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Phosphorylation-Dependent Formation of a Quaternary Complex at the c-*fos* SRE

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The rapid and transient induction of the human proto-oncogene c-*fos* in response to a variety of stimuli depends on the serum response element (SRE). In vivo footprinting experiments show that this promoter element is bound by a multicomponent complex including the serum response factor (SRF) and a ternary complex factor such as Elk-1. SRF is thought to recruit a ternary complex factor monomer into an asymmetric complex. In this report, we describe a quaternary complex over the SRE which, in addition to an SRF dimer, contains two Elk-1 molecules. Its formation at the SRE is strictly dependent on phosphorylation of S-383 in the Elk-1 regulatory domain and appears to involve a weak intermolecular association between the two Elk-1 molecules. The influence of mutations in Elk-1 on quaternary complex formation in vitro correlates with their effect on the induction of c-*fos* reporter expression in response to mitogenic stimuli in vivo.

Both viral and cellular Fos proteins have been implicated in the regulation of genes involved in tumor invasiveness (15, 21). These findings have been corroborated by the observation that skin tumors do not progress efficiently in mice lacking Fos (40). The functional nature of Fos as a transcription factor suggests that its constitutive presence in cells leads to the deregulated expression of its target genes. In resting cells, the cellular c-*fos* gene is transcriptionally inactive but can be induced rapidly and transiently by a large number of extracellular stimuli. Critical for this response is the serum response element (SRE). Several proteins have been shown to interact with the SRE in vitro (reviewed in reference 48). However, the DNA footprint observed on the central and left part of the SRE in vivo is most closely reproduced in vitro by the binding of the serum response factor (SRF) and a ternary complex factor (TCF) (16, 44). Several TCFs, i.e., Elk-1, SAP1, and ERP/NET/SAP2, which belong to the *ets* family of transcription factors, have been cloned (4, 11, 20, 27, 38). While SRF binds as a dimer to the central CArG box of the SRE, TCFs have been detected only as monomers in this nucleoprotein complex. Moreover, TCFs are unable to bind to the SRE directly, presumably because their recruitment involves limited DNA contacts and extensive interactions with SRF (35, 43).

Numerous agents that stimulate c-*fos* transcription act through a conserved signalling cascade that involves sequential activation of the small G-protein Ras, Raf-1 kinase, the mitogen-activated protein (MAP) kinase activators MEK/MKK, and the MAP kinases ERK1 and ERK2 (25, 36). The latter phosphorylate and activate the TCF Elk-1 (8, 9, 22, 26, 31). Transcription of the c-*fos* gene requires the interaction of TCFs with SRF at the SRE. Mutations in the SRE that prevent TCF binding but leave the SRF binding site intact have been reported to prevent c-*fos* induction in response to several mitogens (12, 44). Recently, we and others have also demonstrated that Elk-1 phosphorylation by members of the stress-activated protein kinase subfamily of MAP kinases serves to mediate c-*fos* induction in response to cellular stress (10, 51). In other instances, c-*fos* induction by growth factors through the SRE has been reported to be independent of TCF binding (12, 18, 19, 24). Thus, there seem to be several ways in which extracellular signals utilize the SRE to elicit c-*fos* transcription.

Elk-1 is phosphorylated by members of the MAP kinase family at multiple sites. This event has been associated with an increase in Elk-1 activity manifested both as enhanced ternary complex formation and as elevated transcriptional activation (8, 9, 22, 31). These effects are difficult to differentiate. Although in an in vivo footprinting study, binding of TCF and SRF at the c-*fos* SRE was interpreted to be constitutive (16), increases in ternary complex formation upon growth factor stimulation or exposure of cells to stress are clearly observed in extracts from different cell lines (9, 10, 29, 45).

We now report the identification and characterization of a quaternary complex formed on the SRE. This complex is composed of a dimer of SRF and two molecules of Elk-1, which appear to interact with each other as well as with SRF. Formation of the quaternary complex is dependent on the presence of serine or glutamate at position 383 in the Elk-1 carboxy-terminal regulatory domain and correlates with the potential of Elk-1 mutants to mediate induction of c-*fos* expression in response to mitogens.

MATERIALS AND METHODS

Cell culture and extract preparation. NIH 3T3 and NIH 3T3.HER$_c$ cells were grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum. Cells were transfected by the standard DNA-calcium phosphate coprecipitation procedure and processed as described previously (26). Cells were transfected with 4 μg of the various chloramphenicol acetyltransferase (CAT) reporters and, where indicated, with 4 μg of pSG.GalElk or the corresponding expression vectors for the Gal-Elk mutants (26). Transfection efficiency was monitored by measuring the β-galactosidase activity resulting from cotransfection of 1 μg of pCH110 (Pharmacia) per plate, and the quantity of extract for each point in the CAT assay was normalized according to the relative transfection efficiency. The relative CAT activities given represent the averages of at least three independent experiments and were quantified with the aid of a Fujix BAS 1000 bioimaging analyzer.

Plasmid constructs. pGEX.ElkC was constructed as follows. pBSElk-1 (8) was cut with *Pst*I, and the 3′ ends were removed with the Klenow fragment. Sequences encoding the carboxy-terminal region of Elk-1 were then excised by digestion with *Xba*I. The fragment was ligated into pGEX.KG digested with *Xba*I and *Sma*I. The vector expresses the 210 carboxy-terminal amino acids of Elk-1 fused to glutathione *S*-transferase (GST) upon isopropylthiogalactopyranoside (IPTG) induction.

Vectors for the expression of ERK1, ERK1K→R (ERK1 with lysine changed to arginine), ERK2, and ERK2K→R in bacteria were provided by M. H. Cobb

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and have been described elsewhere (39). The vectors for cell-free transcription and translation of Elk-1 derivatives are based on pBluescript KS^+. pBS.Elk-1 has been described elsewhere (8). pBO.ElkC, pBO.Elk285, pBO.Elk325, and pBO.Elk350 were constructed by transferring Elk-1 coding sequences as *Eco*RI-*Hin*dIII fragments from the corresponding pQElkC, pQElk285, pQElk325, and pQElk350 derivatives into pBO, a pBluescript derivative containing the rabbit β-globin gene 5′ untranslated sequences downstream of the T3 promoter (35). pQElkC has been described elsewhere (8). pQElk285, pQElk325, and pQElk350 were produced by inserting Elk-1 sequences downstream of the *Bgl*I, *Ava*I, and *Bsr*FI sites, respectively, in frame with the ATG codon of pQE60 (Qiagen). ElkCΔ24, ElkCΔ19, and ElkCΔ29 were constructed by deleting Elk-1 sequences between the restriction sites *Nae*I-*Bsm*I (Δ24), *Bsm*I-*Bsr*I (Δ19), and *Bsr*I-*Bsp*120I (Δ29) in pBO.ElkC.

The sequences of the oligonucleotides used to create the SRE40 and PalRE (palindromic SRE) binding sites were as follows: 5′-CTAGATACACAGGAT GTCCA (PalRE.L), 5′-TATATGGACATCCTGTGTAT (PalRE.R), 5′-CTA GTTACACAGGATGTCCATATATGGACATCTGCGTCAT (SRE40U), and 5′-CTAGATGACGCAGATGTCCATATATGGACATCCTGTGTAA (SRE40L). Each oligonucleotide pair was cloned into the *Xba*I site of pBluescript, in the case of PalRE as an inverted repeat, from which the DNA probes were subsequently excised. They were also inserted into the *Xba*I site of ΔF10HCAT, a deletion derivative of pF711CAT (8, 47), to produce PalRECAT and SRE40CAT. The construction of el_2-CAT and of pSG.GalElk and its derivatives has been described (8, 26). pSG.GalElk383Q was constructed by transferring the *Apa*I fragment from pQElk383Q into pSG.GalElkΔApa. The construction of pQElk-1 and its derivatives has also been described elsewhere (8). Substitution of S-383 in pQElk-1 with glutamine was carried out in a manner analogous to that used for the other mutations (26). The sequence of the mutagenic PCR primer used was 5′-CTGCAACCCATTGCGCCCCG.

Standard recombinant DNA technology was used in all cases. Further details are available upon request.

Kinase reactions. Elk-1 was phosphorylated either with an ERK1 preparation from insulin-treated HIRcB cells as described previously (8) or with recombinant enzymes. To this end, ERK1 and ERK2 and their kinase-defective K→R variants were overexpressed from NpT7-5 plasmids in *Escherichia coli* and purified by chromatography on Ni^{2+}-agarose as described previously (39). R4F is a constitutively active mutant of MAP kinase kinase 1 (MKK1) which contains negatively charged residues at both activating phosphorylation sites and an internal amino-terminal deletion (30). ERKs (400 ng) were activated by incubation with 24 ng of R4F.MKK1 in 50-μl reaction mixtures containing 25 mM Tris-HCl (pH 7.2), 10 mM $MgCl_2$, 500 nM okadaic acid, 5 mM *p*-nitrophenyl phosphate, 1 mM dithiothreitol (DTT), 0.1 mM EGTA, and 250 μM ATP for 1 h at 37°C. Subsequently, 5- to 10-μl aliquots of the recombinant kinases were used to phosphorylate Elk-1 for gel retardation analysis.

Gel retardation. Elk-1 phosphorylation and binding reactions were performed as described previously (8). The SRE oligonucleotide probe used throughout has been described elsewhere (42). The SRE40 and PalRE probes were prepared from 100-bp DNA fragments of the respective pBluescript derivatives and end labelled with the Klenow fragment. Antisera (1 μl) were added to the binding reaction mixtures (where indicated), which were then incubated for 10 to 20 min at room temperature prior to addition of the radiolabelled probe. After an additional 10 min, samples were loaded onto 5% polyacrylamide gels cast in 0.8× Tris-borate-EDTA.

Antisera. Antisera were raised in rabbits by injection of antigens mixed with Freund's adjuvant by standard procedures (14). The Elk-1 antiserum has been described elsewhere (8). Anti-ERK serum 83/877 was generated by immunization with a mixture of full-length ERK1 and ERK2. Peptide antiserum 73/993 was raised against ERK1 amino acids 305 to 327 synthesized on preloaded, lysine-branched Tentagel resin (3). Antiserum Y691 was generated in the laboratory of M. H. Cobb.

Protein interaction assays. The experiments were performed so as to incorporate modifications to a procedure described previously (13). Prior to the association reaction, GST or GST-ElkC (C-terminal region of Elk-1) bound to glutathione-agarose beads (Sigma) was incubated with rabbit reticulocyte lysate in 25 mM Tris HCl (pH 7.2)–1 mM DTT–0.1 mM EGTA–0.1 mM sodium orthovanadate–1 μM okadaic acid–100 μM ATP for 45 min at 37°C. The beads were then washed and equilibrated in 200 μl of buffer D (5) containing 1 mM DTT and 30 μg of bovine serum albumin, to which [^{35}S]methionine-labelled Elk-1 proteins expressed in rabbit reticulocyte lysate were added. After incubation for 1 h at room temperature, the beads were washed in buffers D, C (5), and D plus 0.5% Nonidet P-40. The beads were then eluted with reduced glutathione, and the eluates separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10% gels).

RESULTS

A novel nucleoprotein complex over the SRE. Previous analysis of Elk-1 function revealed that ternary complex formation with SRF at the c-*fos* SRE is susceptible to regulation by phosphorylation (8, 9). More recently we have observed that conditions allowing efficient phosphorylation of Elk-1 in vitro lead to the formation of a novel DNA-protein complex, which can be detected in standard nondenaturing gels (cQ [Fig. 1a]). In the experiment shown, $core^{SRF}$ (SRF amino acids 133 to 222) and Elk-1 were overexpressed in and purified from bacteria. However, the ERK1 used to phosphorylate Elk-1 was purified from eukaryotic cells. When phosphorylated by less active fractions of ERK1, Elk-1 generates only the ternary complex (result not shown; see Fig. 2). An analogous complex is formed with full-length SRF and phosphorylated Elk-1 (Fig. 1b). Here, bacterially expressed ERK1 and ERK2 activated in vitro were used to phosphorylate Elk-1, but the SRF was isolated from HeLa cells. The novel complex will be referred to as a quaternary complex.

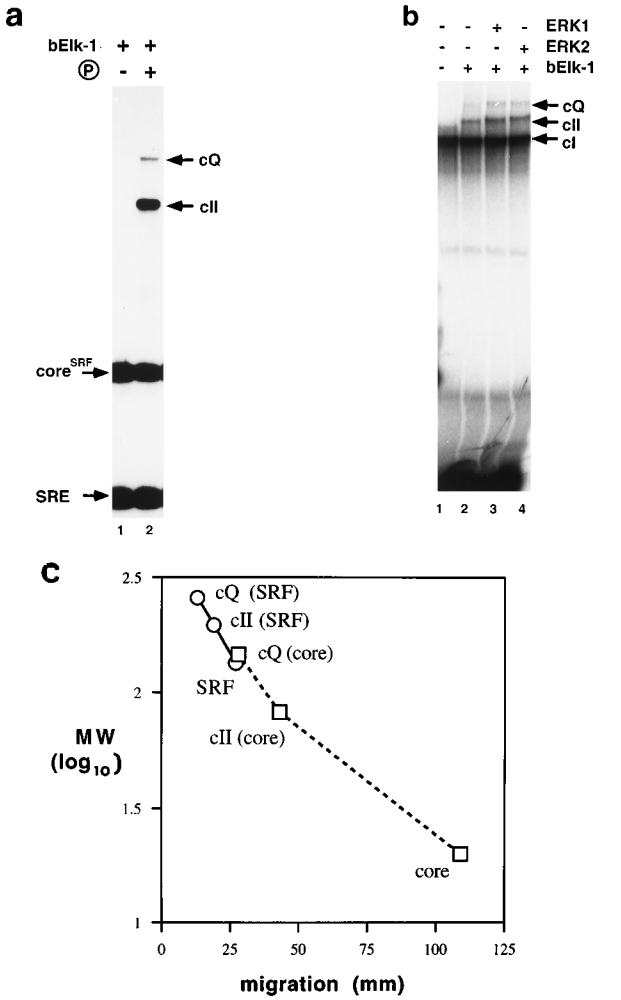

FIG. 1. Formation of quaternary complexes by phosphorylated Elk-1. (a) Bacterially expressed Elk-1 (bElk-1) unphosphorylated (lane 1) or phosphorylated with ERK1 purified from eukaryotic cells (lane 2) was incubated with bacterially expressed $core^{SRF}$ and a radiolabelled SRE probe. The ternary complex (cII) and quaternary complex (cQ) are indicated. (b) A nuclear extract from HeLa cells infected with a recombinant vaccinia virus directing the overexpression of SRF was incubated with the SRE probe alone (lane 1), with bacterially expressed, unphosphorylated Elk-1 (lane 2), or with Elk-1 phosphorylated by ERK1 (lane 3) or by ERK2 (lane 4). Bacterially expressed ERK1 and ERK2 were activated by prior incubation with R4F.MKK1, also purified from bacteria. The ternary and quaternary complexes observed in lane 2 are attributed to the presence of both Elk-1 and ERKs in the nuclear extract. (c) The migrations of the complexes in panels a and b plotted against their respective predicted molecular weights (MW). The quaternary complexes with $core^{SRF}$ and SRF are assigned molecular masses of 144 and 278 kDa, respectively.

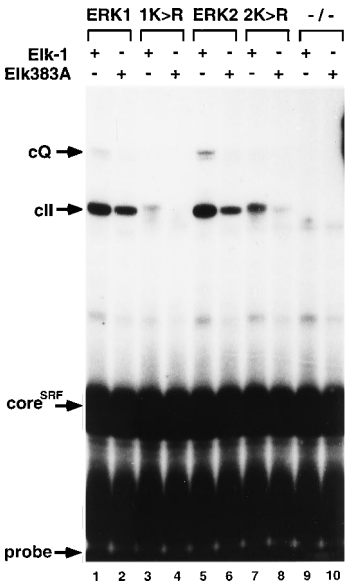

FIG. 2. Activation of Elk-1 in a linked-kinase system. Bacterially expressed Elk-1 or Elk383A was first incubated with the MAP kinases indicated, which had been phosphorylated and activated by R4F.MKK1 (see Materials and Methods) and then with bacterially expressed core^{SRF} and a radiolabelled SRE probe. Mock Elk-1 phosphorylation reactions are shown in lanes 9 and 10. Note that the K→R mutants of ERK1 and ERK2 are not completely inactive, as they stimulate weakly the formation of the ternary complex (lanes 3 and 7).

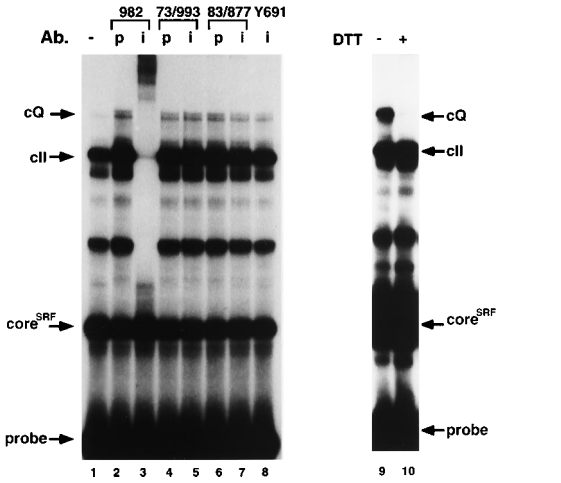

FIG. 3. Disruption of the quaternary complex. Bacterially expressed Elk-1 phosphorylated with ERK1 purified from eukaryotic cells was incubated with bacterially expressed core^{SRF} and a radiolabelled SRE probe (lane 1). Antibodies (Ab.) as indicated (p, preimmune; i, immune) were added to the binding reaction mixture 10 min prior to the addition of the SRE probe. No preimmune serum was available for Y691. The DTT concentration in the DNA binding reaction mixture was 20 mM (lane 10).

One possible composition for the quaternary complex is a dimer of SRF or core^{SRF} associated with two molecules of phosphorylated Elk-1. As an initial test for this hypothesis, the migrations of the different complexes in Fig. 1a and b were compared. As these are native gels, migration does not necessarily correlate linearly with molecular weight. However, the composition of most of the complexes is known, and this information can be used to provide standards. As shown in Fig. 1c, the relative migration of each quaternary complex corresponds to that predicted for complexes of the appropriate molecular weights. The anomalous migration of the core^{SRF} dimer in this gel system has been documented previously (41). In some cases, the novel complex appears in two distinct species (see below). These complexes presumably contain a dimer of core^{SRF} and two different Elk-1 phosphoisomers. Thus, the migration of the quaternary complexes is consistent with their proposed composition.

Quaternary complexes form with SRF and Elk-1. An alternative explanation for the formation of the quaternary complex is the involvement of an unidentified factor that may have been carried over either from the phosphorylation reaction as the ERK1 preparation used to phosphorylate Elk-1 was purified from eukaryotic cells (2) or from the SRF fraction derived from HeLa cells. To rule out this possibility, formation of the quaternary complex was reconstituted exclusively with recombinant proteins. To this end, a constitutively active recombinant MKK1 mutant (R4F.MKK1) was used to activate either ERK1 or ERK2 (30). Both ERK1 and ERK2 activated by this method still induce quaternary complex formation by Elk-1 (Fig. 2, lanes 1 and 5). In contrast, kinase-defective mutants of ERK1 and ERK2, which activate Elk-1 poorly as judged by ternary complex formation (Fig. 2, lanes 3 and 7), do not allow quaternary complex formation. After incubation with ERK1 and ERK2 activated by R4F.MKK1, an Elk-1 mutant in which serine 383 was converted to alanine (Elk383A) also fails to form a quaternary complex. Thus, the involvement of an unidentified cellular factor in the formation of the quaternary complex can be ruled out. These results also suggest that phosphorylation of serine 383 in the carboxy terminus of Elk-1 is a prerequisite for the formation of this complex (see also below).

It has been reported that MAP kinases copurify with TCFs over several chromatographic steps (52). Thus, it also seemed possible that ERK1 or ERK2 is an additional component of the quaternary complex. To address this point, we made use of specific antibodies. When an antiserum raised against an amino-terminal fragment of Elk-1 was added to the binding reaction, it resulted in the removal of both the ternary complex and the quaternary complex from their original positions and in the appearance of several complexes of lower mobility (Fig. 3, lane 3). Thus, the quaternary complex clearly contains Elk-1. However, three different polyclonal antibodies raised against MAP kinases failed to disrupt the quaternary complex (Fig. 3, lanes 5, 7, and 8), strongly implying that MAP kinases are not components of this DNA-protein complex.

The results presented above are consistent with the hypothesis that the quaternary complex is formed by a dimer of SRF and two molecules of Elk-1. However, they do not distinguish between the following possibilities. The two Elk-1 molecules may bind independently on either side of SRF. Alternatively, in the apparent absence of sequence-specific DNA contacts for the second Elk-1 molecule, formation of the quaternary complex may rely on additional protein-protein interactions. In this context, we observe that, unlike core^{SRF} or the ternary complex, the quaternary complex is susceptible to elevated concentrations of DTT in the binding reaction mixtures (Fig. 3, lane 10). Although we have no explanation for this phenomenon at present, it suggests that the quaternary complex is stabilized by interactions that are not required for ternary complex formation.

Intermolecular association of Elk-1. Given the foregoing observation, we sought to establish if Elk-1 molecules are able to interact with each other in solution. To this end, an in vitro interaction assay was used (13). The carboxy-terminal region of Elk-1 (amino acids 215 to 428) was expressed as a GST fusion

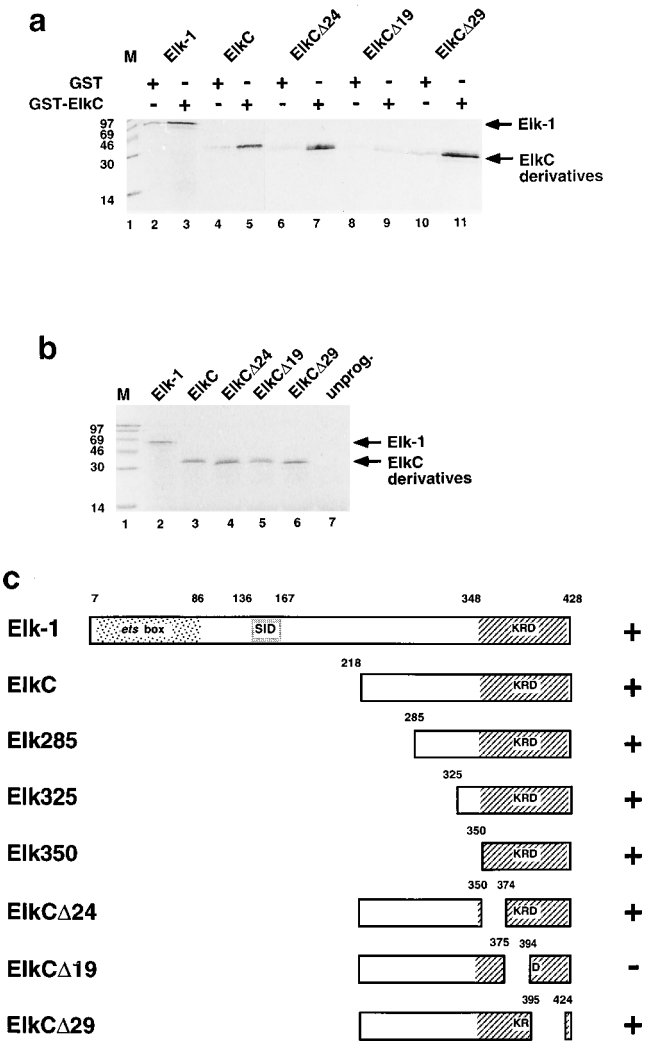

FIG. 4. Intermolecular interactions between Elk-1 proteins. (a) The GST interaction assay was performed with either GST or GST-ElkC (indicated) as described in Materials and Methods. Migrations of Elk-1 and the ElkC derivatives are indicated. (b) ^{35}S-methionine-labelled Elk-1 derivatives (as indicated) were expressed with equal efficiency in rabbit reticulocyte lysates. Sizes of standards (lane 1 in panels a and b) are indicated in kilodaltons. (c) Structures of Elk-1 and the deletion mutants used. The *ets* box is the DNA-binding domain; SID, the SRF interaction domain; KRD, the kinase-regulated domain. Interaction with GST-ElkC is indicated at the right.

(GST-ElkC) in bacteria, bound to glutathione-agarose beads, and preincubated under conditions that are known to activate Elk-1 for ternary complex formation (9). Various ^{35}S-labelled deletion mutants of Elk-1, expressed by cell-free transcription and translation, were then incubated with the bound GST-ElkC. The beads were washed thoroughly, and the association of Elk-1 derivatives was monitored by SDS-PAGE and autoradiography. A variable degree of association of Elk-1 with GST is observed, but as shown in Fig. 4a (compare lanes 2 and 4 with lanes 3 and 5), under the conditions used, both Elk-1 and ElkC associate considerably more efficiently with GST-ElkC than with GST alone. Thus, a weak interaction between Elk-1 molecules can be detected in solution.

ElkC lacks amino-terminal amino acids 1 to 212. Further deletions of Elk-1 were created and tested in the GST-ElkC association assay (Fig. 4c). The shortest Elk-1 derivative that could be expressed and tested, Elk350, consisted of the carboxy-terminal 78 amino acids of Elk-1 and was still able to interact with GST-ElkC (not shown). Therefore, to examine the importance of this carboxy-terminal region of Elk-1 for the interaction more precisely, a series of internal deletion mutants of ElkC was created. Of the three mutants expressed (Fig. 4b) and analyzed, only ElkCΔ19 failed to interact with GST-ElkC (Fig. 4a, lane 9). Thus, although similar deletions at adjacent sites in ElkC have no effect, the removal of amino acids 375 to 394 from ElkC impairs its association with GST-ElkC, suggesting that these residues may be required for the intermolecular association of Elk-1.

Augmented quaternary complex formation on a palindromic SRE. Elk-1 exhibits spatial flexibility when forming ternary complexes over the left part of the SRE. The minimal *ets*-binding motif GGA can be located between 5 and 27 bp from the central CArG box and is utilized even when in the inverted orientation (49). By these criteria, two GGA motifs are present on the right side of the CArG box in the SRE probe used in the assays described above. These might constitute low-affinity sites for the binding of an additional Elk-1 protein over the right side of the SRE. Thus, an oligonucleotide probe lacking both GGA motifs was generated and tested for the formation of the quaternary complex. However, it still allowed recruitment of a second Elk-1 molecule to the SRE. Indeed, the only SRE probe that we were able to generate that does not support formation of the quaternary complex lacks all sequences to the right of the SRE (results not shown). This implies that although sequence-specific base contacts to the right of the SRE are not involved in quaternary complex formation, at least some DNA contacts are required to incorporate a second Elk-1 molecule into the quaternary complex. Although we cannot completely rule out that some degree of sequence-specific binding is required by the second Elk-1 molecule, we infer from these observations that the weak protein-protein interactions between Elk-1 proteins detected in solution may compensate for inadequate sequence-specific DNA-protein interactions in the formation of the quaternary complex.

In the absence of SRE mutants that specifically impair quaternary complex formation, we reasoned that the introduction of a consensus *ets* site to the right of the SRE would augment quaternary complex formation. To this end, oligonucleotides encoding the sequences shown in Fig. 5a (SRE40 and PalRE) were cloned into plasmid vectors and subsequently prepared as DNA probes. Their ability to support complex formation was tested with core^{SRF} and Elk-1 phosphorylated by ERK2. As shown in Fig. 5b, quaternary complex formation is much more efficient with the PalRE probe. Moreover, as shown in Fig. 5c, there is a nonlinear increase in quaternary complex formation as Elk-1 is titrated into the binding reaction. This is indicative of cooperative binding between two Elk-1 molecules at the PalRE.

The PalRE was introduced into the c-*fos* promoter, and its ability to confer serum inducibility on a reporter plasmid was compared with that of the control SRE40 sequence and the native SRE. As shown in Fig. 5d, the PalRE mediated a serum response more than double that of either the control or the native SRE. This result is consistent with a role for the quaternary complex in SRE function, as its stabilization on the PalRE in vitro correlates with enhanced expression in vivo.

Quaternary complex formation requires phosphorylation of serine 383. The effects of mutations at individual phosphoacceptor sites in Elk-1 on ternary complex formation and transcriptional activation have been analyzed previously (8). Mutations at three sites in Elk-1 that are phosphorylated by ERK1 and ERK2 (S383A, S389A, and S422A) impair ternary com-

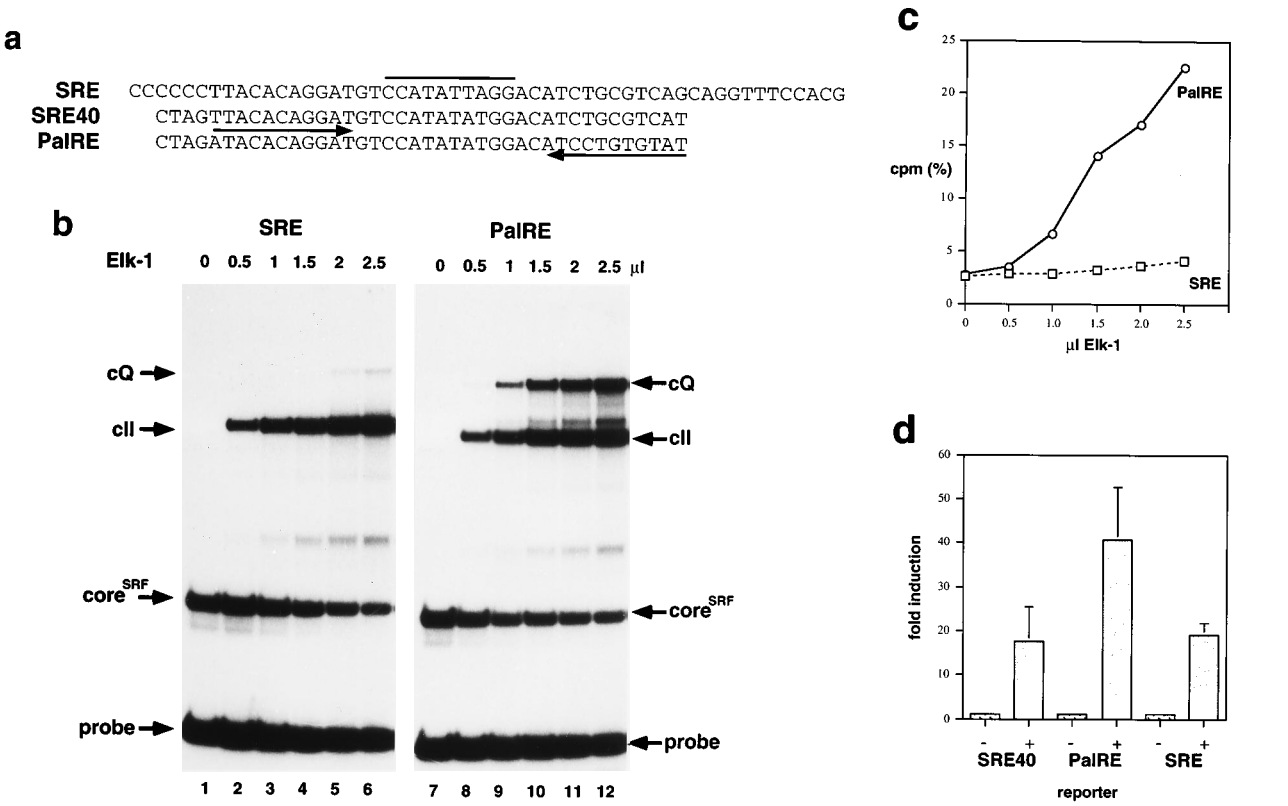

FIG. 5. Stabilization of quaternary complex on a palindromic SRE. (a) Sequences of the SRE probes (upper strands only). The CArG box is overlined, and the palindromic *ets* sites in the PalRE are indicated with converging arrows. (b) Increasing amounts of Elk-1 activated by the linked-kinase system were incubated with core^{SRF} and the SRE40 or PalRE probe as indicated. (c) Quaternary complex formation was quantified with a Fujix BAS 1000 bioimaging analyzer. Each point is expressed as a percentage of the set plotted against the amount of Elk-1. (d) NIH 3T3 cells were transfected with SRE40CAT, PalRECAT, or pF711CAT (SRE) as indicated, starved for 20 h, and treated with 15% fetal calf serum. Results similar to those shown have been obtained independently by Mueller (34).

plex formation. However, only the mutation of serine 383 to alanine is observed to abrogate the response of Elk-1 to mitogens, although it does not abolish ternary complex formation (8, 22, 26, 31). It was therefore of interest to test the ability of the individual Elk-1 phosphorylation mutants for quaternary complex formation.

Elk-1 proteins bearing mutations at each of the nine potential MAP kinase phosphorylation sites were phosphorylated with ERK1 and tested for complex formation. Under the stringent DNA-binding conditions used, complex formation by Elk-1 and all mutant proteins is dependent on phosphorylation (Fig. 6; compare odd- and even-numbered lanes). As reported previously, none of the mutations completely inhibit the formation of the ternary complex (8). However, the mutant Elk383A, which has the severest effect on Elk-1-mediated transactivation, fails to form the quaternary complex (lane 14; see also Fig. 2). This result is also consistent with a role for the quaternary complex in SRE function.

Quaternary complex formation and transcriptional activation. In some cases, negatively charged amino acids have been shown to circumvent the functional requirement for protein phosphorylation at serine residues (6, 7). Indeed, conversion of serine 383 in Elk-1 to glutamate is compatible with transcriptional activation by Elk-1 in response to mitogenic and stress-activated signals (10, 26, 51). Therefore, the ability of each of such Elk-1 mutant proteins to form the quaternary complex was investigated. In this experiment, the amount of each mutant in the binding reaction was first titrated to produce the same level of ternary complex. This rules out that quaternary complex formation could be influenced by the amount of ternary complex available. As shown in Fig. 7a, a glutamate residue in place of serine 383 allows efficient quaternary complex formation. In contrast, replacement of serine 383 with glutamine impairs formation of the quaternary complex, demonstrating that a correctly positioned negative charge at residue 383 is essential for Elk-1 to form the complex.

The behavior of these Elk-1 mutants with respect to formation of the quaternary complex in vitro provided a means to investigate further the requirement for its formation in intact cells. Mutant SREs that do not allow recruitment of endoge-

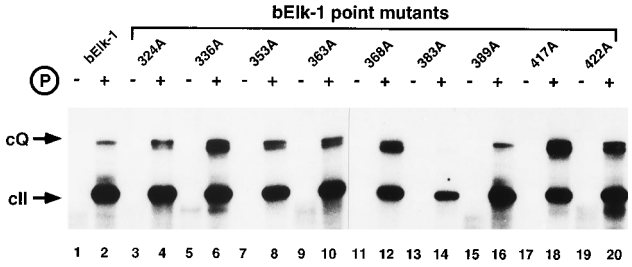

FIG. 6. Quaternary complex formation by Elk-1 phosphorylation site mutants. Bacterially expressed Elk-1 (bElk-1) and the mutants indicated were phosphorylated with ERK1 purified from eukaryotic cells and incubated with bacterially expressed core^{SRF} and the radiolabelled SRE probe. The formation of complexes was monitored by gel retardation. Only the upper part of the gel is shown. The presence of two forms of the quaternary complex seen with several of the mutants is presumably due to the presence of Elk-1 phosphoisomers.

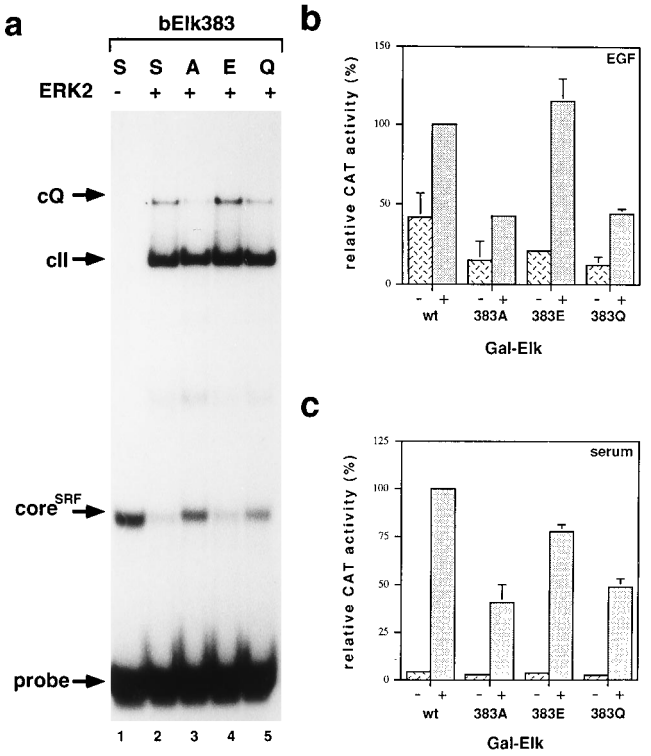

FIG. 7. Quaternary complex formation and transactivation by bacterially expressed Elk-1 S-383 (bElk383) mutants. (a) Various amounts of the Elk-1 mutants (labelled above the lanes in single-letter code) were activated with ERK2 in a linked-kinase reaction and incubated with core^{SRF} and the SRE probe to give a constant level of ternary complex formation. Under these conditions, the variations in quaternary complex formation due to the individual point mutations normalized for the respective ternary complexes are as follows: S, 1; A, 0.39; E, 1.34; and Q, 0.54. (b) NIH 3T3.HER$_c$ cells, which overexpress the human EGF receptor, were transfected with el$_2$-CAT and Gal-Elk expression vectors as indicated. After serum starvation for 24 h, cells were treated with 10 nM EGF (+) or not treated (−) and harvested 8 h later. The high background level of CAT activity observed is due to the exogenous EGF receptor expression (compare with NIH 3T3 cells in panel c). wt, wild type. (c) NIH 3T3 cells were transfected with el$_2$-CAT and Gal-Elk expression vectors as indicated. After serum starvation for 24 h, cells were treated with 15% fetal calf serum (+) or not treated (−) and harvested 8 h later.

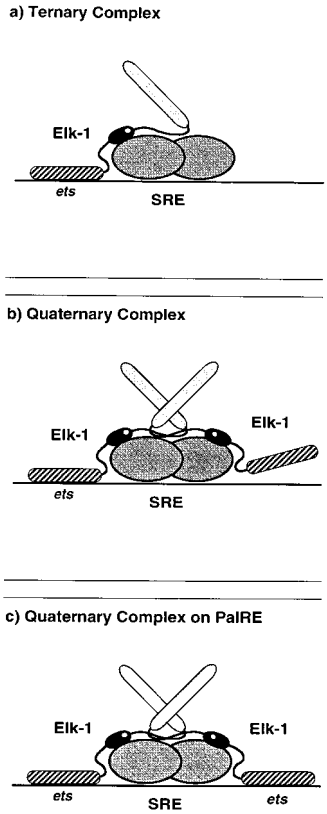

FIG. 8. Diagrammatic representation of the complexes described. (a) The ternary complex consists of a SRF dimer (grey) and the recruited TCF, in this case Elk-1. The *ets* domain of Elk-1 (striped balloon) binds a consensus sequence (GGA) at the left of the SRE. The SRF interaction domain (black) contacts a defined region in the DNA-binding core of SRF, and the kinase-regulated domain is depicted above the SRF (light grey balloon). (b) Recruitment of the second Elk-1 molecule into the quaternary complex at the SRE occurs in the absence of an *ets*-binding site, although some DNA backbone contacts are required. It also appears to depend on Elk-Elk interactions mediated by a small region of Elk-1 that encompasses the S-383 phosphorylation site. (c) A symmetrical SRE with two *ets* sites augments formation of the quaternary complex.

nous TCFs to the c-*fos* SRE have been identified. One example is the el-SRE (44). Reporters bearing this mutation are poorly inducible by serum and by agents that stimulate protein kinase C-dependent pathways. This mutant SRE can be rescued by the coexpression of Gal-Elk proteins as they bind to the el-SRE in an SRF-dependent manner (8). NIH 3T3.HER$_c$ cells were transfected with Gal-Elk proteins harboring serine, alanine, glutamate, or glutamine at residue 383. Their ability to rescue the response of the c-*fos* el$_2$-CAT reporter to epidermal growth factor (EGF) was tested. As described previously, overexpression of the EGF receptor in NIH 3T3 cells gives rise to a background level of mitogenic signalling which reduces the level of induction that can be achieved (8). However, the conversion of serine 383 to alanine impairs the EGF-inducible expression of the reporter. Moreover, a glutamate residue but not a glutamine residue is compatible with EGF-inducible expression (Fig. 7b).

In addition, the same Gal-Elk mutants were tested for the ability to mediate a response to serum in NIH 3T3 cells. Mutations that impair quaternary complex formation reduced the serum response, whereas a glutamate residue permitted an induction (Fig. 7c). Thus, the capacity of the mutant Elk-1 proteins to form quaternary complexes in vitro correlates qualitatively with the ability of their Gal-Elk counterparts to mediate the inducible expression of a c-*fos* reporter.

DISCUSSION

A novel complex observed at the c-*fos* SRE has been characterized. It consists of a dimer of SRF and two molecules of Elk-1 that seem to be arranged symmetrically (Fig. 8). Its formation is induced upon efficient phosphorylation of Elk-1 by MAP kinases and requires a negative charge at serine 383, a major phosphorylation site in Elk-1. Amino acids 375 to 394 of Elk-1, which encompass serine 383, appear to be involved in mediating a weak intermolecular association between Elk-1 molecules that may serve to regulate quaternary complex formation. In three different instances, formation of the quaternary complex in vitro correlates with the efficient activation of the c-*fos* promoter in vivo.

A quaternary complex containing SRF and Elk-1. Several pieces of evidence support the conclusion that the ternary complex is converted to the quaternary complex by the recruitment of a second Elk-1 molecule. We were able to compare the mobilities of the quaternary complexes formed with a dimer of either SRF or core^{SRF} with the mobilities of four other com-

plexes of known composition in the same gel system. Their relative mobilities are consistent with the presence of two molecules of phosphorylated Elk-1 in the quaternary complexes. Initially it also seemed conceivable that a contaminating eukaryotic factor was involved in the formation of the quaternary complex. This possibility was ruled out by reproducing the quaternary complex solely with bacterially expressed proteins. Furthermore, when ERK1 or ERK2 were replaced by their respective inactive counterparts, the quaternary complex was not detected, demonstrating that Elk-1 phosphorylation is a prerequisite for its formation. The only other possibility, namely, that ERK1 or ERK2 were physical participants in the quaternary complex, was ruled out by the observation that three different MAP kinase antibodies failed to affect its formation or migration. Thus, we conclude that the quaternary complex contains only SRF (or core^{SRF}) and Elk-1.

Quaternary complex formation through cooperative interactions. Elk-1 exhibits a large degree of flexibility in its interaction over the left part of the SRE. It can recognize the GGA motif at various distances from the central CArG box of the SRE. Elk-1 also forms a ternary complex when the *ets* motif is present in an inverted orientation or on the opposite face of the DNA helix (49). We found that the quaternary complex also manifests a lax sequence requirement. Initially we inferred the presence in the DNA probe of one or more cryptic *ets*-binding sites to the right of the SRE. However, only the truncation of the DNA probe immediately downstream of the SRE, which does not affect ternary complex formation, prevented the formation of the quaternary complex (result not shown). Therefore, some interactions between Elk-1 and the DNA to the right of the SRE must be required. Our inability to find mutations within or adjacent to the SRE that perturb formation of the quaternary complex prompted us to introduce a second *ets* site to the right of the SRE with the aim of enhancing its formation. The PalRE designed to this end efficiently stabilizes the quaternary complex in vitro. Moreover, the titration of Elk-1 revealed that quaternary complex formation on the PalRE increases in a nonlinear fashion compared with its formation at the SRE, indicative of cooperative binding of the second Elk-1 molecule to the ternary complex.

We also observe that the quaternary complex is susceptible to elevated concentrations of DTT in the binding reactions, whereas both the complex between the SRE and core^{SRF} and the ternary complex are unaffected. This finding also suggests that formation of the quaternary complex is dependent on interactions that are not required for ternary complex formation and argues against the possibility that the two Elk-1 molecules bind independently of each other, one on either side of the SRF dimer. In that case, they would be equally insusceptible to elevated DTT concentrations. In summary, these results suggest that quaternary complex formation on the native SRE relies primarily on protein-protein interactions.

Elk-Elk interactions. Although it is generally thought that TCFs interact with DNA as monomers, the majority of transcription factors described to date function as homodimers, heterodimers, or multimers (46). We were thus prompted to look for an interaction between Elk-1 molecules. A widely used GST fusion-based association assay was adopted and modified in two ways. First, the glutathione agarose-bound GST-ElkC was preincubated with reticulocyte lysate to allow posttranslational modification of the Elk-1 carboxy-terminal domain (9). Second, GST-ElkC and the Elk-1 derivatives were subsequently incubated together under conditions known to permit quaternary complex formation as assayed by gel retardation. A weak but reproducible association between GST-ElkC and several labelled Elk-1 derivatives was detected under these conditions. One small deletion in the carboxy-terminal domain of ElkC (Δ19) was found to impair this interaction, whereas two similar deletions of neighboring sequences had no effect.

Elk-1 is phosphorylated at five sites in vitro by ERK1, and the same sites are phosphorylated in vivo upon growth factor stimulation of cells (8, 22, 31). Three of these sites, S-383, S-389, and S-422, have been implicated in the regulation of SRF-dependent Elk-1 binding as mutations to alanine impair, but do not abolish, ternary complex formation (8). Amino acids 375 to 394 of Elk-1 bridge the MAP kinase phosphorylation sites at S-383 and S-389. By testing a series of nine Elk-1 mutants each lacking a potential phosphorylation site, we could demonstrate that despite the variation in ternary complex formation, only the mutation of S-383 to alanine severely impaired quaternary complex formation.

The ability of negatively charged residues to replace functionally phospho-acceptor sites in proteins has precedents (6, 7, 26, 32, 37). Here we replaced serine 383 with the residues glutamate and glutamine to test their effects on formation of the quaternary complex. In these experiments, the amount of Elk-1 protein incubated in the binding reactions was adjusted to give equal amounts of ternary complex, thereby controlling for the different capacities of the mutants to form ternary complexes. Under these conditions, serine and glutamate at position 383 allow similar levels of quaternary complex whereas alanine reduces complex formation threefold. The importance of the negative charge provided by phosphoserine or glutamate at this site is underlined by the inability of glutamine to substitute functionally for serine 383.

Other residues between positions 375 and 394 (F-378 and W-379) are also essential for Elk-1, as their mutation to alanine abolishes signal-dependent transactivation. As yet no model has been proposed for their function (38). Taken together, these results suggest that this region of Elk-1 allows the phosphorylation-dependent association of two Elk-1 molecules and thereby the establishment of a quaternary complex at the SRE.

Quaternary complex formation and transcriptional activation. The finding that Elk-1 does not require specific base contacts to form the quaternary complex restricts analysis of its functional relevance in vivo. This difficulty notwithstanding, three sets of data support the notion that quaternary complex formation correlates with SRE function. First, the PalRE, which stabilizes the quaternary complex in vitro, is induced by serum more than twice as efficiently as the native SRE. A similar palindromic SRE has been shown independently to be superinducible by tetradecanoyl phorbol acetate (34). The hypothesis that this reflects stabilization of the transcriptionally active complex in vivo is currently under evaluation. In this context, it is noteworthy that as transient expression of Fos protein is ensured by several independent mechanisms (1), an SRE in the c-*fos* promoter that stabilizes an active transcription complex might be counterproductive. By comparison, the Egr-1 promoter, another immediate early-gene promoter, contains an SRE that is functional only when two flanking *ets* sites are present (33).

Second, numerous phosphorylation site mutants of Elk-1 have been examined previously for the ability to mediate transcriptional activation in response to mitogenic signals. Consistently, mutation of serine 383 to alanine impaired transactivation the most (8, 22, 31). We observe that this mutation has the severest negative effect on quaternary complex formation in vitro. Third, quaternary complex formation by different point mutants of serine 383 in vitro correlate with their transcriptional activation potential in vivo. Thus, we have established a clear correlation between the ability of Elk-1 to form a qua-

ternary complex and its capacity to mediate the transcriptional activation in response to EGF and serum.

A common feature of ets family proteins is that they require additional factors to support DNA interactions and bring about transcriptional activation (28, 50). In addition to ternary complex formation at the SRE, Elk-1 is also capable of direct DNA binding at different sites. One example is the E-74 site in ectdysone-inducible promoters. However, a single E-74 site is poorly responsive in transcription assays (23). This situation is mirrored by chimeric Elk-1 proteins with altered DNA-binding specificities, in which the *ets* domain has been replaced by a different DNA-binding domain, such as that of Gal4 or LexA (26, 31). In such cases, a single Gal4 or LexA binding site also gives a weak transcriptional response. A third example is seen in the rescue of artificial or mutated SREs by expression of the appropriate chimeric Elk-1 proteins. Again a single site is poorly responsive to serum or EGF (8, 17), whereas tandem copies of artificial SREs mediate efficient responses. Consistent with this finding is the observation of a functional requirement for two *ets* sites flanking the SRE in the Egr-1 promoter (33).

The Elk-Elk interaction that we describe may provide the explanation for the observed failure of individual E-74 sites or artificial SREs to support a transcriptional response to serum or EGF. Clearly, the presence of at least two Elk-1 molecules would be required for the Elk-Elk association. In the case of artificial SREs, tandem copies, which function as efficiently as a single wild-type SRE (8), would provide one SRF-binding site flanked by two Gal4 or LexA sites. Conceivably such chimeric Elk-1 proteins have a more constrained DNA-binding requirement that is satisfied by this constellation.

The detection of a complex at the SRE containing two Elk-1 molecules and the indication of its functional relevance are particularly evocative in the light of the existence of multiple TCFs. To date, Elk-1, SAP1, and ERP/NET/SAP2, all of which are capable of binding to the c-*fos* SRE in the presence of SRF, have been cloned (4, 11, 16, 20, 27). It is conceivable that the intermolecular association between Elk-1 molecules described here can also occur between different TCFs. A further implication of our findings is that the association of two Elk-1 molecules may promote a conformation that facilitates essential interactions with basal transcription factors. These possibilities are currently under investigation.

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