The EMBO Journal vol.11 no.1 pp.97 - 105, 1992

Casein kinase II phosphorylation increases the rate of serum response factor-binding site exchange

Richard M.Marais, J.Justin Hsuan[1], Caroline McGuigan[2], Judy Wynne and Richard Treisman

Imperial Cancer Research Fund, PO Box 123, Lincoln's Inn Fields, London WC2A 3PX and [1]Ludwig Institute for Cancer Research, 91 Riding House St, London W1P 8BT, UK

[2]Present address: EMBL, Postfach 1022.40, D6900 Heidelberg, FRG

Communicated by Richard Treisman

Recombinant baculoviruses were used to express wild-type serum response factor (SRF) and a mutant, SRF.CKIIA, which lacks all four serine residues in the major casein kinase II (CKII) site at residues 77–90. Purified recombinant SRF binds DNA with an affinity and specificity indistinguishable from that of HeLa cell SRF, and activates transcription *in vitro*. Comparative phosphopeptide analysis of the wild-type and mutant proteins demonstrated that the wild-type protein is phosphorylated at the major CKII site in insect cells. Dephosphorylation of recombinant SRF does not affect its affinity for the c-*fos* SRE, and results in only a 3-fold reduction in binding to the synthetic site ACT.L. However, dephosphorylation does cause a large decrease in the rates of assocation with and dissociation from either site. These effects are due solely to phosphorylation at the major CKII site: the binding properties of the SRF.CKIIA mutant are identical to those of dephosphorylated wild-type SRF, and CKII phosphorylation *in vitro* converts dephosphorylated wild-type SRF from a slow-binding to a fast-binding form without significantly changing binding affinity. CKII phosphorylation thus acts to potentiate SRF–DNA exchange rates rather than alter equilibrium binding affinity.

Key words: casein kinase II/serum response factor/DNA binding/baculovirus/phosphorylation

Introduction

When susceptible cells are treated with growth factors or mitogens, transcription of a large family of cellular 'immediate-early' genes is rapidly and transiently induced without the need for prior protein synthesis (Lau and Nathans, 1985, 1987; Almendral *et al.*, 1988; Bravo *et al.*, 1988). The promoters of many of these genes contain a common regulatory sequence, the serum response element (SRE), which binds a ubiquitous nuclear phosphoprotein, serum response factor (SRF; Treisman, 1985, 1986; Gilman *et al.*, 1986; Prywes and Roeder, 1986; Greenberg *et al.*, 1987). In many cells, SRE mutations that reduce or abolish SRF binding have parallel effects on inducibility of transcription by different growth factors, consistent with the idea that SRF binding is required for the transcriptional response (reviewed by Treisman, 1990). At the c-*fos* SRE, SRF acts to facilitate binding of another protein, $p62^{TCF}$, which *in vitro* exhibits no detectable DNA binding activity (Shaw *et al.*, 1989); in some cell lines, formation of this ternary complex appears to be required for linkage of c-*fos* transcription to particular signalling pathways (Shaw *et al.*, 1989; Graham and Gilman, 1991; Malik *et al.*, 1991).

One potential mechanism by which SRE function could be modulated in response to growth factor stimulation is by regulation of SRF binding. However, initial studies showed that in extracts from many cell types SRF DNA binding activity remains unchanged upon growth factor stimulation (Prywes and Roeder, 1986; Treisman, 1986; Greenberg *et al.*, 1987). Consistent with this, genomic footprinting analysis of the c-*fos* SRE in several cell types *in vivo* suggests that SRF (or a protein with a similar footprint) is bound throughout stimulation (Herrera *et al.*, 1989; Konig, 1991; V.Bardwell and R.Treisman, unpublished data). By contrast, other studies have suggested that binding affinity of SRF is regulated by phosphorylation. For example, in A431 cells a 3- to 10-fold increase in extractable SRE binding activity was reported following stimulation with EGF (Prywes and Roeder, 1986; Prywes *et al.*, 1988); furthermore, phosphatase treatment of SRF in either crude extracts or partially purified preparations, leads to a large apparent reduction in SRE binding activity (Prywes *et al.*, 1988; Boxer *et al.*, 1989; Schalasta and Doppler, 1990).

The N-terminal region of SRF contains an evolutionarily conserved consensus sequence for CKII (human residues 77–90, SGSEGDSESGEEEE; Norman *et al.*, 1988; Mohun *et al.*, 1991). A previous study showed that CKII can phosphorylate recombinant SRF renatured from bacterial cell lysates, causing an apparent increase of over 20-fold in its DNA binding affinity (Manak *et al.*, 1990). This observation is intriguing since CKII activity is regulated in response to growth factors (Sommercorn *et al.*, 1987; Klarlund and Czech, 1988; Ackerman and Osheroff, 1989; Carroll and Marshak, 1989; Ackerman *et al.*, 1990), and microinjection of CKII into REF-52 cells induces c-*fos* expression (Gautier-Rouvière *et al.*, 1991). The major CKII site lies outside the minimal region of SRF required for sequence-specific DNA recognition, which is located between residues 133 and 223 (Norman *et al.*, 1988).

In this paper we present an analysis of the effect of phosphorylation on DNA binding by SRF using purified recombinant wild-type and mutant proteins expressed in insect cells by baculovirus vectors. We show that SRF is phosphorylated at the major CKII site *in vivo*; however, in contrast to previous studies, we show that the major effect of phosphorylation is not to increase equilibrium affinity of SRF for its binding sites but to increase the rate at which the protein exchanges with them.

Results

Expression and purification of recombinant SRF

In order to produce recombinant SRF for biochemical studies we used baculovirus vectors to overexpress wild-type and mutant forms of the protein in insect cells. Two recombinants were studied: SRF.WT, which expresses the full length wild-type SRF open reading frame; and SRF.CKIIA, which expresses a SRF mutant in which each of the four serines in the major CKII consensus site is changed to alanine. Analysis of extracts prepared from cells infected with either recombinant virus showed that their nuclei contained up to 10 000-fold more extractable SRE-specific DNA binding activity than cells infected with nonrecombinant virus (data not shown). The maximum synthesis of recombinant protein occurred between 36 and 48 h post-infection (p.i.), with SRF levels reaching a maximum at 72 h p.i. (data not shown). Both SRF.WT and SRF.CKIIA proteins could be purified from salt−detergent lysates of infected cells by DNA affinity chromatography (see Materials and methods). During this procedure, a polypeptide of M_r ~65 000 was quantitatively removed from the cell lysate (Figure 1A, compare lanes 1 and 2). This coincided with the quantitative removal of both SRE-specific DNA binding activity and anti-SRF immunoreactive material (data not shown). The SRF was eluted from the SRE column with salt, yielding protein at a final purity of >99% (Figure 1A, lane 4). Comparison of recombinant proteins with partially purified HeLa cell SRF on SDS−PAGE showed that SRF.WT and HeLa SRF migrate with virtually identical mobilities, although the SRF.WT band is somewhat more diffuse; by contrast, SRF.CKIIA has a higher mobility (Figure 1A, compare lanes 5 and 6).

Biochemical properties of recombinant SRF

We first measured the relative DNA binding affinities of SRF.WT and HeLa SRF. Equal amounts of protein as determined by immunoblot analysis were tested for binding to the synthetic SRE ACT.L (Treisman, 1987) by gel electrophoretic mobility-shift assay. This analysis showed that SRF.WT has a binding affinity within 2-fold of authentic HeLa SRF (Figure 1B, compare lanes 1−4 with 5−8). The K_d of SRF.WT was determined by Scatchard analysis and found to be $2-3\times10^{-11}$ M, in agreement with previous reports (Prywes and Roeder, 1987; Schroter *et al.*, 1990). The binding affinity of SRF.CKIIA was ~3-fold lower than that of SRF.WT (data not shown, see Figures 3B and 4B). Binding competition analysis using various synthetic and naturally occurring SRE variants indicated that the sequence specificity of SRF.WT is indistinguishable from that of HeLa SRF; both SRF.WT and SRF.CKIIA efficiently recruited the accessory protein $p62^{TCF}$ to the c-*fos* SRE *in vitro* (data not shown).

We next examined the ability of SRF.WT to stimulate transcription *in vitro* in SRF-depleted HeLa cell extracts. Two templates were used in each reaction: one contains a test promoter comprising a TATA element linked to either the ACT.L SRE or the nonbinding mutant ACT.L* SRE; the other template contains a reference promoter comprising a TATA element alone. In this system efficient activity of the test promoter is dependent on both an intact SRE and exogenous SRF (Norman *et al.*, 1988). Addition of saturating amounts of SRF (5 ng) to the system stimulated the SRE-containing promoter by 5- to 7-fold, but had no effect on the activity of the mutant SRE [Figure 1C, compare lanes 5 and 6 (no SRF) with 3 and 4 (5 ng SRF)]. The degree of stimulation is similar to that seen with purified HeLa SRF

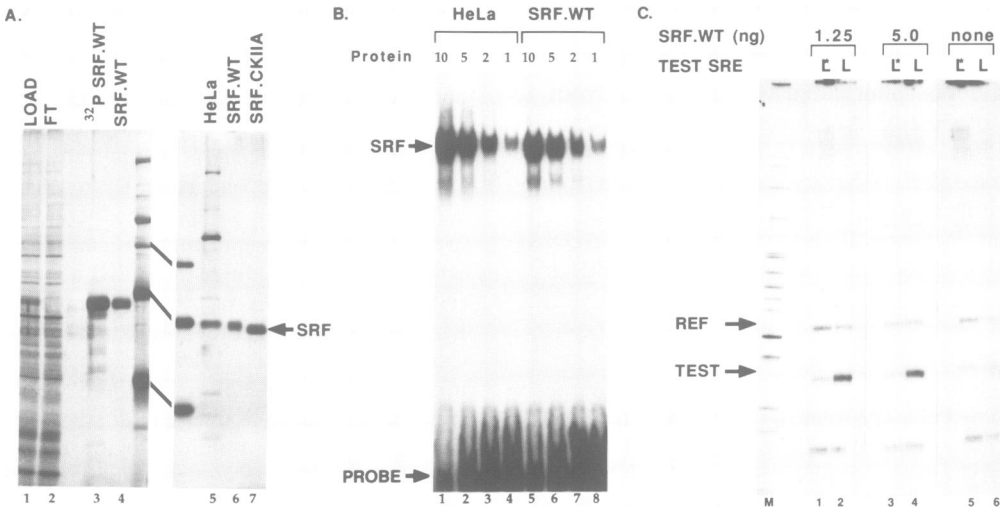

Fig. 1. Purification and properties of recombinant baculovirus SRF (**A**) Silver stained 8% SDS−polyacrylamide gel of fractions from the purification. Lane 1, total lysate of SRF.WT infected cells. Lane 2, flowthrough from the tandemly linked Q-Sepharose, nonspecific DNA and specific DNA affinity columns; lane 3, *in vivo* ^{32}P-labelled SRF.WT purified using a single column method; lane 4, purified SRF.WT; lane 5, partially purified HeLa SRF using the same method as for recombinant SRF; lane 6, purified recombinant SRF.WT; lane 7, purified recombinant SRF.CKIIA. The indicated standard mass markers are 96, 67 and 45 kDa. (**B**) Comparison of HeLa SRF and SRF.WT binding affinities. Equal amounts of protein, as determined by immunoblot analysis, were compared by mobility-shift assay with the ACT.L probe. The relative amount of SRF added is indicated above the lanes. Lanes 1−4, HeLa SRF; lanes 5−8, SRF.WT. (**C**) Recombinant SRF activates transcription in HeLa cell extracts. *In vitro* transcription reactions were performed and analysed by primer extension. The test promoters contained either the nonbinding ACT.L* SRE (lanes 1, 3 and 5) or the high affinity ACT.L SRE (lanes 2, 4 and 6). Lanes 1 and 2 contained 1.25 ng SRF.WT; lanes 3 and 4 contained 5 ng SRF.WT; and lanes 5 and 6 no SRF. Test and reference signals are indicated.

or SRF produced by cell-free translation (Norman *et al.*, 1988, data not shown); maximum transcription was achieved at comparable concentrations of recombinant SRF.WT and target template DNA.

The major CKII site is phosphorylated in vivo

The difference in mobility of the SRF.WT and SRF.CKIIA proteins on SDS–PAGE suggested that SRF.WT is phosphorylated *in vivo* at the major CKII site. To investigate phosphorylation directly, we prepared recombinant SRF labelled with ^{32}P *in vivo*. Both proteins are phosphorylated, but SRF.CKIIA has a greatly diminished phosphate content relative to SRF.WT (Figure 2A, compare lanes 1 and 2 with 3 and 4). Phosphoamino acid analysis revealed that label was exclusively present as phosphoserine (data not shown).

We next compared the radiolabelled peptides generated from the two proteins by CNBr digestion: this procedure should generate a large N-terminal peptide, comprising residues 2–105, that contains the major CKII site. Equal amounts of protein were digested and the peptides separated by SDS–PAGE and transferred to PVDF membrane. Coomassie staining confirmed that both forms of SRF generated a peptide of apparent M_r 21 000 (data not shown), although only the peptide from SRF.WT contained ^{32}P (Figure 2B, compare lanes 1 and 2). The membrane-bound peptide was then subjected to Edman degradation sequencing which confirmed that it was derived from the N-terminus of SRF. Although we were unable to obtain sequence data through the CKII consensus, our data indicated that residues Ser16 and Ser21 are not phosphorylated. Equal amounts of each protein were also subjected to exhaustive digestion with trypsin and the radiolabelled peptides compared by reverse phase HPLC. The results show that for SRF.WT the majority of the label is present in a single tryptic phosphopeptide which is absent in digests of SRF.CKIIA (Figure 2C). Taken together, these data suggest that the N-terminal CKII consensus is the major *in vivo* phosphorylation site of SRF.WT in insect cells.

To obtain an estimate of the stoichiometry of phosphorylation at the CKII site, we examined the effect of phosphatase treatment and rephosphorylation by CKII. Purified *in vivo* ^{32}P-radiolabelled SRF.WT was treated with calf intestinal alkaline phosphatase (CIAP) and repurified from the phosphatase by DNA-affinity chromatography. In a parallel experiment, an identical sample was mock treated and repurified. Under our dephosphorylation conditions, CIAP treatment results in virtually complete removal of phosphate from the protein (Figure 3A, compare lanes 1 and 2). Comparison of the preparations by immunoblot analysis showed that neither treatment caused any significant degradation of either sample; however, dephosphorylation decreased the apparent M_r of SRF.WT from 65 000 to 63 000 (Figure 3, compare lanes 3 and 4). This large discrete

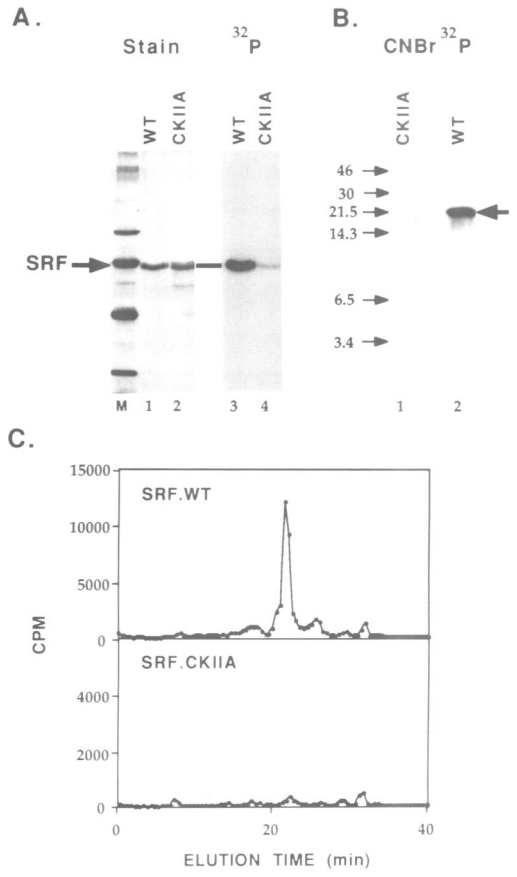

Fig. 2. Recombinant SRF is phosphorylated *in vivo* at the major CKII site. (**A**) *In vivo* phosphate labelling of SRF.WT and SRF.CKIIA. Purified *in vivo* labelled preparations of SRF.WT (lanes 1 and 3) and SRF.CKIIA (lanes 2 and 4) were analysed by 8% SDS–PAGE, stained with silver (lanes 1 and 2), and autoradiographed (lanes 3 and 4). Molecular mass markers are 96, 67, 45 and 31 kDa. (**B**) Autoradiograph of equal amounts of ^{32}P-labelled SRF.CKIIA and SRF.WT which was digested to completion with CNBr and resolved by 20% SDS–PAGE. Lane 1, SRF.CKIIA; lane 2, SRF.WT. The positions of molecular mass markers are shown. (**C**) Tryptic phosphopeptide maps. Equal amounts of *in vivo* ^{32}P-labelled SRF.WT (upper panel) and SRF.CKIIA (lower panel) were digested with trypsin and fractionated by reverse phase HPLC.

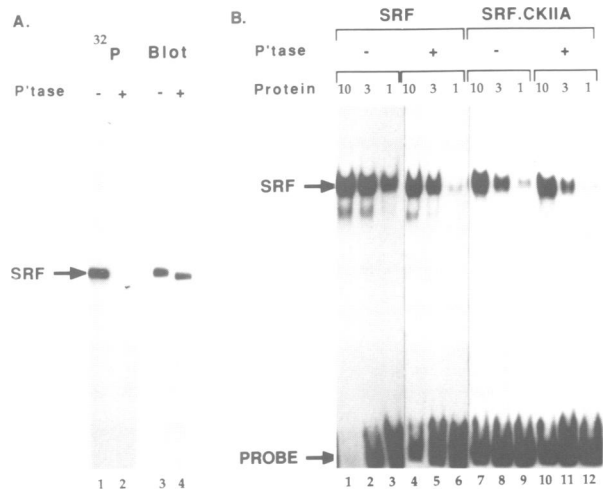

Fig. 3. Dephosphorylation of recombinant SRF. (**A**) *In vivo* ^{32}P-labelled SRF.WT was dephosphorylated (lanes 2, 4) or mock treated (lanes 1, 3) and analysed by 8% SDS–PAGE followed by autoradiography (lanes 1, 2) or immunoblot analysis (lanes 3, 4). The position of SRF is indicated. (**B**) DNA binding asssay of dephosphorylated SRF. ^{32}P-labelled SRF.WT (lanes 1–6) or SRF.CKIIA (lanes 7–12) were mock treated (lanes 1–3 and 7–9) or phosphatase treated (lanes 4–6 and 10–12) and tested for DNA binding by mobility shift assay with the ACT.L probe. The relative amounts of added SRF, as determined by immunoblot analysis is indicated above each lane.

change in mobility observed in the immunoblot analysis indicates that recombinant SRF.WT must be stoichiometrically phosphorylated *in vivo*. In an alternative approach, we compared the relative abilities of untreated and phosphatase-treated SRF.WT to be labelled with ^{32}P by CKII *in vitro*. As a control for background and incorporation into a weak CKII consensus at residues 251–258, we also performed a similar analysis of SRF.CKIIA. Prior dephosphorylation of SRF.WT increases the amount of phosphate incorporated by 3-fold over background (data not shown). We conclude that the majority of available sites are phosphorylated in insect cells *in vivo*.

Dephosphorylation of recombinant SRF does not affect its DNA binding affinity

The results presented above show that recombinant SRF.WT protein, which has a DNA binding affinity comparable to that of HeLa SRF, is phosphorylated *in vivo* at the major CKII consensus. Since previous reports have suggested that phosphorylation of this CKII site increases SRF.WT DNA binding affinity by >20-fold, we investigated the effect of dephosphorylation on DNA binding affinity of SRF.WT. Radiolabelled SRF.WT was dephosphorylated and repurified by DNA affinity chromatography as described above. Dephosphorylation did not affect the chromatographic behaviour of the protein: the dephosphorylated protein was quantitatively retained on the affinity column, as measured by immunoblot assay, and was eluted under the same conditions (data not shown). We then compared the equilibrium binding affinity of the different SRF preparations by gel mobility-shift assay using the ACT.L probe. Dephosphorylated SRF.WT bound ACT.L with an apparent affinity 3-fold lower than phosphorylated SRF (Figure 3B, compare lanes 1–3 with 4–6). By contrast, dephosphorylation of SRF.CKIIA had no effect on its apparent M_r (data not shown), and no effect on its DNA binding affinity, which is comparable to that of dephosphorylated SRF.WT (Figure 3B, compare lanes 7–9 with 10–12). These data show that although recombinant SRF.WT is phosphorylated at the major CKII site in insect cells *in vivo*, removal of these phosphates has only a small effect on its binding affinity. As we will show below, phosphorylation by CKII *in vitro* does not increase the binding affinity of SRF.WT.

Binding assay conditions apparently affect SRF binding

We were puzzled by the above results since phosphorylation of SRF by CKII has been reported to increase its binding affinity by up to 20-fold (Manak *et al.*, 1990). We therefore compared SRF binding under both our conditions, which include 3 mM spermidine, and conditions similar to those used by others. Binding to both the ACT.L and c-*fos* SRE probes was tested in the presence or absence of 3 mM spermidine, with either 2 mM EDTA or 2 mM Mg^{2+}.

Dephosphorylated SRF.WT was prepared by CIAP treatment and, in parallel, SRF.WT and SRF.CKIIA were subjected to mock phosphatase treatment. All three preparations were repurified by DNA affinity chromatography and quantified by immunoblot analysis, and binding was tested by gel mobility-shift assay. The binding activity of phosphorylated SRF.WT was comparable under each of the four assay conditions (Figure 4A, lanes 1–8). By contrast, the binding activity of dephosphorylated SRF.WT appeared to vary with the assay conditions. As shown above, in the presence of spermidine the binding affinity of dephosphorylated SRF.WT was apparently ~3-fold lower than that of phosphorylated SRF.WT (compare lanes 1 and 2 with 9 and 10). However, in the absence of spermidine, the apparent affinity of dephosphorylated SRF.WT was ~50-fold lower than that of phosphorylated SRF.WT (compare lanes 11–14 with 3–6). The binding properties of SRF.CKIIA under the various conditions were identical to those of the dephosphorylated SRF.WT (Figure 4A, compare lanes 9–16 with 17–24).

We next compared binding to the c-*fos* SRE probe under the various conditions. As before, phosphorylated SRF.WT

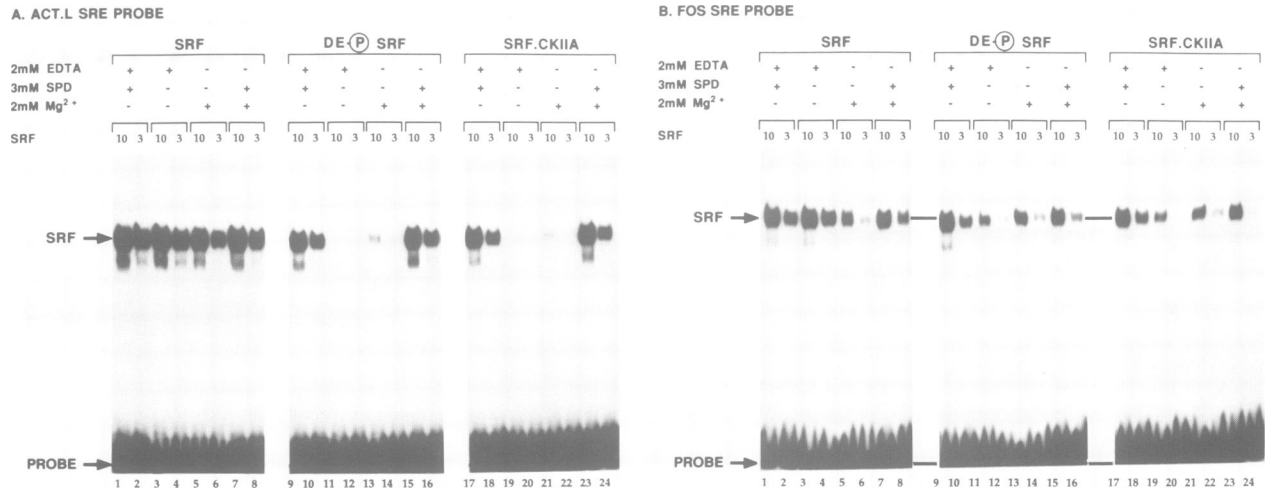

Fig. 4. Effect of binding conditions on apparent SRF binding affinity. (**A**) Analysis using the ACT.L probe. Mock phosphatase-treated SRF.WT (lanes 1–8), phosphatase-treated SRF.WT (lanes 9–16), and mock phosphatase-treated SRF.CKIIA (lanes 17–24) were tested for DNA binding activity in the standard binding assay with the indicated additions. The relative amount of SRF is indicated above the lanes. Lanes 1, 2, 9, 10, 17 and 18, 2 mM EDTA with 3 mM spermidine; lanes 3, 4, 11, 12, 19 and 20, 2 mM EDTA; lanes 5, 6, 13, 14 and 21, 22, 2 mM $MgCl_2$; lanes 7, 8, 15, 16, 23 and 24, 2 mM $MgCl_2$ with 3 mM spermidine. (**B**) Analysis using the c-*fos* SRE probe. Reactions were as above but using the c-*fos* SRE as probe.

exhibited comparable binding affinity under each set of conditions, although in the presence of Mg^{2+} and the absence of spermidine, binding was slightly reduced (Figure 4B, lanes 1–8). However, in contrast to the ACT.L site, binding to the c-*fos* SRE appeared relatively insensitive to the phosphorylation state of the protein, with only a 3- to 5-fold decrease in affinity observed in the absence of spermidine (compare lanes 1–8 with 9–16). As before, the behaviour of SRF.CKIIA under the various conditions was identical to that of the dephosphorylated SRF.WT protein (compare lanes 9–16 with 17–24).

Phosphorylation increases the rate of DNA binding

Although our standard binding reaction involves a 75 min incubation, one explanation for the differences in binding behaviour described above is that complex formation had failed to reach equilibrium prior to gel loading. We therefore analysed the kinetics of complex formation between DNA and the various preparations of SRF in detail. Binding reactions were prepared at various times and loaded onto the gel simultaneously, thereby giving a measure of the rate of association of SRF with the SRE.

Phosphorylated SRF.WT associated with the c-*fos* SRE probe rapidly in the absence of spermidine, reaching equilibrium within 5 min of addition of protein (Figure 5A, lanes 1–7). By contrast, dephosphorylated SRF.WT reached equilibrium much more slowly, taking between 60 and 180 minutes; the SRF.CKIIA mutant behaved identically (Figure 5A, compare lanes 8–14 with 7–21). Although addition of spermidine to the binding reactions increased the rates of binding, the differential between the dephosphorylated and phosphorylated proteins was maintained (Figure 5A, compare top and bottom panels). Similar results were obtained when we examined binding to the ACT.L probe (Figure 5B, compare upper and lower panels, lanes 1–7). Interestingly, binding rates for the ACT.L probe were much lower than those observed with the c-*fos* SRE probe: the SRF.CKIIA and dephosphorylated SRF.WT both required over 8 h to reach equilibrium, and

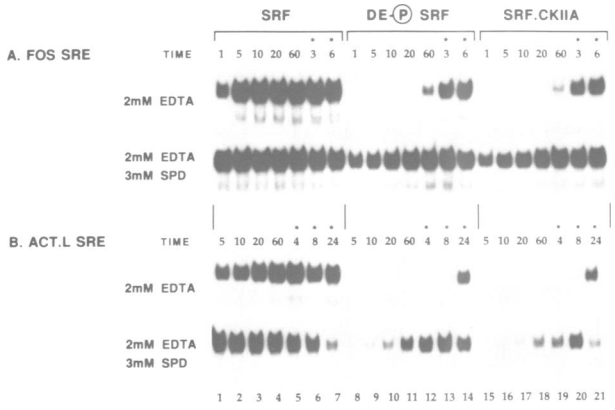

Fig. 5. Phosphorylation increases initial rate of SRF binding. On-rate analysis was performed with equal amounts of mock phosphatase-treated SRF.WT (lanes 1–8), phosphatase-treated SRF.WT (lanes 9–16), and mock phosphatase-treated SRF.CKIIA (lanes 17–24). The time between addition of SRF to the reactions and gel loading is indicated above the lanes in minutes (unmarked) or hours (marked with an *). (**A**) Binding to the c-*fos* SRE probe with 2 mM EDTA (upper panel) or 2 mM EDTA with 3 mM spermidine (lower panel). (**B**) Binding to the ACT.L SRE probe with 2 mM EDTA (upper panel) or 2 mM EDTA with 3 mM spermidine (lower panel).

even with spermidine present, equilibrium binding took between 4 and 8 h to establish (Figure 5B, lanes 8–21; note that at late time points some loss of binding activity occurs in all samples).

These results show that the effect of dephosphorylation of SRF.WT, or mutation of the major CKII site, is to reduce the rate at which SRF can associate with its target DNA sequence. Thus, with short incubations, in the absence of spermidine, dephosphorylated SRF appears to exhibit greatly reduced binding affinity; at true equilibrium, however, the affinities of dephosphorylated and phosphorylated SRF.WT are similar.

Phosphorylation decreases the stability of SRF – SRE complexes

In the previous sections we demonstrated that phosphorylation has only a small effect upon equilibrium DNA binding of SRF, yet greatly potentiates the initial rate of complex formation. A corollary of this is that phosphorylation should also increase the rate at which SRF dissociates from its binding sites. We therefore evaluated the apparent SRF dissociation-rate by gel mobility-shift assay. Binding reactions were set up and allowed to reach equilibrium; a large excess of unlabelled competitor SRE DNA was then added and the system was allowed to re-equilibrate for various times prior to gel loading. In this assay, the rate of disappearance of labelled complex is a measure of the rate of SRF exchange on the DNA and therefore of the stability of the SRF–SRE complex.

First, we measured the stability of complexes formed between phosphorylated SRF.WT and the c-*fos* SRE. In this case, addition of the competitor resulted in virtually complete loss of the signal within 1 h (Figure 6A, lanes 1–7, upper panel); in the presence of spermidine, exchange was even

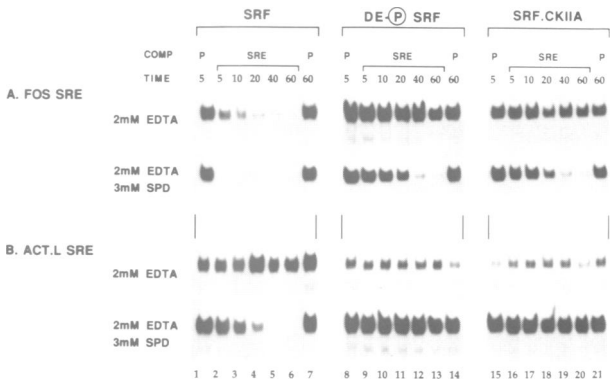

Fig. 6. Phosphorylation increases the rate of SRF–DNA exchange. Exchange-rate analysis using equal amounts of mock phosphatase-treated SRF.WT (lanes 1–8), phosphatase-treated SRF.WT (lanes 9–16), and mock phosphatase-treated SRF.CKIIA (lanes 17–24). Binding reactions were allowed to reach equilibrium: competitor plasmid DNA (COMP), equivalent to 100 times molar excess was then added to each reaction at the indicated times, in minutes, prior to gel loading. Each reaction received either specific (SRE) or nonbinding pUC12 (P) competitor. (**A**) Analysis using the c-*fos* SRE probe with 2 mM EDTA (upper panel) or 2 mM EDTA and 3 mM spermidine (lower panel). Lanes 1, 7, 8, 14, 15 and 21, competition with nonbinding pUC12 DNA; lanes 2–6, 9–13 and 16–20, competition with c-*fos* SRE plasmid pDYAD. (**B**) Analysis using the ACT.L SRE probe with 2 mM EDTA (upper panel) or 2 mM EDTA with 3 mM spermidine (lower panel). Lanes 1, 7, 8, 14, 15 and 21, competition with nonbinding pUC12 DNA; lanes 2–6, 9–13 and 16–20, competition with ACT.L plasmid pACT.L .

more rapid, the system re-equilibrating within a few minutes (Figure 6A, lanes 1–7; lower panels). As predicted, complexes formed between the c-*fos* SRE and dephosphorylated SRF were significantly more stable than those formed between the c-*fos* SRE and phosphorylated SRF. Dephosphorylated SRF exhibited negligible exchange with competitor over a 1 h period in the absence of spermidine; as before, spermidine facilitated exchange (compare lanes 8–14 with 1–8). We obtained similar results with the ACT.L probe, although the exchange rates were slower than with the c-*fos* SRE (Figure 5B). SRF.CKIIA behaved identically to dephosphorylated SRF.WT with both SRE probes (Figure 6, compare lanes 8–14 with 15–21).

The kinetic effects are entirely due to casein kinase II phosphorylation

We demonstrated above that dephosphorylation of SRF.WT lowers both the on- and off-rates of SRF–DNA complex formation without significantly affecting equilibrium binding affinity. Moreover, in all the experiments, SRF.CKIIA, which lacks all four serines at the major CKII consensus site, behaves in a manner indistinguishable from dephosphorylated SRF.WT. These observations imply that phosphorylation by CKII is necessary to modulate the kinetics of DNA binding. To demonstrate that CKII phosphorylation is sufficient for this modulation, we performed *in vitro* phosphorylation studies with purified CKII.

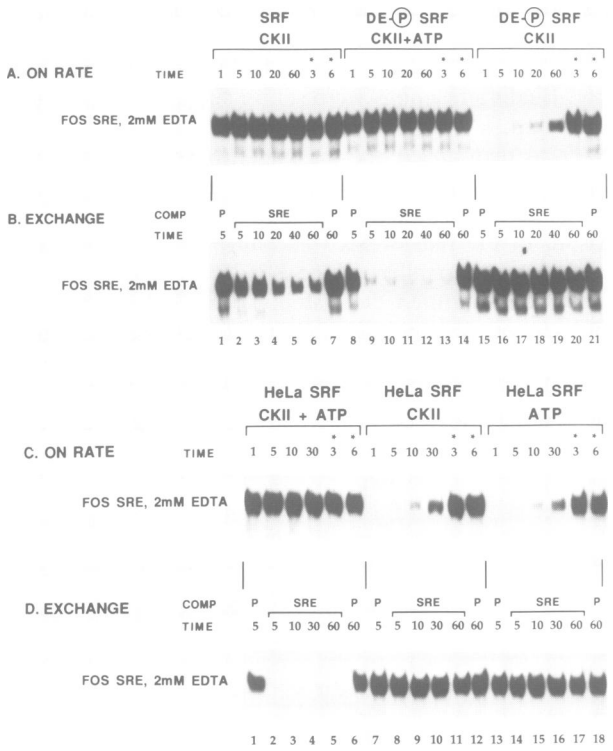

Fig. 7. CKII phosphorylation affects the kinetics of both Sf9 and HeLa SRF. On-rate and exchange-rate measurements were performed for SRF.WT or HeLa SRF using the c-*fos* SRE with 2 mM EDTA. The time between addition of SRF to the reactions and gel loading is indicated above the lanes in minutes (unmarked) or hours (marked with an *). The exchange rate analyses were performed as in Figure 6 with the indicated competition times (COMP) in min. Competition was with specific competitor (SRE) or non-binding pUC12 DNA (P). (**A** and **B**) Analysis with recombinant SRF.WT. Mock phosphatase-treated SRF.WT (lanes 1–7) or phosphatase-treated SRF.WT (lanes 8–21) was treated with CKII alone (lanes 1–7 and 15–21) or with CKII in the presence of ATP (lanes 8–14). (**A**) SRF.WT on-rate measurements. (**B**) SRF.WT exchange-rate measurements. Lanes 1, 7, 8, 14, 15 and 21, competition with nonbinding pUC12 DNA; lanes 2–6, 9–13 and 16–20, competition with c-*fos* SRE plasmid pDYAD. (**C** and **D**) Analysis with HeLa SRF. HeLa SRF was treated with CKII and ATP (lanes 1–6), CKII alone (lanes 7–12) or ATP alone (lanes 13–18). (**C**) HeLa SRF on-rate measurement. (**D**) HeLa SRF exchange rate measurement. Lanes 1, 6, 7, 12, 13 and 18, competition with nonbinding pUC12 DNA; lanes 2–5, 8–11 and 14–17, competition with c-*fos* SRE plasmid pDYAD.

SRF.WT was treated with CIAP or mock treated and repurified. Treatment of the dephosphorylated SRF.WT with CKII in the presence of ATP resulted in efficient phosphorylation of the protein, as measured by incorporation of radiolabel; by contrast treatment with CKII or ATP alone led to no phosphorylation (data not shown). The preparations were quantified by immunoblot analysis and DNA binding activity was investigated using the c-*fos* SRE probe. First, we measured the rate of complex formation. Mock CKII treatment of either phosphorylated or dephosphorylated SRF.WT had no effect on their DNA binding kinetics (compare Figure 7A, lanes 1–7 and 8–14 with Figure 5A, lanes 1–14). However, upon phosphorylation by CKII, dephosphorylated SRF.WT was converted to a fast binding form which formed DNA complexes of slightly lower mobility than the *in vivo* phosphorylated SRF.WT purified from infected cells (Figure 7A, compare lanes 8–14 with 1–7 and 15–21). Comparison of binding reactions at late time points, when true equilibrium is established, showed that phosphorylation by CKII did not significantly increase the affinity of SRF for the c-*fos* SRE (Figure 7A, compare lanes 7, 14 and 21).

Next, we evaluated the effect of CKII phosphorylation on the apparent off-rate of SRF using the exchange competition assay. Mock CKII treatment of dephosphorylated SRF.WT had no effect on its exchange rate (compare Figure 7B, lanes 15–21 with Figure 6A, lanes 8–14). Phosphorylation by CKII converted dephosphorylated SRF from the slow exchanging form to one which exchanged even more rapidly than the *in vivo* phosphorylated SRF.WT purified from the infected cells (Figure 7B, compare lanes 8–14 with 1–7). We also tested whether CKII phosphorylation had any effect on binding of SRF.CKIIA: no changes in either equilibrium binding or binding kinetics were observed (data not shown). We conclude that phosphorylation of SRF at the major CKII site alone is sufficient to modulate its rate of exchange with the SRE.

HeLa SRF binding kinetics are modulated by CKII

To rule out the possibility that the properties we observe are due to a novel modification of SRF produced in insect cells by the recombinant baculovirus, we tested whether similar results could be observed with HeLa cell SRF. For these analyses we used the substantially pure preparation of HeLa SRF shown in Figure 1A, lane 5. This preparation displayed the low association rates characteristic of dephosphorylated SRF.WT, and furthermore, CIAP treatment did not alter these rates (data not shown), suggesting that this preparation of HeLa SRF was not phosphorylated at the CKII site (we cannot say whether this reflects lack of phosphorylation *in vivo*, or loss of phosphate during purification).

Next, we phosphorylated the partially purified HeLa SRF with CKII, and compared its DNA binding properties with

those of the untreated preparation. When treated under conditions which did not lead to phosphorylation, HeLa SRF bound the c-*fos* SRE at rates similar to those of dephosphorylated SRF.WT (Figure 7C, lanes 7–12 and 13–18, compare with Figure 7A, lanes 15–21). However, phosphorylation by CKII converted HeLa SRF to a form that bound DNA extremely rapidly (Figure 7C, lanes 1–6). We also tested the effect of CKII phosphorylation on the stability of the HeLa SRF:SRE complex. Unphosphorylated HeLa SRF complexes were stable and unaffected by addition of competitor DNA (Figure 7D, lanes 7–12 and 13–18). By contrast, HeLa SRF phosphorylated by CKII formed unstable complexes which disappeared rapidly in the competition assay (Figure 7D, lanes 1–6). Thus CKII phosphorylation affects HeLa SRF similarly to recombinant SRF.

Discussion

Properties of recombinant SRF

By several biochemical criteria, recombinant SRF produced in insect cells is indistinguishable from HeLa cell SRF: it binds DNA with similar affinity and specificity, forms ternary complexes with $p62^{TCF}$ and activates transcription *in vitro*. The protein is modified by both phosphorylation and *O*-glycosylation, as is HeLa SRF (Prywes *et al.*, 1988; Ryan *et al.*, 1989; Schroter *et al.*, 1990); we will show elsewhere that the recombinant SRF carries four *N*-acetyl glucosamine moieties at positions C-terminal to the DNA binding domain (A.Reason, R.Marais, R.Treisman and A.Dell, manuscript in preparation).

The sites of phosphorylation of mammalian SRF have not been rigorously characterized, although it appears from tryptic phosphopeptide mapping that the major CKII site in HeLa SRF is phosphorylated (Manak *et al.*, 1990). Comparative phosphopeptide analysis of recombinant wild-type SRF and a mutant lacking all four serines at this site showed that the major CKII site is efficiently phosphorylated in infected insect cells. It appears that SRF is stoichiometrically phosphorylated, since we see a quantitative shift in the mobility of SRF.WT by SDS–PAGE upon dephosphorylation. Consistent with this, dephosphorylation of SRF.WT greatly increases its ability to act as a phosphate acceptor in the presence of CKII. At present we cannot say which of the four serines within the site is phosphorylated. However, we suspect more than one site, since although phosphorylation is stoichiometric, the binding kinetics of the *in vivo* phosphorylated wild-type protein can be enhanced further by CKII treatment. This suggests that at least a proportion of the protein is incompletely phosphorylated *in vivo*.

CKII phosphorylation alters binding kinetics, not affinity

We demonstrate here that CKII phosphorylation of SRF acts to increase both the on- and off-rates for interaction of the protein with the c-*fos* SRE and the synthetic symmetrized ACT.L SRE, but has little effect on binding affinity. Similar results were obtained with two other binding sites, ACT.R and the *Xenopus laevis* γ actin SRE (unpublished data). These kinetic effects are reminiscent of increases in the rates, but not affinity, of antibody–antigen interaction during maturation of the immune response (Foote and Milstein, 1991). We conclude that phosphorylation of SRF is not required for DNA binding *per se*. This result apparently contradicts previously published reports which suggested that phosphorylation of SRF is a prerequisite for high affinity binding (Prywes *et al.*, 1988; Boxer *et al.*, 1989; Schalasta and Doppler, 1990) and that phosphorylation by CKII increases DNA binding affinity by a factor of 20 (Manak *et al.*, 1990, 1991). Under the binding conditions used in those studies the kinetics of SRF association with the SRE are very slow and therefore the binding assays presented were possibly not at equilibrium; indeed we have been able to reproduce those effects under appropriate binding conditions (see Figure 4).

Our studies address the effects of phosphorylation at the major CKII site. It remains possible that other modifications occur in HeLa cells which act in concert with CKII phosphorylation to potentiate binding affinity. However, it is clear that the kinetic effects we observe with recombinant SRF cannot be an artefact of aberrant protein processing in insect cells since we can reproduce them by treatment of partially purified HeLa SRF with CKII. The complete loss of DNA binding activity previously observed upon phosphatase treatment of crude extracts (Prywes *et al.*, 1988; Boxer *et al.*, 1989; Schalasta and Doppler, 1990) may possibly be due either to the binding conditions used, or to the sensitivity of dephosphorylated SRF to unidentified factors in these extracts which destroy its binding activity.

Mechanism of CKII function

Since we observe little change in equilibrium binding affinity, phosphorylation cannot significantly affect SRF dimerization; rather, phosphorylation must lower the activation energies for association with and dissociation from DNA, perhaps by facilitating a conformational change required during DNA binding. The CKII site that mediates the kinetic effects that we observe is located ~50 amino acids to the N-terminal side of the DNA binding domain and is not required for DNA binding (Norman *et al.*, 1988). Regulation of DNA binding properties by phosphorylation at sites apart from the DNA binding domain has been previously observed in proteins such as SV40 large T (Schneider and Fanning, 1988), Myb (Luscher *et al.*, 1990) and c-jun (Boyle *et al.*, 1991; T.Curran, personal communication). An attractive idea is that the CKII site can interact directly with residues in the DNA binding domain. At present, direct physical assays for such interactions are lacking, but two observations suggest that the N-terminal part of the SRF molecule may be flexible. First, the DNA binding region of the bacterial lexA repressor can be fused at the N-terminus of the entire SRF reading frame to generate a protein which can bind to both the SRE and a lexA operator *in vitro* and *in vivo* (S.John and R.Treisman, manuscript in preparation). Second, neither the length nor the primary sequence, apart from a CKII site, is conserved between human and *X.laevis* SRF (Mohun *et al.*, 1991), suggesting that the CKII site may not lie in a conserved structure.

Two interaction models can be envisaged. First, the nonphosphorylated CKII site might sterically prevent interaction of the DNA binding domain with DNA. This seems unlikely, since phosphorylation has little effect on binding affinity. Second, the phosphorylated CKII site might interact with the DNA binding domain, and thereby somehow function to increase the association rate with DNA. In this respect it is intriguing that addition of spermidine to DNA binding reactions has a qualitatively similar effect on

DNA binding to phosphorylation at the major CKII site. Perhaps the effect of both phosphorylation and polyamine addition on the rate of DNA binding is mediated by their charge screening properties, phosphorylation via the ability of phosphate group(s) at the CKII site to interact with the exposed basic residues of the DNA binding domain, and polyamines via their ability to screen negative charges on the DNA backbone. These screening effects might lower the activation energy required to remove hydration shells that must occur as complexes form.

Mechanism of SRF–DNA interaction

We show here that the kinetics of SRF binding can vary in a sequence-dependent fashion. In particular, the ACT.L site exhibits particularly low exchange rates. A simple means to rationalize these observations is that binding occurs in a two stage process: an initial site-recognition event and a subsequent isomerization event involving changes in conformation of the initial complex. According to this view, although the rate of the initial event may be diffusion controlled, the isomerization step might be rate limiting at particular binding sites. We and others have used circular permutation analysis to show that binding of SRF to DNA results in DNA bending, to a degree independent of the affinity of the site (R.Treisman, unpublished data, cited in Treisman, 1987; Gustafson *et al.*, 1989). It is tempting to speculate that the slow kinetics of SRF:ACT.L interaction reflects a much higher activation energy for bending of the ACT.L sequence compared to the other sites.

The role of CKII in SRF function

The presence of a regulatory CKII site that potentiates SRF–DNA exchange rates is of interest given the observations that CKII activity has been observed to increase upon stimulation of a cell by growth factors (Sommercorn *et al.*, 1987; Klarlund and Czech, 1988; Ackerman and Osheroff, 1989; Carroll and Marshak, 1989; Ackerman *et al.*, 1990). Furthermore, microinjection of CKII into REF52 cells can induce c-*fos* expression (Gautier-Rouvière *et al.*, 1991). The time taken to achieve maximal activation of CKII in several systems examined appears to be somewhat less than that taken for the activation of immediate-early gene transcription brought about by growth factors. These studies measured total CKII activity and thus might not have distinguished subpopulations of activity that show more rapid activation kinetics; moreover, such approaches might miss regulated translocation of the enzyme to the nucleus upon factor stimulation (Cochet *et al.*, 1991; Je Yu *et al.*, 1991). Our data suggest that CKII phosphorylation may facilitate SRF–SRE exchange *in vivo*, and it will be interesting to evaluate whether sub-populations of SRF with different kinetic properties are detectable in cell extracts *in vitro*. We have observed no effects of SRF CKII phosphorylation on the affinity or kinetics of SRF/p62^{TCF} interaction *in vitro*, and it therefore appears unlikely that the primary function of CKII phosphorylation is to regulate this interaction (R.Marais and R.Treisman, unpublished data). Genomic footprinting experiments show that SRF is apparently bound to the SRE throughout growth factor stimulation (Herrera *et al.*, 1989); we therefore propose that regulated phosphorylation might serve either to allow replacement of inactive pre-stimulation complexes with active ones, or to facilitate binding of newly synthesized SRF (Misra *et al.*, 1991).

Materials and methods

Viruses and cell culture

To construct SRF.WT, the intact wild-type SRF open reading frame was inserted into the baculovirus transfer plasmid pAcC4, and recombinant virus was generated by standard techniques (Summers and Smith, 1987). The mutant SRF.CKIIA, in which serines 77, 79, 83 and 85 are substituted by alanines, was generated by PCR with oligonucleotides GCTCGGCGCCCAGCTCCTCCTCCTCGCCTGCCTCTGCGTCG and CCCCGGCGCCCACCGCGGGAGCTCTCTACGCAGGCGCAGAGG; the PCR product was digested with *Nar*I and used to replace the *Nar*I fragment in the SRF cDNA. The SRF.CKIIA recombinant virus was generated by a novel *in vivo* recombination system which will be described elsewhere (G.Patel, K.Nasmyth and N.C.Jones, manuscript submitted). Culture of Sf9 cells (ATCC accession number CRL 1711), viral infections and plaque assays were as described (Summers and Smith 1987). For large scale infections, 5×10^8 Sf9 cells were infected at 4 p.f.u./cell in 50 ml complete medium for 60 min, and diluted into 500 ml of complete medium in a 2000 ml spinner culture flask. For small scale infections 2.5×10^6 cells per 5 cm Petri dish were infected at 4 p.f.u./cell for 60 min and incubated in 5 ml medium. For radiolabelling experiments, 5×10^6 infected cells were washed into 2 ml unsupplemented phosphate-free medium 40 h after infection and incubated with 0.8 mCi [^{32}P]orthophosphate (PBS 13, Amersham) for 8 h.

Preparation of cell lysates

SRF expressed in Sf9 cells is soluble and was quantitatively extracted with high ionic strength buffer containing neutral detergents. All procedures were carried out at 4°C. Infected cells were harvested 48 h post infection by centrifugation at 200 *g* for 5 min and resuspended at 5×10^7/ml in lysis buffer (20 mM HEPES pH 7.9, 5 mM EDTA, 10 mM EGTA, 1.5 M KCl, 1.0% Triton X-100, 0.1% NP40, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 5 μg/ml pepstatin, 1 mM benzamidine, 50 μg/ml PMSF, 1 mM DTT). Following sonication for three 10 s pulses (DAWE Soniprobe, microprobe at setting 4), the lysate was diluted with 4 vol of buffer D0.0 (20 mM HEPES pH 7.9, 0.2 mM EDTA, 0.2 mM EGTA, 0.1% NP40, 10% glycerol, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 5 μg/ml pepstatin, 1 mM benzamidine, 50 μg/ml PMSF, 1 mM DTT) and clarified by centrifugation at 47 000 *g* for 30 min. For radiolabelled extracts 5×10^6 infected cells were resuspended in 200 μl of lysis buffer, vortex-mixed for 30 s, diluted with 800 μl D0.0, and clarified in a microfuge (10 min, 13 000 r.p.m.).

Purification of SRF

SRF was purified by a modificiation of published procedures (Treisman, 1987) involving sequential chromatography on Q-Sepharose, the nonspecific DNA column FOS.L* and the SRF-specific DNA affinity column FOS.L. The Q-Sepharose column was required to adsorb residual genomic DNA from the load fraction which otherwise caused the rapid deterioration of the agarose support of the DNA affinity columns. The FOS.L* column was used to adsorb high affinity DNA binding proteins prior to loading the ACT L column. At 0.3 M KCl, SRF does not bind to the Q-Sepharose or FOS.L* columns, but is quantitatively retained on the FOS.L affinity resin.

Clarified cell extract was loaded at 4 ml/h onto the three columns linked in tandem in the order: Q-Sepharose (5 ml), FOS.L* (1 ml), FOS.L (1 ml). The columns were equilibrated in buffer D0.3 (buffer D0.0 containing 0.3 M KCl). After loading, the FOS.L column was uncoupled and washed with 15 ml D0.3 and 15 ml D0.5 (buffer D0.0 containing 0.5 M KCl). The SRF was eluted at 1 ml/h with 20 ml D1.8 (buffer D0.0 containing 1.8 M KCl); 0.2 ml fractions were collected. Fractions containing SRF were detected by mobility-shift or dye-binding assays, pooled, dialysed against 100 vol buffer D0.1 (buffer D containing 0.1 M KCl), and stored in aliquots at −70°C. The final yield of SRF was ~125 μg per 5×10^8 infected cells.

^{32}P-labelled SRF was purified by chromatography on FOS.L* and FOS.L columns. Clarified lysate was passed over a 0.3 ml FOS.L* column in a 1 ml syringe under gravity. The flow through was loaded onto an 0.3 ml FOS.L column, which was washed with 2 ml D0.3, and 2 ml D0.5. The SRF was eluted with 1 ml D1.8; 0.1 ml fractions were collected; fractions containing SRF were identified by radioactivity, pooled, dialysed against buffer D0.1 and stored at −70°C.

For small scale repurification of SRF following phosphatase treatment, samples were loaded under gravity onto a 10 μl FOS.L column equilibrated with D0.3 in a 1 ml Gilson pipette tip. The column was washed with 50 μl buffer D0.3, and the SRF eluted with 40 μl D1.5 (buffer D containing 1.5 M KCl). The eluate was diluted with 160 μl buffer D0.0 and stored at −70°C. All the buffers contained 1 mg/ml ovalbumin as carrier.

Phosphorylation studies

For dephosphorylation, SRF (0.5 μg) was incubated with 15 μl of calf intestinal alkaline phosphatase (molecular biology grade, 22 U/ml, Boehringer

Mannheim) in 150 μl phosphatase buffer (25 mM Na borate pH 9.5, 1 mM $MgCl_2$, 0. 1 mM $ZnCl_2$, 0.1% NP40, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 5 μg/ml pepstatin, 1 mM benzamidine, 50 μg/ml PMSF, 1 mM DTT) at 37°C for 90 min. In mock treated samples, the phosphatase was replaced by 15 μl 10 mg/ml ovalbumin. After treatment, the samples were brought to 20 mM EGTA, 10 mM K_2HPO_4, 1 mM L-cysteine, 20% glycerol, 50 mM HEPES pH 7.0, 300 mM KCl, in a final volume of 200 μl, and purified by affinity chromatography.

For CKII phosphorylation, dephosphorylated, repurified SRF (5 ng) was incubated with casein kinase II (1 μl) in 20 μl kinase buffer (50 mM HEPES pH 7.5, 10 mM $MgCl_2$, 1 mM EGTA, 0.1 mM ATP, 0.1% NP40, 100 mM NaCl, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 5 μg/ml pepstatin, 1 mM benzamidine, 50 μg/ml PMSF, 1 mM DTT, 1 mg/ml ovalbumin) at 37°C for 30 min. The reaction was stopped by addition of EDTA to 20 mM. For radiolabelling, [γ-^{32}P]ATP (200 c.p.m./pmol) was used.

For tryptic phosphopeptide mapping, ~2 μg of [^{32}P]SRF was digested with 0.5 mg trypsin and analysed using a RP-300 column (ABI) as described (Hsuan *et al.*, 1989). Analytical CNBr analysis was performed on ~0.5 μg [^{32}P]SRF as described and loaded onto a 20% polyacrylamide gel (Hsuan *et al.*, 1989). For sequence analysis, peptides from ~2 μg [^{32}P]SRF fragmented by CNBr were resolved on a 15% gel. After transfer to PVDF (Problot) membrane, peptide was sequenced using an ABI 477 automated sequencer using fast cycles (J.J.Hsuan, N.Totty and M.D.Waterfield, in preparation).

Gel mobility-shift assay

DNA binding reactions were as described (Treisman, 1986) except with 3 mM rather than 5 mM spermidine, 2 mM EDTA in place of $MgCl_2$, 2 μg *Msp*I-cut pUC12 as non-specific competitor DNA, and 0.5 mg/ml ovalbumin (Sigma, grade III) as protein carrier; binding probes were *Bam*HI–*Bst*NI fragments from pACT.L (ACT.L SRE) or pDYAD (c-*fos* SRE) (Treisman, 1987). Reactions were incubated for 75 min at room temperature and complexes resolved on a 4% polyacrylamide gel in 0.5×TBE. Variations from this protocol are specified in the figure legends. For on-rate estimation, binding reactions were set up without SRF, which was added to individual reactions at various times; the samples were loaded onto the gels at the same time. For exchange-rate estimation, complete binding reactions were allowed to reach equilibrium; unlabelled *Msp*I-digested competitor plasmid (1 μg: equivalent to a 100-fold molar excess of the binding site) was then added to the reactions at various times, and all samples loaded onto the gel at the same time. For Scatchard analysis, gel mobility-shift assays were performed with varying concentration of ACT.L probe and the amount of complexed and free probe determined by using an Ambis Scanner. Two independent analyses gave values of 3×10^{-11} and 1.9×10^{-11}.

Miscellaneous

Proteins were resolved by SDS–PAGE (Laemmli, 1970); silver staining was as described (Ansorge, 1985). Protein concentration determinations were performed using a Bradford protein assay kit (Bio-Rad). For SRF quantification by immunoblot, we used an antiserum directed against the C-terminal 18 amino acids of SRF (D.Hancock, R.Marais, G.Evan and R.Treisman, unpublished). *In vitro* transcription analysis was performed as described (Norman *et al.*, 1988). The FOS.L and FOS.L* columns were prepared by concatenation of the annealed oligonucleotide pairs FOS.L (AATTGGATGCCCATATTAGGGCATCT and AATTAGATGCCCT-AATATGGGCATCC) and FOS.L* (AATTGGATGCCGATATTACGG-CATCT and AATTAGATGCCGTAATATCGGCATCC), biotinylated with Photoprobe biotin (Vector Labs) and bound to streptavidin agarose (Gibco-BRL). These columns contained 1–2 mg of oligonucleotide per ml of resin.

Acknowledgements

We thank Jim Woodgett (LICR) for preparations of purified casein kinase II and Nick Totty (LICR) for amino acid sequence determinations. We are grateful to members of the laboratory and colleagues at the ICRF for helpful comments on the manuscript.

References

Ackerman,P. and Osheroff,N. (1989) *J. Biol. Chem.*, **264**, 11958–11965.
Ackerman,P., Glover,C.V. and Osheroff,N. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 821–825.
Almendral,J.M., Sommer,D., Macdonald,B.H., Burckhardt,J., Perera,J. and Bravo,R. (1988) *Mol. Cell. Biol.*, **8**, 2140–2148.
Ansorge,W. (1985) *J. Biochem. Biophys. Meth.*, **11**, 13–20.
Boxer,L.M., Prywes,R., Roeder,R.G. and Kedes,L. (1989) *Mol. Cell. Biol.*, **9**, 515–522.
Boyle,W.J., Smeal,T., Defize,L.H., Angel,P., Woodgett,J.R., Karin,M. and Hunter,T. (1991) *Cell*, **64**, 573–584.
Bravo,R., Zerial,M., Toschi,L., Schurmann,M., Muller,R., Hirai,S.I., Yaniv,M., Almendral,J.M. and Ryseck,R.P. (1988) *Cold Spring Harb. Symp. Quant. Biol.*, **2**, 901–905.
Carroll,D. and Marshak,D.R. (1989) *J. Biol. Chem.*, **264**, 7345–7348.
Cochet,C., Balogh,A., Chambaz,E.M. and Filhol,O. (1991) *J. Cell. Biochem. (Suppl)*, **15B**, 121.
Foote,J., and Milstein,C. (1991) *Nature*, **352**, 530–532.
Gautier-Rouvière,C., Basset,M., Blanchard,J.-M., Cavadore,J.-C., Fernandez,A. and Lamb,N.J.C. (1991) *EMBO J.* **10**, 2921–2930
Gilman,M.Z., Wilson,R.N. and Weinberg,R.A. (1986) *Mol. Cell. Biol.*, **6**, 4305–16.
Graham,R. and Gilman,M. (1991) *Science*, **251**, 189–192.
Greenberg,M.E., Siegfried,Z. and Ziff,E.B. (1987) *Mol. Cell. Biol.*, **7**, 1217–1225.
Gustafson,T.A., Taylor,A. and Kedes,L. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 2162–2166.
Herrera,R.E., Shaw,P.E. and Nordheim,A. (1989) *Nature*, **340**, 68–70.
Hsuan,J.J., Totty,N. and Waterfield,M.D. (1989) *Biochem. J.* **262**, 659–663.
Je Yu,I., Spector,D.L., Bae,Y-S., and Marshak,D.R. (1991) *J. Biol. Chem.*, in press.
Klarlund,J.K. and Czech,M.P. (1988) *J. Biol. Chem.*, **263**, 15872–15875.
Konig,H. (1991) *Nucleic Acids Res.*, in press.
Laemmli,U.K. (1970) *Nature*, **227**, 680–685.
Lau,L.F. and Nathans,D. (1985) *EMBO J.*, **4**, 3145–3151.
Lau,L.F. and Nathans,D. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 1182–1186.
Luscher,B., Christenson,E., Litchfield,D.W., Krebs,E.G. and Eisenman,R.N. (1990) *Nature*, **344**, 517–522.
Malik,R.K., Roe,M.W. and Blackshear,P.J. (1991) *J. Biol. Chem.*, **266**, 8576–8582.
Manak,J.R. and Prywes,R. (1991) *Mol. Cell. Biol.*, **11**, 3652–3659.
Manak,J.R., de Bisschop,N., Kris,R.M. and Prywes,R. (1990) *Genes Dev.*, **4**, 955–967.
Mohun,T.J., Chambers,A.E., Towers,N. and Taylor,M.V. (1991) *EMBO J.*, **10**, 933–940.
Misra,R.P., Rivera,V.M., Wang,J.M., Fan,P-D., and Greenberg,M. (1991) *Mol Cell. Biol.* **11**, 4545–4554.
Norman,C., Runswick,M., Pollock,R. and Treisman,R. (1988) *Cell*, **55**, 989–1003.
Prywes,R. and Roeder,R.G. (1986) *Cell*, **47**, 777–784.
Prywes,R. and Roeder,R.G. (1987) *Mol. Cell. Biol.*, **7**, 3482–3489.
Prywes,R., Dutta,A., Cromlish,J.A. and Roeder,R.G. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 7206–7210.
Ryan,W.J., Franza,B.J. and Gilman,M.Z. (1989) *EMBO J.*, **8**, 1785–1792.
Schalasta,G. and Doppler,C. (1990) *Mol. Cell. Biol.*, **10**, 5558–5561.
Schneider,J. and Fanning,E. (1988) *J. Virol.*, **62**, 1598–1605.
Schroter,H., Mueller,C.G., Meese,K. and Nordheim,A. (1990) *EMBO J.*, **9**, 1123–1130.
Shaw,P.E., Schroter,H. and Nordheim,A. (1989) *Cell*, **56**, 563–572.
Sommercorn,J., Mulligan,J.A., Lozeman,F.J. and Krebs,E.G. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 8834–8838.
Summers,M.D. and Smith,G.E. (1987) *Texas Experimental Station Bulletin.* No. 1555.
Treisman,R. (1985) *Cell*, **42**, 889–902.
Treisman,R. (1986) *Cell*, **46**, 567–574.
Treisman,R. (1987) *EMBO J.*, **6**, 2711–2717.
Treisman,R. (1990) *Semin. Cancer Biol.*, **1**, 47–58.

Received on September 17, 1991; revised on October 29, 1991