

# Ternary complex formation over the *c-fos* serum response element: p62<sup>TCF</sup> exhibits dual component specificity with contacts to DNA and an extended structure in the DNA-binding domain of p67<sup>SRF</sup>

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The serum response element (SRE) plays an essential role in the transcriptional regulation of proto-oncogene *c-fos*. A ternary complex, consisting of transcription factors p67<sup>SRF</sup> and p62<sup>TCF</sup> bound to the SRE is present in several cell types and its formation has been correlated with inducibility of the gene in different cells by serum, epidermal growth factor and phorbol esters. Interaction of p62<sup>TCF</sup> with the SRE *in vitro* exhibits both a degree of sequence specificity and a strict dependence on the presence of bound p67<sup>SRF</sup>. A 90 amino acid DNA-binding domain of p67<sup>SRF</sup> (core<sup>SRF</sup>) suffices for ternary complex formation. DNase I footprinting and UV-mediated DNA–protein crosslinking experiments presented here show that direct DNA contacts are made by p62<sup>TCF</sup> with the 5' sequence of the SRE and thus explain the sequence dependence of ternary complex formation. Additionally, analysis of ternary complex formation by chimeras of core<sup>SRF</sup> and the related yeast protein ArgRI as well as comprehensive mutagenesis of non-conserved residues between the two proteins has yielded a core<sup>SRF</sup> mutant specifically unable to interact with p62<sup>TCF</sup> and demonstrates that an extended structure in core<sup>SRF</sup> is required for this interaction. Thus p62<sup>TCF</sup> exhibits dual component specificity in ternary complex formation over the *c-fos* SRE.

**Key words:** ArgRI protein/proto-oncogene/serum inducibility/transcriptional regulation/UV-mediated DNA-protein cross-linking

## Introduction

Proto-oncogene *c-fos* is a paradigm for cellular immediate early genes that are induced when cells are stimulated to pass from G<sub>0</sub> to G<sub>1</sub> by agents such as growth factors and mitogens (Treisman, 1990 and references therein). In the case of *c-fos*, the induction is rapid and transient, RNA levels reaching a peak by 30 min and decaying to basal levels by 1 h. Inducibility is conferred upon the gene by a promoter element with pronounced dyad symmetry, referred to as the Serum Response Element (SRE) (Treisman, 1985, 1986; Gilman *et al.*, 1986). A factor, p67<sup>SRF</sup>, which binds to the central part of this element, referred to as the CARG box (Minty and Kedes, 1986), has been identified, purified (Prywes and Roeder, 1987; Schröter *et al.*, 1987; Treisman, 1987) and cloned as a cDNA (Norman *et al.*, 1988).

Other proteins bind to the SRE. A 62 kDa factor has been identified that binds excentrically over the element (Ryan *et al.*, 1989) and another factor of similar apparent molecular

mass, p62<sup>TCF</sup>, is also able to bind to the SRE, but only in the presence of p67<sup>SRF</sup>, thereby forming a ternary complex (Shaw *et al.*, 1989a). p62<sup>TCF</sup> has been shown recently to be closely related, if not identical, to the *elk1* gene product (Hipskind *et al.*, 1991) or SAP1 (Dalton and Treisman, 1992), which are *ets*-like proteins (Gegonne *et al.*, 1987; Watson *et al.*, 1988). Recent data have identified the presence of other binding sites superimposed over the SRE that bind the C/EBP-related transcription factor rNFIL-6, which appears to be responsible for the SRE-dependent cAMP inducibility in PC12 cells (Metz and Ziff, 1991a) and the helix–loop–helix protein rE12 (Metz and Ziff, 1991b). Contiguous with the SRE downstream is a consensus AP-1 binding sequence centred at position –295 (FAP site), which displays transcriptional activator potential *in vivo*, phorbol ester and cAMP responsiveness (Fisch *et al.*, 1989; Shaw *et al.*, 1989b; Velcich and Ziff, 1990) and binding to transcription factors AP-1 and CREB/ATF *in vitro* (Shaw *et al.*, 1989b).

*In vivo* footprinting experiments performed with several cell lines indicate that the ternary complex forms over the SRE and remains stable during induction of the *c-fos* gene by epidermal growth factor (EGF) or serum (Herrera *et al.*, 1989; König, 1991). They also document the presence of at least one other transcription factor bound stably to the FAP site immediately downstream of the SRE.

The transient nature of the induction is also a property conferred on proto-oncogene *c-fos* by the SRE, which has been shown to mediate the repression of the promoter (Sassone-Corsi and Verma, 1987; König *et al.*, 1989; Shaw *et al.*, 1989b). Repression in the wake of induction has been shown to be a function of FOS, the *c-fos* gene product, acting through the SRE. p67<sup>SRF</sup> has been, as yet, only implicated indirectly. The repressed state of the gene prior to induction, 'poised repression', is also conferred on the gene by the SRE, but in this instance the involvement of p67<sup>SRF</sup> or FOS has neither been demonstrated nor ruled out. In this respect the SRE provides an interesting model system for the study of multi-component transcription factor complexes, the prevalence of which is becoming increasingly apparent (Shaw, 1990).

Formation of the ternary complex by p67<sup>SRF</sup> and p62<sup>TCF</sup> with the SRE has been shown to correlate with induction of the *c-fos* gene by serum in NIH3T3 cells (Shaw *et al.*, 1989a), by phorbol esters in BALB/c 3T3 fibroblasts (Graham and Gilman, 1991) and EGF in human astrocytoma cells (Malik *et al.*, 1991). Other results suggest that under certain conditions, ternary complex formation may be dispensable for induction of *c-fos* by phorbol esters or serum (Rivera *et al.*, 1990; Graham and Gilman, 1991; König, 1991). These observations have relied on mutations in the SRE to modulate p62<sup>TCF</sup> binding although little is known about the specificity requirements for ternary complex formation.

The experiments described in this paper address the

parameters governing the interaction of p62<sup>TCF</sup> with the p67<sup>SRF</sup>-SRE complex. Consistent with the methylation interference and protection patterns diagnostic of p62<sup>TCF</sup> binding (Herrera *et al.*, 1989; Shaw *et al.*, 1989a), an extension of the p67<sup>SRF</sup> DNase I footprint is observed upon binding of p62<sup>TCF</sup>. This is more marked with a p67<sup>SRF</sup> mutant of reduced size, from which is inferred that the extension is unlikely to result from a conformational change in p67<sup>SRF</sup> binding but rather from contacts made by p62<sup>TCF</sup> with the DNA. Furthermore, the DNA-protein contacts allow crosslinking of p62<sup>TCF</sup> to unmodified DNA by UV irradiation. These results indicate that the specificity of ternary complex formation is conferred in part by DNA contacts made by p62<sup>TCF</sup>.

In addition, the analysis of chimaeric p67<sup>SRF</sup>-related proteins in an assay for ternary complex formation and comprehensive mutagenesis of residues implicated in interactions with p62<sup>TCF</sup> reveals that an extended structure within the DNA-binding domain of p67<sup>SRF</sup> is required by p62<sup>TCF</sup> for ternary complex formation. Derivation of a p67<sup>SRF</sup> mutant unable to complex p62<sup>TCF</sup> demonstrates that this extended structure is only perturbed by multiple amino acid substitutions. Thus formation of the ternary complex at the *c-fos* SRE involves dual component specificity on the part of p62<sup>TCF</sup>.

**Results**

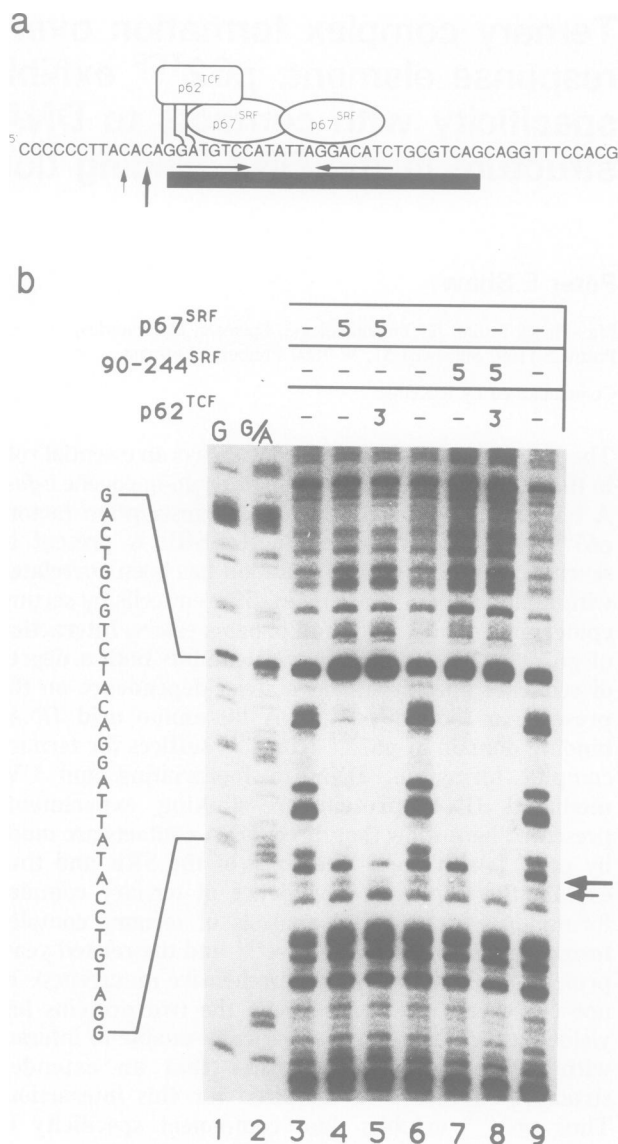
**p62<sup>TCF</sup> extends the p67<sup>SRF</sup>-dependent DNase I footprint over the SRE**

It has been previously observed that p67<sup>SRF</sup> forms an asymmetric DNase I footprint over the SRE, the downstream border extending into the FAP site centred at position -295 and the upstream border corresponding to the 5' limit of the dyad symmetry element (DSE) (Figure 1a; Shaw *et al.*, 1989b). One possible consequence of specific interactions between p62<sup>TCF</sup> and DNA in the ternary complex might be a 5' extension of this footprint. For this reason DNase I footprints produced by p67<sup>SRF</sup> alone and in conjunction with p62<sup>TCF</sup> were compared.

When co-incubated with full-length p67<sup>SRF</sup>, p62<sup>TCF</sup> resulted in only a slight protection beyond the 5' boundary of the p67<sup>SRF</sup> footprint (compare lanes 4 and 5 of Figure 1b). However, when a truncated form of p67<sup>SRF</sup> consisting of amino acid 90-244 (90-244<sup>SRF</sup>) substituted full-length p67<sup>SRF</sup> in the binding reactions, the p62<sup>TCF</sup>-dependent extension to the footprint was pronounced (lanes 7 and 8 of Figure 1b). This result is consistent with p62<sup>TCF</sup> binding in close association with the 5' flanking sequences of the SRE, as are the differences in the methylation interference and protection patterns observed previously (Herrera *et al.*, 1989; Shaw *et al.*, 1989a). However, none of these results exclude the possibility that upon binding p62<sup>TCF</sup> induces a conformational change in p67<sup>SRF</sup> that causes the increased DNA protection observed, but the fact that deletion of 70% of p67<sup>SRF</sup> (90-244<sup>SRF</sup>) resulted in an increase in the p62<sup>TCF</sup>-specific DNase I protection makes this explanation less probable.

**p62<sup>TCF</sup> can be cross-linked to unmodified DNA by UV irradiation**

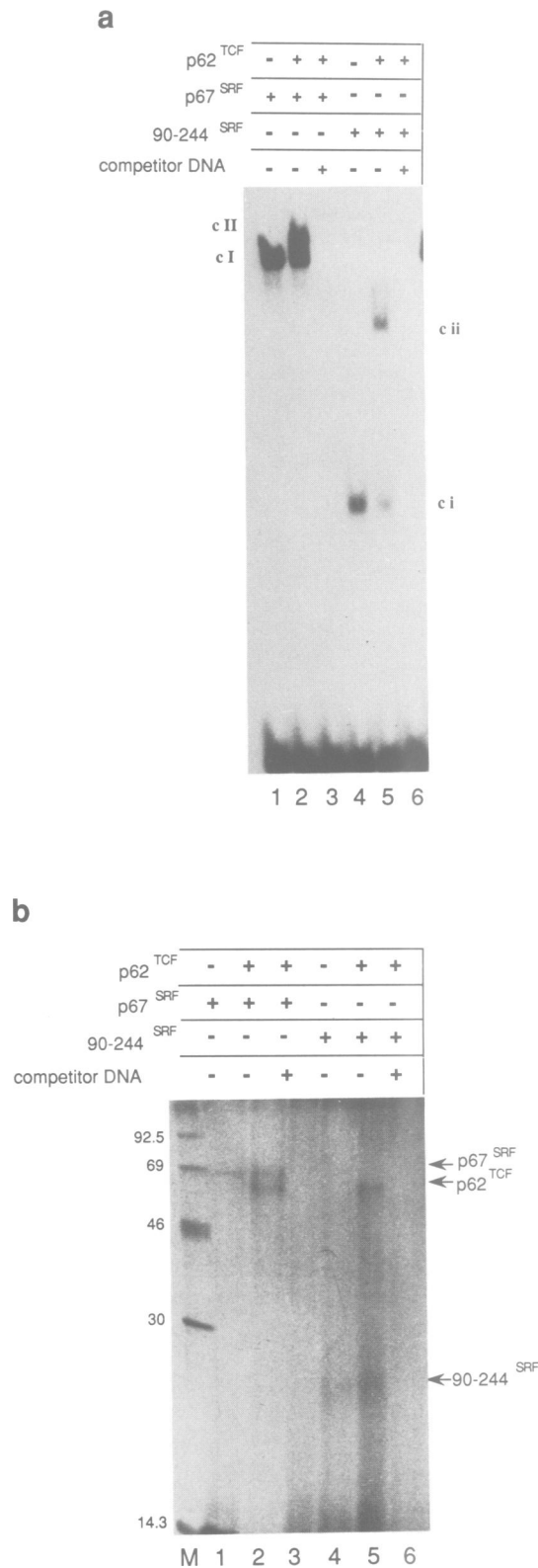
A stringent demonstration of DNA-protein interaction is the formation of covalent DNA-protein adducts upon irradiation by UV light. To this end an internally labelled



**Fig. 1. (a)** Schematic diagram of the ternary complex. The sequence represents the upper strand of the human *c-fos* promoter from positions -332 to -289 and corresponds to the sequence used as the probe in the gel retardation experiments. The horizontal arrows indicate the dyad symmetry element and the shaded box corresponds to the extent of the p67<sup>SRF</sup> footprint. Vertical arrows point to sites of DNase I cuts reduced upon p62<sup>TCF</sup> binding. **(b)** p62<sup>TCF</sup> modifies the DNase I footprint of SRE-bound p67<sup>SRF</sup>. The DNA probe used for the footprinting analysis of the upper strand is described in Materials and methods. 90-244<sup>SRF</sup> was prepared as an unfractionated nuclear extract from HeLa cells infected with recombinant vaccinia virus expressing the protein. p62<sup>TCF</sup> was prepared as an enriched fraction according to the procedure described previously (Schröter *et al.*, 1990). Numbers above the lanes refer to fraction volumes (μl). Arrows to the right of the figure correspond to the vertical arrows in (a).

SRE probe was prepared in which the two guanosine residues (corresponding to positions -319 and -320 of the human *c-fos* promoter) were <sup>32</sup>P-labelled (see Materials and methods) and mixed with full-length or truncated p67<sup>SRF</sup> and p62<sup>TCF</sup>. After incubation either with or without specific competitor DNA and irradiation, a fraction of each sample was analysed by gel retardation (Figure 2a), the remainder was treated with DNase I and the adducts subsequently analysed by SDS-PAGE (Figure 2b).

Formation of a ternary complex, as confirmed by gel



**Fig. 2.** p62<sup>TCF</sup> can be cross-linked to unmodified DNA by UV irradiation. The binding reactions were prepared as for gel retardation (1.5  $\mu$ l of each protein fraction) but scaled up four fold, of which one-quarter was analysed by gel retardation (a) after UV irradiation. The positions of the different complexes are indicated. Unlabelled probe was added as specific competitor DNA at a 100-fold molar excess. (b) Analysis of the radiolabelled DNA-protein adducts on a 10% SDS-polyacrylamide gel. After fixing and drying, the gel was exposed for 14 days.

retardation (Figure 2a, lanes 2 and 5), correlated with the appearance of a labelled DNA-protein adduct with an apparent molecular mass of  $\sim$ 62 kDa (Figure 2b, lanes 2 and 5), which was susceptible to cold competitor DNA (lanes 3 and 6) and independent of the species of p67<sup>SRF</sup> in the complex. Taken with the DNase I footprinting, methylation interference and protection results, this result is entirely consistent with a direct interaction between p62<sup>TCF</sup> and the 5' flanking sequences of the SRE.

#### The SRE-bound core domain of ArgRI does not permit ternary complex formation by p62<sup>TCF</sup>

The use of N-terminal and C-terminal deletions introduced into p67<sup>SRF</sup> demonstrated that a minimal core of  $\sim$ 90 amino acids is capable of forming dimers and binding to the SRE (Norman *et al.*, 1988). It has also been shown that the DNA binding domain corresponds to a relatively protease-insensitive domain that retains the ability to direct ternary complex formation by p62<sup>TCF</sup> (Schröter *et al.*, 1990).

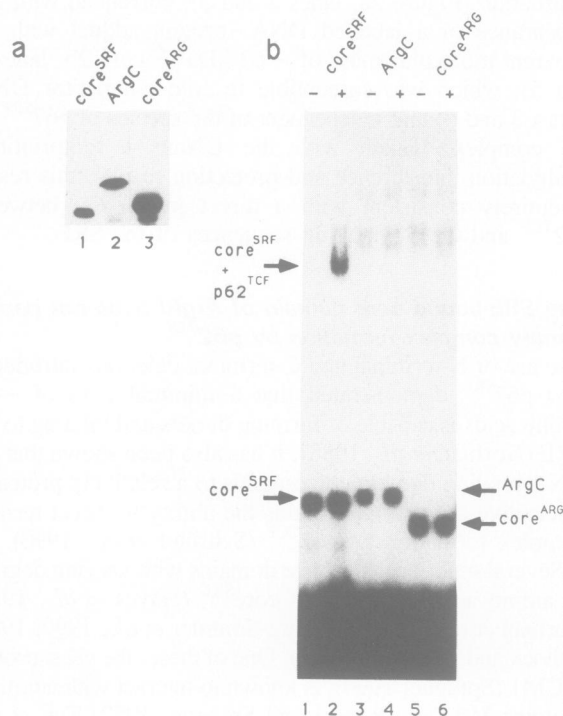
Several other proteins have domains with varying degrees of amino acid similarity to core<sup>SRF</sup> (Hayes *et al.*, 1988; Norman *et al.*, 1988; Schwarz-Sommer *et al.*, 1990, 1992; Pollock and Treisman, 1991). One of these, the yeast protein MCM1 (Sprague, 1990), is known to interact with auxiliary proteins MAT $\alpha$ 1 (Bender and Sprague, 1987; Tan *et al.*, 1988), MAT $\alpha$ 2 (Keleher *et al.*, 1988, 1989), STE12 (Errede and Ammerer, 1989; Primig *et al.*, 1991) and with a fourth, recently described factor, SFF (Lydall *et al.*, 1991). It has also been shown that p67<sup>SRF</sup> and MCM1 bind to each other's recognition site (Hayes *et al.*, 1988). Another related yeast protein, ArgRI, is known to regulate arginine metabolism in conjunction with two other gene products, ArgRII and ArgRIII (Dubois *et al.*, 1987). The region of ArgRI that bears homology to the 90 amino acid DNA-binding domain of p67<sup>SRF</sup> (core<sup>SRF</sup>) maps to the C-terminal half of the protein.

Expression of the C-terminal fragment of ArgRI (residues 67-177, clone ArgC) in a rabbit reticulocyte lysate and subsequent analysis by gel retardation with an SRE probe revealed the formation of a DNA-protein complex (Figure 3b, lane 3) with similar migration to core<sup>SRF</sup> (lane 1), whereas the unprogrammed lysate produced no complex (not shown). The specificity of this interaction was confirmed by the removal of 11 C-terminal amino acids from ArgC to leave a core domain of 100 amino acids (core<sup>ARG</sup>), which formed a complex with the SRE (lane 5) of higher mobility than the ArgC complex, as would be expected, and with slightly reduced efficiency. Therefore the 100 amino acid core domain of ArgRI is able to form dimers and bind to the SRE in an analogous fashion to core<sup>SRF</sup>.

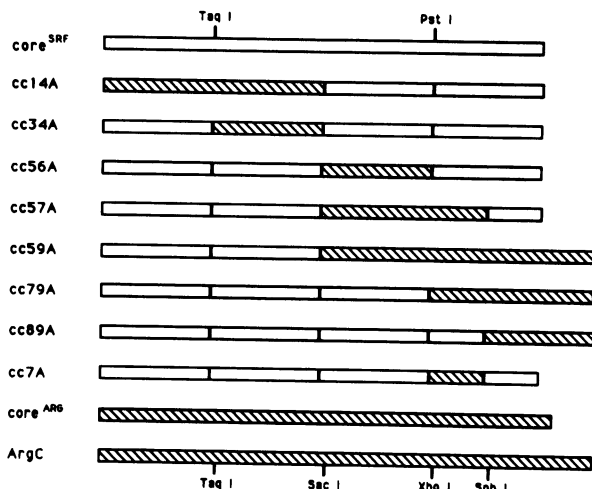
It was of interest to see if core<sup>ARG</sup> was also able to interact with p62<sup>TCF</sup>. Incubation of p62<sup>TCF</sup> with either truncated form of ArgRI (ArgC or core<sup>ARG</sup>) failed to give rise to a ternary complex (lanes 4 and 6) under conditions that allowed efficient ternary complex formation with core<sup>SRF</sup> (lane 2). Thus core<sup>SRF</sup> and core<sup>ARG</sup> differ qualitatively in their capacity to form a ternary complex with p62<sup>TCF</sup>.

#### Analysis of ternary complex formation by core<sup>SRF</sup>-core<sup>ARG</sup> chimaeras

To determine if the interaction of p67<sup>SRF</sup> and p62<sup>TCF</sup> could be attributed to a discrete region of core<sup>SRF</sup>, a series of core<sup>SRF</sup>-core<sup>ARG</sup> chimaeras was constructed (see Figure 4)



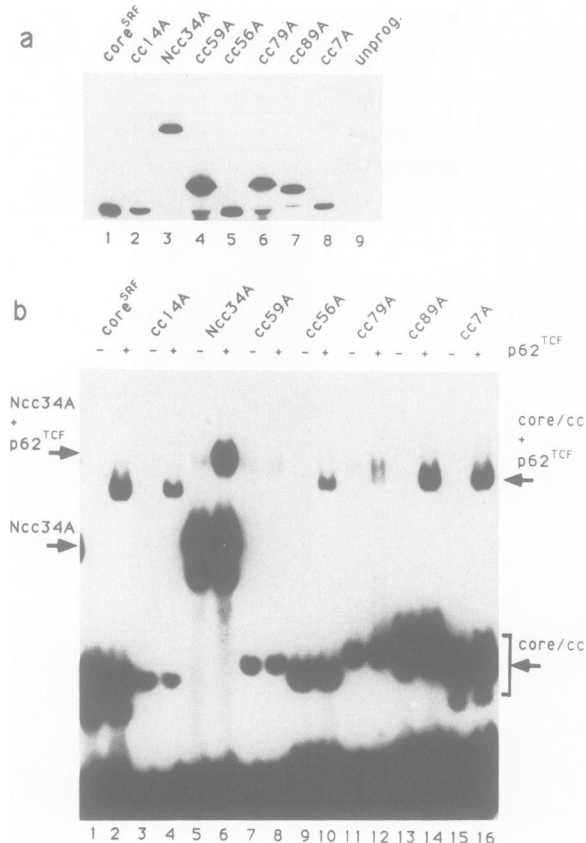
**Fig. 3.** Analysis of SRE binding and ternary complex formation by ArgRI deletion mutants. (a) Expression of the proteins in reticulocyte lysates was compared after electrophoresis through an 11% SDS-polyacrylamide gel and subsequent autoradiography. (b) Analysis by gel retardation of complexes formed with the SRE in the presence (+) and absence (-) of the enriched fraction of p62<sup>TCF</sup>. The positions of the different complexes are indicated by labelled arrows.



**Fig. 4.** Diagrammatic presentation of core<sup>SRF</sup>, core<sup>ARG</sup>, ArgC and the core<sup>SRF</sup>-core<sup>ARG</sup> chimaeras described in this study. Open rectangles correspond to sequences derived from p67<sup>SRF</sup> and hatched rectangles denote ArgRI sequences. Strategic restriction sites present in core<sup>SRF</sup> and core<sup>ARG</sup> are indicated at the top and bottom of the diagram respectively.

and their capacity to support ternary complex formation was tested by gel retardation analysis.

The core<sup>SRF</sup>-core<sup>ARG</sup> chimaeras expressed in reticulocyte lysates bound to the *c-fos* SRE (Figure 5b) with the exception of cc57A (not shown). In this experiment a chimaera with the N-terminal sequences of p67<sup>SRF</sup> (Ncc34A) was substituted for cc34A (Figure 4). Two of the

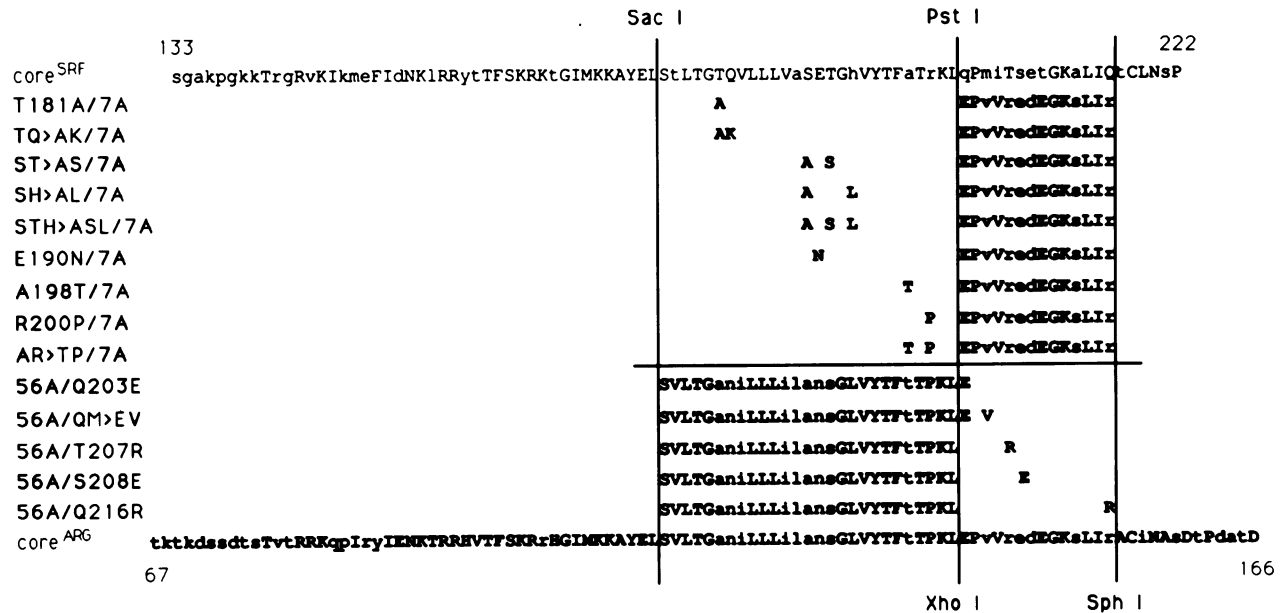


**Fig. 5.** Analysis of SRE binding and ternary complex formation by core<sup>SRF</sup>-core<sup>ARG</sup> chimaeras. (a) Expression of the proteins in reticulocyte lysates was compared after electrophoresis through an 11% SDS-polyacrylamide gel and subsequent autoradiography. The migration of proteins cc59A and cc79A in this gel is distorted by occlusion effect. (b) Analysis by gel retardation of complexes formed with the SRE in the presence (+) and absence (-) of p62<sup>TCF</sup>. The positions of the different complexes are indicated by labelled arrows.

chimaeras, cc59A and cc79A, displayed reduced binding affinity comparable to that of core<sup>ARG</sup> and ArgC (Figure 4 and Figure 5b, lanes 7, 8, 11 and 12) and cc56A binding was impaired to a lesser extent (Figure 5, lanes 9 and 10; Figure 7c, lanes 11 and 12). The binding of cc14A was uncharacteristically low in the experiment shown and was otherwise seen to bind with the affinity of core<sup>SRF</sup> (data not shown; A.D.Sharrocks, H.Gille and P.E.Shaw, submitted).

Three of the chimaeras displayed impaired ternary complex formation with p62<sup>TCF</sup>. cc59A, in which p67<sup>SRF</sup> residues 175-222 are replaced by ArgRI residues 112-177, failed to support detectable p62<sup>TCF</sup> binding (Figure 5b, lane 8). cc56A and cc79A, which each carry half the ArgRI fragment present in cc59A in place of the corresponding portions of core<sup>SRF</sup>, formed ternary complexes with reduced efficiency (Figure 5a, lanes 10 and 12). The ArgRI moiety of chimaera cc79A was further subdivided to give chimaeras cc89A and cc7A (Figure 4) but neither of these chimaeras showed reduced ternary complex formation comparable to cc79A (Figure 5b, lanes 11-16).

These results imply that either a discrete motif, partially disrupted in chimaeras cc56A and cc79A, may suffice to direct ternary complex formation or more than one determinant in the C-terminal half of core<sup>SRF</sup>, perhaps extensive structure, may be recognized by p62<sup>TCF</sup>. Alternatively, rather than core<sup>SRF</sup> providing a specific interface



**Fig. 6.** Diagrammatic presentation of the point mutant chimaeras of core<sup>SRF</sup> used in this study. The amino acid sequence of core<sup>SRF</sup> is given at the top of the figure and that of core<sup>ARG</sup> in bold at the bottom, both in single letter code. Numbers refer to the first and last residues of core<sup>SRF</sup> and core<sup>ARG</sup> with respect to full-length p67<sup>SRF</sup> and ArgRI. The vertical lines indicate the borders of the ArgRI segments in the chimaeras cc56A and cc7A, on which the clones are based. The ArgRI sequences and additional point mutations present in each clone are indicated in bold.

recognised by p62<sup>TCF</sup>, structural determinants in this region of core<sup>ARG</sup> may block ternary complex formation by p62<sup>TCF</sup>.

#### **DNA-bound core<sup>SRF</sup> provides an extended structural motif required for ternary complex formation by p62<sup>TCF</sup>**

In an attempt to distinguish between the possibilities outlined above and to produce a core<sup>SRF</sup> derivative specifically unable to support ternary complex formation by p62<sup>TCF</sup>, two reciprocal sets of core<sup>SRF</sup> mutants were constructed. One set combined the chimaera cc56A and single residue exchanges in the core<sup>SRF</sup> moiety that had been replaced by core<sup>ARG</sup> sequences in cc7A. The other set was conceptually similar but based, conversely, on cc7A. These 'point mutant chimaeras' are presented diagrammatically in Figure 6. They were expressed with equal efficiency in reticulocyte lysates (Figure 7a) and tested for ternary complex formation with p62<sup>TCF</sup> in gel retardation analyses, which are shown in Figures 7b and c. The individual residue exchanges did not affect ternary complex formation by p62<sup>TCF</sup>, nor did they grossly affect DNA binding (results not shown; Sharrocks *et al.*, submitted), but they did show effects in the context of the chimaeras. An additional chimaera, cc57A (see Figure 4), was constructed and tested but, as mentioned above, it failed to bind to the SRE (result not shown). Only one of the point mutant chimaeras was similarly unable to bind the SRE (TQ>AK/7A, lanes 5 and 6 of Figure 7b). This result is due to the combined effect as cc7A bound DNA efficiently (Figure 5b, lanes 15 and 16) and the double residue exchange alone (TQ>AK) had only slightly impaired DNA binding (result not shown; Sharrocks *et al.*, submitted).

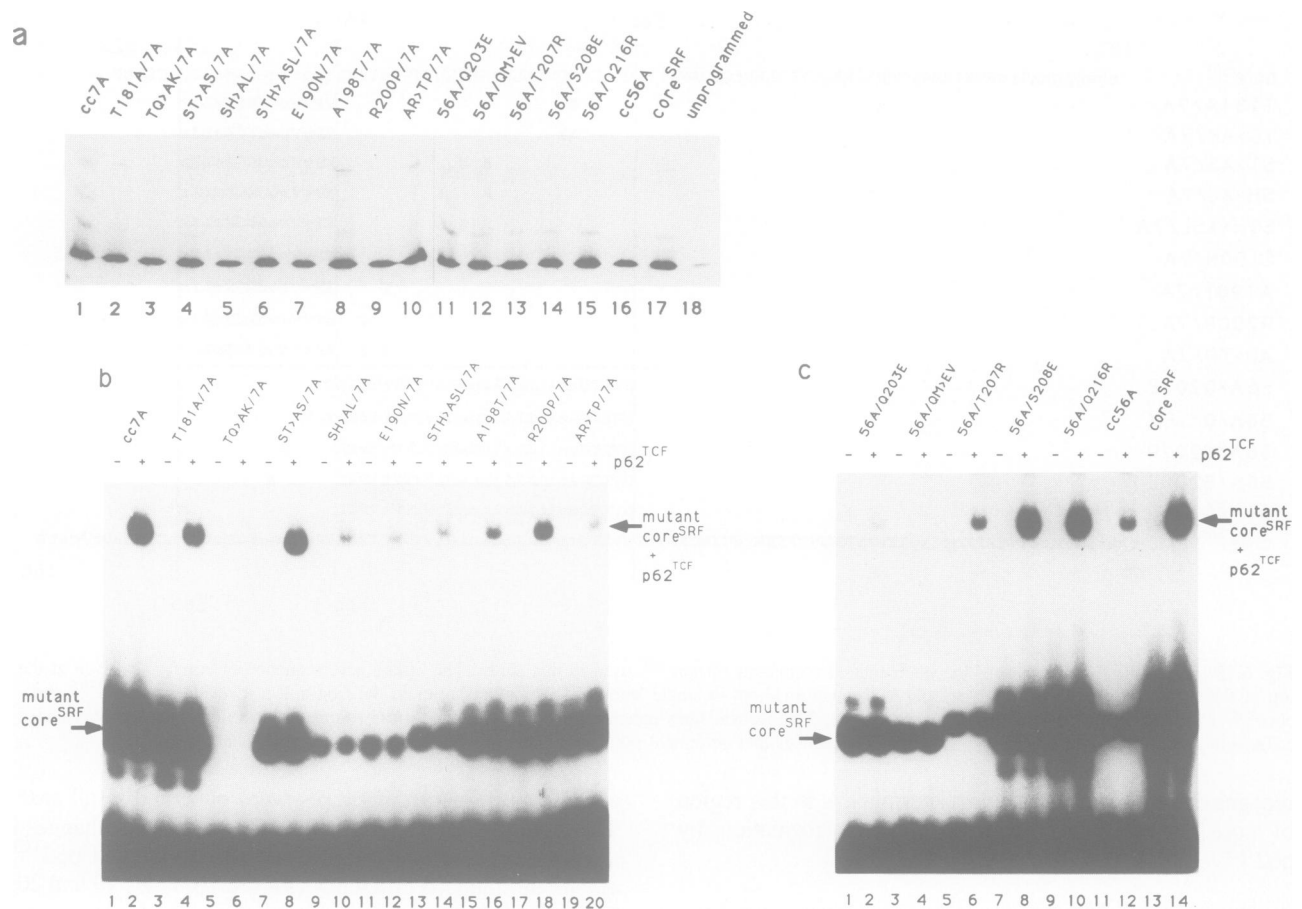
The other cc7A-derived point mutant chimaeras bound to the SRE and directed ternary complex formation by p62<sup>TCF</sup> with varying efficiency. The implications of this variation are discussed below but explicitly, despite the numerous

alterations, ternary complexes were observed in all cases. The mutant AR>TP/7A, with all 11 possible exchanges in a 19 residue segment (Figure 6) still complexed p62<sup>TCF</sup>, albeit with reduced efficiency (Figure 7b, lanes 19 and 20). The effect of additional exchanges towards the N-terminal on ternary complex formation could not be assessed because they abolish SRE binding (SHAR>ALTP/7A, STHAR>ASLTP/7A; results not shown). However, the additional exchanges alone (SH>AL/7A, STH>ASL/7A) and other exchanges towards the N-terminal (T181A/7A, ST>AS/7A, E190N/7A) did not abolish binding to the SRE or ternary complex formation with p62<sup>TCF</sup>.

The cc56A-derived point mutant chimaeras all bound to the SRE with affinities comparable to or higher than cc56A. However, the effects of the additional residue exchanges on ternary complex formation were extremely varied. Whereas ternary complex formation was severely impaired with mutant 56A/Q203E (Figure 7c, lane 2) and abolished with mutant 56A/QM>EV (lane 4), mutants 56A/S208E and 56A/Q216R bound to the SRE and directed ternary complex formation with the efficiency of core<sup>SRF</sup> (lanes 8 and 10). Thus, the only point mutant chimaera generated that specifically lacks the ability to direct ternary complex formation by p62<sup>TCF</sup>, contains 14 exchanges spread across 29 residues, which constitute almost one-third of core<sup>SRF</sup>. These results rule out the existence of a discrete motif partially disrupted in the chimaeras cc56A and cc7A and demonstrate that p62<sup>TCF</sup> relies instead on an extended structure in the C-terminal region of core<sup>SRF</sup> for ternary complex formation.

#### **Discussion**

The experiments presented in this paper address the formation of the ternary complex by p67<sup>SRF</sup> and p62<sup>TCF</sup> with the *c-fos* SRE. The conjecture that both DNA-protein and protein-protein interactions govern p62<sup>TCF</sup> binding is



**Fig. 7.** Analysis of SRE binding and ternary complex formation by core<sup>SRF</sup> point mutant chimaeras. (a) Expression of the proteins in reticulocyte lysates was compared after electrophoresis through 11% SDS-polyacrylamide gels and subsequent autoradiography. (b) Analysis by gel retardation of complexes formed with cc7A-derived point mutant chimaeras and the SRE in the presence (+) and absence (-) of p62<sup>TCF</sup>. The different complexes are identified by labelled arrows. (c) Parallel analysis of complexes formed by cc56A-derived point mutant chimaeras with the SRE in the presence and absence of p62<sup>TCF</sup> as indicated. The positions of the different complexes are identified by labelled arrows.

borne out by the results, which also provide new insights into the specific interactions between p67<sup>SRF</sup> and p62<sup>TCF</sup> that determine complex formation.

#### **Evidence for DNA contacts by p62<sup>TCF</sup>**

It is known that ternary complex formation is associated with extended methylation interference and protection patterns at the SRE and that mutations in the upstream flank of the SRE compromise p62<sup>TCF</sup> binding (Shaw *et al.*, 1989a; Rivera *et al.*, 1990; Graham and Gilman, 1991). From the DNase I footprinting experiments with the truncated form of p67<sup>SRF</sup>, it seemed unlikely that the extension seen at the upstream border of the footprint with p62<sup>TCF</sup> resulted from a conformational change by p67<sup>SRF</sup> upon binding of p62<sup>TCF</sup>, but rather from direct contacts made by p62<sup>TCF</sup> with the DNA. This inference is supported by the UV-mediated DNA-protein cross-linking experiments, which allowed purified p62<sup>TCF</sup> bound in a ternary complex with either full-length or truncated p67<sup>SRF</sup>, to be cross-linked to radio-labelled, but otherwise unmodified, DNA. In this instance the two forms of p67<sup>SRF</sup> served as gratuitous positive controls for cross-linking. Taken with the previous results showing that mutations in the left flank of the SRE abolish ternary complex formation (Shaw *et al.*, 1989a; Rivera *et al.*, 1990; Graham and Gilman, 1991), this result indicates

that one parameter governing ternary complex formation is direct contacts made by p62<sup>TCF</sup> with the DNA.

It has been reported recently that members of the *ets*-like family of proteins are the active components in protein fractions first identified and referred to as p62<sup>TCF</sup> (Hipskind *et al.*, 1991; Dalton and Treisman, 1992). Other members of this extensive family of transcription factors have been demonstrated to bind DNA and this function is known to be encoded by the *ets*-box itself (Boulukos *et al.*, 1989; Wasylyk *et al.*, 1990; Fisher *et al.*, 1991). This is entirely consistent with the conclusion that p62<sup>TCF</sup> makes direct contacts with the SRE.

#### **SRE-bound core<sup>ARG</sup> fails to direct p62<sup>TCF</sup> binding**

In the absence of p67<sup>SRF</sup>, no interaction of p62<sup>TCF</sup> with the SRE can be detected. Thus p67<sup>SRF</sup> must provide a function required by p62<sup>TCF</sup> for DNA binding. In this respect the DNA binding domain of p67<sup>SRF</sup> is sufficient as it promotes ternary complex formation efficiently *in vitro* (Schröter *et al.*, 1990). It was conceivable that p67<sup>SRF</sup> might allow p62<sup>TCF</sup> to bind by a non-specific mechanism, which would explain why the smallest fragment of p67<sup>SRF</sup> to retain DNA binding activity still functions in this respect. If this were so, other DNA binding proteins might substitute for p67<sup>SRF</sup>, given an appropriately positioned DNA binding site. This



is not the case as the DNA-binding domain of ArgRI, which is closely related to that of p67<sup>SRF</sup> (Norman *et al.*, 1988), binds to the SRE thus supporting the contention that the two DNA binding domains share extensive structural homology, but is not able to direct ternary complex formation. It has been previously reported that whereas full-length ArgRI is able to bind to the SRE, a core domain of ArgRI cannot bind in an analogous fashion to core<sup>SRF</sup> (Mueller and Nordheim, 1991). This conflicting result may be due to the use of an alternative C-terminal truncation to the one chosen for core<sup>ARG</sup> described in this paper.

#### **p62<sup>TCF</sup> recognises extensive core<sup>SRF</sup> structure**

core<sup>ARG</sup> is unable to promote ternary complex formation by p62<sup>TCF</sup>, which is consistent with the notion that specific interactions occur between p62<sup>TCF</sup> and p67<sup>SRF</sup>. This observation provided a means to examine how core<sup>SRF</sup> is able to mediate the interaction. Initially chimaeric core<sup>SRF</sup> proteins containing fragments of core<sup>ARG</sup> allowed the interaction with p62<sup>TCF</sup> to be mapped, with low resolution, to the C-terminal half of core<sup>SRF</sup>. As the chimaeras cc56A and cc79A both show impaired p62<sup>TCF</sup> binding, it seemed that the interaction might require either a restricted motif in the region of core<sup>SRF</sup> compromised in both chimaeras, or more extensive structure embodied in the C-terminal portion of core<sup>SRF</sup>.

The results obtained with two complementary sets of point mutant chimaeras show that extended structure in core<sup>SRF</sup> is required for ternary complex formation. Although several point mutant chimaeras displayed reduced ternary complex formation, only the point mutant chimaera 56A/QM > EV retained the ability to bind the SRE, but could not bind p62<sup>TCF</sup> (Figure 6; Figure 7c, lanes 3 and 4). Notably, none of the complementary cc7A point mutant chimaeras, all of which contain the QM > EV exchanges, identified residues among those exchanged 'en bloc' in the cc56A point mutant chimaeras that similarly abolished p62<sup>TCF</sup> binding.

The impaired ternary complex formation observed with the point mutant chimaeras SH > AL/7A, STH > ASL/7A and E190N/7A, was unlikely to have been due solely to weak DNA binding as p62<sup>TCF</sup>, which has been shown to bind synergistically to p67<sup>SRF</sup> and the SRE (Schröter *et al.*, 1990), complexes efficiently with mutants of core<sup>SRF</sup> displaying reduced DNA binding affinity. A limiting amount of p62<sup>TCF</sup> recruits as much of the mutant proteins as of core<sup>SRF</sup> into a ternary complex (A.D.Sharrocks, unpublished observations). However, this point is incidental to the result that ternary complex formation can tolerate the introduction of numerous core<sup>ARG</sup> residues into core<sup>SRF</sup>. The one core<sup>SRF</sup> mutant generated that specifically lacked the ability to direct ternary complex formation by p62<sup>TCF</sup> contained 14 exchanges spread across almost one-third of the protein. Furthermore, other point mutant chimaeras identified compensatory exchanges (S208 to E and Q216 to R) that rescued the weaker complex formation of cc56A. In the context of core<sup>SRF</sup>, these point mutations all had little or no effect on ternary complex formation. Thus, alterations distributed over a region encompassing >40% of core<sup>SRF</sup> were seen to affect ternary complex formation by p62<sup>TCF</sup>.

It was shown recently that ArgRI (Arg80) could be engineered to direct ternary complex formation by p62<sup>TCF</sup> by replacing an eight amino acid segment of ArgRI with the corresponding segment from the DNA binding domain of

p67<sup>SRF</sup> (Mueller and Nordheim, 1991). Similarly, replacement of the same segment of p67<sup>SRF</sup> with the analogous fragment of the yeast protein MCM1 was shown to confer upon core<sup>SRF</sup> the ability to direct ternary complex formation with the yeast protein STE12 as well as p62<sup>TCF</sup>, a capacity otherwise specific to MCM1 (Primig *et al.*, 1991). From these results it was inferred that these residues in a C-terminal subdomain of core<sup>SRF</sup> play a critical role in mediating ternary complex formation with p62<sup>TCF</sup> (Mueller and Nordheim, 1991). This is inconsistent with the results presented here and the conclusion that an extended structure is required for the interaction of p62<sup>TCF</sup> and warrants clarification.

Throughout much of the C-terminal region of their DNA binding domains, p67<sup>SRF</sup> and MCM1 are more similar to each other than to ArgRI at the amino acid sequence level. For example, the segment of ArgRI in cc56A and the segment of core<sup>SRF</sup> that it replaces, differ at 12/27 residues, whereas MCM1 and core<sup>SRF</sup> differ at only six residues over the same region. In contrast, in the region encompassing the amino acids proposed to be critical for ternary complex formation in both MCM1 and p67<sup>SRF</sup> (Mueller and Nordheim, 1991), ArgRI is more like MCM1 (6/19 differences) than p67<sup>SRF</sup> (10/19). Indeed, the residues in MCM1 described as critical for interaction with STE12 (TRSTPKFE) are common to ArgRI (TRTTPKLE) with two exceptions, one of which is a conservative change (S to T) and the other (L139) is present in p67<sup>SRF</sup> (L202). Nevertheless, ArgRI is unable to interact with STE12 (Mueller and Nordheim, 1991), which strongly implies that another characteristic of ArgRI is incompatible with ternary complex formation. For the same reason, the amino acid exchanges between the core domains of MCM1 and p67<sup>SRF</sup>, which allow core<sup>SRF</sup> to interact with STE12, replace the amino acid residues in p67<sup>SRF</sup> described as critical for p62<sup>TCF</sup> binding with ArgRI residues because MCM1 and ArgRI differ at only one of these positions and this is a conservative difference (S to T). But the specificity of core<sup>SRF</sup> does not change to that of core<sup>ARG</sup> as there is no loss of ternary complex formation with p62<sup>TCF</sup> (Mueller and Nordheim, 1991). These results do not demonstrate that the amino acids in this region of core<sup>SRF</sup> are critical for ternary complex formation with p62<sup>TCF</sup>.

In summary, although ArgRI can be engineered to bind p62<sup>TCF</sup> weakly by replacement of three ArgRI residues with their counterparts from core<sup>SRF</sup>, more extensive modification is required to abolish the interaction of core<sup>SRF</sup> with p62<sup>TCF</sup>. The point mutant chimaera described in this study that is unable to interact with p62<sup>TCF</sup>, contains 14 exchanges spread across one-third of the protein. Fewer changes were ineffective, indicating that an extended structure present in core<sup>SRF</sup> is recognised by p62<sup>TCF</sup>, which constitutes the second component required for the specific interaction of p62<sup>TCF</sup> at the SRE.

#### **Possible structure and function for core<sup>SRF</sup> interface with p62<sup>TCF</sup>**

Structural predictions assign a high degree of  $\beta$ -strand to the C-terminal half of the DNA binding domain of p67<sup>SRF</sup> (Norman *et al.*, 1988; Pollock *et al.*, 1991). A similar structural assignment is obtained for MCM1 but, notably in the case of ArgRI, a permuted structure is assigned (A.D.Sharrocks and P.E.Shaw, unpublished observations).

C.D. measurements on core<sup>SRF</sup> are consistent with this assignment of  $\beta$ -strand (A.D.Sharrocks and P.E.Shaw, unpublished results). The region of core<sup>SRF</sup> (and MCM1) directed away from the DNA may thus consist of  $\beta$ -strands with intervening turns. Alternating residues in the  $\beta$ -strands would present side groups on the surface of core<sup>SRF</sup> and these, in conjunction with residues in the turns, might provide an extended surface amenable to interaction with p62<sup>TCF</sup>.

A recent report has shown that a region of the *c-Ets-1* protein serves as an intramolecular regulator for DNA binding (Lim *et al.*, 1992). This region is lacking in the *v-ets* oncogene. Similarly, C-terminal truncations of SAP-1, a protein akin to p62<sup>TCF</sup>, enable the protein to bind DNA independently of p67<sup>SRF</sup>, again suggestive of a regulatory domain governing DNA binding (Dalton and Treisman, 1992). Conceivably, the p67<sup>SRF</sup> structure required for ternary complex formation by p62<sup>TCF</sup> serves to overcome such a negative regulatory mechanism. This suggests a general means whereby the specificity of interactions between structurally constrained DNA binding domains and simple recognition sequences can be enhanced (Smith and Johnson, 1992). The point mutant chimaera described in this study that is unable to interact with p62<sup>TCF</sup> may prove helpful both in characterizing such a mechanism and in understanding the importance of p62<sup>TCF</sup> for the regulation of *c-fos* expression.

## Materials and methods

### DNase I footprinting

DNase I footprinting was performed as described (Lee *et al.*, 1987) but with the modification incorporated previously (Shaw *et al.*, 1989b). The DNA probe used spanned the *c-fos* promoter from positions -360 to +70 (the *Pst*I-HindIII fragment of -EPF) and was prepared and labelled as described previously (Shaw *et al.*, 1989b).

p67<sup>SRF</sup> was enriched by wheatgerm agglutinin chromatography as described (Schröter *et al.*, 1990). An enriched fraction of p62<sup>TCF</sup> and vaccinia-expressed 90-244<sup>SRF</sup> were provided by H.Schröter and R.A.Hipskind respectively.

### UV-mediated DNA-protein cross-linking

The radiolabelled DNA probe was prepared in a two step fill-in reaction with the Klenow fragment of DNA polymerase. Oligonucleotides with the following nucleotide sequences: 5' GCCGTGGAAACCTGCTGACGC-AAGAGTCCTAATGGACATCCTGTGTAAGG and 5'CCTTACACA, were allowed to anneal and served as template and primer, respectively, in the presence of [ $\alpha$ -<sup>32</sup>P]dGTP and Klenow enzyme. After incubation at 4°C for 1 h under standard reaction conditions, which allowed the incorporation of maximally two radiolabelled guanosine residues, unincorporated nucleotides were removed by gel filtration through Bio-Gel P30 resin (Bio-Rad) and the radiolabelled oligonucleotide duplex was precipitated in ethanol. The synthesis of a completely double-stranded, internally labelled duplex was achieved in a second reaction in the presence of all four unlabelled deoxyribonucleotide triphosphates.

p67<sup>SRF</sup>, 90-244<sup>SRF</sup> and p62<sup>TCF</sup> were allowed to form complexes with the DNA probe under conditions described previously for gel retardation (Schröter *et al.*, 1987). The samples were pipetted onto microscope slides with cavities and irradiated with UV for 15 min on ice. 75% of the sample was recovered, treated with DNase I (0.5 mg/ml) for 6 min and precipitated with 2 vol of 20% TCA. After washing with acetone, the samples were dissolved in Laemmli buffer and analysed by SDS-PAGE (10% acrylamide) and subsequent autoradiography. (The remaining 25% of each sample was analysed by gel retardation.)

### Plasmid constructs

*pcore*<sup>SRF</sup>. This was a gift from C.G.F.Mueller and has been described elsewhere (Mueller and Nordheim, 1991).

*pcore*<sup>ARG</sup>. This is a T3-directed expression clone for the 100 amino acid core of ArgRI, constructed with PCR-derived DNA amplified from

yeast genomic DNA with the following primer pair: 5'CAACTCCTG-CAGCCACCATGACGAAAATAAGG; 5'CGGCGACTAGACGG-TAGCGTC and cloned as a *Pst*I-SpeI fragment into pBluescript. The coding sequences were subsequently excised as a HindIII-NoI fragment and inserted into *pcore*<sup>SRF</sup> in place of *pcore*<sup>SRF</sup> coding sequences.

*pArgC*. This was constructed by ligating the N-terminal HindIII-XhoI fragment of *pcore*<sup>ARG</sup> with the C-terminal XhoI-Sau3AI fragment of ArgRI (excised from pQKS1, a gift from E.Dubois) into HindIII-BamHI cut *pcore*<sup>SRF</sup>, in place of *pcore*<sup>SRF</sup> coding sequences, thus adding the 11 C-terminal amino acids of ArgRI to *pcore*<sup>ARG</sup>.

*pcc14A*. This was constructed by replacing the C-terminal SacI fragment of *pcore*<sup>ARG</sup> with the C-terminal SacI fragment of *pcore*<sup>SRF</sup>.

*pNcc36A*. This was constructed from PCR-derived DNA, amplified from *pcore*<sup>ARG</sup> with the following primer pair: 5'GGTACATCGATAATA-AGACC, 5'CACCGGCTGCAGCTTAGCGC and cloned into pBluescript as a *Cl*aI-PstI fragment. C-terminal sequences from *pcore*<sup>SRF</sup> were added as a *Pst*I-SacI fragment and in a third step, N-terminal sequences of p67<sup>SRF</sup> were added as a HindIII-TaqI fragment. This clone is described as it was used as an intermediate in the construction of other clones used in the paper.

*pNcc34A*. This was constructed in an analogous fashion to *pcc14A* by replacing the C-terminal SacI fragment of *pNcc36A* with the C-terminal SacI fragment of *pcore*<sup>SRF</sup>.

*pcc56A*. This was constructed by replacing the C-terminal SacI fragment of *pcore*<sup>SRF</sup> with the C-terminal SacI fragment of *pNcc36A*.

*pcc57A*. This was constructed by replacing the N-terminal HindIII-XhoI fragment of *pAR>TP/7A* with the equivalent fragment of *pcc56A*.

*pcc59A*. This was constructed by replacing the C-terminal SacI fragment of *pcore*<sup>SRF</sup> with the equivalent fragment of *pArgC*.

*Pcc7A*. This was constructed by replacing the N-terminal HindIII-SphI fragment of a *pcore*<sup>SRF</sup> derivative into which a SphI site was introduced (as described for *pcc89A* below), with the analogous N-terminal fragment from *pcc79A*.

*pcc79A*. This was constructed by replacing the N-terminal HindIII-XhoI fragment of *pArgC* with the analogous fragment from *pcore*<sup>SRF</sup> into which a XhoI site was introduced by PCR amplification from *pcore*<sup>SRF</sup> with the following primer: 5'CATGGGCTCGAGTTTTTCGGG.

*pcc89A*. This was constructed by replacing the N-terminal HindIII-SphI fragment of *pArgC* with the analogous *pcore*<sup>SRF</sup> fragment into which a SphI site was introduced by PCR amplification with the following primer: 5'AGTGGATCCTATGGCGAGTTGAGGCATGCTGAATC.

*pT181A/7A*, *TQ>AK/7A*, *pE190N/7A*, *pA198T/7A*, *pR200P/7A* and *pAR>TP/7A*. These were constructed by inserting PCR derived HindIII-XhoI fragments from the corresponding *pcore*<sup>SRF</sup> point mutants (not shown; Sharrocks *et al.*, submitted) in place of the equivalent fragment of *pcc7A*.

*pST>AS/7A*, *SH>AL/7A* and *pSTH>ASL/7A*. These were constructed by replacing the SacI fragment of *pcore*<sup>SRF</sup> with equivalent fragments derived from *pcc7A* in a two-step PCR procedure with the following mutagenic primer: 5'GGTGGCCGCTGAG(AT)CAGGCC(AT)TGTG as described previously (Sharrocks and Shaw, 1992).

*p56A/Q203E* and *p56A/QM>EV*. These were constructed by inserting PCR-derived XhoI-XbaI fragments from the corresponding *pcore*<sup>SRF</sup> point mutants (not shown; Sharrocks *et al.*, submitted) in place of the equivalent fragment of *pcc59A*.

*p56A/T207R*, *p56A/S208E* and *p56A/O216R*. These were constructed by replacing the HindIII-PstI fragment of *pcor56A* with fragments excised from the corresponding *pcore*<sup>SRF</sup> point mutants (not shown; Sharrocks *et al.*, submitted).

All clones derived from PCR-amplified DNA were verified by sequence analysis.



**In vitro translation and gel retardation analysis**

All plasmids were linearized 3' of the gene and transcribed with T3 RNA polymerase (Stratagene) according to the instructions supplied with the enzyme. Translation reactions were performed in 10  $\mu$ l reactions containing 5  $\mu$ l rabbit reticulocyte lysate (Promega) according to the manufacturer's instructions.

Gel retardation analyses were performed as described previously (Schröter *et al.*, 1987) except that 45  $\mu$ g/ml poly(dIC) was present in each binding reaction. The enriched fragment of p62<sup>TCF</sup> was provided by H.Schröter and its preparation has been described elsewhere (Schröter *et al.*, 1990). The oligonucleotide probe sequence is presented in Figure 1a.

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