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Two distinct cellular phosphoproteins bind to the c-*fos* serum response element

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Induction of c-*fos* transcription by serum growth factors requires a 20 bp DNA sequence termed the serum response element (SRE). The SRE is a binding site for a nuclear protein, serum response factor (SRF). There is as yet no direct evidence that links SRF activity to SRE function. We show here that cells contain a second specific SRE-binding protein. It is distinguished from SRF by its size, chromatographic properties, binding specificity and contacts with nucleotides in the SRE. The presence in cells of two distinct proteins that can bind independently to the SRE suggests that the multiple functions of the SRE may be mediated by distinct proteins.

Key words: c-*fos*/DNA-binding assays/DNA-binding proteins/serum response element/signal transduction

Introduction

Transcription of the c-*fos* proto-oncogene is induced within minutes of stimulation of cell surface growth factor receptors (Greenberg and Ziff, 1984; Kruijer *et al.*, 1984; Muller *et al.*, 1984). Induction is rapid, transient and can occur in the absence of new protein synthesis (Greenberg *et al.*, 1986). c-*fos* transcription is triggered by activation of several intracellular signal transduction pathways, each of which can independently activate transcription of the gene (for review see Curran, 1988). How these different signals are transmitted to the c-*fos* gene is not yet known, but it is clear that multiple sequence elements flanking the gene serve as targets for these signals. One such element, the serum response element (SRE), is required for the response of transfected c-*fos* genes to whole serum (Treisman, 1985, 1986; Gilman *et al.*, 1986; Greenberg *et al.*, 1987; Gilman, 1988) and to purified growth factors (Fisch *et al.*, 1987; Buscher *et al.*, 1988; Gilman, 1988; Sheng *et al.*, 1988; Stumpo *et al.*, 1988). The SRE appears to be the target for multiple intracellular signalling pathways. It is required for response to activators of protein kinase C (Fisch *et al.*, 1987; Buscher *et al.*, 1988; Gilman, 1988; Sheng *et al.*, 1988; Stumpo *et al.*, 1988) and for growth-factor-induced signals independent of protein kinase C (Gilman, 1988). It is not required for response to cAMP or calcium-dependent signals (Buscher *et al.*, 1988; Gilman, 1988; Sheng *et al.*, 1988).

The SRE is a binding site for a specific DNA-binding activity present in nuclear extracts of a variety of cell-types (Gilman *et al.*, 1986; Prywes and Roeder, 1986; Treisman, 1986; Greenberg *et al.*, 1987). This activity, termed SRF, is generally recovered in similar amounts from all cell extracts, regardless of the state of c-*fos* transcription (Gilman *et al.*, 1986; Prywes and Roeder, 1986; Treisman, 1986; Greenberg *et al.*, 1987; Sheng *et al.*, 1988; Stumpo *et al.*, 1988). SRF has been purified from HeLa cells and found to be due to a polypeptide of apparent molecular mass 67 kd (Schroter *et al.*, 1987; Treisman, 1987) or 62 kd (Prywes and Roeder, 1987). Oligonucleotides that constitute *in vitro* binding sites for SRF restore serum responsiveness to both deleted c-*fos* and heterologous promoters (Treisman, 1986; Greenberg *et al.*, 1987; Mohun *et al.*, 1987; Gilman, 1988; Sheng *et al.*, 1988; Siegfried and Ziff, 1989). These observations argue that the SRE operates *in vivo* at least in part as a positively acting element. This view is supported by microinjection experiments in which injected SRE oligonucleotides block c-*fos* induction by serum (Gilman *et al.*, 1988). Yet the serum response conferred by SRE oligonucleotides on heterologous promoters is both transient and superinducible with cycloheximide (Siefried and Ziff, 1989), suggesting that the SRE is also a target for transcriptional repression following serum stimulation. Moreover, Leung and Miyamoto (1989) have recently shown that certain SRE mutations that interfere with SRF binding *in vitro* appear to relieve this repression. Thus, *in vivo* the SRE is a target for both positively and negatively acting factors. Proper transcriptional response via the SRE may therefore involve multiple SRE-binding proteins.

To determine whether cells contain additional SRE-binding proteins that were previously overlooked in studies that relied on the gel mobility-shift assay, we have fractionated nuclear extracts and used a DNA-affinity precipitation assay (Franza *et al.*, 1987) to analyze partially purified fractions. Using this procedure, we have identified a new cellular protein that binds to the c-*fos* SRE. This protein has several properties that are quite distinct from those of SRF, which we also identify in these assays. These observations raise the possibility that regulation of c-*fos* expression through the SRE may involve multiple SRE-binding proteins.

Results

Probes used in the DNA-binding assays

Figure 1 shows the DNA sequence of the mouse c-*fos* gene in the region encompassing the SRE and of a set of synthetic SRE oligonucleotides used in these studies. The wild-type oligonucleotide (SRE-WT, Figure 1) carries the entire 20 bp element together with linker DNA that matches wild-type c-*fos* sequence 1 bp upstream and 2 bp downstream. The mutant oligonucleotide (SRE-PM) differs from wild-type in four positions which correspond to contact points for SRF as defined by methylation interference assays (Gilman *et al.*, 1986; Treisman, 1986). These mutations substantially reduce

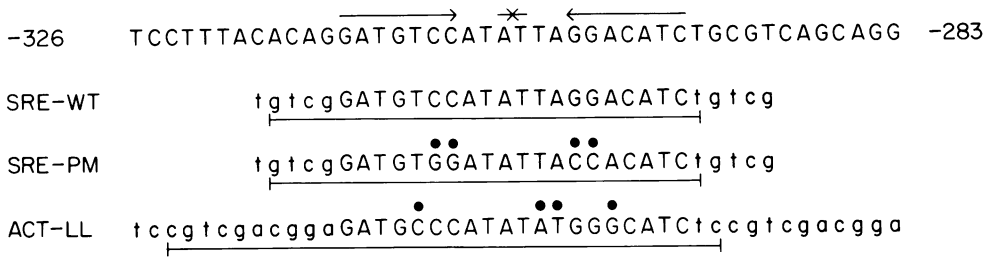

Fig. 1. Sequences of SRE oligonucleotides. The top line shows the sequence of the mouse c-*fos* gene from −326 to −283. The dyad symmetry that comprises the SRE is marked. The bottom three sequences represent the SRE sequences contained within the oligonucleotides used for both the mobility-shift and DNA-affinity precipitation assays. Upper case letters represent sequences corresponding to the SRE. Lower case letters are linker sequences that flank the SREs when the oligonucleotides are ligated. Filled circles mark nucleotides that differ from wild-type sequence. The lines beneath the sequences represent the repeating unit in ligated arrays (see Materials and methods for details).

SRF binding *in vitro* and SRE function *in vivo* (Gilman, 1988). The third sequence (ACT-LL) corresponds to a synthetic symmetrical element derived from the *Xenopus* γ-actin SRE (Mohun *et al.*, 1987) that is a strong binding site for SRF (Treisman, 1987). For the mobility-shift assays, single copies of the oligonucleotide elements inserted into the pUC119 polylinker were excised as larger restriction fragments. For the DNA-affinity precipitation assays, the oligonucleotides were annealed and ligated into tandem arrays. The extent of the repeating unit in these arrays is shown by the lines beneath each sequence in Figure 1.

Two cellular proteins bind specifically to the c-fos SRE

While screening a series of cell lines for SRF activity, we discovered that the human T-lymphoblast line H9 was highly enriched for SRF relative to HeLa cells. A nuclear extract prepared from H9 cells was applied to a heparin−agarose column. At 200 mM KCl, >90% of the extract protein flowed through the column, and we eluted bound proteins with a linear KCl gradient from 200 to 600 mM. Figure 2A shows the profile of this column. When assayed using our standard mobility-shift conditions, a single peak of SRE-specific mobility-shift activity was observed, eluting at ~400 mM KCl peaking in fractions 73−76 (arrowhead, Figure 2B). The other complexes formed in this assay were non-specific and were observed identically with a probe carrying a mutant SRE (Figure 1, data not shown).

We next assayed fractions from the heparin−agarose column using a DNA-affinity precipitation assay (Franza *et al.*, 1987). For this assay, we covalently coupled biotin molecules to oligonucleotides encoding either a wild-type or mutant SRE. The biotinylated oligonucleotides were incubated with column fractions and recovered by association with streptavidin protein coupled to agarose beads. Proteins bound to the biotinylated oligonucleotides were released by boiling in buffer containing SDS, resolved by SDS−PAGE and visualized by silver staining. Figure 2C shows the result of such an assay of selected heparin−agarose fractions. In fractions 74 and 76, we recovered significant amounts of a 67 kd protein with the wild-type SRE oligonucleotide but not with the mutant (lanes 8−11). Smaller amounts of the protein were specifically recovered from fractions 72 and 78 (lanes 6, 7, 12 and 13). Other proteins in these fractions were recovered equally with both oligonucleotides, indicating that they were not specific for the SRE. These fractions corresponded to those containing specific SRE-binding activity in the standard mobility-shift assay (Figure 2B). This observation is consistent with the assignment of the SRE-binding activity to the 67 kd polypeptide, the size previously reported for SRF (Schroter *et al.*, 1987; Treisman, 1987).

In fractions eluting at lower salt, in which no specific SRE-binding activity was observed by the standard mobility-shift assay, the DNAP assay detected a 62 kd protein. This protein was specifically bound by the wild-type SRE oligonucleotide and not by the mutant site in fractions 68 and 70 (Figure 2C, lanes 2−5), fractions that were devoid of SRF activity. Again, several other proteins in these fractions were non-specifically bound, including one that comigrated with the 62 kd protein. This observation suggested that these fractions contained a protein that specifically bound the SRE, yet failed to form stable complexes with the SRE in our standard mobility-shift assay. When we modified the conditions of the mobility-shift assay, however, we detected a specific protein−DNA complex in the fractions enriched for the 62 kd protein (Figure 2D, lanes 2−6). In this assay, the complete binding reaction was incubated for 10 min prior to addition of the labeled probe, and gel electrophoresis was carried out in Tris−borate rather than Tris−glycine buffer. In other experiments, we noted that the two SRE-binding activities also had distinct magnesium and salt optima. These differences may explain why the new activity was not detected under conditions optimal for SRF binding activity. Nevertheless, under the conditions employed in Figure 2D, both complexes formed well. Both of the binding activities and proteins were detected in a variety of cell extracts, including HeLa (data not shown). We conclude from these experiments that there are at least two cellular proteins that specifically bind the c-*fos* SRE. One of these is the activity previously described (Gilman *et al.*, 1986; Prywes and Roeder, 1986; Treisman, 1986; Greenberg *et al.*, 1987), and we confirm its identity as a 67 kd protein (Schroter *et al.*, 1987; Treisman, 1987). The second activity has not been previously noted, and we tentatively assign this activity to a 62 kd polypeptide.

The 62 and 67 kd proteins have distinct binding specificities

To confirm the specificities of the interactions we observed and to support our assignments of the 62 and 67 kd proteins to the corresponding mobility-shift activities, we performed mobility-shift and DNAP assays using the three different SRE-derived probes shown in Figure 1. Figure 3A shows

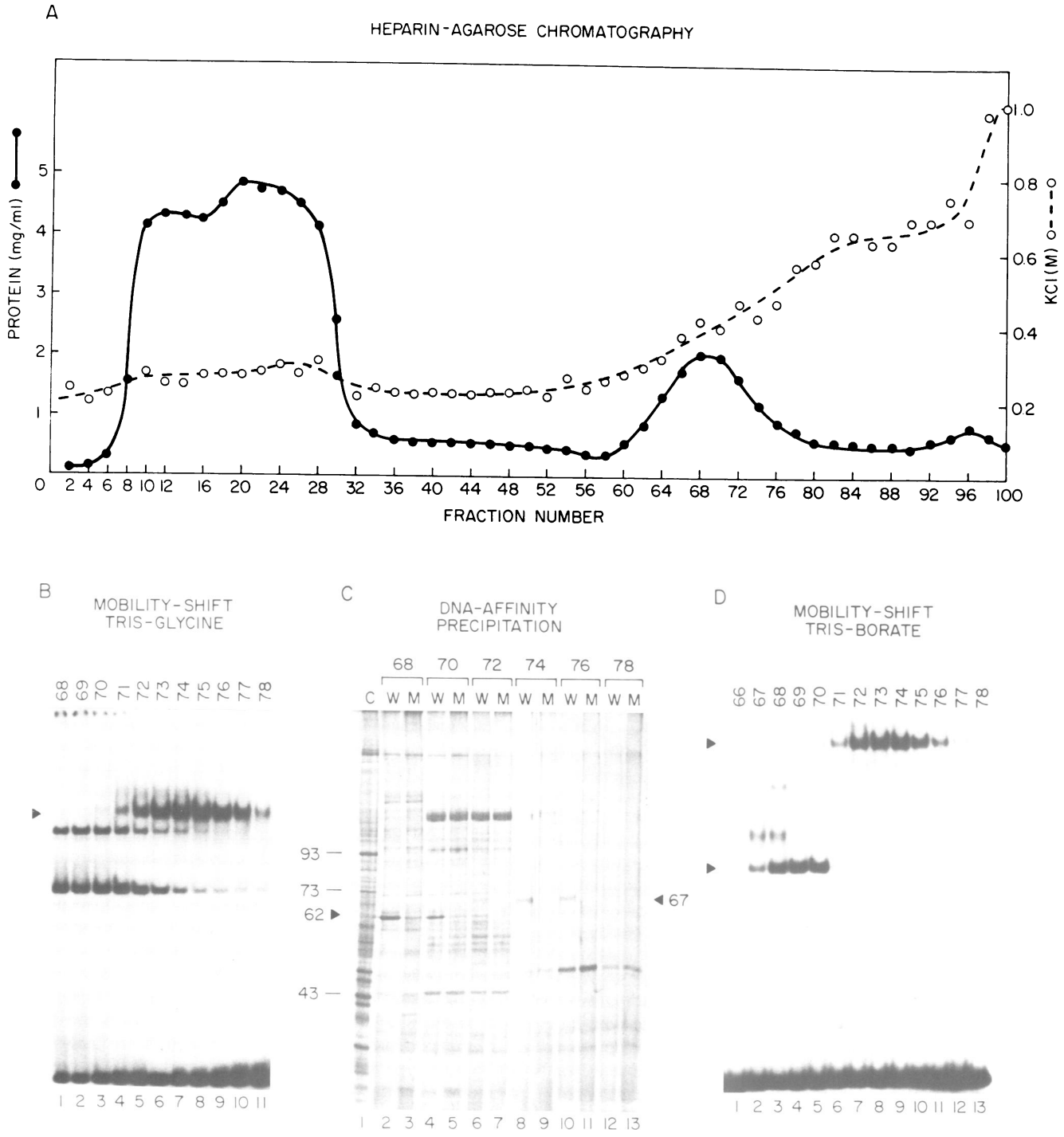

Fig. 2. Heparin–agarose chromatography of H9 cell nuclear extract. (**A**) Column profile. The solid line and filled circles represent the protein concentrations in the corresponding fractions. The broken line and open circles represent the estimated KCl concentrations in the fractions as determined by conductivity measurements. (**B**) Mobility-shift assay of peak fractions. The assay was carried out without a preincubation step, using Tris–glycine electrophoresis buffer. The probe was the wild-type SRE. The arrow indicates the specific complex that formed in these assays. The other bands formed equally well in a parallel assay using a mutant SRE probe (data not shown). (**C**) DNA-affinity precipitation assay of selected fractions from the heparin–agarose column. The indicated fractions were incubated with either ligated wild-type SRE oligonucleotide ('W', **lanes 2, 4, 6, 8, 10** and **12**) or mutant SRE ('M', **lanes 3, 5, 7, 9, 11** and **13**). The arrows indicate proteins specifically recovered with the wild-type oligonucleotide. **Lane 1** contains H9 whole-cell extract. The indicated markers are hsp90 (93 kd), hsp70 (73 kd) and actin (43 kd). Protein bands were visualized by siver staining. (**D**) Mobility-shift assay performed using the preincubation protocol and Tris–borate electrophoresis buffer described in Materials and methods. The probe was the wild-type SRE. The arrows indicate specific complexes that did not form in a parallel assay using the mutant SRE probe (data not shown).

that the SRF activity in fraction 74 bound strongly to the natural c-*fos* SRE and to the synthetic actin site but not to the SRE carrying point mutations that biologically inactivate the element (lanes 4–6). The new activity in fraction 69 bound strongly to the natural c-*fos* SRE and failed to bind detectably to the mutant SRE (lanes 1–3). In contrast to SRF, it bound only weakly to the synthetic actin SRE.

Similar results were obtained with the DNAP assay. When

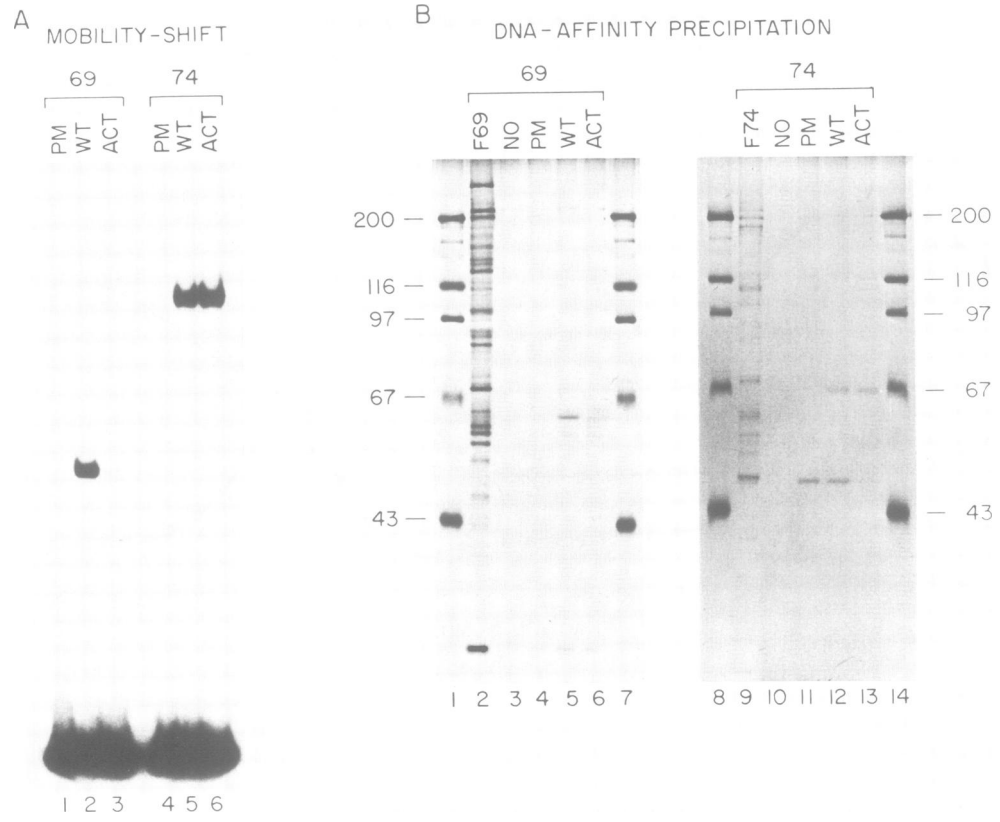

Fig. 3. Binding specificities of the SRE-binding proteins. (**A**) Mobility-shift assays of heparin–agarose fractions 69 (**lanes 1–3**) and 74 (**lanes 4–6**). The probes used were the mutant SRE (**lanes 1** and **4**), the wild-type SRE (**lanes 2** and **5**) and the synthetic actin SRE (**lanes 3** and **6**). (**B**) DNA-affinity precipitation assays of fractions 69 (**left panel**) and 74 (**right panel**). In each panel, **lanes 1** and **7** are molecular weight markers; **lane 2** is 1 μl of the corresponding heparin–agarose fraction; **lane 3** is an assay with no added biotinylated oligonucleotide; **lane 4** is an assay using the mutant SRE oligonucleotide; **lane 5** is an assay using the wild-type SRE oligonucleotide; **lane 6** is an assay using the symmetric actin SRE oligonucleotide. Protein bands were visualized by silver staining.

fraction 69 was assayed, we observed strong recovery of the 62 kd protein with the natural c-*fos* SRE (Figure 3B, lane 5) and weaker recovery with the synthetic actin SRE (lane 6). No 62-kd protein was detected in assays performed in the absence of biotinylated oligonucleotide or with the mutant SRE (lanes 3 and 4). Several faint protein bands bound non-specifically to all three oligonucleotides. When we assayed fraction 74, we recovered similar amounts of the 67 kd protein with the natural c-*fos* SRE and the synthetic actin site (Figure 3B, lanes 12 and 13). No 67-kd protein was recovered with the mutant SRE or in the absence of oligonucleotide (lanes 10 and 11). In addition, we observed a 47 kd protein that was bound to both the mutant and wild-type SRE oligonucleotide and was therefore not specific for the SRE.

Thus, the two specific SRE-binding proteins behaved differently. SRF bound strongly to both the natural c-*fos* SRE and to the synthetic actin SRE, whereas the 62 kd protein bound more strongly to the natural site. Furthermore, neither bound detectably to the mutant SRE. We obtained identical results with both the mobility-shift and DNAP assays, reinforcing the interpretation that the 62 and 67 kd polypeptides are responsible for the mobility-shift activities present in their respective fractions.

The 62 and 67 kd proteins are present in the mobility-shift complexes

To confirm that the 62 and 67 kd proteins were indeed responsible for the complexes formed in the mobility-shift assay, we carried out a UV cross-linking experiment. Here, our strategy was to covalently cross-link bound proteins to isotopically labeled SRE probes prior to electrophoresis on a mobility-shift gel, and then to elute the DNA–protein complexes from the gel, treat them with DNase I and analyze the labeled proteins by SDS–PAGE. Therefore, we visualized directly and specifically only those cross-linked proteins present in the shifted complexes. The results of this experiment are shown in Figure 4. When proteins present in fraction 69, enriched for the 62 kd protein, were cross-linked to a wild-type SRE probe, eluted from the higher-mobility complex and analyzed by SDS–PAGE, we detected one major labeled protein of 62 kd (lane 2). This protein was not detected in the eluate from the same position of the gel in an adjacent reaction with a mutant SRE probe (lane 1). When proteins from fraction 74 were cross-linked to the SRE and eluted from the SRF complex, we detected a single labeled protein of 67 kd (lane 3). This protein was not recovered from the same region of an adjacent gel lane containing protein from a reaction with a mutant SRE probe. These observations strongly support the contention that a 67 kd protein is responsible for the SRF shift whereas a 62 kd protein is responsible for the new activity.

The 62 and 67 kd proteins make different contacts with SRE DNA

The previous experiments indicated that the two SRE-binding proteins have distinct biochemical properties and DNA-binding specificities. To determine whether they make

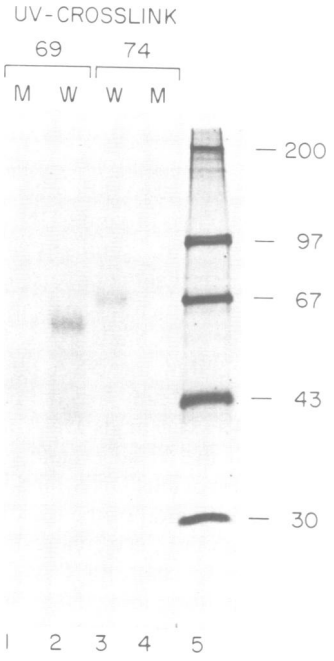

Fig. 4. UV cross-linking assays of the SRE-binding proteins. Proteins in fractions 69 (**lanes 1** and **2**) and 74 (**lanes 3** and **4**) were cross-linked to either a wild-type (**lanes 2** and **3**) or mutant (**lanes 1** and **4**) SRE-containing oligonucleotide. The irradiated binding reactions were loaded onto a mobility-shift gel. The shifted complexes were excised from the reactions using wild-type probe and the corresponding regions were excised from parallel reactions using a mutant SRE probe. Eluted proteins were treated with DNase I and analyzed by SDS–PAGE. Protein bands were visualized by autoradiography. **Lane 5** contains molecular weight markers.

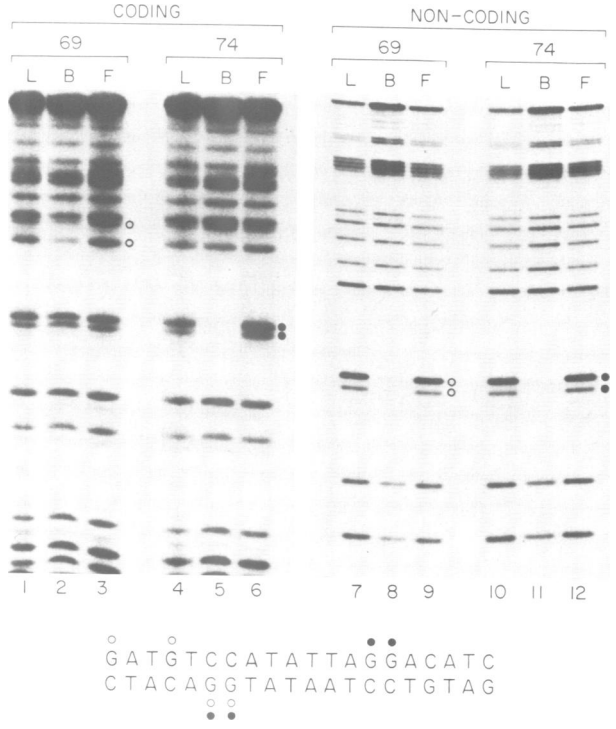

Fig. 5. DMS interference assays for the two SRE-binding proteins. The wild-type SRE fragment was 3′ end-labeled on the coding strand (**lanes 1–6**) or on the non-coding strand (**lanes 7–12**). Assays were performed using either fraction 69 (**lanes 1–3** and **7–9**) or fraction 74 (**lanes 4–6** or **10–12**). The guanidine ladders are shown in **lanes 1, 4, 7** and **10**. Bound fragments are shown in **lanes 2, 5, 8** and **11**. Free fragments are shown in **lanes 3, 6, 9** and **12**. Guanine methylations that interfered with protein binding are suppressed in the bound lane and are indicated by filled circles for the 67 kd protein and open circles for the 62 kd protein. The data are summarized at the bottom of the figure, showing the complete SRE dyad symmetry element.

different contacts with the SRE, we performed dimethyl sulfate (DMS) interference assays. For these assays, a wild-type SRE probe 3′ end-labeled on either the coding or non-coding strand was partially methylated on guanine residues with DMS and used in preparative mobility-shift assays. The DNA in the shifted complexes was eluted, cleaved with piperidine and analyzed on sequencing gels. DNA molecules carrying methyl groups that interfere with protein binding are excluded from the bound complexes, and the corresponding bands in the guanine sequence ladder are consequently under-represented. When DMS interference assays were carried out with an SRF-containing fraction, fraction 74, we observed intererence at a pair of adjacent guanines on the coding region (Figure 5, cf. lanes 5 and 6) and at a second pair of guanines symmetrically situated on the non-coding strand (Figure 5, lanes 11 and 12). This pattern of interference for SRF corresponds with that reported previously for SRF from other cell lines (Gilman *et al.*, 1986; Treisman, 1986; Schroter *et al.*, 1987). DMS interference assays of the 62 kd complex in fraction 69 revealed a different pattern of interference. On the coding strand, no interference was observed at the guanine pair that interferes with SRF binding. Instead, we observed partial interference at residues 10 and 13 bp upstream, and possible weak interference at the adjacent guanine (Figure 5, lanes 2 and 3). On the non-coding strand, the interferences obtained with the 62 kd complex in fraction 69 and with the SRF complex in fraction 74 were identical (Figure 5, lanes 8 and 9).

The results of these assays are summarized at the bottom of Figure 5. Whereas SRF makes symmetrical contacts on both halves of the SRE, the 62 kd protein binds to one half of the dyad symmetry. Moreover, it makes extra contacts with guanine residues on the coding strand not contacted by SRF. Thus, these proteins bind the SRE differently. The nature of these contacts together with the large difference in the mobility of the two protein–DNA complexes in mobility-shift gels (Figures 2D and 3A) suggests that these proteins may bind DNA with different quaternary structures. For example, the 62 kd protein may bind the SRE as a monomer or dimer, whereas SRF binds as a dimer or tetramer.

Both the 62 and 67 kd proteins are phosphorylated in vivo

Because protein kinases are implicated in many of the signal transduction pathways that activate c-*fos* transcription, we asked whether the SRE-binding proteins are phosphorylated *in vivo*. Whole cell extracts prepared from H9 cells labeled with [^{32}P]inorganic phosphate were assayed by DNAP using wild-type and mutant SRE probes. Figure 6 shows that the wild-type probe binds two prominent phosphoproteins of 67 and 62 kd (lane 3) that are not bound in the absence of oligonucleotide (lane 2) or with the mutant SRE probe (lane 4). We cannot confirm directly that these proteins are identical to the proteins we visualized by silver-staining in our heparin–agarose fractions. However, both phosphoproteins are bound by heparin–agarose at 200 mM KCl

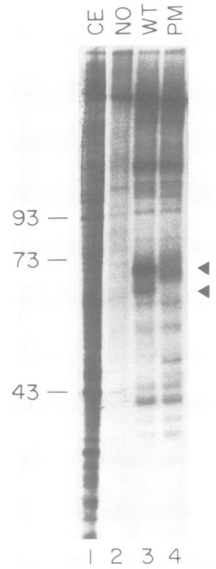

Fig. 6. The SRE-binding proteins are phosphoproteins. H9 cells were metabolically labeled with [^{32}P]inorganic phosphate for 75 min. A whole cell extract was prepared and assayed by DNA-affinity precipitation using no biotinylated oligonucleotide (**lane 2**), the wild-type SRE oligonucleotide (**lane 3**) and the mutant SRE oligonucleotide (**lane 4**). A portion of the extract is loaded in **lane 1**. The arrowheads indicate the phosphoproteins specifically recovered with the wild-type (**lane 3**). Note that in **lane 4** there are proteins specifically recovered with the mutant SRE.

(data not shown). We have not noted any obvious changes in phosphorylation of these proteins detectable on one-dimensional protein gels when we have compared extracts of cells induced for c-*fos* transcription (data not shown). Prywes *et al.* (1988) have recently reported that SRF is phosphorylated *in vivo*.

Discussion

We have shown that H9 cells contain two different proteins that bind specifically to the c-*fos* SRE. Using DNA-affinity precipitation assays and UV cross-linking, we have identified the polypeptide responsible for each activity. These proteins are separable by heparin–agarose chromatography. They have distinguishable affinities for different SRE derivatives and they make distinct contacts with nucleotides in the SRE. Moreover, optimal conditions for their binding to the SRE are different. These substantial dissimilarities in the biochemical properties of the proteins strongly suggest that these proteins are distinct translation products rather than related forms of the same polypeptide.

It is likely that the 67 kd protein we have detected here is responsible for the originally identified SRE binding activity now termed SRF (Gilman *et al.*, 1986; Prywes and Roeder, 1986; Treisman, 1986; Greenberg *et al.*, 1987). We base this conclusion on several observations. First, it gives the symmetrical guanine nucleotide contacts originally observed for this activity (Gilman *et al.*, 1986; Treisman, 1986). Second, it binds well to the synthetic symmetrical actin SRE, as reported for SRF (Treisman, 1987); in contrast to that report, however, we do not find the actin sequence to be a stronger binding site for SRF under new mobility-shift conditions. Third, its size corresponds to that reported for the purified protein by Treisman (1987) and by Schroter *et al.* (1987). Therefore, we confirm the identification of SRF as a 67-kd protein.

The 62 kd protein also binds specifically to the c-*fos* SRE and is abundant in nuclear extracts of all cell types thus far examined. We think it unlikely that it is a modified form of SRF because its biochemical properties are dramatically different. Furthermore, the large difference in the electrophoretic mobilities of the two protein–DNA complexes in mobility-shift gels despite the similarity in mass of the polypeptide chains suggests that the proteins bind DNA with different stoichiometries. Structural analysis of SRF based on its cDNA shows that SRF binds DNA as a dimer and that dimerization is required for DNA binding (Norman *et al.*, 1988). Thus, it is unlikely that the 62 kd protein is a modified form of SRF that binds DNA as a monomer.

Several properties of the 62 kd protein can account for its eluding detection in previous DNA-binding studies with the c-*fos* SRE. First, formation of the 62 kd mobility-shift complex is dependent on the electrophoresis buffer used in the assay. It fails to form stably in Tris–glycine buffer, whereas it is substantially more stable in half-strength Tris–borate buffer (Figure 2B and D). Second, binding of the 62 kd protein to the SRE is significantly more salt-sensitive than is binding of SRF, and assays carried out at 75–100 mM KCl, a concentration range optimal for SRF binding, fail to detect binding of the 62 kd protein. Third, formation of the 62 kd mobility-shift complex is strongly reduced by commonly used plasmid DNA competitors, but not by poly(dI-dC).poly(dI-dC). Fourth, because the 62 kd protein is quite abundant in crude nuclear extracts, small amounts of specific competitor DNAs may not be sufficient to reduce binding of the protein to a labeled SRE probe, thereby leading to its characterization as a 'non-specific' DNA-binding activity.

These biochemical properties, together with the distinct chromatographic behaviors and DNA-binding specificities of the two proteins, probably account as well for the absence of the 62 kd protein from the SRF preparations described by Treisman (1987) and by Schroter *et al.* (1987). By contrast, Prywes and Roeder (1987) described the purification of a HeLa cell SRE-binding protein with a mol. wt of ~62 kd. While their purification procedures could have resulted in substantial recovery of the 62 kd protein we have identified here, the similar sizes of the two SRE-binding proteins precludes a definitive conclusion about the identity of the protein purified by these workers. Nevertheless, our results firmly establish that there are in fact two distinct SRE-binding proteins in cells.

The 62 kd SRE-binding protein we have identified may be related to the MAPF1 activity described by Walsh and Schimmel (1987) that binds to the CC(A/T)$_6$GG element of the chicken skeletal α-actin gene. This element, found in all actin promoters, mediates muscle-specific expression of the α-actin genes (Bergsma *et al.*, 1986; Minty and Kedes, 1986; Muscat and Kedes, 1987; Walsh and Schimmel, 1988). It is similar in structure to the SRE, and in cytoplasmic actin genes these sites bind SRF and function indistinguishably from the c-*fos* SRE (Mohun *et al.* 1987). MAPF1 is expressed in a non-cell type-specific fashion. It binds asymmetrically to the CC(A/T)$_6$GG element, making contacts similar to those we have observed for the 62 kd protein on the SRE (Walsh and Schimmel, 1987). MAPF1 binds to the c-*fos* SRE with properties similar to the 62 kd

protein, and conversely SRF binds to the $CC(AT)_6GG$ elements of the muscle-specific actin gene (Phan-Dinh-Tuy *et al.*, 1988; Walsh, 1989). Therefore, the actin muscle-specific elements and the c-*fos* SRE appear to share structural similarities and common DNA-binding proteins, despite functional differences.

The SRE is a multi-functional element. It is the target of at least two different signal transduction pathways that activate c-*fos* transcription (Gilman, 1988). It confers basal activity on the c-*fos* promoter and on the *Xenopus* γ-actin promoter (Mohun *et al.*, 1987; M.Z.Gilman, unpublished). Of particular interest is the possibility that the SRE may also be the target for repression of c-*fos* transcription following induction with serum (Greenberg *et al.*, 1986; Sassone-Corsi *et al.*, 1988). This possibility is raised by the recent observations that an SRE oligonucleotide confers transient and cycloheximide-superinducible serum responsiveness to a heterologous promoter (Siegfried and Ziff, 1989) and that SRE mutations can raise the level of c-*fos* transcription in serum-deprived cells (Leung and Miyamoto, 1989). Both observations suggest that the SRE can act as an inducible repressor of transcription. Yet microinjection of SRE oligonucleotides into serum-deprived cells blocks c-*fos* induction by serum (Gilman *et al.*, 1988), showing clearly that positively acting factors must also bind to the SRE. Whether these activities are mediated by different SRE-binding proteins is not yet clear. However, our observation that there are indeed distinct SRE-binding proteins in the cell is consistent with this model. A detailed mutational analysis of the SRE will be required to determine whether this model is correct. Our preliminary mutagenesis studies have failed as yet to generate mutations that specifically disrupt binding of each SRE-binding protein alone (R.Graham and M.Z. Gilman, unpublished data). However, we note that the SRE mutations described by Leung and Miyamoto (1989) that interfere with SRF binding and eliminate transcriptional repression *in vivo* fall well outside the binding site we have defined for the 62 kd protein. Therefore, it is possible that by itself SRF acts as a transcriptional repressor *in vivo* whereas the 62 kd protein functions as an activator.

Several eukaryotic transcriptional regulatory elements are now known to bind multiple cellular proteins (for review, see Johnson and McKnight, 1989). These include the octamer element (Staudt *et al.*, 1986), CCAAT box (Chodosh *et al.*, 1988; Hatamochi *et al.*, 1988; Raymond-jean *et al.*, 1988; Santoro *et al.*, 1988), 'AP-1' site (Franza *et al.*, 1988; Hai *et al.*, 1988) and 'NF-κB' site (Franza *et al.*, 1987; Baldwin and Sharp, 1988). The complexities of the potential interactions at each of these sites means that assigning an *in vivo* function to any single DNA-binding protein is very difficult. Detailed mutagenesis of the binding sites coupled with functional assays of the various activities of such sites *in vivo* are required to evaluate the role each protein plays in the regulation of transcription through complex sites like the c-*fos* SRE.

Materials and methods

Extract preparation and heparin–agarose chromatography

Nuclear extracts were prepared from logarithmically growing H9 T-lymphoblasts by a modification of the method of Dignam *et al.* (1983). Nuclei were washed and extracted with buffer containing 300 mM KCl, yielding an extract with a conductivity equivalent to 225 mM KCl. The extract (50 ml, 350 mg protein) was loaded directly onto a 75 ml column of heparin–agarose, prepared by the method of Davison *et al.* (1979). The column was washed with 500 ml of buffer containing 20 mM Hepes, pH 8.0, 0.5 mM EDTA, 1 mM DTT, 1 mM PMSF, 10 mM Na metabisulfite, 25% (v/v) glycerol and 200 mM KCl. Proteins were eluted with a 600 ml gradient of the same buffer ranging from 200 to 600 mM KCl. Additional protein was eluted with the same buffer containing 1 M KCl. For metabolic labeling, H9 cells were incubated for 75 min in serum-free, phosphate-free DME medium containing 22 mCi/ml [^{32}P]inorganic phosphate (Amersham), and extracts were prepared according to Franza *et al.* (1988).

Oligonucleotide probes

The sequences of the probes used in the mobility-shift and DNA-affinity preparation assays are shown in Figure 1. The SRE wild-type (WT) and point mutant (PM) oligos were described in Gilman (1988). The symmetrical actin-derived SRE (Treisman, 1987) was prepared from two overlapping oligonucleotides 5′-CGTCGACGGAGATGCC-3′ and 5′-CATATATGG-GCATCTC-3′. After annealing and ligation, these oligonucleotides yielded actin SREs flanked by *Sal*I sites. A monomer *Sal*I fragment was cloned into pUC119 for use in the mobility-shift assay. For the mobility-shift assays, the cloned oligonucleotides were excised with *Hin*dIII and *Eco*RI, 5′-end-labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP and gel purified as described (Gilman *et al.*, 1986).

For the UV cross-linking assay isotopically labeled bromodeoxy-uridine-substituted probes were prepared by primer extension. An oligo-nucleotide encoding the SRE, 5′-CACAGGATGTCCATATTAGGAC-ATCTGCGTCAGCAGGT-3′, was mixed with a short primer 5′-ACCT-GCTGAC-3′ that annealed to the 3′ end of the longer oligonucleotide. This formed a primed structure which was elongated with Klenow fragment in the presence of 60 μCi [α-^{32}P]dCTP and 1 mM each dATP, dGTP and BrdUTP. The full-length product was gel-purified.

Mobility-shift and DMS interference assays

Mobility-shift assays were carried out as described in Gilman *et al.* (1986), with the following modifications. First, pUC13 DNA and $MgCl_2$ were omitted from the binding reactions, and the poly(dI-dC)·(dI-dC) concentration was 50 μg/ml. Second, except for the experiment in Figure 2B, the binding reactions were preincubated in the absence of labeled probe for 10 min at room temperature. Third, except for the experiment shown in Figure 2B, the electrophoresis buffer contained 45 mM Tris base, 45 mM boric acid, 1 mM EDTA, pH 8.5.

DMS interference assays were done as detailed in Gilman *et al.* (1986), except that bound and free DNA was eluted onto Hybond M&G paper (Amersham). The paper was rinsed twice in water, once in ethanol and incubated in 1 M piperidine for 30 min at 90°C. The paper was discarded and the cleaved DNA purified by repeated lyophilization.

UV cross-linking

Binding reactions were set up with isotopically labeled, bromodeoxyuridine-substituted probes. After incubation, the reactions were transferred to Saran Wrap and illuminated with 302 nm light from a height of 6 cm for 9 min (62 kd protein) or 15 min (67 kd protein). The reactions were fractionated on a Tris–borate mobility-shift gel, and the shifted complexes visualized by autoradiography. The complexes were excised from the gel together with the corresponding regions of adjacent lanes prepared using a mutant probe. Protein was eluted into 350 μl of 40 mM Tris, pH 8.0, 150 mM NaCl, 0.1% (w/v) SDS, 5 mM DTT, 0.1 mM EDTA, 100 μg/ml insulin by shaking at 37°C overnight. The eluted protein was precipitated with acetone, redissolved in 15 μl buffer containing 10 mM Tris, pH 7.4, 50 mM KCl, 5 mM $MgCl_2$, 2.5 mM $CaCl_2$, and treated with 1 U of DNase I for 10 min at room temperature. An equal volume of 2 × SDS–PAGE loading buffer was added. The samples were boiled for 3 min and electrophoresed on a 9% polyacrylamide–SDS gel. The gel was dried and exposed to film for autoradiography.

DNA-affinity precipitation assay

This assay was performed as described by Franza *et al.* (1987). Oligonucleotides were biotinylated with Photoprobe Biotin (Vector Laboratories), phosphorylated with T4 polynucleotide kinase, and ligated into oligomeric arrays. Column fractions (400 μl) were mixed with 1 ml Solution B (Franza *et al.*, 1987) and 120 μg poly(dI-dC)·(dI-dC) (Pharmacia) and incubated for 15 min at room temperature. Biotinylated oligonucleotides (3 μg) were added and incubation continued for 20 min. The reactions were centrifuged for 2 min in a microcentrifuge and the supernatant transferred to ~20 μl washed streptavidin–agarose beads (BRL). The beads were resuspended and the suspensions incubated for 20 min. The beads were recovered by centrifugation and washed twice with ice-cold Solution B containing 50 mM KCl. The washed beads were boiled for 2 min in 30 μl Solution D (Franza

et al., 1987). The supernatant solution was recovered and the beads were boiled again in Solution D. The two supernatant solutions were pooled and a portion was loaded onto a 9% polyacrylamide–SDS gel. Proteins were visualized by silver staining.

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Note added in proof

Recently, Shaw *et al.* (*Cell*, **56**, 563–572, 1989) have identified a 62 kd protein that forms a ternary complex with SRF and SRE-containing DNA. We also detect this activity in our extracts and agree with these workers on its size and binding properties (Ryan *et al.*, unpublished). This activity separates completely from the 62 kd SRE-binding protein upon heparin–agarose chromatography (Figure 2). Therefore, the two 62 kd proteins are not identical, although we cannot rule out the possibility that they are related.