Treisman, manuscript in preparation). In the ternary complex with SRF, the Elk-1 C-terminal region cooperates with the SRF C-terminal activation domain to regulate transcription (Hill *et al.*, 1993). These studies demonstrate that at least part of the serum-inducible transcriptional activition of the SRE occurs via the MAP kinase pathway and is dependent on the SRF/TCF ternary complex. Nevertheless, several observations suggest that at least in some cells activation of the SRE can occur independently of TCF binding. For example, in mouse BALB/c 3T3 and rat cardiac myocyte cells, c-*fos* SRE mutations that block TCF binding *in vitro* block transcriptional induction resulting from activation of protein kinase C (PKC) by phorbol myristate acetate (PMA), but do not significantly reduce the transcriptional response to serum stimulation (Graham and Gilman, 1991; Sadoshima and Izumo, 1993). In NIH3T3 cells, by contrast, such mutations have been reported to cause either severe reductions in serum inducibility (Shaw *et al.*, 1989) or to leave regulation by either serum- or PMA-induced activation of PKC unaffected (Konig, 1991). In addition, several immediate-early gene promoters contain SRF binding sites that apparently lack associated Ets motifs (Mohun *et al.*, 1987; Latinkic *et al.*, 1991).

In this paper we present evidence for TCF-independent

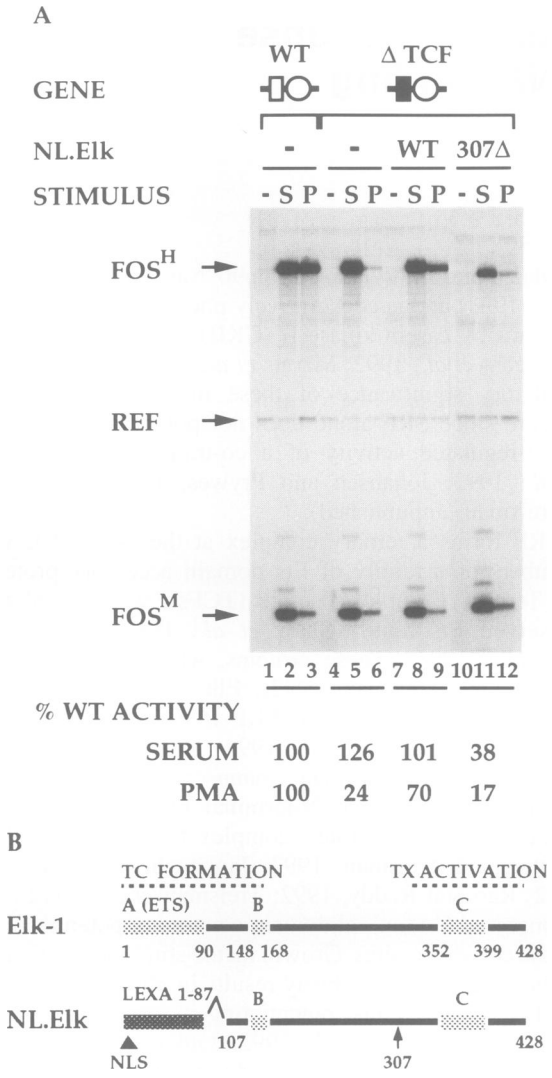

Fig. 1. (**A**) TCF binding at the c-*fos* SRE is required for PMA- but not serum-induced transcriptional activation. NIH3T3 cells were transfected with pF711, which contains the intact human c-*fos* gene including 711 base pairs of 5′ flanking sequence (lanes 1–3) or pF711ΔTCF, a derivative in which the Ets motif of the single SRE is changed to a LexA half-operator (lanes 4–12; for sequence see Figure 2A). α-Globin plasmid πSVHSα118 (2.5 μg) was co-transfected as an internal reference together with 1 μg of the following expression plasmids: MLV128β (control) (lanes 1–6), MLVNL.Elk (lanes 7–9), MLVNL.Elk 307Δ (lanes 10–12). Total cell RNA was prepared either before stimulation (lanes 1, 4, 7 and 10) or after a 30 min stimulation with either 15% FCS (lanes 2, 5, 8 and 11) or 50 ng/ml PMA (lanes 3, 6, 9 and 12). Nuclease-protected products of 287 nucleotides (transfected human c-*fos*, FOS^H, exon 1), 132 nucleotides (α-globin exon 1; REF) and ~60 nucleotides (partially protected endogenous mouse c-*fos*, FOS^M, exon 1) are indicated by arrows. The samples in lanes 10–12 were analysed on a separate gel. Symbols representing the SRE and mutant SRE are shown above: the wild-type SRF binding site is shown as an open circle; the TCF binding site as an open rectangle; the LexA half-operator as a filled rectangle. The data in the table below are averaged from four independent experiments. Induced activities of the transfected wild-type gene by serum and PMA were taken as 100%; the actual level of activity induced by PMA ranged from 41 to 69% that induced by serum in the four experiments. The low level of inducibility of the endogenous gene by PMA relative to serum compared with the transfected gene is under investigation. (**B**) Structures of Elk-1 and the altered specificity mutants NL.Elk and L.Elk (Hill *et al.*, 1993). Elk-1/SAP-1 homology regions A, B and C (Dalton and Treisman, 1992) are indicated by light shaded boxes, the LexA DNA binding domain as a dark shaded box, and the nuclear localization signal (NLS) in NL.Elk by a black triangle. For *in vitro* studies L.Elk was used, which is as NL.Elk, but lacks the NLS. Functional domains are indicated; the position of the truncation in MLVNL.Elk 307Δ that removes the activation domain is indicated by an arrow.

regulation of transcription by SRF. We show that in NIH3T3 cells serum-induced activation of the c-*fos* SRE is largely independent of the ternary complex, and that overexpression of SRF can restore TCF-independent activation to an inactive SRE derivative with low affinity for SRF. The DNA binding domain of SRF is instrumental in mediating TCF-independent transcriptional regulation in response to serum and requires direct contact with an AT-rich SRE to do so. Our data suggest that both efficient TCF-independent activation by SRF and the function of the SRF/TCF ternary complex require a specific configuration of the SRF DNA binding domain on DNA. We propose that serum-induced transcriptional activation by SRF requires interaction of its DNA binding domain with a novel accessory factor.

Results

Serum-activated c-fos transcription in NIH3T3 cells does not require TCF binding

We first evaluated the contribution of TCF binding to transcriptional activation of the c-*fos* SRE in mouse NIH3T3 cells. We examined the behaviour of a mutant human c-*fos* gene which cannot form the TCF/SRF ternary complex at its SRE. This gene, c-*fos*ΔTCF, contains a LexA half-operator in place of the SRE Ets motif, a mutation which allows efficient SRF binding, but prevents recruitment of TCF *in vitro* (Hill *et al.*, 1993). Mouse NIH3T3 cells were transfected with the mutant gene or a wild-type control and their transcription in response either to serum stimulation or to PMA-induced activation of PKC was analysed using an RNase protection assay. The wild-type gene was strongly activated after a 30 min stimulation with either agent (Figure 1A, lanes 1–3). Mutation of the TCF binding site had very little effect on the response to serum, but reduced the PMA response to, on average, 24% that of the wild-type gene (Figure 1A, compare lanes 1–6). Both responses were substantially blocked by a mutation that blocked SRF binding (data not shown; see also Treisman, 1990). Similar results were previously obtained by others using mouse BALB/c 3T3 and rat cardiac myocyte cells (Graham and Gilman, 1991; Sadoshima and Izumo, 1993).

To prove directly that the ΔTCF mutation blocks the PMA response by preventing ternary complex formation, we tested whether PMA regulation can be restored by NL.Elk, an altered specificity Elk-1 protein that binds the LexA half-operator in an SRF-dependent fashion (Figure 1B; Hill *et al.*, 1993). The NL.Elk protein restored the PMA response of the c-*fos* ΔTCF mutant, but left the serum response unaffected (Figure 1A, compare lanes 5 and 6 with 8 and 9). Expression

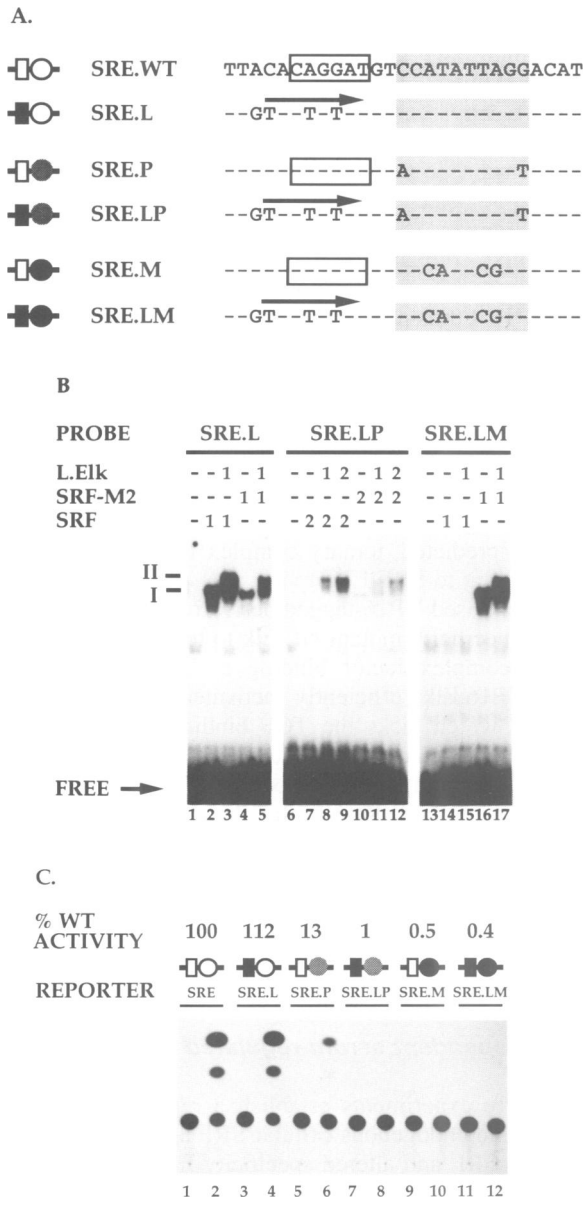

Fig. 2. SRE.LP binds SRF weakly *in vitro* and is transcriptionally inactive *in vivo*. (**A**) Sequences of the intact c-*fos* SRE (SRE.WT) and its derivatives. In SRE.L, SRE.LP and SRE.LM the LexA half-site (arrow) replaces the Ets motif (boxed). SRE.P and SRE.LP have C→A transversions at the outermost base pairs of the SRF binding consensus motif (shaded box); in SRE.M and SRE.LM a high-affinity MCM1-specific site (Wynne and Treisman, 1992) replaces that for SRF (Hill *et al.*, 1993). In all cases dashes indicate identity to SRE.WT. The symbols for the various SRE derivatives are as in Figure 1. The SRF binding sites are shown schematically as circles: open (SRE.WT, SRE.L); shaded (SRE.P, SRE.LP); filled (SRE.M, SRE.LM). The TCF binding site is shown as an open rectangle for the wild-type Ets motif (SRE.WT, SRE.P, SRE.M) and filled for the LexA half-operator (SRE.L, SRE.LP, SRE.LM). (**B**) Binding and ternary complex formation by SRF and altered specificity derivative SRF-M2 at the mutated SREs. Proteins were produced by *in vitro* translation and analysed by gel mobility shift assay using the indicated SRE probes, of equal specific activity. Binding reactions contained either 4 μl unprogrammed lysate (lanes 1, 6 and 13) or the indicated amounts of SRF, SRF-M2 and L.Elk lysate. Complex I, SRF or SRF-M2 alone; complex II, ternary complexes with L.Elk. (**C**) Serum inducibility of the variant SREs. Cells were transfected with SRE-controlled TKCAT reporter genes as indicated. CAT activity was measured in extracts from transfected serum-deprived cells (odd lanes) or cells stimulated for 8 h with 15% FCS (even lanes). The data displayed are from a single representative experiment and data averaged from four independent experiments are shown above. Induced CAT activity is expressed relative to that of the intact c-*fos* SRE (100%).

of NL.Elk had no effect on regulation of the wild-type c-*fos* gene in this assay (data not shown). An NL.Elk deletion mutant lacking the Elk-1 C-terminal regulated activation domain was incapable of mediating the PMA response; intriguingly, this protein also significantly inhibited the response to serum stimulation (Figure 1A, lanes 10–12). This inhibitory effect requires recruitment to the SRE, since expression of this mutant NL.Elk protein had no effect on the transcriptional regulation of a transfected wild-type c-*fos* gene which has no cognate binding site (data not shown).

The differential effect on signalling observed in these experiments shows that the ΔTCF mutation must effectively block ternary complex formation in NIH3T3 cells *in vivo*. PKC-induced transcription is substantially TCF dependent and can be mediated by the altered specificity Elk-1 protein. In contrast, serum-induced transcription is largely TCF independent, but does require SRF binding.

Experimental strategy

The experiments described above strongly suggest that in NIH3T3 cells SRF can mediate serum-induced signals in the absence of a TCF. This view apparently contradicts our previous study of transcriptional activation by SRF using the altered DNA binding specificity SRF derivative SRF-M2 (Hill *et al.*, 1993). SRF-M2 contains multiple sequence changes in its DNA binding domain that allow it to bind the inactive mutant SREs SRE.M and SRE.LM (Figure 2A; Hill *et al.*, 1993). Serum-regulated activation by these SREs is restored by SRF-M2, but only when bound in a ternary complex with a TCF (Hill *et al.*, 1993). A simple resolution of this apparent paradox is that transcriptional activation by SRF-M2 is compromised either by the sequence changes in its DNA binding domain or by the sequence of SRE.LM itself. To test this idea, we developed an assay in which transcriptional activation by wild-type SRF could be analysed. A new mutant SRE was designed whose affinity for SRF is low enough such that *in vivo* it can only be effectively bound by SRF when the protein is overexpressed. The ability of SRF derivatives to restore serum-regulated transcriptional activation to reporter genes controlled by this SRE was then tested.

The new SRE derivative, SRE.P (for Poor affinity for SRF; Figure 2A), retains the AT-rich core of the c-*fos* SRE, but contains transversions at the conserved outermost base pairs of the SRF binding consensus sequence. To allow study of the role of ternary complex factors at this site using our altered specificity TCF mutants, and to prevent cooperation of SRF with endogenous TCFs, we converted the Ets motif bound by TCF to a LexA half-operator (SRE.LP, for LexA operator; Figure 2A). In the following sections we show that SRE.LP binds SRF weakly *in vitro*, and that SRE.LP-controlled reporter genes are uninducible *in vivo* in NIH3T3 cells. We then show that the regulated activity of SRE.LP can be restored by overexpression of exogenous SRF and altered specificity TCF.

SRE.LP binds SRF weakly in vitro and is uninducible in vivo

We used the gel mobility shift assay to evaluate DNA binding and ternary complex formation by SRF and the altered specificity mutant SRF-M2. Both proteins bind the SRE.LP probe, but very weakly, forming ~30-fold less complex in the mobility shift assay than wild-type SRF binding to the c-*fos* SRE (Figure 2B, compare lanes 2, 7 and 10). Similar results were obtained with an SRE.P probe (data not shown). As previously shown, the altered specificity mutant SRF-M2 efficiently binds the SRE.LM site, which is not bound detectably by wild-type SRF (Hill *et al.*, 1993; Figure 2B, lanes 14 and 16). Both proteins formed ternary complexes with the altered specificity L.Elk protein at SRE.LP; in both cases binding appeared cooperative, as judged by the increase in total complex formation in the presence of L.Elk (Figure 2B, lanes 6–12). Similar data were obtained using wild-type Elk-1 and its cognate probe SRE.P (data not shown).

The above experiments show that *in vitro* SRE.LP binds SRF weakly and that this binding can be enhanced by ternary complex formation. To test whether the response of SRE.LP and SRE.P to serum stimulation reflects their affinity for SRF, we used reporter genes in which the HSV thymidine kinase (TK) promoter is controlled by two copies of each SRE. In these reporters the SRE does not activate transcription in response to PMA, so only serum stimulation was tested (see below): a representative experiment is shown and the results summarized in Figure 2C. As observed in the c-*fos* promoter experiments above, TCF binding was not required for an efficient serum response by the c-*fos* SRE in NIH3T3 cells (Figure 2C, lanes 1–4). Similar results were obtained in HeLa cells (data not shown). In contrast, SRE.P activated transcription some 8-fold less effectively than the intact c-*fos* SRE; this response was entirely dependent on an intact TCF binding site since SRE.LP, in which it is replaced by the LexA half-operator, was inactive (Figure 2C, lanes 5–8). Reporters that cannot bind SRF *in vitro*, such as SRE.M and SRE.LM, were uninducible (Figure 2C, lanes 9–12; Hill *et al.*, 1993). Taken together these data suggest that SRE.LP cannot effectively bind SRF *in vivo*, but that SRF binding can be facilitated by ternary complex formation. In addition, the weak activation of SRE.P suggests that cellular SRF and TCF concentrations are insufficient to allow saturation of this site *in vivo*.

SRE.LP binds SRF weakly in vivo

To examine SRF and TCF binding to SRE.LP independently of serum stimulation, we tested activation of SRE.LP by VP16 activation domain-tagged SRF and Elk-1 derivatives. Transcriptional activation by such constitutively active fusion proteins allows DNA binding by SRF and TCFs to be detected even under conditions in which SRE activity remains uninduced (Dalton and Treisman, 1992).

We first compared the ability of SRF.VP16 to bind SRE.LP sites *in vivo*; the results are shown in Figure 3A. SRF.VP16 efficiently activated the c-*fos* SRE, but only weakly activated the low-affinity SRF site in SRE.LP (Figure 3A, lanes 1–4). This weak activation could be dramatically increased when NL.Elk was co-expressed with SRF.VP16 (Figure 3A, lanes 5–7). Activation of SRE.LP by SRF.VP16 could also be potentiated by NL.Elk307Δ, which lacks the Elk-1 C-terminal activation domain (Figure 3A, compare lane 4 with lanes 8–10). Thus, as predicted, ternary complex formation facilitates SRF binding to SRE.LP *in vivo*.

We next used VP16-tagged derivatives of Elk-1 and the altered specificity mutant NL.Elk to test the specificity of ternary complex factor binding at SRE.LP. In starved cells, VP16.Elk efficiently activated SRE.P, but not SRE.LP, which lacks the TCF binding site; conversely, NL.Elk.VP16 efficiently activated SRE.LP, but not SRE.P which lacks the LexA half-operator (Figure 3B, lanes 1–6). Moreover, at SRE.LP the degree of activation by NL.Elk.VP16, but not VP16.Elk, could be increased by overexpressing SRF (data not shown). These experiments show that the Elk-1 derivatives can form a ternary complex with SRF at SRE.LP in a sequence-specific manner, and indicate that SRF overexpression would not result in recruitment of endogenous TCFs to SRE.LP.

TCF-independent serum-regulated activation by SRF

The above experiments establish a reporter gene which cannot bind endogenous cellular SRF and TCF, but which can bind SRF and altered specificity Elk-1 when overexpressed. We next tested the ability of SRF and TCF derivatives to restore serum-regulated transcriptional activation to SRE.LP; a representative experiment is shown in Figure 4. Overexpression of SRF alone restored serum-induced activation of SRE.LP to, on average, 8% of that of the intact SRE, showing that SRF is able to activate transcription in the absence of TCF (Figure 4A, lanes 5–10). Expression of NL.Elk, but not Elk-1 itself, also restored serum-induced activity of SRE.LP, to on average 18% of the activity of the wild-type SRE, reflecting its ability to form a ternary complex with endogenous SRF (Figure 4A, lanes 11–16; data not shown). Expression of both proteins activated SRE.LP significantly more efficiently than either protein alone, reaching on average ~40% of the activity of the intact c-*fos* SRE (Figure 4A, lanes 17–22). In contrast, the truncation mutant NL.Elk307Δ, which lacks the Elk-1 C-terminal activation domain, could not restore serum-regulated activity to SRE.LP, even though it can form a ternary complex with SRF at SRE.LP *in vivo*. Moreover, when co-expressed with SRF, NL.Elk307Δ suppressed activation below the level obtained with SRF alone (Figure 4B; see Discussion).

We compared the ability of SRF and NL.Elk to restore

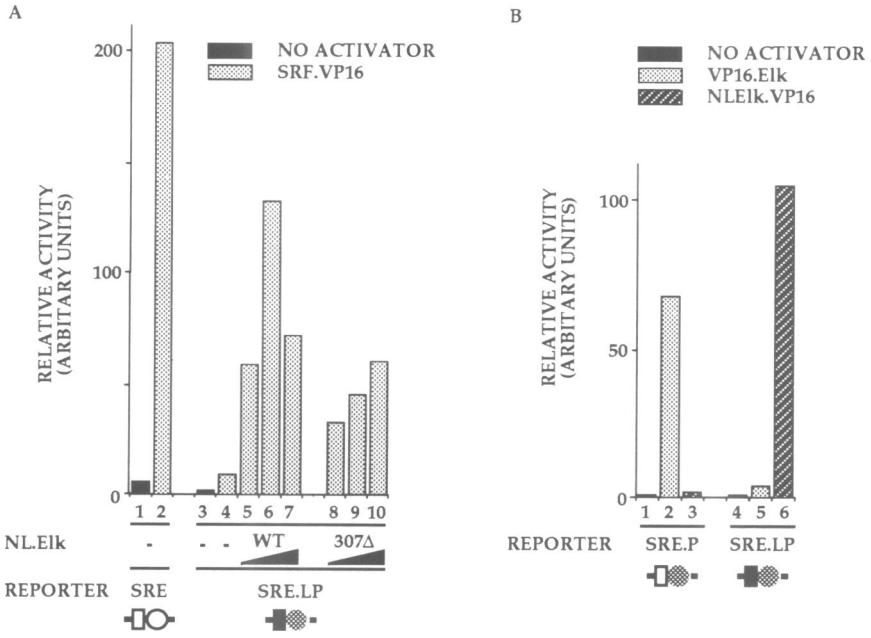

Fig. 3. SRF- and TCF binding properties of SRE.LP *in vivo*. (**A**) SRF can be recruited to SRE.LP *in vivo* by ternary complex formation. TKCAT reporter genes were controlled by two copies of the c-*fos* SRE (lanes 1 and 2) or SRE.LP (lanes 3–10). The symbols are as in Figure 2. Transactivating plasmids were either MLV128β (control) (lanes 1 and 3) or MLVSRF.VP16 (0.3 μg) alone (lanes 2 and 4), or with increasing amounts (0.1, 0.3 or 1 μg) of MLVNL.Elk (lanes 5–7) or MLVNL.Elk 307Δ which encodes the C-terminal truncation mutant of NL.ELK (lanes 8–10). CAT activity was measured in extracts from cells that had been serum starved for 40 h. (**B**) Sequence-specific activation of SRE.LP and SRE.P by Elk-1 derivatives. TKCAT reporter genes were controlled by SRE.P (lanes 1–3) or SRE.LP (lanes 4–6) and were co-transfected with 0.1 μg MLV128β (lanes 1 and 4) or MLVVP16.Elk (lanes 2 and 5) or MLVNL.Elk.VP16 (lanes 3 and 6).

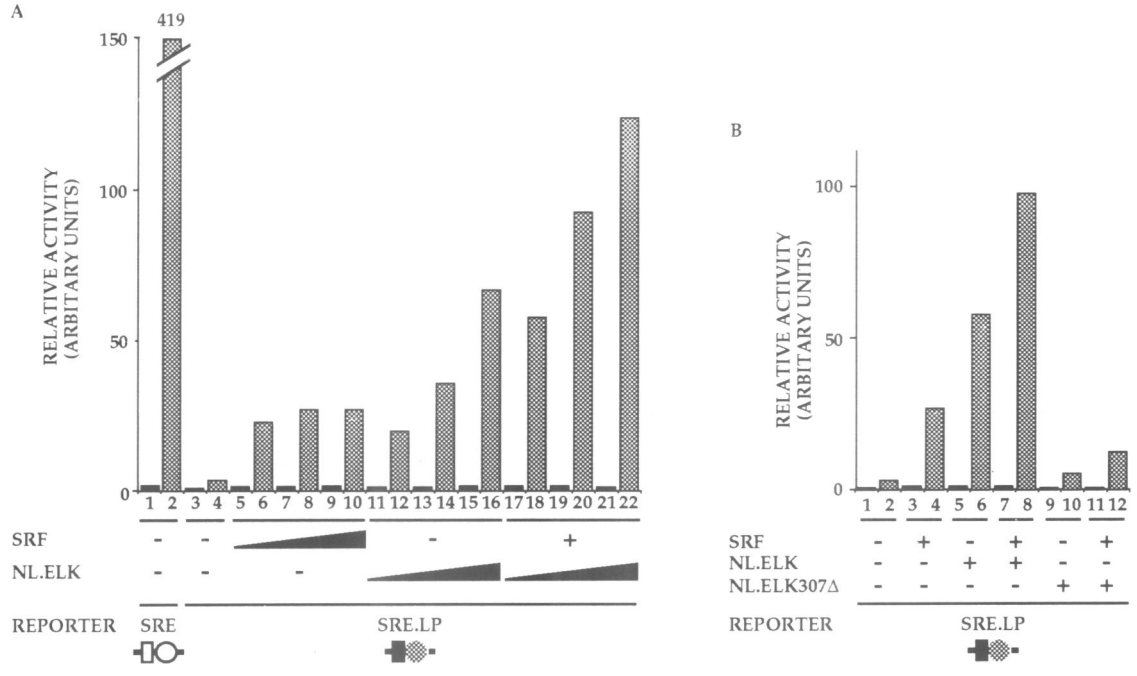

Fig. 4. Activation of SRE.LP by SRF and the Elk-1 derivative NL.Elk. (**A**) Cells were transfected with TKCAT reporter genes controlled by two copies of the c-*fos* SRE (lanes 1 and 2) or SRE.LP (lanes 3–22). The symbols are as in Figure 2. The transactivating plasmids were as follows: lanes 1–4, MLV128β (control); lanes 5–10, increasing amounts (30, 100, 300 ng) of MLVSRF; lanes 11–16, increasing amounts (10, 30, 100 ng) of MLVNL.Elk; lanes 17–22, 300 ng MLVSRF with increasing amounts (10, 30, 100 ng) of MLVNL.Elk. CAT activity was measured in extracts from transfected serum-deprived cells (odd lanes) or cells stimulated for 8 h with 15% FCS (even lanes). The value for serum-induced activation of the SRE (lane 2) is shown above the bar. The data presented are from a single representative experiment. Relative to the wild-type SRE (100%), the levels of restored serum-induced activation of SRE.LP by overexpression of SRF and/or NL.Elk were as follows (average of five independent experiments): 300 ng MLVSRF, 8%; 100 ng MLVNL.Elk, 18%, 300 ng MLVSRF + 100 ng MLVNL.Elk, 38%. (**B**) The C-terminal truncation mutant of NL.Elk inhibits SRF-dependent activation of SRE.LP in response to serum. The reporter gene and activation plasmids are as indicated: 300 ng MLVSRF expression plasmid were used and 100 ng MLVNL.Elk or MLVNL.Elk 307Δ. The data are from a single representative experiment.

Table I. Restoration of serum-induced transcription of mutant SREs by SRF and Elk-1 derivatives

Reporter[a]	Activator[b]	Activity relative to wild-type SRE (%)[c]	
SRE	–	100	
SRE.P	–	11	
	SRF	39	
SRE.LP	–	1	
	SRF	7	(8)
	NL.Elk	10	(18)
	SRF + NL.Elk	29	(38)
SRE.LM	–	0.5	
	SRF-M2	0.8	
	SRF-M2 + NL.Elk	9	
	SRF	1	
	SRF + NL.Elk	4	

The values in parentheses are from five independent experiments: 300 ng MLVSRF and 100 ng NL.Elk was used.
[a]TKCAT reporter genes were controlled by two copies of SRE derivatives as indicated.
[b]100 ng of each activator plasmid was tested.
[c]The values are serum-induced transcriptional activation expressed relative to the wild-type SRE (100%) and are from a single representative experiment.

transcriptional activation to the SRE.LP reporter with that achieved by our altered specificity SRF, SRF-M2 and NL.Elk at SRE.LM (Table I; Hill *et al.*, 1993). The activity of SRE.LP in the presence of SRF alone was comparable to that of the altered specificity ternary complex at SRE.LM. However, SRF-M2 by itself could not restore serum-induced activity to SRE.LM, even though its affinity for this sequence is much greater than that of SRF for SRE.LP (Table I; see Figure 2B). Together, SRF and NL.Elk restored the activity of SRE.LP to a level ~4 times that obtained by co-expression of SRF-M2 and NL.Elk at SRE.LM (Figure 4A; Table I). SRF overexpression increased the activity of the weak SRE.P site to the level achieved by co-expression of NL.Elk and SRF with SRE.LP.

SRF is sufficient to mediate a response to serum, but not to PMA

We demonstrated above that the PMA response of the c-*fos* SRE is predominantly TCF dependent. To test the effect of SRF and NL.Elk expression on PMA-inducible activity of SRE.LP, we used a reporter gene in which two copies of the c-*fos* SRE or SRE.LP control a minimal human c-*fos* promoter containing 124 base pairs of 5′ flanking sequence. In contrast to the TK-based reporter genes, in these reporters the c-*fos* SRE mediated a PMA response, although this was weak compared with the serum response; SRE.LP was defective in both responses (Figure 5, lanes 1–6). SRF overexpression partially restored only serum regulation, while NL.Elk overexpression restored the serum-induced response to the same extent as SRF, but in addition restored the PMA response (Figure 5, lanes 7–12). Co-expression of both proteins efficiently restored both responses (Figure 5, lanes 13–15). These results demonstrate that in NIH3T3 cells SRF alone can activate transcription in response to serum stimulation, but not in response to activation of PKC.

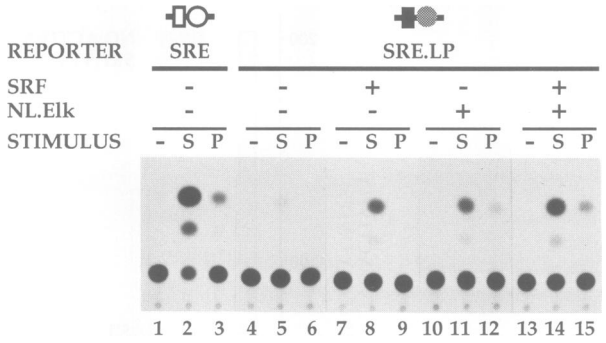

Fig. 5. SRF is sufficient to mediate a response to serum, but not to PMA. FosCAT reporter genes controlled by two copies of either the wild-type c-*fos* SRE (lanes 1–3) or SRE.LP (lanes 4–15) were transfected with 1 μg (MLV128β) (control) (lanes 1–6) or activator plasmids as indicated (MLV SRF, 300 ng; MLVNL.Elk, 100 ng). The SRE symbols are as in Figure 2. CAT activity was measured in extracts from unstimulated cells (lanes 1, 4, 7, 10 and 13), or cells stimulated for 8 h with either 5% FCS (lanes 2, 5, 8, 11 and 14) or 50 ng/ml PMA (lanes 3, 6, 9, 12 and 15). The data are from a single representative experiment.

Functional regions of SRF

We used a set of SRF mutants (shown schematically in Figure 7) to investigate which regions of SRF mediate TCF-independent transcriptional activation. The SRF-CKII and SRF-103 point mutants contain alanine substitutions at the phosphoacceptor serines in the N-terminal CKII and pp90^{rsk} sites, respectively (Marais *et al.*, 1992; Rivera *et al.*, 1993), while the deletion mutants SRF52Δ114 and SRF-265Δ, respectively, delete either all the N-terminal phosphorylation sites or the C-terminal activation domain (see Hill *et al.*, 1993). Two DNA binding domain mutants were examined: SRF-198/203, in which SRF residues 198–203 (the region implicated in TCF recruitment; Mueller and Nordheim, 1991; Shaw, 1992) are replaced by the corresponding residues from the SRF-related yeast protein ARG80; and the altered specificity mutant SRF-M2, in which SRF basic region residues 133–166 are replaced with the corresponding residues from MCM1 (Hill *et al.*, 1993).

We first characterized the DNA binding properties of these mutants and tested their abilities to form ternary complexes with Elk-1 derivatives both *in vitro* and *in vivo*. Except in the case of the C-terminal deletion mutant SRF-265Δ, extracts prepared from transfected cells expressing either these mutants or wild-type SRF contain comparable levels of protein, as judged by DNA binding assays with the c-*fos* SRE probe (Figure 6A) or by immunoblotting (data not shown). Analysis of the DNA binding domain mutants with an SRE.LP probe also revealed comparable amounts of DNA binding activity (Figure 6B, lanes 4, 7 and 10). Whole-cell extracts containing SRF-M2 or wild-type SRF form ternary complexes with recombinant L.Elk with equal efficiency, while SRF-198/203 extracts exhibit ~4-fold reduced ternary complex activity, as expected from previous studies (Figure 6B, lanes 4–12; Mueller and Nordheim, 1991; Shaw, 1992). We also tested the ability of SRF-198/203 and SRF-M2 to activate SRE.LP *in vivo* as mutant SRF−VP16 fusion proteins: in this assay, SRF-198/203.VP16 activated SRE.LP more efficiently than did SRF.VP16, confirming that it binds SRE.LP

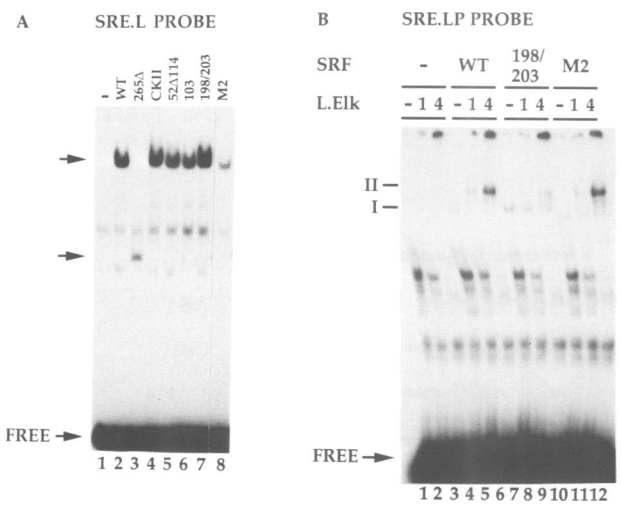

Fig. 6. Characterization of SRF mutants. (**A**) SRE binding activities of SRF mutants expressed in NIH3T3 cells, assayed by gel mobility shift assay. Cells were transfected with MLV128β (lane 1) or MLV expression plasmids (3 μg) encoding SRF or mutant derivatives (shown schematically in Figure 7) as indicated (lanes 2–8). Whole-cell extracts were prepared from serum-deprived cells and assayed for binding to the SRE.L probe. The arrows indicate the SRF complexes. (**B**) SRF DNA binding domain mutants bind SRE.LP and form ternary complexes with L.Elk. Whole-cell extracts were prepared from serum-deprived cells transfected with MLV128β (lanes 1–3), or expression plasmids encoding SRF mutants as indicated (lanes 4–12) and assayed for binding to the SRE.LP probe either alone (lanes 1, 4, 7 and 10) or with 1 or 4 μl *in vitro* translated L.Elk as indicated. Complexes of SRF derivatives alone are denoted I, and ternary complexes with L.Elk are denoted II.

Table II. Activation of SRE.LP by VP16-tagged SRF derivatives

Reporter[a]	Activator[b]	Relative activity (arbitary units)
SRE.LP	–	2
	SRF.VP16	10
	SRF-M2.VP16	2
	SRF-198/203.VP16	23

[a]TKCAT reporter gene was controlled by two copies of SRE.LP.
[b]0.3 μg activator plasmid was tested.

in vivo; however, the activity of the SRF-M2.VP16 fusion protein was very low (Table II; see Discussion). Finally, to test ternary complex formation *in vivo*, we compared the abilities of SRF, SRF-198/203 and SRF-M2 to restore activity to SRE.LM in a ternary complex with NL.Elk. In this assay, both wild-type SRF and SRF-198/203 restore activity to ~50% of the level observed with the altered specificity mutant SRF-M2 (data not shown; see Table I), indicating that although SRF-198/203 is reduced in its ability to form ternary complexes *in vitro*, it is not defective in ternary complex formation *in vivo*.

We then tested the ability of the SRF mutants to mediate TCF-independent activation of SRE.LP in response to serum. Mutations in the N-terminal region had no significant effect (Figure 7, left-hand side; proteins A, C–E). In contrast, deletion of the C-terminal region lowered activity some 3-fold (Figure 7, left-hand side; proteins A and B); although this mutant yielded less c-*fos* SRE binding activity than wild-type SRF, reduced activation was still observed when the amount of expression plasmid transfected was adjusted to compensate for this. Neither DNA binding domain mutant restored activation above the background level observed in the absence of transfected SRF (Figure 7, left-hand side; proteins F and G). Similar results were obtained with the altered specificity mutant SRF-M1 (Hill *et al.*, 1993) in which only 10 residues between 142 and 166 are substituted by those found in MCM1 (data not shown). These results indicate that specific residues in the SRF DNA binding domain are essential for TCF-independent activation. Each mutant was then co-expressed with NL.Elk to test its activity in the context of the ternary complex at SRE.LP. The effects of the various mutations in this situation were comparable to those observed in the absence of NL.Elk (Figure 7, right-hand side; proteins A–G). Both DNA binding domain mutations reduced activation to levels below those obtained with NL.Elk alone, indicating that they must compete with endogenous SRF for binding to the SRE.LP target. Taken together, these data indicate that the integrity of the SRF DNA binding domain is of crucial importance for the full transcriptional activation by the SRF/Elk-1 ternary complex.

Efficient activation by SRF requires direct binding to DNA

Having demonstrated the importance of the SRF DNA binding domain for efficient transcriptional activation, we investigated whether its contact with DNA was important for the activation process. We previously found that a LexA–SRF fusion protein can bind a LexA operator efficiently, but only weakly activates transcription in response to serum (S.John and R.Treisman, unpublished). We compared this activation with the ability of SRF to activate the SRE.LP reporter (Figure 8A). Wild-type SRF activated the SRE.LP reporter ~16-fold more efficiently than the LexA–SRF fusion protein activated the LexA operator controlled reporter gene (Figure 8A, compare lanes 3 and 4 with 7 and 8). Gel mobility shift analysis of cell extracts using LexA and SRE probes indicated that the LexA–SRF fusion protein and wild-type SRF produced comparable levels of LexA operator and c-*fos* SRE binding activity respectively (Figure 8B, compare lanes 2–4 with 9–11); moreover, the SRF DNA binding domain of LexA–SRF is folded correctly, since the protein binds the c-*fos* SRE and the LexA operator equally well (Figure 8B, compare lanes 5–7 with 9–11). Consistent with this the LexA–SRF protein could activate SRE.LP with efficiency similar to that of SRF itself (data not shown). These results suggest that efficient transcriptional activation by SRF requires that its DNA binding domain is specifically bound to DNA.

Discussion

We have developed a novel inactive SRE variant, SRE.LP, whose activity is dependent upon overexpression of SRF and altered specificity Elk-1. Together, these proteins can restore serum-regulated transcriptional activation to a level within 3-fold that of the intact c-*fos* SRE. We used this system to show that: (i) SRF can regulate transcriptional activation independently of ternary complex factors such as Elk-1; (ii) transcriptional activation by SRF requires

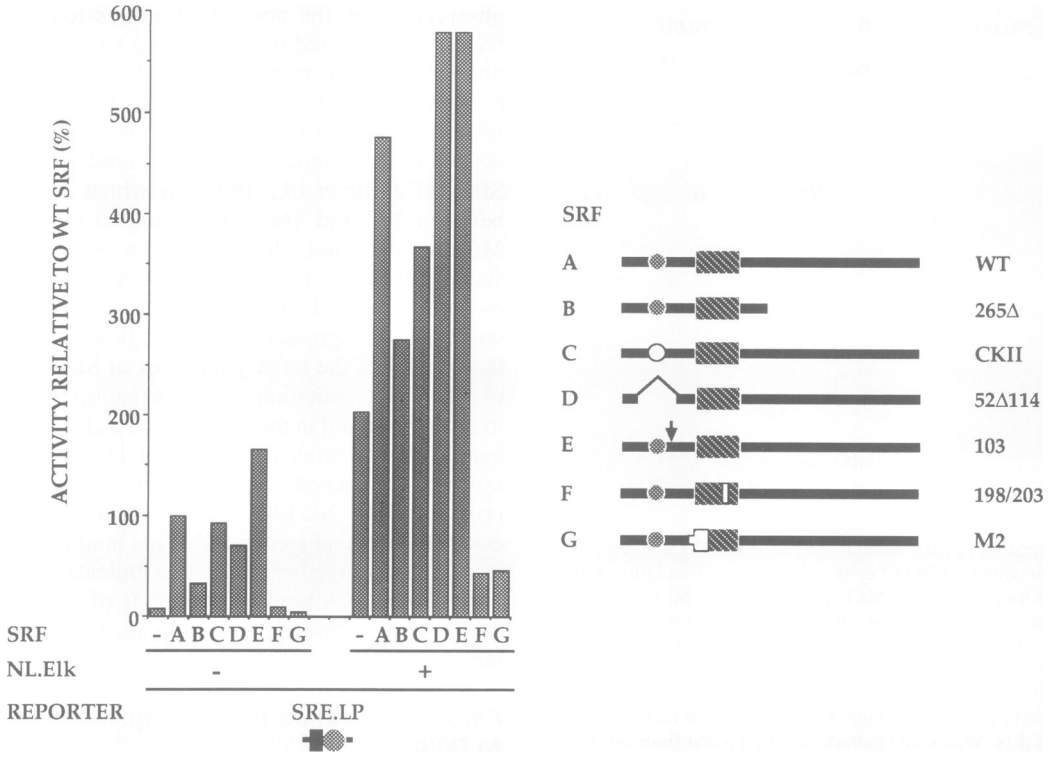

Fig. 7. Activation of SRE.LP by SRF mutants in response to serum. Cells were transfected with the TKCAT reporter gene controlled by two copies of SRE.LP with 300 ng expression plasmid encoding wild-type or mutant SRF derivatives with or without 100 ng plasmid encoding NL.Elk. The data are expressed as levels of serum-activated transcriptional activation relative to the level of activation of SRE.LP by wild-type SRF alone (100%), and are averages of at least two experiments, quantitated as described in Materials and methods. SRF mutants are shown schematically. The CKII site is shown as a shaded circle and the MCM1 homology region/DNA binding domain as a shaded box. (**A**) wild-type SRF; (**B**) C-terminal truncation mutant, 265Δ; (**C**) CKII mutation (serines 77, 79, 83 and 85 mutated to alanine); (**D**) N-terminal deletion 52Δ114; (**E**) serine 103 mutated to alanine, arrow; (**F**) SRF 198/203 (three point mutations in region 198–203 SRF ATRKLQ→TTPKLE, white box); (**G**) SRF-M2 (region replaced by MCM1 sequences shown by white box).

sequences within its DNA binding domain; (iii) efficient transcriptional activation by both SRF and the ternary complex requires that the SRF DNA binding domain directly contact DNA. We previously used altered specificity derivatives of SRF and Elk-1 to demonstrate serum-regulated transcriptional activation by the SRF/Elk-1 ternary complex. In contrast to the c-*fos* SRE, however, activity of this reconstituted complex is absolutely dependent on the TCF moiety, and it is also relatively inefficient (Hill *et al.*, 1993). These properties apparently arise because the altered specificity SRF derivative is defective in mediating serum-induced transcriptional activation.

Regions of SRF required for regulated transcriptional activation

Two kinds of experiment suggest that the intact SRF DNA binding domain, bound to DNA, is required for optimal TCF-dependent and TCF-independent regulation in response to serum stimulation. First, when tethered to DNA by a heterologous DNA binding domain, regulated transcription by SRF is barely detectable, in agreement with previous experiments (Johansen and Prywes, 1993). In contrast, such fusion proteins and SRF itself restore serum-regulated activation when targeted to an appropriate SRE via the SRF DNA binding domain. Second, mutations within the DNA binding domain itself inhibit both TCF-independent activation and activity of the ternary complex (see Figure 9). These mutants, in which amino acids in

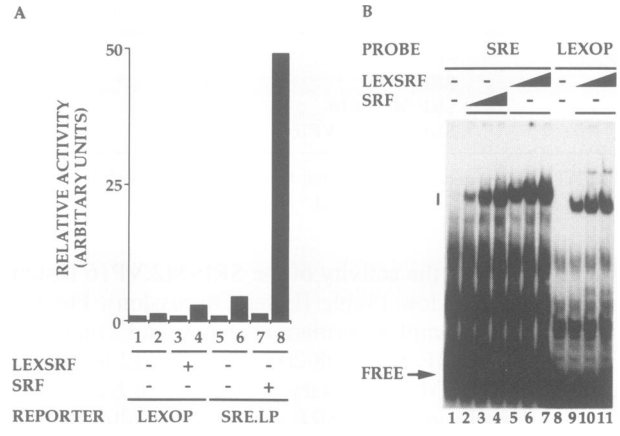

Fig. 8. SRF must be bound to DNA via its own DNA binding domain to activate transcription. (**A**) SRF cannot efficiently mediate transcriptional activation in response to serum when tethered to DNA via a LexA DNA binding domain. TKCAT reporter genes were controlled by two LexA operators (lanes 1–4) or two copies of SRE.LP (lanes 5–8). Activator plasmids (0.3 μg) were as indicated. CAT activity was measured in extracts from transfected serum-deprived cells (odd lanes) or cells stimulated for 8 h with 15% FCS (even lanes). (**B**) Binding activities of SRF and LexSRF. Whole-cell extracts were prepared from cells transfected with MLV128β (lanes 1 and 8), MLVSRF (0.3, 1, 3 μg) (lanes 2–4) or MLVLexSRF (0.3, 1, 3 μg) (lanes 5–7 and 9–11), and were analysed for binding in gel mobility shift assays to the probes as indicated. The vertical line indicates SRF or LexSRF complexes.

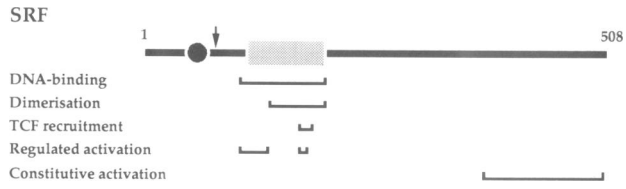

Fig. 9. Functional regions in SRF. The molecule is shown schematically: shaded box, region of homology with MCM1 (residues 143–222) (Norman *et al.*, 1988); shaded circle, constitutively phosphorylated CKII sites (serines 77, 79, 83 and 85) (Janknecht *et al.*, 1992; Marais *et al.*, 1992); arrow, growth-factor regulated $pp90^{rsk}$ phosphorylation site at Ser-103 (Rivera *et al.*, 1993). Residues 133–222, DNA binding domain (Norman *et al.*, 1988); residues 198–210 are required for TCF recruitment (Mueller and Nordheim, 1991; Shaw, 1992; Hill *et al.*, 1993); residues 414–508, constitutive transcriptional activation domain (Prywes and Zhu, 1992; Johansen and Prywes 1993; Liu *et al.*, 1993). The regions of the DNA binding domain essential for serum-induced transcriptional regulation span residues 133–166 and 198–203. For discussion, see the text.

the DNA binding domain are replaced by the corresponding amino acids from the yeast SRF-related proteins ARG80 and MCM1, bind SRE.LP as efficiently as wild-type SRF. The failure of the altered specificity SRF-M2 mutant to activate transcription is not a result of its inherently low affinity for SRE.LP *in vivo*, since this protein cannot restore TCF-independent transcription even to a high-affinity binding site such as that used in our previous experiments (Hill *et al.*, 1993). The region encompassing the two mutations also inhibits the activity of the SRF C-terminal activation domain in Gal4–SRF fusion proteins (Johansen and Prywes, 1993). The mechanism by which the DNA binding domain contributes to transcriptional activation is discussed further below.

The integrity of the SRF N-terminal region is required neither for TCF-independent signalling nor for full activity of the ternary complex in our assay. Deletions and point mutations that remove or disrupt phosphorylation sites within this region do not affect transcriptional regulation; thus, neither constitutive phosphorylation of SRF at the CKII site (Manak *et al.*, 1990; Janknecht *et al.*, 1992; Marais *et al.*, 1992) nor its growth factor-regulated phosphorylation at serine 103 (Rivera *et al.*, 1993) contribute to regulated transcriptional activation. It remains possible that N-terminal sequences outside those investigated here do contribute to activation, but these sequences are not evolutionarily conserved. As we observed previously, the SRF C-terminal activation domain potentiates transcriptional activation some 2- to 3-fold (Hill *et al.*, 1993), but does not confer regulation by itself as a Gal4 fusion protein (S.John and R.Treisman, unpublished data; Johansen and Prywes, 1993). The SRF C-terminal activation domain is phosphorylated at DNA-dependent protein kinase sites (Liu *et al.*, 1993), and interacts both with the basal transcription factor TFIIF (Zhu *et al.*, 1994) and the HTLVI transactivator protein Tax (Fujii *et al.*, 1992).

There is considerable similarity between the properties of SRF and those of the yeast protein MCM1, which is 70% identical to SRF within its DNA binding domain (Norman *et al.*, 1988). Although the C-terminal region of MCM1 does contribute to transcriptional activation (Sengupta and Cochran, 1991; Bruhn *et al.*, 1992), its DNA binding domain appears sufficient for transcriptional activation and interaction with accessory factors (Christ and Tye, 1991; Primig *et al.*, 1991; Bruhn *et al.*, 1992). Moreover, as with SRF, mutations which block activation without affecting DNA binding by MCM1 have been identified (Bruhn and Sprague, 1994).

Mechanism of transcriptional activation

Our results imply that in the absence of TCF, serum-induced signals must modify either SRF itself or a non-TCF accessory factor that interacts with it; moreover, the mutagenesis studies suggest that this modification or interaction is required for the full activity of the ternary complex as well. Since the SRF DNA binding domain does not appear to be modified following growth factor stimulation, we prefer the notion that an as yet unidentified factor is the target for a serum-activated signalling pathway, and that this factor recognizes the DNA-bound SRF DNA binding domain. This model is similar to the 'recognition factor' model invoked to explain the involvement of the MyoD basic region in myogenesis (Weintraub *et al.*, 1991). The relatively inefficient and completely TCF-dependent activation obtained with our altered specificity ternary complex (Hill *et al.*, 1993) would reflect failure to recruit this putative factor. Our mutational analysis implicates two regions of the SRF DNA binding domain in transcriptional activation. We speculate that mutations in these regions alter the conformation of SRF on DNA such that it is not capable of interaction with the putative recognition factor. One region, spanning SRF residues 198–203, is also involved in interaction with the TCFs (Mueller and Nordheim, 1991; Shaw, 1992; Hill *et al.*, 1993), suggesting that multiple cofactors may compete for a common surface of the SRF DNA binding domain. Such a competition might explain our observation that although TCF binding *per se* is not required for serum-regulated transcription, a TCF that lacks its activation domain interferes with serum-induced SRE activity. The SRF-related yeast protein MCM1 also uses this region of its DNA binding domain to interact with multiple unrelated accessory factors (Mueller and Nordheim, 1991; Primig *et al.*, 1991; Bruhn and Sprague, 1994).

What might the 'recognition factor' be? Three non-TCF proteins have been implicated in SRE function, including Phox1 (Grueneberg *et al.*, 1992), TFIIF (Zhu *et al.*, 1994) and CBP (Arias *et al.*, 1994). TFIIF is particularly intriguing, since its RAP74 subunit can interact with both the SRF C-terminal activation domain and the DNA binding domain (Zhu *et al.*, 1994). However, it is unlikely that this interaction is itself sufficient for regulated transcriptional activation, because neither our LexA–SRF fusion protein nor a Gal4–SRF C-terminal fusion protein is responsive to serum induction when bound to LexA or Gal4 binding sites, respectively (Johansen and Prywes, 1993; S.John and R.Treisman, unpublished data). The Phox1 homeodomain protein can potentiate binding of SRF to the *c-fos* SRE *in vitro* (Grueneberg *et al.*, 1992), while microinjection of anti-CBP antibodies inhibits induced activation of SRE-, TRE- and CRE-controlled lacZ reporter genes, which might be expected if CBP were a cofactor (Arias *et al.*, 1994). We are currently

investigating these factors and searching for factors that will potentiate the activity of the SRE *in vivo*.

Role of the AT-rich SRE core

Our results indicate that the AT-rich centre of the SRE is important for full activation by the ternary complex. Previously, we showed that the altered specificity SRF derivative SRF-M2 can restore TCF-dependent serum-dependent activation to the GC-rich MCM1 binding site-derived SRE.LM. Although wild-type SRF can also partially restore activity to this site as part of the ternary complex, the activity of this complex is somewhat less than that achieved by SRF-M2, and weak compared with activation by the ternary complex at the intact *c-fos* SRE. It therefore appears that full activity of the ternary complex requires that SRF binds to an SRE containing an AT-rich centre.

Two kinds of role for the AT-rich centre of the SRE can be envisaged. First, it might allow another protein to bind the SRF–DNA complex: the sequence specificity of such a factor would have to be fairly relaxed since the sequence of this region is not conserved among SREs. This kind of interaction has a precedent in the simultaneous interaction of HMG I(Y) and NF-κB with the minor and major grooves of the IFN-β PRDII promoter element, which faciliates assembly of a multiprotein promoter complex (Thanos and Maniatis, 1992; Du *et al.*, 1993). Although HMG I(Y) binds AT-rich sequences, it apparently does not bind the *c-fos* SRE (Eckner and Birnstiel, 1989); however, another possibility is that the AT-rich SRE reflects a requirement for interaction of SRF with proteins such as Phox-1 (Grueneberg *et al.*, 1992). Alternatively the AT-rich sequences might allow the SRF–DNA complex to adopt a particular structure. As judged by the circular permutation DNA binding assay, the SREs discussed here are similarly bent by both wild-type and the mutant SRFs (our unpublished observations; see Gustafson *et al.*, 1989). A more attractive idea is that the AT-rich SRE centre might allow SRF to assume an 'active' conformation, which we discuss below.

The role of DNA contact

We showed that the SRF DNA binding domain must be directly in contact with DNA for regulated transcriptional activation to occur. DNA contact via the SRF DNA binding domain is also required for growth factor-independent activation of transcription by SRF in the presence of myogenic factors such as MyoD (our unpublished data). These observations strongly suggest that upon binding DNA the SRF DNA binding domain undergoes a structural change that enables regulated transcriptional activation to occur. Prywes and co-workers have proposed that DNA binding is required to relieve an inhibitory effect of SRF residues 141–203, within the DNA binding domain, upon the activity of the C-terminal activation domain (Johansen and Prywes, 1993). Our data are consistent with this idea; however, the dispensability of the C-terminal activation domain for regulated transcription leads us to propose that SRF–DNA contact must lead to the exposure of an activation function associated with the DNA binding domain itself, in addition to any role it may have in potentiating the activity of the SRF C-terminal domain. DNA-induced conformation changes in SRF may be important for the recruitment of additional regulatory factors.

Although there is much evidence for DNA-induced structural changes in DNA binding domains, direct evidence that this can be of functional significance is far more limited. Two persuasive examples are the DNA-induced active conformation of the NF-κB p50 dimer (Fujita *et al.*, 1992) and the DNA-induced conformational change in the Oct-1 POU domain which allows its recognition by the HSV VP16 protein (Walker *et al.*, 1994). Intriguingly, there is also circumstantial evidence that DNA-induced conformation changes play a role in transcriptional activation by the SRF-related yeast protein MCM1. Proteolytic clipping experiments indicate a correlation between a particular DNA sequence-dependent conformation of the MCM1 binding domain and its ability to activate transcription (Tan and Richmond, 1990). Moreover, some MCM1 mutants are defective for transcription, but unimpaired in known protein and DNA binding interactions (Bruhn and Sprague, 1994). However, there is as yet no direct proof that a particular conformation of MCM1 is necessary for transcriptional activation.

Requirement for a particular conformation of the SRF DNA binding domain for activation may explain a puzzling observation concerning the SRF–VP16 fusion proteins. We found that although SRF and its altered specificity derivative SRF–M2 both bind the low-affinity site SRE.LP equally well *in vitro*, only the wild-type SRF.VP16 fusion protein can efficiently activate this site *in vivo*, even though SRF-M2.VP16 strongly activates transcription via its cognate site SRE.LM (our unpublished observations). This result is unlikely to reflect failure to bind SRE.LP *in vivo*, because SRF-M2 can efficiently antagonize the recruitment of endogenous SRF to this site by a co-transfected Elk-1 derivative. We suggest that the conformation of the mutant SRF-M2 DNA binding domain on DNA may be incompatible with transcriptional activation by the VP16 moiety, and are currently investigating this further.

TCF-independent signalling to the SRE

In NIH3T3 and HeLa cells, serum-induced transcriptional activation by the *c-fos* SRE is substantially independent of TCF binding, while PMA-induced activation requires TCF; this confirms and extends previous findings in mouse BALB/c 3T3 and rat cardiac myocytes (Graham and Gilman, 1991; Sadoshima and Izumo, 1993). Serum can therefore signal to the SRE in two ways: TCF-independent activation of SRF, and phosphorylation of TCFs via activation of the MAP kinase pathway (Gille *et al.*, 1992; Hill *et al.*, 1993; Janknecht *et al.*, 1993; Marais *et al.*, 1993; Zinck *et al.*, 1993; Kortenjann *et al.*, 1994). Since both serum and PMA activate ERKs 1 and 2, and ERK 2 is the best candidate for the Elk-1 kinase *in vivo*, the TCF-independent signal must either be mediated by a distinct kinase or require both the ERKs and an additional kinase. Some indication that this may be the case comes from the observation that although Raf-1 activation of the c-*fos* SRE is largely TCF dependent and is mediated via the MAP kinase pathway (Kortenjann *et al.*, 1994), dominant negative Raf mutants fail to block SRE activation by serum (Miltenberger *et al.*, 1993). We are currently

investigating the mechanism of TCF-independent signalling to the SRE in more detail.

Materials and methods

Plasmids

All DNA manipulations were carried out by standard techniques and the plasmid structures were verified by sequencing. Plasmids based on MLVβplink and T7βplink (Dalton and Treisman, 1992) were used for expression in mammalian cells and for *in vitro* transcription respectively. The following plasmids were as described previously: MLVSRF, MLVSRF-M2, MLV128β, T7SRF-M2, MLVNL.Elk, MLVNL.Elk 307Δ and T7L.Elk (Hill *et al.*, 1993), pF711, πSVHSα118 (Treisman, 1985), MLVElk-1 (Marais *et al.*, 1993), T7SRF (Norman *et al.*, 1988). SRF-CKII, SRF-52Δ114 and SRF-265Δ are as the SRF-M2 derivatives described in Hill *et al.* (1993), but with wild-type SRF. In SRF-198/203, codons 135–140 of ARG80 replace codons 198–203 of SRF. In SRF-103, serine 103 is replaced by an alanine (Rivera *et al.*, 1993). SRF.VP16 encodes (SRF codons 1–412)−(VP16 codons 410–490) (Dalton and Treisman, 1992). SRF-M2.VP16 and SRF-198/203.VP16 encode, respectively (codons 1–412 of SRF-M2 or SRF-198/203)−(VP16 codons 410–490). LexSRF encodes (LexA codons 1–87)−(PVEASA)−(SRF codons 1–508). VP16.Elk-1 was constructed from VP16/SAP1 (Dalton and Treisman, 1992); Elk-1 codons 1–428 replace SAP1 codons 1–310. NL.Elk.VP16 is as NL.Elk, but residues 308–428 of Elk-1 are replaced by -(RTRD)−(VP16 codons 410–490). pF711ΔTCF is a derivative of pF711 (Treisman, 1985) in which the region containing the Ets motif of the SRE, ACACAGGAT, contains four changes that convert it to a LexA half-operator GTACTGTAT.

TKCAT reporter plasmids controlled by two copies of the wild-type SRE, SRE.M, SRE.LM and LEXOP, were described previously (Hill *et al.*, 1993; Marais *et al.*, 1993). The reporter plasmids controlled by SRE.L, SRE.P and SRE.LP contain two copies of the oligonucleotide pairs 1/2, 4/6 and 5/6, respectively (see below). In all cases except SRE.LP, the orientation of the mutant SREs was opposite to that found in the *c-fos* gene.

Reporter genes SRE_2.F124/CAT and SRE.LP_2.F124/CAT, in which two copies of either the c-*fos* SRE or SRE.LP, respectively, were placed upstream of a minimal c-*fos* promoter, were derived from the corresponding TKCAT plasmid in which TK promoter sequences − 75 to +51 were replaced by c-*fos* sequences − 124 to +42 from p222/CAT (Treisman, 1985).

Oligonucleotides

1. GGATCTAGATGTACTGTATGTC (SRE.L and SRE.LM top strand).
2. GGATCTAGAGATGTCCTAATATGGACATACAGTAC (SRE.L bottom strand).
3. GGATCTAGAGATGTCCCGATTGGGACATACAGTAC (SRE.LM bottom strand)
4. GGATCTAGACAGGATGTACATATTAGTAC (SRE.P top strand).
5. GGATCTAGATGTACTGTATGTACATATTAGTAC (SRE.LP top strand).
6. GGATCTAGAGATGTACTAATATGTAC (SRE.P and SRE.LP bottom strand). The SRF/MCM1 binding sites have been underlined. SRE.L (1/2), SRE.LM (1/3), SRE.P (4/6) and SRE.LP (5/6) were annealed, rendered double stranded and cut with *Xba*I before cloning.

Cell culture, transfections and RNA preparation

NIH3T3 cells were maintained and transfected by the diethylaminoethyl (DEAE)−dextran method as described previously (Hill *et al.*, 1993). For transfections with CAT reporter genes, the transfected DNA included 4 μg reporter plasmid, 1 μg MLVlacZ (transfection efficiency control) and expression plasmids as indicated in the figure legends. The amount of expression plasmid was held constant by addition of plasmid MLV128β. After transfection, cells were incubated in DME/0.5% fetal calf serum (FCS) for 40 h before stimulation with 15% FCS, 5% FCS or 50 ng/ml PMA (final concentration) for 8 h. Extracts were prepared and assayed for CAT and β-galactosidase activity as previously described (Hill *et al.*, 1993). For RNase protection experiments 90 mm dishes (1.2×10^6 cells) were transfected with 10 μg p711 or p711ΔTCF, 2.5 μg πSVHSα118 (transfection efficiency control; Treisman, 1985) and 1 μg of expression plasmid as indicated in the legend to Figure 1. After 40 h in DME/0.5% FCS, cells were stimulated with either 15% FCS or 50 ng/ml (final concentration) PMA for 30 min. Total cell RNA was prepared as follows using a method of S.Goodbourn (unpublished). Cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed in 4 M guanidinium thiocyanate, 0.5% sodium *N*-lauroylsarcosine, 0.1 M 2-mercaptoethanol, 25 mM sodium citrate (500 μl/plate). Samples were sonicated and mixed with 50 μl 2 M Na acetate (pH 4) and 500 μl acid phenol; following the addition of 100 μl $CHCl_3$, the aqueous phase was precipitated with propan-2-ol. The pellet was washed with 95% ethanol, resuspended in 200 μl 10 mM Tris−HCl (pH 7.5), 1 mM EDTA, 0.1% SDS and centrifuged to remove debris. Nucleic acids were recovered by ethanol precipitation, redissolved in 100 μl 50 mM Tris−HCl (pH 7.5), 1 mM EDTA, 5 mM $CaCl_2$, 12.5 mM $MgCl_2$, 1 mM dithiothreitol containing 20 U placental RNase inhibitor and treated for 30 min at 37°C with 1.35 U DNase I (Worthington; RNase-free, 2.7 U/μl). RNA was recovered by ethanol precipitation following phenol/chloroform extraction and RNase protection mapping was as described previously (Treisman, 1985).

Quantitation of RNase protection assays and CAT assays

Acetylated chloramphenicol was quantitated by phosphorimage analysis of the TLC plates. Where samples were off the linear scale in the CAT assay, they were re-assayed for the same time using lower amounts of extract. The data presented in Figures 3, 4 and 8 and Table II are as relative activities in arbitary units. The range of serum-induced activity of SRE_2TKCAT in these units was 203–661 in 10 independent experiments. The data presented are not corrected for β-galactosidase activity (for discussion see Hill *et al.*, 1993).

The RNase protection gels were quantitated using a PhosphorImager. The amount of c-*fos* mRNA was quantitated relative to the α-globin reference. Induced activity of the wild-type gene by serum and PMA was taken as 100%.

Gel mobility shift assay

[^{35}S]Methionine-labelled translated proteins were prepared and quantitated as described previously (Norman *et al.*, 1988). Preparation of whole-cell extracts (WCE) from unstimulated cells and gel mobility shift assays were carried out as described previously (Marais *et al.*, 1993). In the gel mobility shift assays, poly (dI-dC)·poly (dI-dC) competitor DNA was used at 100 μg/ml for *in vitro* translated proteins and 150 μg/ml for WCE. Binding probes SRE.L, SRE.LM and SRE.LP were synthesized by polymerase chain reaction (PCR) using oligonucleotides 1/2, 1/3 and 5/6, respectively. The c-*fos* SRE probe used in Figure 8 was as previously described (Hill *et al.*, 1993). The LexOP probe which contained two LexA operators was synthesized by PCR using M13 forward and pBLCAT.R primers (Treisman *et al.*, 1992) with LexOP_2TKCAT as template.

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