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Spatial flexibility in ternary complexes between SRF and its accessory proteins

Richard Treisman, Richard Marais and Judy Wynne

Transcription Laboratory, Imperial Cancer Research Fund, PO Box 123, Lincoln's Inn Fields, London WC2A 3PX, UK

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We investigated the sequence requirements for ternary complex formation by the transcription factor SRF and its Ets domain accessory factors Elk-1 and SAP-1. Ternary complex formation is specified by an SRF consensus site $CC(A/T)_6GG$ and a neighbouring Ets motif (C/A)(C/A)GGA(A/T), which is contacted by Elk-1/SAP-1. Both the spacing of these sequences and their relative orientation can be substantially altered with little effect on the efficiency of ternary complex formation. Efficient ternary complex formation by Elk-1 is mediated by the B box, a conserved 21 amino acid region located 50 residues C-terminal to the Ets domain, which also acts to inhibit autonomous DNA binding. Binding studies with the isolated Ets domains indicate that ternary complex formation compensates for low affinity Ets domain-DNA interactions. Several naturally occurring SREs containing Ets motifs at different locations to that in the human c-*fos* SRE allow SAP-1 and Elk-1 recruitment *in vitro*. We discuss the mechanism of ternary complex formation.

Key words: DNA binding protein/Elk-1/p62-TCF/SAP-1/serum response factor

Introduction

The Serum Response Element (SRE) is a regulatory sequence which mediates the transcriptional response of many cellular immediate-early genes to growth factor stimulation, and binds the ubiquitous transcription factor SRF (for review see Norman *et al.*, 1988; Treisman, 1990). SRE mutations that block SRF binding abolish the response of the SRE to a variety of stimuli (Treisman, 1986; Fisch *et al.*, 1987; Greenberg *et al.*, 1987; Treisman, 1987). *In vitro*, SRF can form a ternary complex at the c-*fos* SRE with an accessory protein(s), p62/TCF: in this complex the protein contacts DNA to the 5′ side of SRF, although it does not bind the SRE by itself (Shaw *et al.*, 1989). Genomic footprinting studies suggest that such a ternary complex is present at the c-*fos* SRE *in vivo* (Herrera *et al.*, 1989), while in certain cell lines p62/TCF recruitment is required for response of the SRE to extracellular signals (Shaw *et al.*, 1989; Graham and Gilman, 1991; Konig, 1991; Malik *et al.*, 1991).

We recently used an *in vivo* cloning strategy to identify SAP-1, an SRF accessory protein (Dalton and Treisman, 1992). SAP-1 is related to Elk-1 (Rao *et al.*, 1989), which also acts as an SRF accessory protein (Hipskind *et al.*, 1991; A.Rogers and R.Treisman, unpublished data). Both proteins contain N-terminal Ets domains (Karim *et al.*, 1990), and the c-*fos* SRE sequences contacted by p62/TCF match the Ets domain binding consensus core motif GGA(A/T) (Karim *et al.*, 1990; Wasylyk *et al.*, 1990). SRE mutations that disrupt either the SRF or Ets domain binding sites prevent the recruitment of Elk-1 and SAP-1 to the c-*fos* SRE both *in vivo* and *in vitro* (Hipskind *et al.*, 1991; Dalton and Treisman, 1992; J.Wynne, C.Hill, S.Dalton and R.Treisman, unpublished observations). Efficient ternary complex formation at the c-*fos* SRE requires the SAP-1/Elk-1 Ets domain and an additional conserved sequence, box B, linked to its C-terminus by a non-conserved spacer of ~50 residues; in SAP-1, box B also acts to inhibit autonomous binding to the c-*fos* SRE (Dalton and Treisman, 1992; A.Rogers and R.Treisman, unpublished data). One simple interpretation of these observations is that box B is required for interaction with SRF, while the Ets domain contacts DNA (Dalton and Treisman, 1992).

Although Ets motifs are found in many SREs, their location with respect to the SRF site is not fixed. For example, the *Xenopus* and chicken c-*fos* SREs contain Ets motifs located 3 bp further from the SRF site than that in the human c-*fos* SRE (Fujiwara *et al.*, 1987; Mohun *et al.*, 1989). We therefore investigated the DNA sequence requirements for ternary complex formation by SRF accessory proteins in more detail. In this paper we present evidence that ternary complex formation occurs via a 'grappling hook' mechanism, in which the only essential contacts between the accessory proteins and SRF are mediated by the B box. This allows substantial variation in both the relative spacing and the orientation of the DNA sequences contacted by the two proteins.

Results

Selection of Elk-1 binding sites

To investigate sequence requirements for ternary complex formation, we used the site selection method shown in Figure 1A to purify DNA capable of forming ternary complexes from a pool of randomized oligonucleotides (Blackwell and Weintraub, 1990; Pollock and Treisman, 1990). For site selection we used an oligonucleotide comprising 32 bp of random sequence DNA abutting the preferred core SRF binding site CCATATAAGG (Pollock and Treisman, 1990), flanked by defined primer sequences for the PCR (see Figure 1A). The large extent of random sequence was chosen because in preliminary experiments we had observed efficient recruitment of SAP-1 or Elk-1 to Ets core motifs up to 12 bp from the SRF binding site (data not shown). Ternary complexes were formed between the radiolabelled oligonucleotides, the SRF derivative SRF[133-265] and intact Elk-1 protein produced by *in vitro* translation, and resolved by gel electrophoresis (the truncated SRF protein was used to allow maximum resolution of

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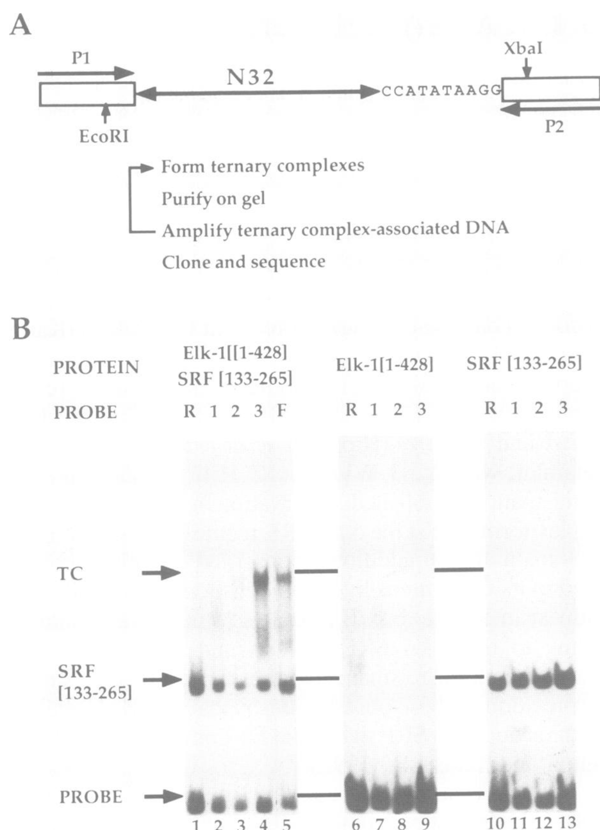

Fig. 1. (**A**) Site selection protocol. The structure of the randomized oligonucleotide is shown. The sequence of the SRF binding site is shown, and the primer sequences are represented by boxes. The steps in site selection are summarized below. (**B**) Gel mobility-shift analysis of ternary complex-selected DNA. Binding reactions were set up using radiolabelled selected DNA with either 5 ng BV.SRF[133–265] together with 1 μl Elk-1[1–428] rabbit reticulocyte lysate (lanes 1–5), 1 μl Elk-1[1–428] rabbit reticulocyte lysate alone (lanes 6–9) or 5 ng BV.SRF[133–265] alone (lanes 10–13). Probes were prepared from the randomized oligonucleotide pool (lanes 1, 6 and 10) or DNA purified by one (lanes 2, 7 and 11), two (lanes 3, 8 and 12) and three (lanes 4, 9 and 13) rounds of selection. As a control, a probe of similar specific activity containing human c-*fos* promoter sequences including the SRE was used in a binding reaction with 0.5 ng BV.SRF[133–265] and Elk-1[1–428] (lane 5). The positions of the SRF[133–265] complex and the SRF[133–265]–Elk-1[1–428] ternary complex (TC) are indicated. The smeariness in lanes 6 and 7 arises from partial proteolysis of the Elk-1 protein and was not observed in the original selection.

ternary complexes). The DNA associated with ternary complexes was recovered from the gel, amplified using the PCR, and used for further rounds of selection.

Labelled DNA from each round of selection was used to monitor progress of the experiment in gel mobility-shift assays with Elk-1 and SRF[133–265] either alone or in combination (Figure 1B). Ternary complex formation on the randomized oligonucleotide was barely detectable, although it binds SRF[133–265] efficiently (Figure 1B, lanes 1 and 10). During the selection, the efficiency of ternary complex formation increased until it was comparable with c-*fos* SRE (Figure 1B, compare lanes 1–4 with lane 5). The affinity of the selected DNA for SRF[133–265] showed little change (Figure 1B, lanes 10–13), while specific binding of Elk-1 protein alone was not detectable at the concentration of protein used for selection (Figure 1B, lanes 6–9). Thus in general, the affinity of Elk-1 for DNA must be substantially increased by interactions with SRF[133–265] in the ternary complex. We obtained similar results when we tested SAP-1 and SRF[133–265] in ternary complex assays using the Elk-1 selected DNA as probes (data not shown).

Table I shows oligonucleotide sequences present after three rounds of selection by ternary complex formation. The majority of the sequenced oligonucleotides (44/51) contain the core 'Ets motif', GGA(A/T), 13 sequences containing two motifs; no obvious sequence pattern is apparent between the Ets motif and the SRF site. It is striking that the Ets motifs do not occur at a fixed position relative to the SRF site, although almost half are within 5 bp of it (14/31 sites). Moreover, a significant minority, 8/31, of the sequences contain Ets motifs in the inverted orientation relative to that in the c-*fos* SRE (Table I). A consensus Ets motif, derived from analysis of 31 single sites, reveals the consensus (C/A)(C/A)GGA(A/T)(A/G) (see Table I). The frequency of occurrence of the Ets motif, and its preferred orientation, strongly suggests that it is the major determinant for Elk-1 recruitment by SRF.

The Ets motif participates in ternary complex formation

To verify that the Ets motif in the selected sites interacts with Elk-1, we studied protein–DNA interactions in the ternary complexes in more detail. Probes were chosen containing Ets motifs in either orientation, both close to and distant from the SRF CCATATAAGG motif, and partially carboxyethylated using diethylpyrocarbonate (DEPC). In sites 1.3-40 and the *Xenopus laevis* c-*fos* SRE, the Ets consensus is located in the positive orientation, separated from the SRF site by 16 and 5 bp respectively, while in site 1.3-38, the Ets motif is in inverted orientation 3 bp from the SRF site. Modified probes, radiolabelled on either DNA strand, were used for ternary complex formation with SRF[133–265] and either Elk-1 (sites 1.3-38 and 1.3-40) or SAP1[1–310] (*Xenopus* SRE). DNA associated with ternary complexes or SRF[133–265] alone was purified by mobility-shift gel and cleaved by piperidine. The cleavage pattern was compared with that of free DNA on the sequencing gels shown in Figure 2.

In each case, modification of bases within the Ets or SRF consensus sequences interferes with ternary complex formation, even when they are inverted (site 1.3-38; Figure 2A, lanes 1–3 and 7–9) or separated by more than one helical turn (site 1.3-40; Figure 2A, lanes 4–6 and 10–12). As expected, in the *Xenopus* c-*fos* SRE, whose Ets consensus motif is 3 bp to the 5′ side of its position in the human sequence, SAP-1–DNA interactions are correspondingly shifted (Figure 2B, lanes 1–10; cf. Dalton and Treisman, 1992). In cases where the Ets consensus and the SRF site are non-contiguous, modification of the intervening bases has no effect on ternary complex formation (Figure 2A lanes 5 and 11; Figure 2B, lane 4). In the *Xenopus* SRE, carboxyethylation of certain adenine bases within the AT rich core of the SRF site interferes strongly with binding of SRF alone, but has a smaller effect on ternary complex formation (Figure 2B, compare lanes 8 and 9). The data, summarized in Figure 2C, show that the Elk-1/SAP-1 Ets domain and SRF contact DNA at their respective sequence motifs in the ternary complexes, irrespective of their relative position and orientation.

Table I. Ternary complex-selected DNAs

"NORMAL" (c-fos) GGA(A/T) MOTIFS

Clone	Probe	Sequence
1.3-1	E	gaattcgcctcTCACACTACATGCACGAGCCTAACAACAGGAAccatataaggactctaga
1.3-11.3		gaattcgcctcCCCATACAGAACGGGCTCCTTATCAGGAACTGccatataaggactctaga
1.3-22.1		gaattcgcctcCGATGGCTGAAAATAGAACCGAAACGGAAAGAccatataaggactctaga
1.3-23	F	gaattcgcctcTCAGCACCAGACATCACCGCCAACCGGAAGGTccatataaggactctaga
1.3-33.1		gaattcgcctcTGCACACCACGTAATATTGTGGGCGGGAAGTAccatataaggactctaga
1.3-33.2		gaattcgcctcTTTAGTTGAGCAAAGCCTGCACTCCGGAAAGCccatataaggactctaga
1.3-2.1		gaattcgcctcTCATACGGACCTTACGGTACGGCCGGAATAGTccatataaggactctaga
1.3-6		gaattcgcctcACCATTGACGCAAATGTCCCTGGCGGAAGTCAccatataaggactctaga
1.3-35		gaattcgcctcACGATCATTCGACTATTAGCAACTGGAAGCTAccatataaggactctaga
1.3-14		gaattcgcctcCGAGCTAGATGAACACTCTAACCGGAAATTAAccatataaggactctaga
1.3-26	G	gaattcgcctcCATTGCGAAAGCCGCCCAATGCCGGAAATTGCccatataaggactctaga
1.3-10		gaattcgcctcCAAAACCAGCTTTGAGCCGGACGGATGTACCGccatataaggactctaga
1.3-16	Q	gaattcgcctcCAGCGGTGCTGCAGCCAAACCGGaAATGATGTccatataaggactctaga
1.3-4	H	gaattcgcctcAACTTCACCGTCCGTGTCCCGGAAGTCGTGTTccatataaggactctaga
1.3-29		gaattcgcctcTGTACGCTGGGCAGGGCCGGAAACCGACATCCccatataaggactctaga
1.3-12		gaattcgcctcCCGTTACTTGGGGCGGAACCAGNTTTAGACACccatataaggactctaga
1.3-18	M	gaattcgcctcTCTACTAGCTCCCCGGAAAACGCTGcATACTGAccatataaggactctaga
1.3-3.2		gaattcgcctcCCCCTTTGCACAGGAAGCTTCAGAAAACACCAccatataaggactctaga
1.3-40	T	gaattcgcctcAGGGACGCAACCGGATATAAATGGGACACTGTccatataaggactctaga
1.3-46		gaattcgcctcAACCTGCTAGCGGATGTCAGAAGNTAGGTTGaccatataaggactctaga
1.3-5		gaattcgcctcTTgGTACACCGGAAGCACCTAATGGGGCGTATccatataaggactctaga
1.3-44		gaattcgcctc GACGGAACGGGCgAGATCTCAACTACCGTGAccatataaggactctaga
1.3-21	P	gaattcgcctcCGGAAGCTCTGACAAAGAGGTGAAGCATAAGAccatataaggactctaga

"INVERTED" GGA(A/T) MOTIFS

Clone	Probe	Sequence
1.3-38	D	gaattcgcctcAATAATCTATAAATATCTAGGACTTCCGTTGAccatataaggactctaga
1.3-39		gaattcgcctc TTTAGCGCCAACAAGCATGACTTCCTTATGAccatataaggactctaga
1.3-11.2		gaattcgcctcTCTCGGCTTGAGAGGTGAGACTTCCGGCCTACccatataaggactctaga
1.3-45.1		gaattcgcctcCAAACAGTGTACTTCCGACGCGCAGAGAGTGAccatataaggactctaga
1.3-28		gaattcgcctcTCCTACGTAACCTTCCTTAGGGACACTAGACAccatataaggactctaga
1.3-25.2		gaattcgcctcGAGCAGACATCCCGGACATAAGGCTCTTACGCccatataaggactctaga
1.3-37	A	gaattcgcctcATTTCCGGCTCACAAATATACGTCACGCTAGAccatataaggactctaga
1.3-24.2		gaattcgcctcTTCCGATTAGACAACGAAGAACGCGTGAACTAccatataaggactctaga

TWO GGA(A/T) MOTIFS

Clone	Probe	Sequence
1.3-13	J	gaattcgcctcCCGTGAGGTACCACTTCCGGAATGGCTTAACGccatataaggactctaga
1.3-27	L	gaattcgcctcCTGATCATCCACCGGAAGAGCTAATGTAATNGccatataaggactctaga
1.3-36		gaattcgcctcCGAATATTCAAATCCCAACCCCGGAAACCCGCccatataaggactctaga
1.3-32		gaattcgcctcCTCGTCGCTAAGGCCCGGAAATCCACGCAGGAccatataaggactctaga
1.3-2.2		gaattcgcctcAACGACAGAGCGGAAGATACTTGGGGGATCATccatataaggactctaga
1.3-24.1		gaattcgcctcTCCGTTATGACGTATCCGGAGTGTCGGAATTAccatataaggactctaga
1.3-34		gaattcgcctcGGTAGGCGGATAGCGCTTTGAGCGGATGCAATccatataaggactctaga
1.3-7	K	gaattcgcctcCCAGACATaATACCCGGAAGCTTCCTGTAGTAccatataaggactctaga
1.3-11.		gaattcgcctcCGATTCCGGAAATACAACCGTAGGAGATCAGTccatataaggactctaga
1.3-45.2		gaattcgcctcAAATCCTGTCTCACCAAACCGTCATCCGATTTccatataaggactctaga
1.3-41		gaattcgcctcTTACCCAACGGAAGATCATCCTCCTGAGCACTccatataaggactctaga
1.3-22.2		gaattcgcctcAGCCCTGCTTCCCACTTCCTGCACAGACCGGAccatataaggactctaga
1.3-43		gaattcgcctcTTCCGGAAAGTCACTAGCGACCAGCGCCCGAAccatataaggactctaga

NO SITES: 7

CONSENSUS (SINGLE MOTIFS ONLY: 31 SITES)

T	11	5	5	5	2	1	-	-	-	4	-	16	7	6
C	12	11	9	3	20	24	-	-	-	-	3	8	10	11
G	5	10	7	11	3	1	31	31	-	-	17	5	6	8
A	3	5	10	12	6	5	-	-	31	27	11	2	8	8
			ac	ag	CA	CA	G	G	A	AT	ga	tc		

The sequences of 51 oligonucleotides remaining in the pool after three rounds of selection were determined, and the 44 sequences which contain the GGA(A/T) motif in either orientation are shown. The ternary complex consensus hexamer and the SRF CCATATAAGG motif are underlined in each sequence. For compilation of the consensus sequence we omitted sequences containing two motifs, since it is likely that one of the matches represents a chance occurrence of the motif rather than a true interaction site. Moreover, since the probability of this sequence arising by chance in non-selected contaminating DNA is relatively high, it remains possible that even some of the single site oligonucleotides represent spurious contaminants. Thus single copies of the motif do not necessarily represent selected sequences. The letters next to some sequences denote those used as probes in the binding studies. 'Normal' orientation is the same as that found at the c-*fos* SRE.

Fig. 2. Carboxyethylation interference analysis of ternary complexes. Interference patterns are shown in (A) and (B), and the data are summarized in (C). To facilitate comparison the locations of the CC(A/T)6GG and Ets motifs are indicated by shaded boxes and arrows respectively. **(A)** Elk-1[1−428] and SRF[133−265] interactions with probes 1.3-38 and 1.3-40. Proteins were produced by recombinant baculovirus. Lanes 1−6, top strand; lanes 7−12, bottom strand. Lanes 1, 4, 7 and 10, SRF[133−265] complexed DNA; lanes 2, 5, 8 and 11, SRF[133−265]−Elk-1[1−428] ternary complex DNA; lanes 3, 6, 9 and 12, uncomplexed DNA. **(B)** SAP-1[1−310] and SRF[133−265] interactions with the *Xenopus* c-*fos* gene SRE. SRF[133−265] was expressed by recombinant baculovirus and SAP-1[1−310] by *in vitro* translation. Lanes 1−5, bottom strand; lanes 6−12, top strand. Lanes 1 and 6, uncomplexed DNA; lanes 2 and 7, free DNA from SRF[133−265] binding reactions; lanes 3 and 8, SRF[133−265] complexed DNA; lanes 4 and 9, SRF[133−265]−SAP-1[1−310] complexed DNA; lanes 5 and 10, free DNA from SRF[133−265]−SAP-1[1−310] binding reactions. **(C)** Summary of interference data. The probe sequences are shown with positions of interference with ternary complex formation indicated next to the sequence and positions of interference with SRF[133−265] complex formation indicated away from the sequence. Symbol size is indicative of strength of the interference.

Fig. 3. Comparison of ternary complex formation by isolated selected sites and the c-*fos* SRE. Binding reactions were set up with selected oligonucleotide or the c-*fos* SRE and either Elk-1[1−428] baculovirus lysate alone (50 cell equivalents; panel **A**), SRF[1−508] alone (0.2 ng; panel **B**), or Elk-1[1−428] together with purified recombinant SRF[1−508] (panel **C**). The probe sequences are shown in panel **D**, with the CCATATAAGG and Ets motifs respectively shown boxed and shaded. Probes B, C, N, R, S and T were from DNAs isolated following two rounds of selection (data not shown). The complexes moving slightly more slowly than the free probe arise from a buffer component.

Properties of isolated ternary complex oligonucleotides

We next compared the efficiency of ternary complex formation at different sites with that at the human c-*fos* SRE, using a panel of the selected sequences. The probes shown in Figure 3D were synthesized at equal specific activity using the PCR and tested for binding of SRF[1−508] and Elk-1, either alone or together, using the gel mobility-shift assay. In these experiments we used SRF and Elk-1 proteins produced by recombinant baculoviruses (Marais *et al.*, 1992; R.Marais, unpublished data). None of the sites efficiently bound Elk-1 at the concentrations used in these assays (Figure 3A, lanes 1−22; but see below). In contrast, all probes bound SRF[1−508] with efficiencies comparable with the c-*fos* SRE, small variations in affinity probably arising from sequence differences next to the CCATATAAGG motif (Figure 3B, lanes 1−22).

In order to investigate the effect of different site spacings on the efficiency of ternary complex formation, we tested

nine probes in which the Ets motif CCGGAA is located between 27 and 5 bp from the SRF CCATATAAGG motif, at intervals of 2−6 bp (probes F−P, Figure 3D). It is striking that SRF recruits Elk-1 to each probe with an efficiency somewhat greater than the c-*fos* SRE, regardless of spacing between the Ets and SRF motifs (Figure 3C, compare lanes 7−15 with lanes 5 and 16). Moreover, the efficiency of ternary complex formation appears largely independent of helical pitch, although there is a small apparent periodic variation at intervals of about one helical turn (Figure 3C, compare lanes 7−15, 17 and 20). The broadly comparable affinities of these sites regardless of the spacing between the Ets and SRF motifs is consistent with the wide variety of spacings observed in the selected oligonucleotide population.

Although the majority of selected Ets motifs occur in the same orientation as that in the c-*fos* SRE, a significant minority are in the inverted orientation. To compare efficiency of ternary complex formation at the inverted motifs we tested four probes in which inverted Ets motifs are located between 3 and 24 bp from the SRF site. Probes containing the Ets motifs CCGGAA and TCGGAA located 24 and 11 bp from the SRF site formed ternary complexes as efficiently as the c-*fos* SRE (Figure 3C, compare lanes 1 and 2 with lane 5), while sites comprising an ACGGAA motif located 8 or 3 bp from the SRF site showed lesser or greater efficiency (Figure 3C, compare lanes 3 and 4 with lane 5). The variation in ternary complex formation observed with these probes is at least partly attributable to variation in the affinity of these oligonucleotides for SRF (compare Figure 3B and C lanes 3 and 4).

To examine the effect of variations in the Ets motif sequence upon ternary complex formation, we used probes containing different Ets motifs at comparable distances from the SRF CCATATAAGG motif. Of four Ets sites located either 15 or 16 bp from the SRF site, CCGGAT or CCGGAA formed ternary complexes with an efficiency greater than the c-*fos* SRE (Figure 3C, compare lanes 20 and 22 with 16); in contrast, a probe containing a TGGGAA motif exhibited virtually no ternary complex formation, while one containing the sequence TAGGAA formed ternary complexes with intermediate efficiency (Figure 3C, compare lanes 19−22; note low SRF affinity of probe S, lane 19). A similar result was obtained with probes containing Ets motifs 7 bp distant from the CCATATAAGG motif: at this location, a site containing the sequence ACGGAT formed ternary complexes less efficiently than one containing the CCGGAA motif (Figure 2C, compare lanes 17 and 18).

Taken together, these data suggest that Elk-1 (and SAP-1) interacts with SRF and DNA largely independently of the spacing or orientation of the Ets and SRF motifs, and that no particular intervening sequences are required. However, the efficiency of ternary complex formation is governed by the primary sequence of the Ets motif in addition to the affinity of DNA for SRF. We shall show below that the effects of variation of the sequence of the Ets motif upon ternary complex formation at least partly reflects its affinity for the Elk-1 or SAP-1 Ets domain.

Ternary complex formation always requires Box B

At the c-*fos* SRE, ternary complex formation by both SAP-1 and Elk-1 requires Box B, a short sequence located C-terminal to the Ets domain [Elk-1 residues 148−168; SAP-1 residues 136−156 (Dalton and Treisman, 1992; A. Rogers and R.Treisman, unpublished data; compare Figure 4A lane 2 with Figure 4B lane 2). To test whether Box B is required for recruitment of Elk-1 to distant Ets motifs, we tested deletion derivatives of Elk-1 in which Box B is either intact (Elk-1[1−205]) or truncated (Elk-1[1−156]) in ternary complex assays with members of the probe panel. As with the intact proteins, Elk-1[1−205] efficiently forms ternary complexes with probes containing Ets motifs in either orientation at a variety of distances from the SRF site (Figure 4A, lanes 3−8; see Figure 4C). No complexes characteristic of autonomous Elk-1[1−205] binding are observed even though these binding reactions contain excess probe (Figure 4A, lanes 3−8). A strikingly different result was obtained when these probes were tested with an equivalent amount of Elk-1[1−156], in which the B Box is truncated. In this case, efficient ternary complex formation did not occur with any of the probes (Figure 4B, lanes 3−8; compare Figure 4A). However, probes containing the Ets motif CCGGAA, and to a lesser extent those containing an ACGGAA motif, showed significant amounts of a complex migrating faster than SRF[133−265]. This complex is also formed in the absence of the SRF derivative, suggesting it

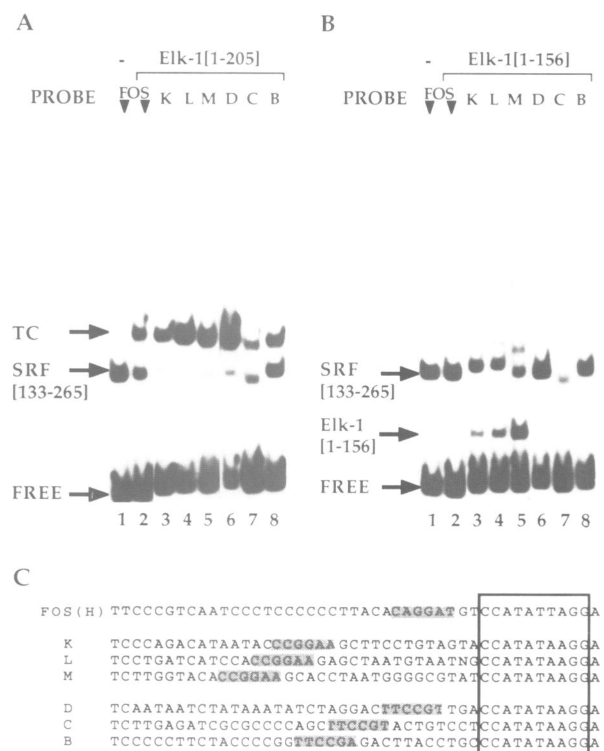

Fig. 4. Ternary complex formation requires Elk-1 Box B regardless of the relative positions of the Ets and SRF motifs. (**A**) Ternary complex formation by Elk-1[1−205] (Box B^+). Binding reactions were set up with SRF[133−265] either alone (lane 1), or with Elk-1[1−205] (lanes 2−8). The truncated SRF derivative was used to improve complex resolution. (**B**) Ternary complex formation by Elk-1[1−156] (Box B^-). Binding reactions were set up with SRF[133−265] either alone (lane 1), or with Elk-1[1−156] (lanes 2−8). Equal amounts of each Elk-1 derivative were used as assessed by SDS−PAGE analysis. The positions of the ternary complexes (TC), the SRF[133−265] complex, and the Elk-1[1−156] complex are indicated. The variation in mobility of the SRF[133−265] complex probably arises from differential bending of the different probes. (**C**) Binding probes. The probe sequences are shown with the Ets motif shaded and the SRF CCATATAAGG motif boxed. Probe designations are as in Figure 3.

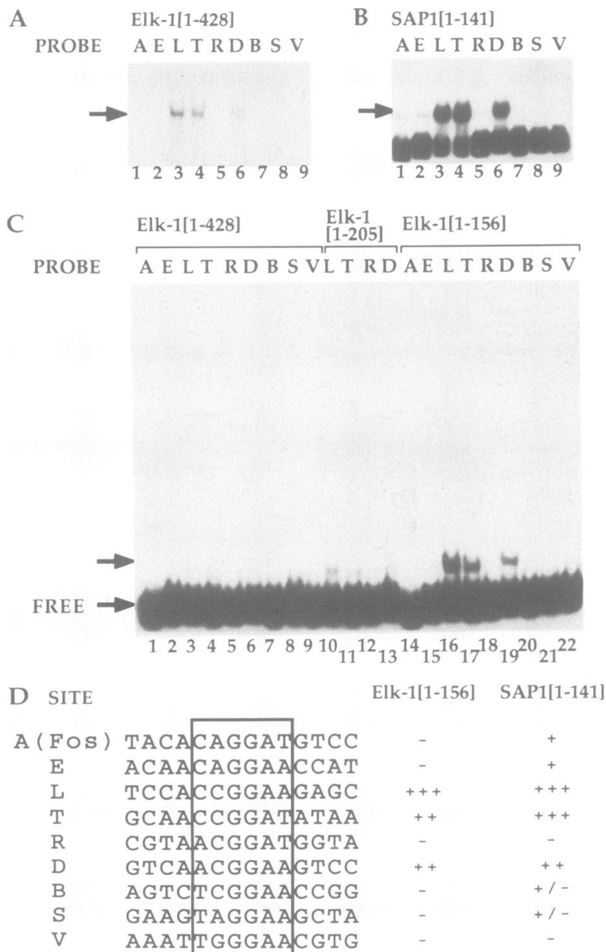

Fig. 5. Autonomous DNA binding by Elk-1 and SAP-1 derivatives. A set of probes containing different Ets motifs was tested for binding Elk-1 and SAP-1 derivatives. **(A)** Elk-1[1−428] exhibits weak DNA binding activity. Extracts of SF9 cells infected with recombinant baculovirus expressing Elk-1[1−428] (2500 cell equivalents; compare Figure 3A) were tested for binding to the c-*fos* SRE (probe A, lane 1) or selected oligonucleotide probes (lanes 2−9). Free probes were run off the gel; the arrow indicates the position of the protein−DNA complex. **(B)** Binding specificity analysis of SAP-1[1−141]. Binding reactions contain 1 μl rabbit reticulocyte lysate programmed with SAP-1[1−141], together with the c-*fos* SRE (probe A, lane 1) or selected oligonucleotide probes (lanes 2−9). Free probes run at the base of the panel and the protein−DNA complex is arrowed. **(C)** Elk-1 C-terminal sequences inhibit autonomous binding. Probes from the panel in part D were tested for binding with equal amounts of either Elk-1[1−428] (lanes 1−9), Elk-1[1−205] (lanes 10−13), or Elk-1[1−156] (lanes 14−22), as judged by SDS−PAGE. The Elk-1[1−156] complex is indicated by the arrow. **(D)** Summary of data. The context of each Ets motif is shown together with a summary of the binding data obtained with Elk-1[1−156] and SAP-1[1−141]. The probe designations are as in Figure 3, except for the c-*fos* probe, designated A.

arises from autonomous binding of Elk-1[1−156] (data not shown; see below). The small amount of ternary complex formation by the Elk-1[1−156] derivative mirrors the degree of independent DNA binding with each protein (Figure 4B, compare lanes 2−8). We conclude that cooperative interaction of Elk-1 with SRF requires box B sequences, irrespective the relative position and orientation of the DNA sequences contacted by the DNA binding domains.

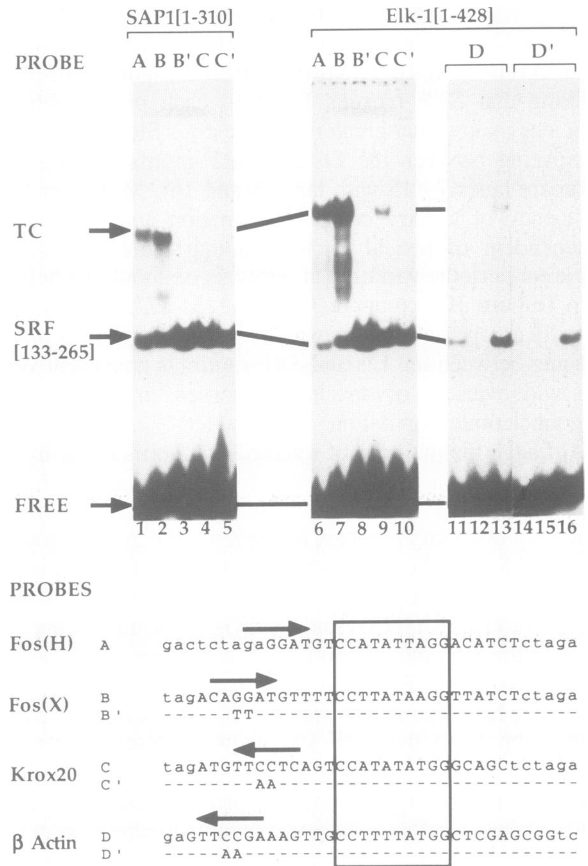

Fig. 6. Recruitment of Elk-1 and SAP-1 to natural variant SREs. SAP-1[1−310] (lanes 1−5) or Elk-1[1−428] (lanes 6−16) produced by *in vitro* translation, were tested for ternary complex formation with SRF[133−265]. The probes used are shown below the figure with the SRF motif boxed and the Ets motifs shown as arrows; the double point mutation probes are shown as dashed lines with the mutations indicated. The probes contained the human c-*fos* SRE (lanes 1 and 6), wild-type *Xenopus* SRE (lanes 2 and 7), mutant *Xenopus* SRE (lanes 3 and 8), wild-type krox20 upstream SRE (lanes 4 and 9), mutant krox20 upstream SRE (lanes 5 and 10), wild-type β actin TATA proximal SRE (lanes 11−13) and mutant β actin TATA proximal SRE (lanes 14−16). Lanes 1−5, 6−10, 13 and 16, ternary complexes with SRF[133−265]; lanes 11 and 14, SRF[133−265] alone; lanes 12 and 15, Elk-1[1−428] alone. The positions of ternary complexes (TC) and SRF[133−265] complexes are indicated. Binding of SAP-1[1−310] to the krox20 SRE is clearly visible on the original autoradiogram.

Autonomous DNA binding by Elk-1 and SAP-1

To investigate autonomous DNA binding by the Elk-1 and SAP-1 proteins in more detail, we performed DNA binding studies with probes containing different Ets motifs. We showed in Figure 3 that each of these probes, with the exception of probe V, directs efficient ternary complex formation by Elk-1[1−428] and SRF, at Elk-1 concentrations at which no autonomous binding is detectable. However, when a larger amount of Elk-1[1−428] is used in the reactions, weak autonomous binding was detectable, but only to probes containing the Ets motifs CCGGAA, CCGGAT or ACGGAA (Figure 5A, lanes 1−9).

We next used Elk-1 deletion derivatives produced by *in vitro* translation to test whether the Elk-1 C-terminal sequences inhibit the autonomous DNA binding activity of the Elk-1 Ets domain, as previously reported for SAP-1 (Dalton and Treisman, 1992). Equal amounts of

Elk-1[1-428], Elk-1[1-205] and Elk-1[1-156] as judged by SDS-PAGE were tested for DNA binding. Neither Elk-1[1-428] nor Elk-1[1-205] efficiently bound any of the probes tested, although weak binding was detectable on long exposure of the gels (Figure 5C, lanes 1-13). In contrast Elk-1[1-156], which comprises only the Ets domain linked to a truncated B box, efficiently bound probes containing the CCGGAA, CCGGAT and ACGGAA motifs (Figure 5C, lanes 14-22). Similar results were obtained with SAP-1[1-141], a derivative of the SAP-1 analogous to Elk-1[1-156] (Figure 5B). This protein efficiently bound probes containing the CCGGAA, CCGGAT and ACGGAT motifs, albeit with different relative affinity from Elk-1[1-156]; however, it also weakly bound probes containing other Ets motifs, such as the CAGGAT sequence in the *c-fos* SRE (Figure 5B lanes 1-9 with Figure 5C lanes 14-22).

We conclude that the isolated Ets domains of SAP-1 and Elk-1 have substantially similar but not identical sequence specificities, and that the C-terminal sequences of both proteins prevent their efficient autonomous DNA binding. Moreover, since ternary complex formation can occur at Ets motifs which do not interact efficiently with the isolated Elk-1 or SAP-1 Ets domains, it must to some extent compensate for suboptimal interactions between the Ets domain and DNA.

Ternary complex formation at natural SREs

The results presented above demonstrate that cooperation in ternary complex formation by SRF and its accessory proteins is not crucially dependent on the relative positions of the DNA sequences contacted by the two proteins. We therefore examined binding of Elk-1[1-428] and SAP-1[1-310] to three naturally occurring SREs which contain Ets motifs located at different positions from that in the human *c-fos* SRE. The probes chosen were the *Xenopus c-fos* SRE, in which the Ets motif is 3 bp 5′ to its position in the human SRE; and two SREs containing inverted Ets motifs, the krox20 proximal (Chavrier *et al.*, 1989) and β actin TATA proximal (Kawamoto *et al.*, 1989) SREs. As controls, probes containing GG to TT transversions in the Ets motif, which should abolish interaction with the Ets domain, were also tested. The results are shown in Figure 6. As expected from the DEPC interference analysis described above, in the presence of SRF[133-265] both SAP-1 and Elk-1 efficiently bound the wild-type but not the mutant *Xenopus c-fos* SRE (Figure 6, compare lanes 1-3 and 6-8). Both proteins also formed ternary complexes with the krox20 and β actin SREs, but less efficiently than with the *Xenopus c-fos* SRE; however, in both cases ternary complex formation was abolished by mutation of the Ets motif (Figure 6, compare lanes 4 and 5, and 9-16; data not shown). Taken together, the data show that in each case it is the variant Ets motif, rather than the sequences adjacent to the SRF binding site, that specifies ternary complex formation at these SREs *in vitro*.

Discussion

In this paper we have shown that the SRF accessory proteins Elk-1 and SAP-1 exhibit striking flexibility in their DNA binding properties with SRF. Ternary complex formation is specified by an SRF binding site together with the Ets motif consensus (C/A)(C/A)GGA(A/T), and can occur cooperatively even when the sequences contacted by the two proteins are inverted with respect to each other, or moved apart over two helical turns of DNA.

Our findings may provide a partial explanation for the puzzling observation that although p62/TCF binding appears important for the function of the human *c-fos* SRE, its binding site is not conserved in other SREs: of the many SRE sequences characterized to date, only the zif268/egr1 SRE2 (bp-96; Tsai *et al.*, 1988) contains an Ets motif at the same position. However, several other SREs do contain Ets motifs, but located at different positions relative to the SRF binding site. These SREs include both krox20 SREs (Chavrier *et al.*, 1989), the chicken and *Xenopus c-fos* SREs (Fujiwara *et al.*, 1987; Mohun *et al.*, 1989), the distal zif268/egr1 SREs (Tsai *et al.*, 1988) and the TATA proximal β actin SRE (Kawamoto *et al.*, 1989). Our *in vitro* DNA binding experiments indicate that ternary complex formation at SREs of this type is indeed directed by the Ets motifs, albeit with variable efficiency. Moreover, the evolutionary conservation, but variable position, of the Ets motif in the *c-fos* SRE suggests that at least in this case a variant motif will specify ternary complex formation *in vivo*. In addition, our preliminary data suggest that at least in transfection experiments, variant Ets motifs can direct binding of Elk-1-VP16 or SAP-1-VP16 fusion proteins to such SREs (J.Wynne and R.Treisman unpublished data; see Dalton and Treisman, 1992). Further experiments, including mutagenesis of the corresponding promoters, are required to establish conclusively the role of ternary complex formation in function of such variant SREs.

A number of SREs do not appear associated with Ets motifs, including those present in the promoters of the *Xenopus* type 5 actin (Mohun *et al.*, 1987), cyr61 (Latinkic *et al.*, 1991) and SRF (R.Pollock, T.Mohun and R.Treisman unpublished data) genes. Since the intact *c-fos* Ets motif has been associated with specific ability to respond to PKC-mediated signals in certain cells (Graham and Gilman, 1991), these SREs may respond to a slightly different spectrum of signals than do SREs which contain Ets motifs. These SREs may recruit Elk-1 and SAP-1 to more distant Ets motifs, or to variant Ets motifs lacking an intact GGA(A/T) core element (see below); alternatively, different SRF accessory proteins might be involved.

One potential consequence of the extreme flexibility of spacing observed in our experiments is that when SRF binding site oligonucleotides are tested using reporter plasmids, cryptic SAP-1 or Elk-1 sites may be adventitiously placed in their vicinity. For example, we have found that SRF binding sites inserted into the polylinker of the reporter plasmid pBLCAT2 (Luckow and Schutz, 1987) can recruit Elk-1 or SAP-1 to an Ets motif at the neighbouring *Bam*HI site (R.Treisman, unpublished observations). It is thus possible that accessory proteins also contribute to signalling effects previously attributed to an isolated SRF site.

Our experiments show that the efficiency of ternary complex formation is a function of the affinity of the SRF binding site and the sequences of the Ets motifs, rather than their relative spacing or orientation. The consensus Ets motif found in ternary complex DNA, (C/A)(C/A)GGA(A/T), is very similar to that recently determined for autonomous

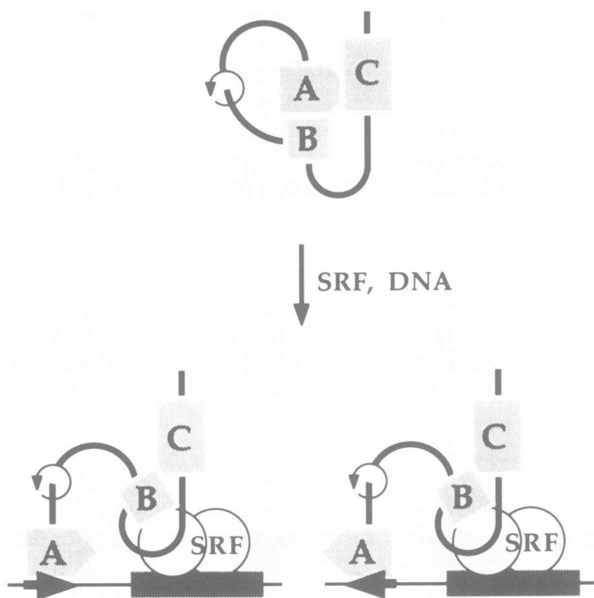

Fig. 7. 'Grappling hook' model for Elk-1 and SAP-1 interactions with SRF. In solution the Ets domain is obscured by Box B and possibly C-terminal sequences resulting in low autonomous DNA binding activity. Ternary complex formation does not involve direct interaction between SRF and the Ets domain, and is mediated by Box B (and possibly other C-terminal sequences). The polypeptide between the Ets domain and Box B is flexible and allows the BoxB region to interact efficiently with SRF to a large extent regardless of the location and orientation of the Ets motif with respect to the SRF binding site.

DNA binding by the c-Ets-1 protein (Fisher *et al.*, 1991; Woods *et al.*, 1992). In contrast, autonomous binding of the isolated Elk-1 and SAP-1 Ets domains indicates a strict preference for the sequences CCGGAA, CCGGAT and ACGGAA. Consistent with a previous report (Rao and Reddy, 1992) we found that the intact Elk-1 protein also binds these sequences, but with a lower affinity probably arising from inhibition by the Elk-1 C-terminal sequences. Ternary complex formation must to some extent compensate for suboptimal DNA interactions by the Ets domain, since Ets motifs that exhibit little if any binding to the isolated Ets domain can efficiently direct Elk-1 (and SAP-1) recruitment. Ternary complex interactions can also compensate for the effects of mutations in the invariant GGA core of the Ets motif, since these still allow inefficient recruitment of Elk-1 or SAP-1 (unpublished data). It will be interesting to test whether, conversely, Elk-1–SAP-1 binding can compensate for suboptimal SRF binding sites.

Almost half the recovered oligonucleotides contain Ets and SRF motifs spaced within 5 bp of each other, suggesting that the selection process results in a clear bias towards close spacing of the DNA contact sites. However, we observed little difference in efficiency of ternary complex formation at closely versus distantly spaced sites. Although this bias may reflect a slightly higher average affinity of more closely spaced sites, it also could arise from selection for compatible sequences between the two sites. For example, selection for DNA deformation or bending may be a contributory factor. However, we cannot discern any sequence constraint on the DNA between the Ets and SRF binding motifs. Further experiments are necessary to address the question of whether ternary complex formation results in DNA deformation in the ternary complex.

The variations in spacing of the DNA contact sites in the Elk-1–SAP-1 ternary complex with SRF is extreme, but not without precedent. In prokaryotic systems, single basepair deletions between the halfsites of the Lac operator actually increase affinity for repressor (Sadler *et al.*, 1983; Simons *et al.*, 1984), while $\gamma\delta$ resolvase halfsites can be spaced over a whole helical turn (for review see Hatfull and Grindley, 1988). In eukaryotes, heterodimers among some members of the nuclear receptor family can recognize halfsites with various orientations and spacings over a helical half-turn (Naar *et al.*, 1991; Umesono *et al.*, 1991; Leid *et al.*, 1992), while the structure of the GABPα/β heterotetramer may allow flexibility in binding site recognition (Thompson *et al.*, 1991). An extreme example of flexibility in DNA binding is found in the yeast MATα2 protein, which forms ternary complexes with the yeast SRF-related protein MCM1 at **a**-specific promoters (for review see Dolan and Fields, 1991). By itself, MATα2 can bind to halfsites separated by up to two helical turns, which can be individually or simultaneously inverted without substantial effects on DNA binding affinity (Smith and Johnson, 1992). In contrast to our findings with Elk-1/SAP-1, MATα2 is restricted to recognition of halfsites with fixed orientation and spacing in its ternary complex with MCM1 (Smith and Johnson, 1992). However, the ternary complex properties of another MCM1 accessory protein, STE12, may be more similar to those of the SRF accessory proteins. This protein does not bind single copies of its recognition sequence effectively, but can do so if MCM1 is bound nearby (Errede and Ammerer, 1989). Although at the STE2 UAS the sequences contacted by the two proteins are separated by 11 bp, in other promoters functionally cooperative MCM1 and STE12 binding sites are spaced differently (Kronstad *et al.*, 1987). Perhaps the STE12 protein exhibits a similar flexibility in ternary complex formation with MCM1 to that observed between SRF and Elk-1/SAP-1.

Taken together with our earlier findings concerning the ternary complex formation properties of SAP-1, our findings suggest a 'grappling hook' model for protein–protein interactions in the ternary complex, as shown in Figure 7. In the absence of SRF, autonomous DNA binding by Elk-1 and SAP-1 is inhibited by their C-terminal sequences: at least part of this inhibition appears to be mediated by homology B, since when this is truncated or bound by anti-Box B antibody, autonomous DNA binding activity is greatly increased (A.Rogers and R.Treisman, unpublished data). A similar masking model has been proposed for c-Ets-1, the prototypic Ets domain protein (Lim *et al.*, 1992). In the ternary complex, essential interactions with SRF are mediated by Box B (and possibly C-terminal sequences), while the Ets domain interacts with the DNA and makes no essential contacts with SRF. According to this model, the sequences between the Elk-1/SAP-1 Ets domains and Box B act as a flexible tether, allowing the location and orientation of the Ets motif to vary with respect to the SRF binding site. Consistent with this idea, the length of this tether sequence, which is rich in proline and glycine, differs between Elk-1 and SAP-1; moreover sequences can be added or deleted in the SAP-1 tether region without affecting ternary complex formation (Dalton and Treisman, 1992). We have observed no differences in ternary complex formation by Elk-1 and SAP-1, but presume that the length of the tether region and

bendability of the intervening DNA will ultimately limit the spacing of sequences contacted in the ternary complex. A prediction of the grappling hook model is that since the Ets domain plays no role in recruitment of the protein into the ternary complex, it should be possible to replace it with a heterologous DNA binding surface: the resulting chimeric protein would then form ternary complexes with SRF, provided the appropriate DNA sequences are provided next to the SRF site. If this is the case, such fusion proteins would greatly aid the study of ternary complex function *in vivo*.

Materials and methods

Plasmids and proteins

Recombinant SRF[1−508] and SRF[133−265] were produced using recombinant baculovirus and purified as previously described (Marais *et al.*, 1992; J.Wynne, R.Marais and R.Treisman, unpublished data). Elk-1[1−428] was produced using recombinant baculovirus and used as a total cell lysate prepared as previously described (Marais *et al.*, 1992). Elk-1 and SAP-1 derivatives were also produced by *in vitro* translation of appropriate cRNAs in reticulocyte lysates. pT7Elk[1−428] contains Elk-1 sequences from codon 1 to a *Bam*HI site 3′ to the translational termination codon inserted between the *Nco*I and *Bam*HI sites of pT7βplink (Dalton and Treisman, 1992). For production of Elk-1[1−428], the plasmid was linearized with *Bam*HI. For production of Elk-1[1−205] and Elk-1[1−156], the plasmid was cut with *Stu*I and *Ava*I respectively.

DNA binding assays

DNA binding assays were in 20 μl reactions containing 12 μl buffer D (20 mM HEPES, pH 7.9, 100 mM KCl, 0.2 mM EDTA, 0.2 mM EGTA, 20% glycerol, 1 mg/ml ovalbumin, 0.1% NP40 and protease inhibitors), 2 μg poly(dIdC)(dIdC), 3 mM spermidine, 1 mM EDTA. Reactions contained either 0.2 ng SRF[1−508] or 2.5 ng SRF[133−265], together with either 1−2 μl reticulocyte lysate programmed with Elk-1 or SAP-1 derivatives, or 50 cell equivalents of whole extract of infected insect cells. Complexes were resolved on 4% 40:1 polyacrylamide gels in 1×TBE. DEPC interference analysis was performed as described (Sturm *et al.*, 1987; Dalton and Treisman, 1992).

Probes for DNA binding studies were generated by PCR, either from oligonucleotide subclones using T7 and KS primers, or from pBLCAT2 derivatives using M13 forward and pBLCAT2R (AAGGCCGGATC-CTCTAG) primers, as described (Pollock and Treisman, 1990). For analysis of naturally occurring SREs the following sequences (Ets and SRF motifs underlined) were inserted at the *Xba*I site of pBLCAT2*, a derivative of pBLCAT2 (Luckow and Schutz, 1987) in which the *Bam*HI site has been destroyed by cleavage with *Bam*HI and treatment with mung bean nuclease to remove the GATC cohesive ends:

SRE AGGATGTCCATATTAGGACATCT
SREx CAGGATGTTTTCCTTATAAGGTTATCT
SREx′ CATTATGTTTTCCTTATAAGGTTATCT
SREk TGTTCCTCAGTCCATATATGGCAGCT
SREk′ TGTTAATCAGTCCATATATGGCAGCT
SREa AGTTCCGAAAGTTGCCTTTTATGGCTCGAGT
SREa′ AGTTAAGAAAGTGCCTTTTATGGCTCGAGT

Binding site selection

Site selections were performed using a variation of established methods (Blackwell and Weintraub, 1990; Pollock and Treisman, 1990) using the random oligonucleotide CAGGTCAGTTCAGCGTCTAGAGTCCTTAT-ATGG-(N)32-GAGGCGAATTCAGTG, rendered double stranded by primed synthesis with primer 1, GCTGCAGTTGCACTGAATTCGCCTC. Radiolabelled oligonucleotide was incubated in a standard gel mobility-shift binding reaction together with 0.5 ng SRF[133−265] and 1 μl reticulocyte lysate containing full length Elk-1 protein. Protein−DNA complexes were resolved on a standard mobility-shift gel, the associated DNA was recovered and amplified by the PCR using primer 1 and primer F (Pollock and Treisman, 1990), and used for further rounds of selection. For sequence analysis, selected oligonucleotides were subcloned in pBS KS+ and sequenced by standard techniques (Bankier *et al.*, 1987; Vieira and Messing, 1987).

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Note added in proof

After submission of this manuscript, Janknecht and Nordheim [*Nucleic Acids Res.*, **20**, 3127–3324 (1992)] also reported the requirement for the Elk-1 Box B region for ternary complex formation at the c-*fos* SRE.