Interaction of Transcription Factors with Serum Response Factor

IDENTIFICATION OF THE Elk-1 BINDING SURFACE*

Yan Ling‡, Adam G. West, E. Claire Roberts, Jeremy H. Lakey, and Andrew D. Sharrocks§
From the Department of Biochemistry and Genetics, The Medical School, University of Newcastle upon Tyne, Newcastle upon Tyne, NE2 4HH, United Kingdom

Serum response elements (SREs) play important roles in transforming extracellular signals into specific nuclear responses. The SRE-binding protein, serum response factor (SRF), plays a pivotal role in this process. Several transcription factors have been shown to interact with SRF and thereby create distinct complexes with different regulatory potentials. The ETS domain transcription factor Elk-1 is one such protein and serves to integrate distinct mitogen-activated protein kinase cascades at SREs. Elk-1 uses a short hydrophobic surface presented on the surface of an α-helix to interact with SRF. In this study we have used site-directed mutagenesis to identify residues in SRF that comprise the Elk-1 binding surface. The Elk-1 binding surface is composed of residues that lie on a hydrophobic surface-exposed groove located at the junction of the MADS box and C-terminal SAM motif. Different residues are implicated in interactions between SRF and the transcription factor Fli-1, indicating that although some overlap with the Elk-1 binding surface occurs, their interaction surfaces on SRF are distinct. Our data are consistent with the hypothesis that the SRF DNA-binding domain acts as docking site for multiple transcription factors that can bind to small surface-exposed patches within this domain.

The serum response element (SRE) within the c-fos promoter has been studied extensively and has developed into a paradigm for how immediate-early genes are regulated. The SRE is bound by the serum response factor (SRF) both in vitro and in vivo (reviewed in Refs. 1–3) and thus plays a pivotal role in regulating transcriptional activation of the c-fos promoter via this element. Multiple diverse extracellular signals are transduced through the SRE including both mitogenic stimuli (e.g. epidermal growth factor) and stress stimuli (e.g. UV light) (reviewed in Ref. 3). At least two alternative pathways exist to transduce these signals via SRF (4, 5). One pathway goes through a ternary complex composed of the SRE, SRF, and a member of the ternary complex factor (TCF) subfamily of ETS domain transcription factors (reviewed in Refs. 2 and 3). This pathway is responsive to the majority of mitogenic and stress stimuli. The second pathway acts via a TCF-independent mechanism and is responsive to alternative serum constituents such as lysophosphatidic acid and upstream activators such as the Rho family of GTPases (6, 7).

The TCF-dependent pathways have been extensively studied. In this complex, the TCF component is the recipient of signals transduced through the mitogen-activated protein kinase cascades (Refs. 8–13 and reviewed in Refs. 14 and 15). The TCFs use both protein-DNA (by their ETS domain) and protein-protein interactions to bind to the binary SRF-SRE complex (reviewed in Ref. 2) as predicted by the “grappling hook” model (1). A short region of the TCF Elk-1 (the B box) has been demonstrated to be sufficient for interaction with SRF (16). Upon binding to SRF, this region is thought to adopt an inducible α-helical structure in which hydrophobic amino acids play a major role (17). The minimum region of SRF required for this interaction with the TCF’s in vitro maps to its minimal core DNA-binding domain (coreSRF) (18). Experiments using chimeric transcription factors have localized the TCF binding surface to the C-terminal half of this domain (18, 19). Moreover, the core domain is sufficient for correct dimerization, sequence-specific DNA binding, and protein-induced DNA bending by SRF (reviewed in Refs. 2 and 20). Recently the structure of this domain has been solved (21) and shown to adopt a novel fold in which the N-terminal portion (MADS box) makes all the DNA contacts. Dimerization is mediated by elements within the MADS box and additional contributions from the C-terminal extension (the SAM motif).

In addition to binding TCFs, the core DNA-binding domain of SRF is sufficient to form DNA complexes with or bind directly to other transcription factors including myogenic basic helix-loop-helix proteins (22), the ETS-domain protein Fli-1 (23, 24) and p65/NF-κB (25). Moreover, a higher order complex involving SRF, Phox, and TFII-I/SP2 and the c-fos SRE has also been identified (26). These diverse transcription factors show little homology with the Elk-1 B box, implying that they use alternative interaction motifs and different surfaces on SRF.

The aim of this study was to identify the TCF interaction surface on SRF. The recent elucidation of the structure of the minimal DNA-binding domain of SRF (21) permits both improved experimental design and interpretation of the biochemical data. By using an extensive mutagenic approach, we have identified a surface-exposed patch on SRF which is involved in interactions with Elk-1. The transcription factor Fli-1 interacts with a different surface on SRF which overlaps the Elk-1 binding region. Taken together our data are consistent with a model in which the SRF DNA-binding domain acts as a docking site with interaction surfaces for multiple transcription factors involved in diverse cellular processes.
MATERIALS AND METHODS

Plasmid Constructs—The following plasmids were constructed for making proteins by \textit{in vitro} transcription/translation. pAS197 encodes the ETS DNA-binding domain and B box of Elk-1 (Elk1–168; amino acids 1–168) (17). pAS305 encodes the N-terminally truncated zebrafish Fli-1 protein, Fli-219–451 (Fli-1 amino acids 219–451). pT3G-SRF encodes the DNA-binding domain of SRF (coreSRF; amino acids 132–222) (18). pAS30 (Y195D), pAS31 (F197D) (28), pAS432 (V187A), pAS626 (S189A), pAS627 (E190A), pAS640 (H193A), pAS641 (T191A/H193A), pAS431 (V194A), pAS430 (T196A), pAS625 (V194A/T196A), pAS493 (T199A), pAS629 (R200A), pAS642 (T196A/R200A), pAS646 (Q203A/T207A), pAS429 (K201A), pAS643 (V194A/K201A), pAS494 (L202A), pAS630 (Q203A), pAS428 (P204A), pAS447 (I206A), pAS459 (T210A), pAS644 (T196A/T207A), pAS635 (V194E), pAS451 (T196E), pAS492 (T199E), pAS629 (A198D), pAS632 (Q203E), pAS637 (Q203E/Q207E), pAS636 (I206D), pAS631 (T207D), and pAS634 (T210D), and pAS633 (Q216E) encode mutant derivatives of coreSRF from pT3G-SRF with the indicated site-directed mutations. pAS433, pAS434, pAS435, pAS439, and pAS52 encode point mutant derivatives of METcoreSRF from pAS37 (29) with site-directed mutations L155A, L155E, Y158A, T166A, and K154E/V144K, respectively. Mutations were introduced by a two-step PCR protocol using a mutagenic primer and two flanking primers as described previously (30). pAS489 encodes Elk-1–168 with the mutation L158P and was also constructed using this two-step PCR protocol. Details of mutagenic primers can be supplied upon request.

The following plasmids were used for protein expression in \textit{Escherichia coli}. pAS76 contains the c-fos SRE cloned into the circular permutation vector pBEND2 (20).

**TABLE I**

| Mutant SRF derivative | 2° complex with SRE | 3° complex with Elk-1 & CEC1 site | GST-SRF pull-down of Elk-1 | aGST-B-box pull-down of SRF | 3° complex with Fli-1 & c-fos SRE |
|------------------------|---------------------|-----------------------------------|---------------------------|----------------------------|-------------------------------|
| L155A                  | ND                  | ND                                | ND                        | ND                         | ND                            |
| L155E                  | ND                  | ND                                | ND                        | ND                         | ND                            |
| Y158A                  | ND                  | ND                                | ND                        | ND                         | ND                            |
| T166A                  | ND                  | ND                                | ND                        | ND                         | ND                            |
| K154E/V144K            | ND                  | ND                                | ND                        | ND                         | ND                            |
| V187A                  | +++++               | +++++                             | ND                        | +++                       | ++++                         |
| S189A                  | +++++               | +++++                             | ND                        | +++                       | ++++                         |
| E196A                  | +++++               | ++                                | ++                       | +                        | +++                           |
| H193A                  | +++++               | +++++                             | ND                        | +++                       | +++                           |
| T191A/H193A            | +++++               | ++                                | ND                        | +++                       | +++                           |
| V194A                  | +++++               | +++++                             | ND                        | +++                       | +++                           |
| T196A                  | +++++               | +++++                             | ND                        | +++                       | +++                           |
| V194A/T196A            | ++                  | ND                                | ND                        | +                        | +                             |
| T199A                  | ++                  | ++                                | ND                        | +                        | +                             |
| R200A                  | +++++               | +++++                             | ND                        | +++                       | +++                           |
| T196A/R200A            | +++++               | +++++                             | ND                        | +++                       | +++                           |
| K201A                  | +++++               | +++++                             | ND                        | +++                       | +++                           |
| V194A/K201A            | ++                  | +                                | ND                        | +                        | +                             |
| L202A                  | +                  | ND                                | +                        | +                        | +                             |
| Q203A                  | +++++               | +++++                             | ND                        | +++                       | +++                           |
| P204A                  | +                  | ND                                | +                        | +                        | +                             |
| T207A                  | +++++               | +++++                             | ND                        | +++                       | +++                           |
| T196A/T207A            | +++++               | +++++                             | ND                        | +++                       | +++                           |
| Q203A/T207A            | +                  | ND                                | +                        | +                        | +                             |
| V194E                  | +++++               | +                                | ND                        | +                        | +                             |
| Y196D                  | +                  | ND                                | +                        | +                        | +                             |
| T196E                  | +                  | ND                                | +                        | +                        | +                             |
| T196K                  | +++++               | +                                | ND                        | +                        | +                             |
| F197D                  | -                   | ND                                | +                        | +                        | +                             |
| A198D                  | -                   | ND                                | +                        | +                        | +                             |
| T199D                  | +                   | ND                                | +                        | +                        | +                             |
| Q203E                  | +++++               | +                                | ND                        | +                        | +                             |
| D206D                  | +                  | ND                                | +                        | +                        | +                             |
| T207D                  | +                  | ND                                | +                        | +                        | +                             |
| T210D                  | +++++               | +                                | ND                        | +                        | +                             |
| Q216E                  | +                  | ND                                | +                        | +                        | +                             |

\* Note range of the assay is from 20 to 100% only. + + is taken as a significant decrease.

\^ These values represent \(5–10\)-fold increases over wild-type SRF.

\# Mutations were introduced into the N-terminally truncated METcoreSRF derivative.

**Protein Production—**\textit{In vitro} transcription and translation of SRF,
Elk-1, and Fli-1 derivatives were carried out with TNT®-coupled reticulocyte lysate system (Promega) according to the manufacturer’s recommendations. 35S-Labeled proteins were analyzed by electrophoresis through 0.1% SDS-12% polyacrylamide gel electrophoresis before visualization and quantification of bands representing intact proteins by phosphorimaging (Fuji BAS-1500 phosphorimager and TINA 2.08e software).

GST fusion proteins were purified from E. coli as described previously (16). The purity and concentration of protein samples were estimated by SDS-polyacrylamide gel electrophoresis.

**Gel Retardation and GST Pull-down Assays—**Pull-down assays for protein-protein interactions with GST-B box and GSTcoreSRF derivatives were carried out essentially as described previously (16).

Gel retardation assays were performed in 12-μl reaction volumes as described previously (28). Assays were carried out on the c-fos SRE (oligonucleotides ADS134 and ADS135; top strand, 5′-CTAGTTTACAGGAATGTCATTTAGGAACATCTGCGTCAGCAGG-3′) or the CECI site (oligonucleotides ADS341 and ADS342; top strand, 5′-CCGGTGAAGCCGGGAAATGTGATCAACTATTTATAGATA-3′) or the CArG box SRF-binding site (CArG box oligonucleotides ADS341 and ADS342; top strand, 5′-CCGGTGAAGCCGGGAAATGTGATCAACTATTTATAGATA-3′). The CArG box SRF-binding sites are underlined, and the Elk-1-binding sites (ets motifs) are shown in bold. The CECI site (combined ets and CArG) consists of the SRE ets motif identified by site selection (31) and the N10 site (29). Elk-1 binds to the ets motif in this site with higher affinity than to the c-fos SRE (31). Protein-DNA complexes were resolved by electrophoresis through nondenaturing 5% polyacrylamide (30% acrylamide, 0.8% bisacrylamide) gels cast in either 0.5×Tris/borate/EDTA (TBE) or 1×TBE for circular permutation assays. Relative DNA binding affinities were calculated by phosphorimager analysis of protein-DNA complexes (Fuji BAS1500; TINA 2.08e software). Experiments were carried out to achieve ≥50% of total DNA binding in protein-DNA complexes. Under these conditions, relative binding affinities within an experiment can be calculated by direct quantification of DNA-protein complexes. The scoring of these relative binding affinities is indicated in the legend to Table I.

**Circular Permutation Analysis—**For circular permutation analysis, DNA fragments were produced by appropriate restriction enzyme digestion of PCR products derived from pAS57e (containing the SRE) and purified as described previously (20, 32). Curve fitting and apparent DNA bend angles were calculated as described previously (32). Bend angles are quoted as the average of three independent experiments. Standard deviations (n = 1) of bend angles are in the range 0.5–1.6°. To show direct visual comparisons of data obtained from proteins that give rise to complexes of differing mobilities, the data were normalized for the fastest mobility complex (μ) (32).

All figures were generated electronically from scanned images of autoradiographic images using Picture Publisher (Micrografx) and Powerpoint (Microsoft) software. Final images are representative of the original autoradiographic images.

**RESULTS**

**Experimental Rationale—**A mutagenic approach was used to identify residues in SRF that are involved in binding Elk-1. Amino acids were either changed to alanines (removing potential interacting side chains) or to charged residues. The latter approach relies on introducing amino acids that will disrupt protein-protein contacts and therefore the residues need not be in direct contact with Elk-1 but might instead be in very close proximity to residues comprising the binding surface. Both these approaches have been used successfully in independent

![Fig. 1. DNA binding by SRF mutant proteins.](image1)

Gel retardation analysis of binary complex formation between wild-type and mutant coreSRF proteins and the c-fos SRE. Equal molar quantities of each mutant protein (indicated above each lane) were used in the binding reactions. The locations of the free SRE and binary (2°) SRF-SRE complexes are indicated.

![Fig. 2. Ternary complex formation by SRF mutant proteins and the c-fos SRE.](image2)

A, diagrammatic representation of the ternary SRF:Elk-1-SRE complex. The c-fos SRE consists of a strong SRF-binding site (CArG box) and a weak Elk-1-binding site (ets motif). Elk-1 (open circles) is recruited to this site by protein-protein interaction between the B box and SRF (shaded circles) thereby supplementing interactions between the Elk-1 ets domain and the ets motif. B, gel retardation analysis of ternary complex formation between wild-type and mutant coreSRF proteins, Elk-1–168 and the c-fos SRE. The amount of each mutant protein used (indicated above each lane) was adjusted to give equal amounts of binary SRF-SRE complex. Equal molar quantities of Elk-1–168 were added to each reaction. The locations of the free SRE, binary (2°) SRF-SRE and ternary (3°) SRF:Elk-1-SRE complexes are indicated.
Elk-1 proteins are sufficient for complex formation in both the pres-
tence of DNA.

A combination of assays were therefore used to assess

the function of mutant proteins in both the presence and ab-

sence of DNA.

studies with virtually identical results to map surface residues

on TATA binding protein which bind to components of the

transcription preinitiation complex (33, 34). The majority of

the mutations introduced were at single amino acid residues. In-
dividual residues in the core DNA-binding domain of SRF could
have multiple roles in addition to protein-protein interactions
including DNA binding, dimerization, or protein-induced DNA
bending. A combination of assays were therefore used to assess

the function of mutant proteins in both the presence and absence of DNA.

Mutations were introduced into the minimal DNA-binding
domain of SRF (coreSRF) and tested for binding to the truncated
Elk-1 protein (Elk-1–168) which contains the ETS DNA-bind-
ing domain and the B box SRF-binding motif. These truncated
proteins are sufficient for complex formation in both the pres-
ence and absence of DNA (16, 17). The results of these assays
are presented below and summarized in Table I.

DNA Binding by SRF Mutants—Initially the mutant SRF
proteins were tested for their ability to bind to the CArG (CC(A+
T) rich) box in the c-fos SRE. The majority of the mutant
proteins bound the SRE with similar efficiency to the wild-type
protein (Fig. 1; Table I). However, the mutants V194A/T196A,
T199A, L202A, and T199D exhibited a reduced affinity for the
SRE (Fig. 1, lanes 8, 9, 14, and 27). Binding of the mutants
P204A, F197D, A198D, and I206D was severely reduced and
not detectable by this assay (Fig. 1, lanes 16, 25, 26, and 29).

To detect further mutations that affect ternary complex for-
mation, we used a second assay system in which a high affinity
ets motif is juxtaposed next to a weak CArG box in the CECI
(combined ets and CArG) site (Fig. 3A). This arrangement
of sites is the opposite that found in the c-fos SRE and allows
recruitment of SRF by Elk-1.3 Under these conditions, protein-
protein interactions are essential for the recruitment of SRF by
Elk-1 into a ternary complex. In contrast to the results on the
c-fos SRE, many of the mutant proteins exhibited a reduced
affinity for the ternary complex formation on the CECI site (Fig.
3B; Table I). The mutant proteins E190A, T191A/H193A,
V194A/T196A, T199A, Y195D, Q203E, T207D, and Q216E
showed significant reductions in complex forming efficiency
(<50% WT binding) (Fig. 3B, lanes 5, 6, 8, 9, 22, 28, 30, and 32;
Table I), whereas binding by the mutant proteins V194A/
K201A, V194E, T196E, and T196K (Fig. 3B, lanes 13, 21, 23,
and 24 and Table I) was barely detectable (<5% WT binding,
not visible in Fig. 3B). No complex formation was observed by
the mutants L202A, P204A, F197D, A198D, T199D, and I206D
(Fig. 3B, lanes 14, 16, 25–27, and 29 and Table I). Moderate
reductions (60% WT binding) were also observed for the mu-
ant proteins H193A, V194A, and I206A (Fig. 3B, lanes 4, 6 and
17 and Table I).

The lack of complex formation by the mutants P204A,
F197D, A198D, and I206D is probably attributable to disrup-
tions in their DNA binding capabilities (see Fig. 1; Table I).
Similarly, the mutant proteins V194A/K201A, L202A, and
T199D exhibit significantly reduced DNA binding activity (Fig.
1; Table I). In contrast, DNA binding by the mutant proteins
V194E, T196E, and T196K is unimpaired, suggesting that the
effect of these mutations is specifically to disrupt SRF-Elk-1
interactions. Likewise, the reductions in complex formation by
the mutants E190A, H193A, T191A/H193A, V194A, V194A/
T196A, T199A, I206A, Y195D, Q203E, and T207D are also likely
to be attributable to losses in protein-protein interactions
with Elk-1.

Taken together these assays suggest key roles for amino
acids Val-194 and Thr-196 in complex formation with Elk-1.
Furthermore, several additional residues, Glu-190, His-193,
Thr-199, Tyr-195, Gin-203, Ile-206, and Thr-207, are identified
as part of or close to the Elk-1 binding surface of SRF.

Fig. 3. Ternary complex formation by SRF mutant proteins at
the CECI site. A, diagrammatic representation of the ternary
SRF-Elk-1-CECI complex. The CECI site consists of a weak
SRF-binding site (CArG box) and a strong Elk-1-binding site (ets motif). Elk-1
(open circles) recruits SRF to this site by protein-protein interactions
between the B box and SRF (shaded circles). B, representative data
from gel retardation analysis of ternary complex formation between
wild-type and mutant coreSRF proteins and the CECI site. Equal molar
quantities of Elk-1–168 and each mutant protein (indicated above each lane) were added to each reaction. The locations of the free CECI site,
binary (2°) Elk-1-CECI and ternary Elk-1-SRF-CECI complexes are indicated.

10509

SRF Protein-Protein Interaction Surfaces

DNA Binding by SRF Mutants—Initially the mutant SRF
proteins were tested for their ability to bind to the CArG (CC(A+
T) rich) box in the c-fos SRE. The majority of the mutant
proteins bound the SRE with similar efficiency to the wild-type
protein (Fig. 1; Table I). However, the mutants V194A/T196A,
T199A, L202A, and T199D exhibited a reduced affinity for the
SRE (Fig. 1, lanes 8, 9, 14, and 27). Binding of the mutants
P204A, F197D, A198D, and I206D was severely reduced and
not detectable by this assay (Fig. 1, lanes 16, 25, 26, and 29).

Ternary Complex Formation with Elk-1 and CArG Boxes—
The ability of the mutant SRF proteins to form ternary DNA-
bound complexes with Elk-1 and the c-fos SRE was tested by
gel retardation analysis. Protein-protein interactions with SRF
are essential for efficient recruitment of Elk-1 into ternary
complexes on this site (16). Assays were carried out using
concentrations of SRF that give equal amounts of binary
SRF-SRE complex. The efficiency of ternary complex formation
was virtually identical for all the mutant SRF proteins tested
(Fig. 2) with the exception of the mutants V194E, T196E, and
T196K which exhibited a reduced efficiency of complex forma-
tion (Fig. 2B, lanes 19, 21 and 22). In the case of V194E no
ternary complex was detectable.

To detect further mutations that affect ternary complex for-
mation, we used a second assay system in which a high affinity
ets motif is juxtaposed next to a weak CArG box in the CECI
(combined ets and CArG) site (Fig. 3A). This arrangement
of sites is the opposite that found in the c-fos SRE and allows
recruitment of SRF by Elk-1.3 Under these conditions, protein-
protein interactions are essential for the recruitment of SRF by
Elk-1 into a ternary complex. In contrast to the results on the
c-fos SRE, many of the mutant proteins exhibited a reduced
affinity for the ternary complex formation on the CECI site (Fig.
3B; Table I). The mutant proteins E190A, T191A/H193A,
V194A/T196A, T199A, Y195D, Q203E, T207D, and Q216E
showed significant reductions in complex forming efficiency
(<50% WT binding) (Fig. 3B, lanes 5, 6, 8, 9, 22, 28, 30, and 32;
Table I), whereas binding by the mutant proteins V194A/
K201A, V194E, T196E, and T196K (Fig. 3B, lanes 13, 21, 23,
and 24 and Table I) was barely detectable (<5% WT binding,
not visible in Fig. 3B). No complex formation was observed by
the mutants L202A, P204A, F197D, A198D, T199D, and I206D
(Fig. 3B, lanes 14, 16, 25–27, and 29 and Table I). Moderate
reductions (=60% WT binding) were also observed for the mu-
ant proteins H193A, V194A, and I206A (Fig. 3B, lanes 4, 6 and
17 and Table I).

The lack of complex formation by the mutants P204A,
F197D, A198D, and I206D is probably attributable to disrup-
tions in their DNA binding capabilities (see Fig. 1; Table I).
Similarly, the mutant proteins V194A/K201A, L202A, and
T199D exhibit significantly reduced DNA binding activity (Fig.
1; Table I). In contrast, DNA binding by the mutant proteins
V194E, T196E, and T196K is unimpaired, suggesting that the
effect of these mutations is specifically to disrupt SRF-Elk-1
interactions. Likewise, the reductions in complex formation by
the mutants E190A, H193A, T191A/H193A, V194A, V194A/
T196A, T199A, I206A, Y195D, Q203E, and T207D are also likely
to be attributable to losses in protein-protein interactions
with Elk-1.

Taken together these assays suggest key roles for amino
acids Val-194 and Thr-196 in complex formation with Elk-1.
Furthermore, several additional residues, Glu-190, His-193,
Thr-199, Tyr-195, Gin-203, Ile-206, and Thr-207, are identified
as part of or close to the Elk-1 binding surface of SRF.

Protein-Protein Interactions between Elk-1 and SRF Mutant

3 E. C. Roberts, unpublished data.
Proteins in the Absence of DNA—Direct protein-protein interactions between SRF and Elk-1 were investigated using the GST pull-down assay. Although Elk-1 and SRF are usually found as DNA-bound complexes, complexes can be detected \textit{in vitro} in the absence of DNA using this assay (16). First, SRF mutants were translated \textit{in vitro} as 35S-labeled proteins and tested for binding to an immobilized protein consisting of GST fused to the Elk-1 B box (GST-B box) (16). Initially, a series of SRF derivatives with mutations in the N terminus of the MADS box were tested. However, none of the tested mutations reduces the binding of SRF to the B box (Table I; data not shown). Further SRF derivatives were tested that harbor mutations in the C-terminal end of the MADS box or within the SAM domain. Of the alanine substitution mutants, the single mutants E190A, T199A, and P204A (Fig. 4A lanes 3, 9, and 16; Fig. 4B) and the double mutants V194A/T196A, T196A/T207A, and Q203A/T207A (Fig. 4A lanes 8, 19, and 21; Fig. 4B) display large decreases in binding to GST-B box (50% wild-type binding). The residues Val-194, Thr-196, and Gln-203 probably play the major roles in the reductions observed in the double alanine mutants as single mutations in these residues also cause reductions in SRF binding to GST-B box (Fig. 4A; lanes 6, 7, and 15; Fig. 4B). Furthermore, the mutant protein I206A shows a moderate reduction in binding to the Elk-1 B box (Fig. 4B, lane 7; 51% WT). The lack of binding by the P204A mutant is probably due to a structural change in SRF (see “Discussion”). However, the residues Glu-190, Val-194, Thr-196, Thr-199, and Ile-206 are directly implicated in binding to Elk-1 which correlates well with their proposed role in ternary complex formation on the CECI site (Fig. 3).

Further residues are implicated in binding the Elk-1 B box from analysis of the mutants with charged residues substitutions. The mutants V194E, Y195D, T196E, T196K, F197D, A198D, and I206D exhibited large decreases in binding to GST-B box (Fig. 4A lanes 21-26 and 29; Fig. 4B). Of these mutants, the structures of F197D, A198D, and I206D are probably severely perturbed (see “Discussion”). However, clear correlations are apparent with the effect of the mutations V194E, T196E, and T196K on complex formation by SRF on the CECI site (Fig. 3; Table I). Moderate reductions in binding to GST-B box could also be observed for Q203E and T207D (Fig. 4A lanes 28 and 30; Fig. 4B) which also correlates with reductions in their ability to recruit SRF to the CECI site (Fig. 3; Table I).

In summary, direct protein-protein interaction assays and DNA-bound ternary complex formation assays both suggest important roles for Glu-190, Val-194, Thr-196, Thr-199, and Ile-206 in forming the interaction surface with Elk-1.

To confirm that the differential interactions observed with the mutant SRF proteins and the isolated Elk-1 B box could be reproduced in its natural context, the interaction assay was repeated with reciprocal constructs in which a selection of the SRF mutants were fused to GST and the truncated Elk-1–168 protein was translated and labeled \textit{in vitro}. As a control to assess levels of nonspecific binding, the mutant Elk-1 derivative Elk-1–168 (L158P) which is unable to bind to SRF (13) was used. Under these conditions, stronger interactions between SRF and Elk-1 are seen than to the isolated B box (Ref. 16 and data not shown).4

In comparison to the wild-type protein, the SRF mutants tested exhibited different levels of binding to Elk-1 (Fig. 5, A and B; Table I). Moreover, consistent with the reciprocal experiment (Fig. 4), the SRF mutant V194E showed the greatest decrease in binding (Fig. 5A, lanes 11 and 12). The mutant T196K also exhibited a significant decrease (Fig. 5A, lanes 15 and 16), and binding by the mutant E190A was also reduced to less than 50% (Fig. 5A, lanes 5 and 6). The other tested SRF mutants all bound to at least 50% of the level exhibited by the wild-type protein.

Taken together, our results demonstrate that in the absence of DNA, the mutations V194E, T196K, and T196E in SRF cause the greatest decrease in protein-protein interactions with

---

4 Y. Ling and A. D. Sharrocks, unpublished data.
interactions between p65/NF-κB and Fli-1 (23, 24). The series of SRF mutant proteins were analyzed by the GST pull-down assay in which the DNA-binding domain of p65/NF-κB was immobilized as a GST fusion protein. In contrast to the reductions in binding to the Elk-1 B box observed in several of the mutant SRF proteins (Fig. 4), no large decreases in binding to p65/NF-κB were observed (data not shown). These results imply that different residues comprise the p65/NF-κB and Elk-1 binding surfaces on SRF.

The ability of Fli-1 to form ternary complexes with SRF and the c-fos SRE was also investigated. In these assays, the quantity of each mutant SRF protein was adjusted to achieve equivalent amounts of binary SRF-SRE complex. An N-terminally truncated Fli-1 protein was used that requires the presence of SRF for recruitment into a ternary complex (Fig. 7A). All the mutant proteins could still form complexes with Fli-1 (Fig. 7; Table I) with the majority exhibiting comparable efficiency to the wild-type protein. However, the mutant proteins T199A, V194E, Y195D, and T196K exhibited reduced ability to bind to the c-fos SRE. Probes were generated by digestion with MluI (lanes 1, 9, 17, and 25), BglII (lanes 2, 10, 18, and 26), XhoI (lanes 3, 11, 19, and 27), EcoRV (lanes 4, 12, 20, and 28), SmalI (lanes 5, 13, 21, and 29), StuI (lanes 6, 14, 22, and 30), RsalI (lanes 7, 15, 23, and 31), BamHI (lanes 8, 16, 24, and 32) (32). The data from each circular permutation experiment are shown graphically below each set of primary data. The relative mobilities of protein-DNA complexes were normalized for differences in complex mobilities (see “Materials and Methods”) and plotted as a function of the position of the center of the SRE from the 5’ end of the probe. Circular permutation data were fitted to a cosine function, and the relative bending angles calculated from these curves are indicated.

### Fig. 5. Interaction of Elk-1 with mutant GST-SRF fusion proteins

A. GST pull-down assay of mutant SRF proteins fused to GST and [35S]-labeled wild-type Elk-1–168 (W) or Elk-1–168 (L158P) (M). Equal molar quantities of each GST-SRF fusion protein were used (indicated above each pair of lanes). 33% of the labeled input proteins is loaded on the gel. B, quantification of the data from pull-down assays with GST-SRF. Data and S.E. represent the average of three independent experiments.

Elk-1. These residues are therefore directly implicated in Elk-1 binding. The mutation of additional residues also causes reductions in direct SRF-Elk-1 interactions, implicating multiple amino acids in forming the SRF-Elk-1 interface.

**Protein-induced DNA Bending Is Unaltered in SRF Mutant Proteins—SRF-mediated DNA bending is observed in the ternary SRF-Elk-1-SRE complex (20). The magnitude of protein-induced DNA bending may affect the ability of SRF to recruit Elk-1 into ternary complexes. Several residues in SRF have been implicated in protein-induced DNA bending (32), including His-193 which is located in the vicinity of several residues investigated in this study (Fig. 8; see Ref. 21). To rule out an effect of the important residues identified in this study on protein-induced bending and hence ternary complex formation, the DNA-bending properties of several mutant proteins were investigated using the circular permutation assay. In this assay, changes in the mobility of protein-DNA complexes as the binding site location is moved with respect to DNA ends is indicative of protein-induced DNA bending. Moreover, this assay has added the advantage in that gross changes in protein structure would also become apparent.

Wild-type SRF induces an apparent DNA bend of 72° (Fig. 6A; 32). Similarly, the SRF mutants V194A/T196A, V194E, and T196E induce apparent DNA bends of comparable magnitude (68°–70°; Fig. 6, B–D). These mutant proteins also bind DNA with a similar efficiency to wild-type SRF (Fig. 1). Collectively, these results demonstrate that DNA binding by the SRF mutants V194A/T196A, V194E, and T196E is unaltered and that the differences in ternary complex formation with Elk-1 (Figs. 2 and 3) and Fli-1 (see below) are attributable to changes in direct protein-protein interactions.

**Complex Formation with the Transcription Factor Fli-1—**In addition to Elk-1, the core DNA-binding domain of SRF has also been demonstrated to form complexes with p65/NF-κB (25) and Fli-1 (23, 24). The series of SRF mutant proteins were tested for interaction with these transcription factors to determine whether their interaction surfaces on SRF differ. First, interactions between p65/NF-κB and the SRF mutant proteins were analyzed by the GST pull-down assay in which the DNA-binding domain of p65/NF-κB was immobilized as a GST fusion protein. In contrast to the reductions in binding to the Elk-1 B box observed in several of the mutant SRF proteins (Fig. 4), no large decreases in binding to p65/NF-κB were observed (data not shown). These results imply that different residues comprise the p65/NF-κB and Elk-1 binding surfaces on SRF.

The ability of Fli-1 to form ternary complexes with SRF and the c-fos SRE was also investigated. In these assays, the quantity of each mutant SRF protein was adjusted to achieve equivalent amounts of binary SRF-SRE complex. An N-terminally truncated Fli-1 protein was used that requires the presence of SRF for recruitment into a ternary complex (Fig. 7A). All the mutant proteins could still form complexes with Fli-1 (Fig. 7; Table I) with the majority exhibiting comparable efficiency to the wild-type protein. However, the mutant proteins T199A, V194E, Y195D, and T196K exhibited reduced levels (~50%) of ternary complex formation (Fig. 7B, lanes 9, 19, 20, and 22). Conversely, significantly enhanced complex formation is observed in the mutant T196A and double mutants V194A/T196A, T196A/R200A, and T196A/T207A (Fig. 7C). These results support the conclusion that different residues comprise the Fli-1 binding surface on SRF.

A. D. Sharrocks, unpublished data.
Protein-protein interactions play a key role in the formation of the ternary nucleoprotein which forms between SRF, Elk-1, and the c-fos SRE (Refs. 16, 18, and 19 and reviewed in Ref. 2). The interaction surface on Elk-1 is provided by residues in the conserved B box which is predicted to indi-
cus an α-helix upon interaction with SRF (17). In this study we have used site-directed mutagenesis to identify residues which comprise the Elk-1 binding surface on SRF.

**The Elk-1 Binding Surface—**Residues in SRF were mutated to either alanine or charged residues. A reduction of interaction upon introduction of an alanine residue was taken as evidence for a direct role of the amino acid in binding to Elk-1. Moderate effects by introduction of charged residues suggest that the residues are close to (but not necessarily part of) the Elk-1-binding motif, whereas severe effects indicate that the residues are probably within the binding surface. Based on these criteria, Glu-190, Val-194, Thr-196, Thr-199, and Ile-206 are identified as residues that are important in forming the binding surface, and Tyr-195, Gln-203, and Thr-207 are located close to the binding surface. Significantly, these residues form a continuous surface-exposed patch, whereas residues that are not thought to be part of this surface (including Thr-210, Gln-216, Val-187, Ser-189, Arg-200, and Lys-201) are located outside this patch (Fig. 8, B–D). Other residues such as Ile-206 whose mutation causes moderate changes in Elk-1 binding are located close to this patch (Fig. 8E). The total loss of Elk-1 binding exhibited by the mutant V194E is consistent with the central location of this residue in the binding surface (Fig. 8, C and D).

This surface-exposed patch is predominantly hydrophobic in character (Fig. 8B) and is consistent with the observation that the residues in Elk-1 which play important roles in SRF binding are hydrophobic in character and are predicted to form a hydrophobic surface presented on the face of an α-helix (17). Together, our data are therefore consistent with a model in which protein-protein interactions between Elk-1 and SRF are mediated by the interaction of the hydrophobic surface of an α-helix formed by the Elk-1 B box and a hydrophobic patch on the surface of SRF. Recently, the structure of the related yeast MADS box protein Mcm1 in a ternary complex with DNA-bound MATα2 has been solved (35). In this complex, a surface-exposed hydrophobic groove forms the binding surface for MATα2. One end of this groove is centered on Val-69 with other defining residues including Phe-72 and Thr-74 toward the opposite end of the groove. The analogous residues in SRF (Val-194, Phe-197, and Thr-199) all lie within the Elk-1 binding surface (Fig. 8, B–D). Furthermore, MATα2 inserts a phenylalanine residue into the hydrophobic pocket surrounding Val-69. As three aromatic residues in Elk-1 are critical for interaction with SRF (17), it is tempting to speculate that one of these residues occupies a similar position in the hydrophobic pocket surrounding Val-194 in SRF. This scenario is consistent with the severe reduction in Elk-1 binding caused by the introduction of a charged residue in place of Val-194.

A previous study has pinpointed residues Ala-198, Arg-200, and Gln-203 as determinants of the differential ability of SRF and the yeast MADS box protein ArgR1 to bind to Elk-1 (18). Furthermore, an additional study has implicated residues spread over a wider area (Ser-189–Met-205) as contributing to their differential Elk-1 binding capabilities (19). Our results are consistent with both studies and implicate residues from within the β1–βII turn, βII and βII–αII turn in forming the Elk-1 binding surface (Fig. 8A). Together with the SRF structure (Ref. 21; Fig. 8, B–D), our results allow a molecular rationale to be applied to explain the results of these previous studies. The insertion of Ala-198 and Gln-203 into ArgR1 is
likely to result in the removal of inhibitory effects of the side chains of the analogous residues (Thr and Glu, respectively). The mutation Q203E inhibits interactions of SRF with Elk-1 (Table I), and the mutation A198T would likely introduce a bulky residue into the center of the Elk-1 binding surface on SRF (Fig. 8, B–D). The combination of these residues in ArgRI would therefore inhibit ternary complex formation.

The mutations of several residues that are predicted to lie within the binding surface, including F197D, A198D, and I206D, had deleterious effects on DNA binding in addition to interaction with Elk-1. Their role in protein-protein interactions could not therefore be unambiguously assessed. The effect of F197D has previously been attributed to disruption of the dimerization interface (21, 28). Similarly, the introduction of charged residues in place of Ala-198 and Ile-206 is likely to have severe structural consequences as these side chains are at least partially buried in the hydrophobic core of the protein (Fig. 8; Ref. 21). The mutation of Pro-204 is also likely to cause a structural change in the C terminus of the DNA-binding domain as this helps define the orientation of the turn between βII and αII structural elements (21).

The mutant protein T191A/H193A exhibits markedly decreased binding in DNA-bound complexes on the CECI site but virtually wild-type binding in pull-down assays, thereby suggesting a role for these residues in DNA-dependent interactions. This SRF mutant exhibits reduced DNA bending properties in comparison to the wild-type protein,6 suggesting that DNA structure may play a role in ternary complex formation. We are currently exploring this possibility further.

Binding Surfaces for Other Transcription Factors—Fli-1 (23, 24) and p65/NFκB (25) can both form complexes with SRF although in the former case, the biological significance is unknown. Testing of these transcription factors in addition to Elk-1 for binding to the panel of SRF mutants identified residues that appear to have different roles in binding each protein, e.g. Thr-199 (Elk-1) and Thr-196 (Fli-1). In contrast, other residues (e.g. Glu-190 and Val-194) play key roles in binding both Elk-1 and Fli-1. None of the mutations caused dramatic reductions (or enhancements) in interaction with p65/NFκB,

---

6 A. G. West and A. D. Sharrocks, unpublished data.
indicating that the tested residues either play different roles or that different residues comprise the p65/NFκB binding surface. The observation that these proteins interact using different surfaces is consistent with the observation that neither Fli-1 nor p65/NFκB exhibits strong homology to the B box SRF interaction motif of Elk-1. Further studies are required to fully define the interaction epitopes of Fli-1 and p65/NFκB, but it is interesting to note that Val-194 appears to form an important part of each interaction surface and is associated within a hydrophobic cleft in the protein (Fig. 8E). Furthermore, removal of the side chain of the neighboring residue Thr-196 significantly enhances (5–10-fold; Fig. 7; Table I) the formation of complexes between Fli-1 and SRE-bound SRF. This mutation removes a hydrophilic side chain and increases the size of the hydrophobic groove on the surface of SRF (Fig. 8, B and E). One possible role for this residue may be to reduce potential interactions with Fli-1 in vivo. Further experiments are required to address the possible physiological significance of this observation.

In summary, we have defined a small surface-exposed patch on SRF that forms the binding interface with the Elk-1 B box. Furthermore, we have demonstrated that other transcription factors bind to different surfaces on SRF which in part appear to overlap the Elk-1 binding surface. The related yeast MADS box protein Mcm1 also binds to multiple co-regulatory proteins (reviewed in Ref. 27). One of these, MATa2, binds to a surface-exposed hydrophobic groove which is located in an analogous position to the Elk-1 binding surface on SRF (35). Our data are consistent with the notion that MADS box proteins act as DNA-bound targets that allow the recruitment of many different transcription factors and hence allow different responses to diverse cellular signals.

**Acknowledgments**—We thank Margaret Bell and Catherine Pyle for excellent technical and secretarial assistance and Bob Liddell for DNA sequencing and oligonucleotide synthesis. We are grateful to members of our laboratory for stimulating discussions and comments on the manuscript. We are grateful to Neil Perkins for reagents and Song Tan and Tim Richmond for communicating data prior to publication.

**REFERENCES**

1. Treisman, R. (1992) *Trends Biochem. Sci.* 17, 423–426
2. Treisman, R. (1995) *EMBO J.* 14, 4905–4913
3. Cahill, M. A., Janknecht, R., and Nordheim, A. (1996) *Curr. Biol.* 6, 16–19
4. Hill, C. S., Wynne, J., and Treisman, R. (1994) *EMBO J.* 13, 5421–5432
5. Johansen, P.-E., and Prywes, R. (1994) *Mol. Cell. Biol.* 14, 5920–5928
6. Hill, C. S., Wynne, J., and Treisman, R. (1995) *Cell* 81, 1159–1170
7. Hill, C. S., and Treisman, R. (1994) *Mol. Cell. Biol.* 14, 5037–5047
8. Price, M. A., Cruzalegui, F. H., and Treisman, R. (1996) *EMBO J.* 15, 6552–6563
9. Whitmarsh, A. J., Shore, P., Sharrocks, A. D., and Davis, R. J. (1995) *Science* 269, 403–407
10. Shatrov, T., Gille, H., and Shaw, P. E. (1996) *Proc. Natl. Acad. Sci. U. S. A.* 93, 11563–11568
11. JANKE, R., and Hunter, T. (1997) *EMBO J.* 16, 1620–1627
12. Cavigelli, M., Dolfi, F., Clarret, F. X., and Karin, M. (1995) *EMBO J.* 14, 5957–5964
13. Treisman, R. (1996) *Curr. Opin. Cell Biol.* 8, 205–215
14. Shallo, A. J., and Davis, R. J. (1996) *J. Mol. Med.* 74, 589–607
15. Shallo, P., and Sharrocks, A. D. (1994) *Mol. Cell. Biol.* 14, 3283–3291
16. Ling, Y., Lakey, J. H., Roberts, E. C., and Shorrocks, A. D. (1997) *EMBO J.* 16, 2431–2440
17. Mueller, C. G. F., and Nordheim, A. (1993) *EMBO J.* 11, 2419–2429
18. Shallo, P. E. (1992) *EMBO J.* 11, 3011–3019
19. Shallo, A. D., and Shore, P. (1995) *Nucleic Acids Res.* 23, 2442–2449
20. Pellegrini, L., Tan, S., and Richmond, T. J. (1995) *Nature* 376, 490–498
21. Groisman, R., Masutani, H., Leibovitch, M. P., Robin, P., Soudant, I., Trouche, D., and Harel-Bellan, A. (1996) *J. Biol. Chem.* 271, 5258–5264
22. Watson, D. K., Roninson, I., Hodge, D. B., Kola, I., Papas, T. S., and Seth, A. (1997) *Oncogene* 14, 233–241
23. Magnaghi-Jaulin, L., Masutani, H., Robin, P., Lipinski, M., and Harel-Bellan, A. (1996) *Nucleic Acids Res.* 24, 1052–1058
24. Franzen, K., Carlson, L., Brown, K., Daucher, M. B., Bressler, P., and Siebenlist, U. (1996) *EMBO J.* 15, 3403–3412
25. Grueneberg, D. A., Henry, R. W., Braver, A., Novina, C. D., Cheriyath, V., Roy, A. L., and Gilman, M. (1997) *Genes Dev.* 11, 2482–2493
26. Shallo, P., and Shallo, A. D. (1995) *Eur. J. Biochem.* 229, 1–13
27. Shallo, A. D., Gille, H., and Shaw, P. E. (1993) *Mol. Cell. Biol.* 13, 123–132
28. Shallo, A. D., Von Hasler, F., and Shaw, P. E. (1993) *Nucleic Acids Res.* 21, 213–221
29. Shallo, P., Whitmarsh, A. J., Bhaskaran, R., Davis, R. J., Waltho, J. P., and Shallo, A. D. (1996) *Mol. Cell. Biol.* 16, 3338–3349
30. Shallo, P., and Shallo, A. D. (1995) *Nucleic Acids Res.* 23, 4698–4706
31. West, A. G., Shore, P., and Shallo, A. D. (1997) *Mol. Cell. Biol.* 17, 2876–2887
32. Bryant, G. O., Martel, L. S., Burley, S. K., and Berk, A. J. (1996) *Genes Dev.* 10, 4961–5004
33. Tang, H., Sun, X., Reinberg, D., and Ebrigt, R. H. (1996) *Proc. Natl. Acad. Sci. U. S. A.* 93, 1119–1124
34. Tan, S., and Richmond, T. (1998) *Nature* 391, 660–666