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Elk-1 can recruit SRF to form a ternary complex upon the serum response element

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ABSTRACT

The initial genomic response to serum growth factors is the transcriptional activation of a set of immediate-early genes. Serum-induced transcriptional activation of several of these genes involves the formation of a ternary complex that includes the serum response factor (SRF), a 62 kDa ternary complex factor (TCF) and a serum response element (SRE). TCF alone does not bind the SRE of the protooncogene c-*fos*, but requires the prior assembly of the SRF–SRE binary complex for it to be recruited into a ternary complex. Here we show that this SRF–SRE binary complex is not an obligatory prerequisite for the formation of a serum responsive ternary complex. We demonstrate that Elk-1, which has properties of TCF, can recruit SRF into a ternary complex on elements that do not support formation of the SRF–DNA binary complex. We also show that for two immediate-early genes, *pip92* and *nur77*, formation of the ternary complex may occur without the prior assembly of SRF–DNA binary complex. Finally, we show that the ability of different sequences to support formation of Elk-1–SRF–DNA ternary complex *in vitro* correlates with their ability to respond to serum growth factors *in vivo*. Our results suggest that a much broader range of DNA sequences than the consensus SRF and TCF binding sites can support ternary complex formation, and by inference, serum induction. Possible implications of these results are discussed.

INTRODUCTION

One of the primary cellular responses to the actions of polypeptide growth factors is the rapid and transient activation of a set of immediate-early genes, which number as many as 100 in mouse 3T3 fibroblasts (1–3). A subset of these genes is co-regulated with the protooncogene c-*fos*: they are transcriptionally activated within minutes of growth factor addition, reaching a maximal level of transcription at 10–30 min and are repressed within 1 h thereafter (1). Among this group of genes, the *c-fos* protooncogene has been studied most extensively with regard to the mechanisms underlying its rapid induction (4–6). Activation of c-*fos* by serum growth factors is mediated through a promoter element known as the serum response element (SRE), a sequence comprised of a CArG box [CC(A/T)$_6$GG] and a weak Ets-like binding site (5,6). The c-*fos* CArG box binds a dimer of a 67 kDa protein, serum response factor (SRF) (7). Induction of c-*fos* by TPA in Balb/c 3T3 (8) or NIH 3T3 cells (9) requires another protein, a 62 kDa ternary complex factor (TCF or p62^{TCF}), which binds at the Ets-like binding site. TCF does not bind to the c-*fos* SRE by itself, but requires the prior formation of the SRF–SRE binary complex (10). At least two Ets family transcription factors, Elk-1 and SAP-1a, have properties of TCF *in vitro* (11,12). Although Elk-1 can bind independently to its own high affinity binding sites characteristic of Ets-domain proteins and can act as a transactivator (13,14), it does not bind to the c-*fos* Ets-like site by itself. SRF interacts physically with Elk-1 (15,16) while bound upon the CArG box, thus recruiting Elk-1 to bind a neighboring Ets-like binding site to form a ternary complex. The distance between the CArG-box and the Ets-site can vary without detracting from the ability of SRF to recruit TCF for ternary complex formation (17).

To gain insight into the diversity of the regulatory mechanisms that control the coordinate activation of immediate-early genes, we have analyzed the regulation of several such genes that are transcriptionally activated with kinetics similar to those of c-*fos*. Among those genes studied are *cyr61*, which encodes an extracellular matrix signaling molecule (18); *nur77*, which encodes a transcription activator of the steroid/thyroid hormone receptor superfamily (19); and *pip92*, which encodes a short-lived, proline-rich cytoplasmic protein of unknown function (20). Our results indicate that the TCF/SRF-mediated mechanism of immediate-early gene activation is surprisingly general, at least in fibroblasts. We initially thought that the immediate-early gene *cyr61* was not regulated through SRF since no CArG box was found within 1.5 kb of the transcriptional start. Further analysis showed that *cyr61* is indeed regulated through a CArG box located far upstream (21). Likewise, initial analyses suggested that *nur77* and *pip92* might be regulated through an SRF-independent mechanism, as there were no recognizable SRF-binding sites in their promoters. Detailed analysis revealed that activation of these genes is mediated through 'mutated' CArG sequences, significantly altered from the consensus such that they are not expected to bind strongly to SRF (22,23).

As suggested by the CArG-box consensus sequence, CC(A/T)$_6$GG, it was thought that the GC base pairs were invariant for SRF binding whereas the ATs in the middle tolerated sequence diversity inasmuch as they were ATs (4). This notion was reinforced by the following observations: (i) in a PCR-based selection experiment carried out with SRF and random oligonuleotides, only sequences that fit the CArG consensus were selected even under low-stringency conditions (24); (ii) SRF directly contacts GG

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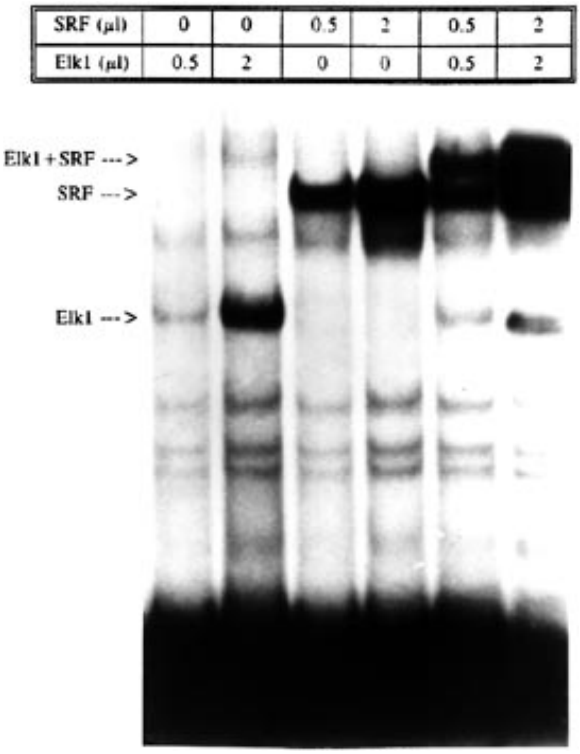

Figure 1. Ternary complex formation upon the *nur77* serum responsive element. Nucleotides –126/–72 of the *nur77* promoter was end-labeled and incubated with various amounts of SRF or Elk-1 translated *in vitro*, and the resulting protein–DNA complexes were subjected to EMSA. The amounts of reticulocyte lysate programmed for *in vitro* translation of either SRF or Elk-1 used in the EMSA are indicated above the corresponding lanes. Elk-1–DNA and SRF–DNA binary complexes, as well as the Elk-1–SRF–DNA ternary complex is indicated by arrows.

dinucleotides within the CArG box, and mutations in those contact points have the most severe effects on the SRF/CArG interaction (25); (iii) the CArG boxes found in the promoters of several immediate-early genes all conform to the CArG consensus (25). It was thus surprising to us that deletion and linker scanning analyses of the *pip92* and *nur77* promoters pinpointed 'CArG-like' sequences, which did not fit the consensus, as being essential for activation (22,23). Both sequences deviate from the CArG consensus significantly: TCTTATATGG for *pip92* and CCTTGTATGG for *nur77*. Neither was expected to bind strongly to SRF (26).

These findings prompted us to propose that in genes such as *nur77* and *pip92*, it is the binding of TCF to a high-affinity Ets-binding site in the vicinity of the 'mutated' CArG box that recruits SRF to form a serum-responsive ternary complex (23). We have tested this hypothesis directly. In this report we show that on elements with high-affinity Ets sites together with CArG-like sites, which may differ from the consensus by 1 or 2 base pair (bp) changes, Elk-1 can recruit SRF to form a ternary complex. We show that the SREs of *pip92* and *nur77* form both SRF–DNA and Elk-1–DNA binary complexes, and also Elk-1–SRF–DNA ternary complex. We also show that elements with mutated CArG boxes that do not bind SRF alone but have strong Ets-binding sites can nevertheless support the formation of the Elk-1–SRF–DNA complex. Moreover, sites that fit the consensus binding sequence for RSRFs (27), a group of transcription factors thought to have binding specificities distinct from those of SRF, can in fact bind SRF and interact Elk-1 to form a ternary complex where neighboring Ets sites are available. These results indicate that the range of DNA sequences that can support the assembly of a SRF–TCF–DNA ternary complex is much broader then originally thought. We suggest that the interaction between SRF and TCF might alter the DNA binding properties of both, allowing them to bind a broader range of sequences then they otherwise can bind individually.

MATERIALS AND METHODS

Cell culture and transfections

NIH 3T3 cells were grown and transiently transfected with the reference plasmid PGKβgal and a test plasmid as described (23). Cells were rinsed in phosphate buffered saline (PBS) 1 day following transfection and allowed to recover in growth medium for one day. Cells were then switched to medium containing 0.5% calf serum. Two days later, cells were either stimulated with 20% calf serum for 3 h or were left unstimulated (quiescent). CAT and β-galactosidase assays were performed as described (28). At least three sets of transfections for each construct were analyzed.

Oligonucleotides and construction of test plasmids

Sequences of oligonucleotides used are as follows: CE, derived from the c-*fos* promoter CArG box), has the sequence gatcCTACCGCCAACCGGAATAGTCCATATAAGGACTCg; mCE, the *pip92* CArG-like sequence and a proximal Ets site (same as 'CP' in ref. 23). The mmCE oligonucleotide differs from mCE by a change in CArG-like site: TCTTATATG*G* changed to TCTTATATG*T*. The mmCmE oligonucleotide differs from mmCE by a mutation in the Ets site: T*C*CGGAAG changed to T*T*CGGAAG. The –274/–249 *nur77* (29) oligonucleotide: gatCCGAGAGGAAAACTATTTATAGATCAg. The lower case nucleotides represent *Bam*HI cohesive ends. Other oligonucleotides used as competitors in EMSA ('Ets', distal and proximal *pip92* Ets sites, and *cyr61* CArG box) were described (23). Double-stranded oligonucleotides were cloned in *Bam*HI site of tk CAT vector (22); direct sequence analysis was carried out to confirm the sequences each clone isolated and used. All tk CAT constructs used in this report have a single copy of an insert in the wild-type orientation.

Electrophoretic mobility shift assay (EMSA)

All probes were double-stranded oligonuleotides described above, except that the *nur77* 126/72 fragment was excised from the corresponding tk CAT construct (22). All probes were labeled by DNA polymerase Klenow fragment fill-in reaction (28). *In vitro* transcription–translation, NIH 3T3 cell nuclear extract preparation, binding reactions and electrophoretic analysis were carried out as described (23). The efficiency of translation of Elk-1 and SRF was approximately equal as determined by ^{35}S-Met incorporation, followed by the SDS–PAGE analysis (data not shown).

RESULTS

SREs of *nur77* and *pip92* form binary complexes with either Elk-1 or SRF, and a ternary complex with both

The SREs of the immediate-early genes *pip92* and *nur77* each consists of a high-affinity Ets-binding site and a CArG-like site,

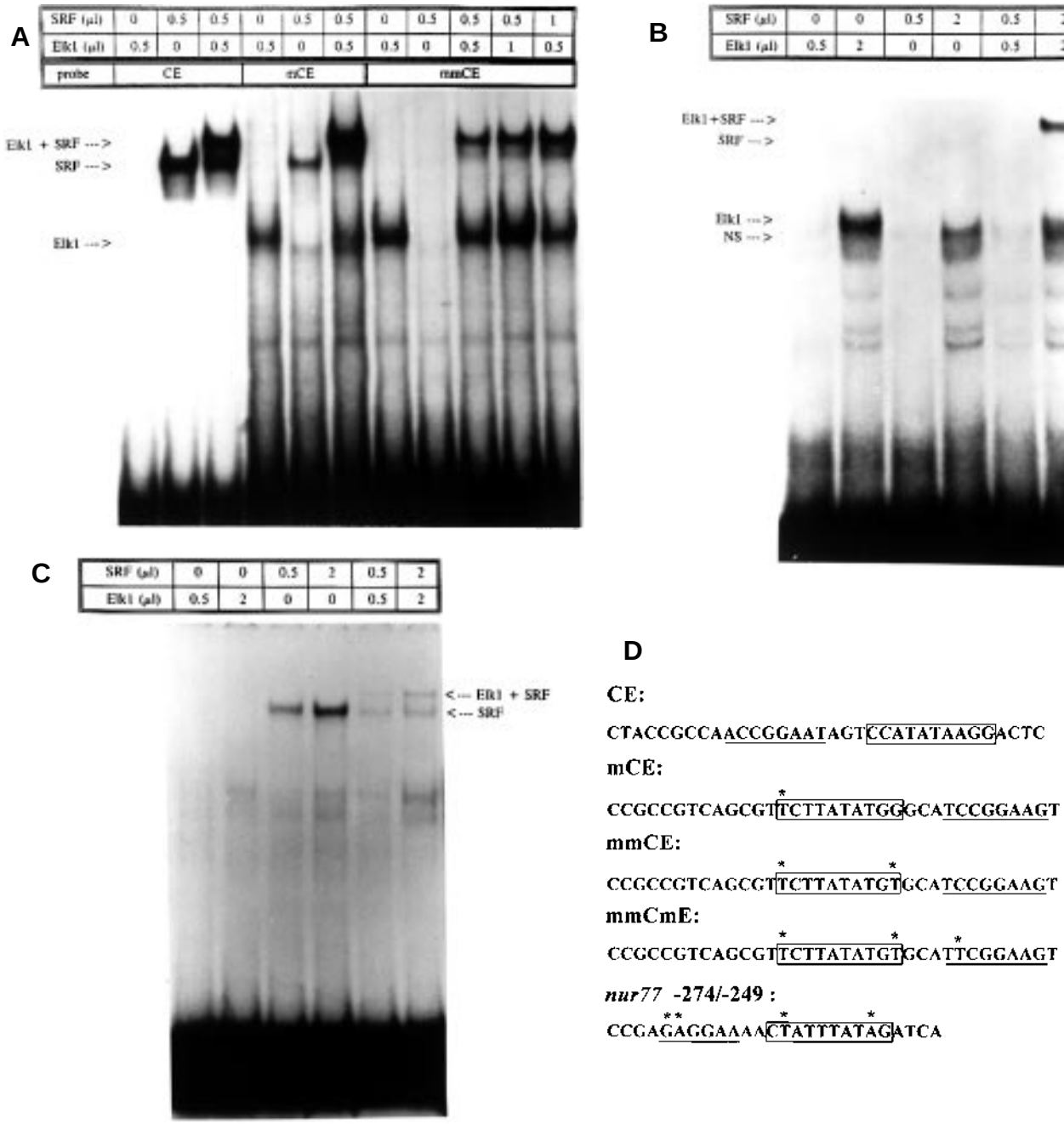

Figure 2. Ternary complex formation upon Ets-CArG sites of various affinities for Elk-1 and SRF. (**A**) CE (c-*fos* SRE), mCE (*pip92* SRE) or mmCE probes were incubated with SRF and/or Elk-1 synthesized *in vitro*, and resulting complexes were analyzed by EMSA. The oligonucleotide probe and the *in vitro* synthesized protein used are indicated above appropriate lanes. (**B**) Similar DNA–protein complex analysis as in (A), except that the probe used is mmCmE, which contains two deviations from the CArG box consensus and one in the Ets site. NS indicates a non-specific complex. (**C**) A similar assay as those shown in (A) and (B), except that the probe is the *nur77* –274/–249 sequence. This sequence contains the RSRF site and a Ets-like binding site. (**D**) Sequences of probes used in EMSA. CArG or CArG-like sequences are boxed; Ets binding sites are underlined. Each deviation from the consensus CArG and Ets sites is marked by an asterisk.

both of which are required for serum induction (22,23). The CArG-like sites are expected to have relatively low affinities for SRF as they deviate from the consensus sequence. We examined the abilities of these SREs to interact with SRF and Elk-1, and to form a ternary complex *in vitro*. We found that both the SRE of *nur77* (Fig. 1) and *pip92* (mCE probe; Fig. 2A) (23) can interact with *in vitro* translated SRF and Elk-1 independently as judged by EMSA. Furthermore, both SREs support the formation of the SRF–Elk-1–DNA ternary complex (Figs 1 and 2). Thus, although these SREs do not have a consensus CArG box, they nevertheless interact with SRF and form a ternary complex with Elk-1; such a ternary complex is necessary for induction of the c-*fos* promoter through protein kinase C and MAP kinase-mediated pathways (8,30–32).

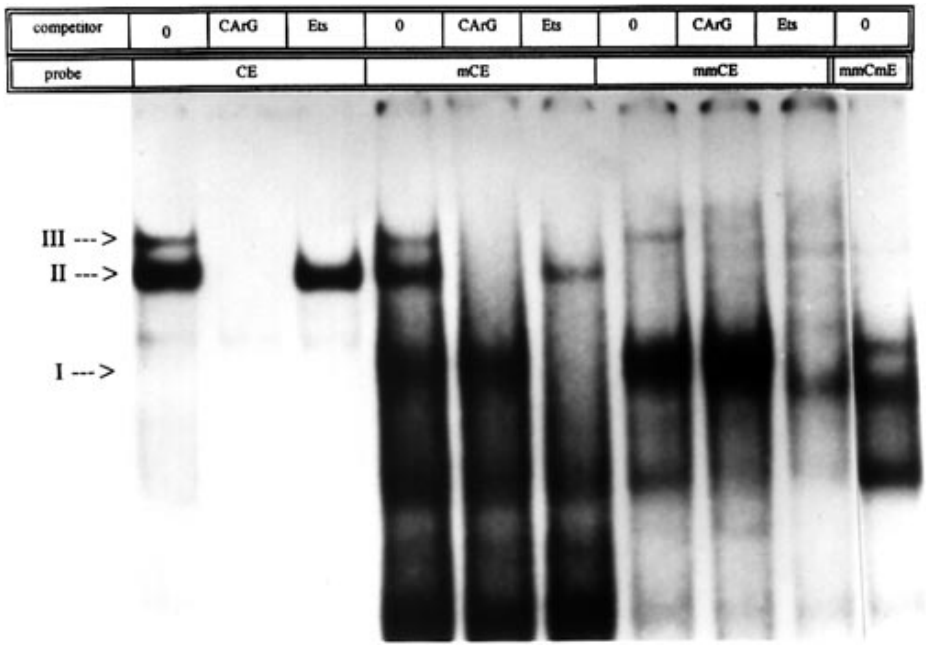

Figure 3. Ternary complex formation of nuclear extract proteins upon various Ets-CArG sites. Nuclear extracts from NIH 3T3 fibroblasts were incubated with probes indicated above the lanes, and binding reactions were subjected to EMSA. Competitor oligonucleotides, where indicated, were present at 200-fold molar excess. I, complex that competed with Ets oligos; II, complex that competes with CArG oligos; III, ternary complex that competes with both Ets and CArG oligos.

Elk-1 can recruit SRF to form a ternary complex

That the *nur77* and *pip92* SREs can form ternary complexes suggested to us that perhaps ternary complex formation need not necessarily occur by SRF recruitment of Elk-1 as in the case of c-*fos*. Specifically, we proposed that in SREs where the SRF-binding sites (CArG boxes) are weak but the Est-binding sites are strong, Elk-1 might be able to recruit SRF to form the ternary complex. To test this hypothesis, we designed a series of oligonucleotides with progressively altered CArG-boxes and a neighboring Ets-binding site (nomenclature for the oligonucleotides: 'C', high-affinity, consensus CArG box: $CC(A/T)_6GG$; 'E', consensus Ets core binding site: CCGGAA; 'm', a deviation from these sequences; see Fig. 2D). In this set of oligonucleotides, CE carries the sequence of the c-*fos* SRE whereas mCE carries the sequence of the *pip92* SRE. Thus mCE has a high-affinity Ets-site and CArG-like sequence with 1 bp deviation from the consensus; mmCE has two significant deviation from the CArG consensus (Fig. 2D). We compared the abilities of the mCE, CE and mmCE oligonucleotides to interact with Elk-1 and SRF and to form the ternary complex. As expected, CE (c-*fos* SRE) binds SRF strongly; while there is no detectable binding to Elk-1, CE also supports formation of the ternary complex in the presence of SRF and Elk-1 (Fig. 2A). Similarly, mCE binds Elk-1 and SRF independently and simultaneously, thus forming the ternary complex. mmCE, however, is unable to bind SRF but is able to bind Elk-1. This is expected since mmCE contains a high-affinity Est-binding site but a CArG-like site with two significant deviations from the consensus. Strikingly, mmCE is still able to form the ternary complex in the presence of both Elk-1 and SRF under the same conditions (Fig. 2A). These results strongly suggest that Elk-1 is able to direct SRF to form a ternary complex. Thus, formation of the SRF–DNA binary complex may not be an obligatory prerequisite for ternary complex assembly.

The above results show that oligonucleotides that do not exhibit detectable binding to SRF alone can nevertheless support the formation of the ternary complex in the presence of Elk-1, given that a strong Ets-binding site exists in the vicinity of the low affinity SRF-binding site. To test the effect of lowering the binding affinity of the Ets-site, we introduced a mutation into the Ets-site of mmCE to create the mmCmE probe (Fig. 2D). Weak Elk-1–DNA binary complex could be detected for mmCmE (Fig. 2B) only when a relatively high amount of Elk-1 was used in the EMSA (2 µl of translation product used in Figure 2B for mmCmE compared with 0.5 µl used in Figure 2A for mmCE). Moreover, in the presence of relatively high amounts of both SRF and Elk-1, a ternary complex can form (Fig. 2B). Thus, although mmCmE does not bind SRF alone and binds Elk-1 with relatively low affinity, it can nevertheless form a ternary complex in the presence of both SRF and Elk-1. This result provides further support for the notion that there is a cooperativity between TCF and SRF in formation of a ternary complex (33).

nur77 RSRF-Ets sites support the formation of SRF–DNA and Elk-1–SRF–DNA complexes

A subfamily of proteins, the related-to-SRF (RSRF) proteins, shares considerable sequence homology with SRF (34). However, the binding sites of SRF and RSRF appear to be distinct: SRF does not bind the RSRF consensus site, $CTA(A/T_4)TAG$, and RSRF does not bind the CArG box (34). The RSRF consensus can be regarded as a subset of CArG boxes with two nucleotide substitutions, i.e. $C\underline{T}(A/T)_6\underline{A}G$ versus $CC(A/T)_6GG$, although the central AT-rich sequence is more limited than the CArG consensus. Thus, the RSRF site deviates from the CArG consensus in a way that is similar to the mmCE oligonucleotide (Fig. 2D), both changing two nucleotides in the GC base pairs. The observation that mmCE can form a ternary complex in the presence of a strong Ets-binding site (Fig. 2A)

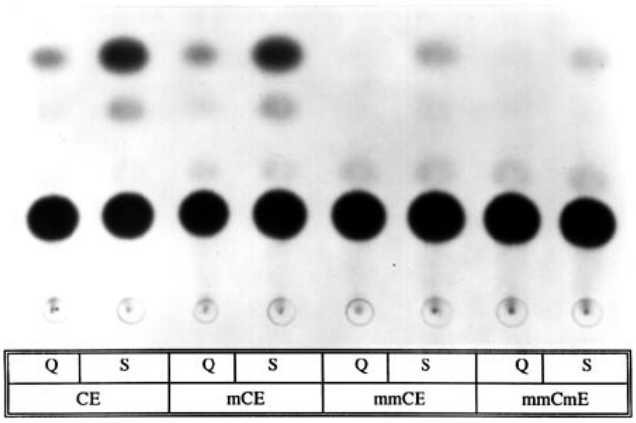

Figure 4. Formation of Elk-1–SRF–DNA complex *in vitro* correlates with serum inducibility in NIH 3T3 cells. NIH 3T3 fibroblasts were transiently transfected with reporter constructs containing indicated oligos cloned in front of the tk basal promoter in tk CAT plasmid, together with PGKβgal reference plasmid. Extracts containing equal amounts of β-galactosidase activity were analyzed for CAT activity. Q, quiescent cells; S, serum stimulated cells.

suggests that the RSRF site might also support ternary complex formation. We therefore tested the possibility that the RSRF site, in the vicinity of an Ets-binding site, might support the formation of an Elk-1–SRF–DNA ternary complex. An oligonucleotide corresponding to the –274/–249 region of the *nur77* promoter was synthesized (Fig. 2D) (22,29). This sequence includes a consensus RSRF site, previously shown to bind RSRF (34), as well as an Ets-like site GAGGAA (see Fig. 2D). Whereas it is expected that this fragment does not bind Elk-1, it is rather unexpected that it is able to bind SRF (Fig. 2C). We are not sure of the reasons underlying the apparent discrepancy between this result and those reported previously (34), although it is possible that in our experiments a much higher concentration of SRF was used than in the previous study. It is also possible that not all 2 bp-mismatch CArG boxes are equivalent, thus SRF may bind the *nur77* RSRF site (Fig. 2C) but not all mmCEs under similar conditions (Fig. 2A).

Remarkably, the *nur77* –274/–249 fragment forms a ternary complex in the presence of both SRF and Elk-1 (Fig. 2C). These results show that a sequence previously thought to interact only with RSRF can bind SRF as well, and suggest the possibility that RSRF sites may interact with neighboring Ets sites to mediate the serum response. This is consistent with the finding that the –278/–174 fragment of *nur77*, though not the major serum responsive element, has been shown to promote some level of serum-responsive transcription (22).

Binding of NIH 3T3 nuclear proteins to Ets-CArG sites

Since the above experiments were carried out with SRF and Elk-1 proteins synthesized *in vitro*, we sought to determine whether similar protein binding patterns occur with NIH 3T3 cell nuclear proteins. The same probes used in Figure 2A and B were incubated with nuclear extracts from NIH 3T3 cells, and the resulting complexes were resolved by non-denaturing PAGE. Specific complexes were identified by their sensitivity to competition by a consensus CArG box or an oligonucleotide with two high-affinity Ets sites. CE, mCE and mmCE all supported the formation of binary and ternary complexes, consistent with interactions with SRF and an Elk-1-like protein. As shown in Figure 3, complexes I, II and III correspond to DNA binding to an Elk-1-like protein (TCF), SRF and the ternary complex, respectively. CE (c-*fos* SRE) forms complexes II and III but not complex I, as expected. mCE (*pip92* SRE) forms both complexes I and II, as well as complex III, which corresponds to the ternary complex that can be competed by either cold CArG or Ets-binding sequences (Fig. 3). The mmCE probe formed complex I and III, but not complex II, consistent with the mmCE binding to Elk-1 but not SRF (Figs 2 and 3). The mmCmE probe formed a very faint, poorly reproducible ternary complex band in some experiments where higher amount of nuclear extract was used (data not shown). These results correlated well with the results obtained with *in vitro* translated SRF and Elk-1 (Fig. 2).

Ternary complex formation *in vitro* correlates with serum responsiveness *in vivo*

To investigate whether formation of ternary complex *in vitro* correlates with serum responsiveness *in vivo*, we cloned the various Ets-CArG sites used in the Figure 2A and B upstream of the herpes simplex virus thymidine kinase (tk) basal promoter driving the CAT reporter gene. The resulting constructs were transiently transfected into NIH 3T3 cells, and their ability to respond transcriptionally upon serum stimulation was quantified using CAT assays. Both CE and mCE, which form the Elk-1–SRF–DNA ternary complex with the highest efficiency among the sites tested in this study, supported strong serum inducibility of the tk CAT reporter gene (Fig. 4) (23). By contrast, mmCE and mmCmE, which form the Elk-1–SRF–DNA ternary complex with lower efficiency than mCE or CE, mediated lower levels of serum responsiveness of the tk CAT reporter (Fig. 4). The tk CAT vector alone provides only a low level of CAT activity that is unaltered by serum stimulation (data not shown) (22,23). We note that mmCE and mmCmE, which do not support the SRF–DNA binary complex in our experiments but form the Elk-1–SRF–DNA ternary complex, mediate a weak serum-induced transcriptional response. This result provides additional evidence that the ternary complex, rather than the SRF–DNA complex alone, is the functional serum-responsive complex (23).

DISCUSSION

Recent studies on the transcriptional activation of immediate-early genes have focused on the importance of a ternary complex, comprised of SRF and TCF bound to SRE, as the integral part of the serum-responsive transcriptional apparatus (16). Since the importance of the ternary complex was first recognized in c-*fos*, where SRF binds strongly to and TCF is recruited by SRF (35), TCF has been regarded as an 'accessory protein' subservient to SRF (36).

Results presented in this report suggest that given the appropriate SRE sequence, TCF can also recruit SRF. A simple explanation for the prominence of SRF in c-*fos* regulation is the presence of a good CArG box that binds SRF strongly. At the same time, the c-*fos* Ets-binding site (CAGGAT) is weak, being quite different from the CCGGAA high-affinity binding consensus (37). In the *pip92* and *nur77* promoter, where the Ets-binding sites (CCGGAA) are strong and the SRF-binding CArG sites are weak, SRF and Elk-1 can bind independently but together they bind synergistically (Figs 1 and 2A). When the CArG box is further weakened by mutation, then Elk1 can apparently recruit SRF to form a ternary complex (Fig. 2). These results indicate that the interaction between SRF and Elk-1 allows them to bind a broader range of DNA sequences than previously thought.

In this report we have shown that Elk-1 can recruit SRF to Ets-CArG elements that do not form detectable SRF–DNA binary

complex (Fig. 2), a situation opposite from the one at the c-*fos* SRE, where SRF recruits TCF (10). In addition, we have shown that the SREs of two immediate-early genes, *pip92* and *nur77*, interact with Elk-1 and SRF individually as well as in the SRF–Elk-1–DNA ternary complex (Figs 1 and 2A) (23). The cooperativity of TCF–SRF interaction (33) most likely allows a great sequence variability of composite Ets-CArG elements in the ternary complex.

The three DNA elements tested in this study, mmCE, mmCmE and *nur77* –274/–249, form a SRF–Elk-1–DNA ternary complex with a low efficiency (Fig. 2). The mmCE and mmCmE elements support only a low level of serum-induced transcription (Fig. 4). *nur77* –278/–174 promoter region, which includes –274/–249 fragment that forms a Elk-1–SRF–DNA ternary complex (Fig. 2C), acts as a weak SRE (22). Such weak elements are unlikely to operate alone as SREs, but may act together with other sites present in the promoters of immediate-early genes. In the case of *nur77* promoter, distal Ets-CArG (RSRF) element at –274/–249 may interact with an Ets-CArG element at –126/–72, which forms ternary complex with a high efficiency (Fig. 1) and is a strong SRE (22). Multiple Ets-CArG (or CArG-like) sites also exist in the promoters of immediate-early genes *zif268* (38), *krox20* (39), β-actin (40) and *pip92* (23).

Our results show that an RSRF site in the *nur77* promoter together with an adjacent Ets site supports formation of the SRF–Elk-1–DNA ternary complex (Fig. 2C). Even though both RSRF and SRF bind distinct high-affinity sites (34), this result shows that they can bind to common (RSRF) CArG-Ets composite sites. Thus, both RSRF and SRF may be able to interact with Ets-site binding factors and form functional ternary complexes.

In this report we have studied an SRF–Elk-1–DNA ternary complex. However, growing evidence suggests that SRF may interact with transcription factors other then those from the Ets family. For example, in the promoter of interleukin-2 α receptor SRF site functions together with a NFκB binding site (41). Recently, Treisman and colleagues showed that in NIH 3T3 cells c-*fos* SRE can retain serum responsiveness in the absence of an Ets site; they suggest that SRF–CArG complex is likely to interact with yet unidentified factor (30). This interpretation is in agreement with our results from this and previous report (23) that suggest that SRF–CArG binary complex is not sufficient for serum responsiveness. These results, taken together with numerous other studies on the role of SRF in serum-induced and muscle-specific transcription, indicate the function of SRF sites depend on the promoter context to a large extent.

Even though a relatively large subset of immediate-early genes may be regulated by a SRF–TCF–DNA ternary complex, this does not imply uniformity of regulation of those genes. The sequence variability of distinct Ets-CArG sites may contribute to the diversity of regulation of distinct genes that carry these sites. For example, those genes with strong Ets and weak CArG-like sites may function in a SRF-TCF mode in one cellular background, and in a SRF-independent mode, through an interaction with any of the Ets family members, in a different cellular background. If AP-1 and SP1 sites are present in promoters of those genes with strong Ets sites, Ets-AP-1 (42) and/or Ets-SP1 (43) composite elements may form and function. Widely different Ets-CArG sites may act as sensors of concentrations of active SRF and Ets family members, thereby leading to the differential regulation of genes in whose promoters they operate.

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