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DNA bending in the ternary nucleoprotein complex at the *c-fos* promoter

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ABSTRACT

Transcriptional induction of the c-*fos* proto-oncogene in response to serum growth factors is mediated in part by a ternary complex that forms on the serum response element (SRE) within its promoter. This complex consists of Elk-1, serum response factor (SRF) and the SRE. Elk-1 is phosphorylated by MAP kinase, which correlates with the induction of c-*fos* transcription. In this study we have investigated the protein-induced DNA bending which occurs during the formation and post-translational modification of the ternary complex that forms at the c-*fos* SRE. Circular permutation analysis demonstrates that the minimal DNA-binding domain of SRF, which contains the MADS box, is sufficient to induce flexibility into the centre of its binding site within the SRE. Phasing analysis indicates that at least part of this flexibility results in the production of a directional bend towards the minor groove. The isolated ETS domains from Elk-1 and SAP-1 induce neither DNA bending nor increased DNA flexibility. Formation of ternary complexes by binding of Elk-1 to the binary SRF:SRE complex results in a change in the flexibility of the SRE. Phosphorylation of Elk-1 by MAP kinase (p42/ERK2) induces further minor changes in this DNA flexibility. However, phasing analysis reveals that the recruitment of Elk-1 to form the ternary complex affects the SRF-induced directional DNA bend in the SRE. The potential roles of DNA bending at the c-*fos* SRE are discussed.

INTRODUCTION

Transcription of the proto-oncogene c-*fos* is rapidly induced by a plethora of extracellular stimuli, including serum growth factors and tetradecanoylphorbol acetate (TPA). These signals are transduced in part through a multicomponent nucleoprotein complex which forms at the serum response element (SRE) within the c-*fos* promoter (reviewed in 1,2). This complex is comprised of two transcription factors, the serum response factor (SRF) and the ternary complex factor $p62^{TCF}$ (3). A combination of DNase I footprinting, methylation interference and methylation protection analyses has confirmed the presence of this complex both *in vitro* (3,4) and *in vivo* (5).

SRF is a dimeric protein which binds to sites containing the consensus sequence $CC(A/T)_6GG$ (6), known as the CArG box. A subfamily of ETS domain proteins, the ternary complex factors (TCFs), includes Elk-1 (7), SAP-1 (8) and ERP/Net (9,10). Elk-1 and SAP-1 possess biochemical activities which are characteristic of $p62^{TCF}$ (8,11). Elk-1 binds to the c-*fos* SRE in conjunction with SRF via an ets motif located upstream from the CArG box to form a ternary complex (11). DNA binding is mediated by the N-terminal ETS domain (12,13). However, Elk-1 is incapable of binding autonomously to the SRE, although it has been shown to bind autonomously to other sites which contain ets motifs (12,14,15). In contrast, a truncated version of SAP-1 which contains the N-terminal ETS domain binds to the SRE *in vitro* (8). A second domain, the B Box, is required for cooperative binding of TCFs to the binary SRF:SRE complex (8,12). This domain is responsible for specific protein–protein interactions with SRF (15).

Elk-1/$p62^{TCF}$ is a nuclear target of the MAP kinase signal transduction pathway (16–18). Phosphorylation occurs at several conserved MAP kinase sites within a domain located towards the C-terminus of the protein (17,19,20). The phosphorylation of $p62^{TCF}$/Elk-1 by MAP kinase stimulates ternary complex formation (16,21) and may also induce a conformational change in one or more components of the ternary complex (17,18). In addition, MAP kinase phosphorylation stimulates Elk-1-mediated transcription *in vivo* (19,20,22).

DNA bending is induced upon binding of a number of eukaryotic transcription factors, including POU domain proteins (23), Myc/Max (24), Fos/Jun (25), Myb (26), YY1 (27) and CREB/CREM (28). However, the precise role of DNA bending is unclear, although it may function in providing recognition specificity or alternatively in modelling promoter architecture. It has been demonstrated using circular permutation analysis that a protein within nuclear extracts bends DNA sequences containing CArG boxes (29). This protein has biochemical characteristics identical to those exhibited by SRF. Such DNA bending may be important in the juxtapositioning of c-*fos* promoter elements. Modulation of SRF-induced bending may therefore have profound effects on the promoter architecture and hence on transcriptional activation.

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The aim of this study was to investigate the effect of Elk-1 binding on the DNA bending exhibited by the SRF:SRE binary complex. Our results demonstrate that the purified SRF DNA-binding domain (core^{SRF}) is sufficient to induce DNA flexibility at the centre of the CArG box. Phasing analysis indicates that at least part of this flexibility results in a directional bend towards the minor groove. DNA bending parameters within the ternary Elk-1:SRF:SRE complex were investigated. As observed in the binary complex, the centre of bending in the ternary complex is located at the centre of the CArG box. However, although DNA flexibility in the ternary complex can be detected, the magnitude of the directional bend is reduced. The overall direction of this reduced bending is apparently towards the major groove. Phosphorylation of Elk-1 by MAP kinase (p42/ERK2) results in a decreased mobility of the ternary complex. Circular permutation analysis indicates that part of this decreased mobility may be due to an increase in the magnitude of DNA flexibility in the ternary complex. The major effect of phosphorylation of Elk-1 by MAP kinase on the mobility of the ternary complex therefore appears to be induction of a change in one of the protein components in this complex.

MATERIALS AND METHODS

Plasmid construction

The plasmid pAS76 was constructed by ligating the two annealed complementary oligonucleotides ADS110 (5′-CTAGACACAG-GATGTCCATATTAGGACATCTGC-3′ and ADS111 (5′-CTA-GGCAGATGTCCTAATATGGACATCCTGTGT-3′) into the *Xba*I site of pBEND2 (30). These oligonucleotides contain the CArG box and the upstream ets site from the c-*fos* SRE. The plasmids pAS145–pAS150 were made by a two step procedure. Duplexes formed by annealing oligonucleotides ADS110 and ADS111 were ligated into the *Xba*I site of the plasmids pSB10, -12, -14, -16, -18 and -20 (31). The orientations of the inserted oligonucleotides were determined by DNA sequencing. Plasmids containing the insert in the same orientation (pSB10SRE–pSB20SRE) were subsequently cleaved with *Bam*HI and religated to eliminate a poly[A:T] tract that is located between the variable linker and the SRE. The identities of the resulting plasmids, pAS145–pAS150 respectively, were confirmed by restriction analysis.

The plasmid pAS136 (encoding SAP-1 amino acids 1–92 with the mutation Q32E) was constructed by two-step PCR mutagenesis (32) using pT7SAP-1A as template (8). The first step utilized the primers T7 (5′-TTAATACGACTCACTAT-3′) and ADS144 (5′-GCCTGCAAAAGCTTGAATTCCCCATCATTA-GAGG-3′). The second step used the product from the first step and the oligonucleotide ADS143 (5′-CGACGGATCCGAATT-CATCAGTTCAAAATCTCTGG-3′). The resulting PCR product was cleaved with *Nco*I and *Bam*HI and ligated into the pBluescript derivative pAS37 (33) which had been cut with the same enzymes. The plasmid pGERK, encoding a fusion of p42/ERK2 MAP kinase with GST was kindly provided by Roger Davis.

Protein production

core^{SRF} was purified from *Escherichia coli* as described previously (15). Elk1-168, which encodes Elk-1 amino acids 1–168, was synthesized by *in vitro* transcription/translation as described previously (15). SAP-92 (SAP-1 amino acids 1–92, encoding the ETS DNA-binding domain with the mutation Q32E) was synthesized by *in vitro* transcription/translation from a DNA template generated by linearization of pAS136 with *Bam*HI. Full-length Elk-1 (amino acids 1–428) was purified as a C-terminally histidine-tagged protein from *E.coli* transformed with the plasmid pQE6/16Elk, as described elsewhere (21). MAP kinase (p42/ERK2) was expressed as a fusion with GST (GST–p42^{MAPK}) and purified from *E.coli* by a modification of the procedure described by Smith and Johnson (34). Cells were lysed and GST–p42^{MAPK} was bound to reduced glutathione–agarose beads in 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM PMSF, 1 mM DTT. Beads were washed in the same buffer and the purified GST–p42^{MAPK} was eluted in 100 mM HEPES, pH 8, 15 mM reduced glutathione. Finally, the purified GST–p42^{MAPK} was dialysed against 20 mM HEPES, pH 8, 20% glycerol, 0.2 mM EDTA, 0.1 mM KCl, 0.5 mM PMSF, 0.5 mM DTT.

Active MEK (MAP kinase kinase) was partially purified from rat L6 cell extracts on a MonoQ column using FPLC (Pharmacia).

Phosphorylation of Elk-1

Phosphorylation of Elk-1 by MAP kinase was carried out in 20 µl reaction mixtures containing ~4 ng GST–p42^{MAPK}, 1 µl MEK extract, 0.1 mM ATP, 0.1 mM sodium vanadate, 40 mM Tris-Cl, pH 7.4, 1 mM DTT, 10 mM $MgCl_2$, 10 µCi [γ-^{32}P]ATP, ~10 ng bacterially expressed Elk-1. Reactions were allowed to proceed for 1 h at 30°C. The presence of ATP, MEK and MAP kinase was required for maximal stimulation of Elk-1 phosphorylation (our unpublished data).

Circular permutation and phasing analysis

For circular permutation analysis, DNA fragments were isolated from pAS76 by digestion with the appropriate restriction enzymes, dephosphorylated by calf intestinal alkaline phosphatase and end-labelled using T4 polynucleotide kinase in the presence of [γ-^{32}P]ATP using standard procedures (35). Alternatively, PCRs were carried out (20 cycles) in the presence of [α-^{32}P]dCTP in 25 µl reaction mixtures containing pAS76 as template and 25 pmol of each of the oligonucleotides ADS188 (5′-CAAGAGGCCCGGCAGTAC-3′) and ADS189 (5′-TTGT-CTCATGAGCGGATAC-3′) as described previously (6). Subsequently, the radioactively labelled PCR products were cleaved with the appropriate restriction enzymes and the 153 bp DNA fragments purified from non-denaturing 10% polyacrylamide gels. DNA binding reactions were carried out essentially as described previously (36), except that 150 mM KCl was included in the binding reactions. The protein:DNA complexes and free DNAs were resolved by electrophoresis through 4 or 5% polyacrylamide gels cast in 1× Tris–borate–EDTA (TBE) buffer (35) and visualized by autoradiography. The magnitude of apparent DNA bending was calculated from the variation in the mobilities of protein:DNA complexes during circular permutation analysis using the formula $\mu M/\mu E = \cos(\alpha/2)$, where μM and μE are the relative mobilities of the core^{SRF} complexes (37) and α is the induced bend angle. The values of μM and μE were calculated from the curve produced after fitting the data to a cosine function (38). Curve fitting was carried out using the program Fig.P (Biosoft).

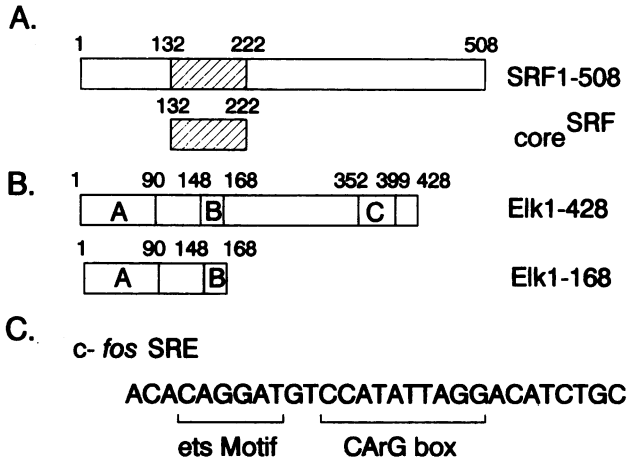

Figure 1. Diagrammatic representation of the components comprising the ternary complex. (**A**) SRF is represented by a rectangle. The segment corresponding to the minimal DNA-binding domain (core^{SRF}) is hatched. Amino acid numbers are given above the diagram. (**B**) Elk-1 is represented by a rectangle. The locations of the A (DNA-binding), B (SRF interaction) and C (MAP kinase target) domains are indicated. The composition of the C-terminally truncated derivative Elk1-168 is also indicated. Amino acid numbers are given above the diagram. (**C**) The DNA sequence of the c-*fos* SRE used in this study. The location of the CArG box and ets motif within the SRE are indicated.

For phasing analysis, DNA fragments were synthesized by PCR using the primers ADS230 (GAAATTAATACGACTCAC) and ADS231 (TATGTATCATACACATAC) and plasmids pAS145–pAS150 as template. DNA binding reactions and subsequent gel electrophoresis were performed as above. The relative mobilities of free DNA and protein:DNA complexes were determined by measuring the distances travelled from the origin of the gel.

RESULTS

A domain of SRF of ~90 amino acids (core^{SRF}) is sufficient for dimerization, DNA binding and interaction with Elk-1 (Fig. 1A) (15,39,40). Core^{SRF} was used in the following experiments in order to enable the clear resolution of DNA:protein complexes. For the same reason, a truncated version of Elk-1, Elk1-168 (Fig. 1B) (15) was also used in circular permutation assays. This protein lacks the regulatory C-terminal domain and binds with high affinity in ternary complexes with core^{SRF} and the SRE (12,15).

Core^{SRF} induces DNA bending at the c-*fos* SRE

Circular permutation analysis (41) was used to determine whether core^{SRF} induces DNA bending. This assay depends on the fact that DNA bending is a determinant of its mobility in native polyacrylamide gels. The migration of a DNA fragment is at its least when a bend occurs at the centre, whilst it is at its greatest when the bend is located at the end. Both the location and apparent degree of DNA bending can be determined using this assay. However, the circular permutation assay cannot unambiguously demonstrate bending in a defined direction (38). For this reason, we refer to bend angles calculated using this method as apparent bend angles and refer to changes in DNA conformation as changes in DNA flexibility. A series of binding sites of identical sequence and length were constructed in which the position of the c-*fos* SRE varies with respect to the end of the fragment (Fig. 2A).

The results of the circular permutation analysis are consistent with the notion that core^{SRF} bends DNA upon forming binary complexes (Fig. 2B and C). Binary core^{SRF} complexes in which the SRE is located towards the centre of the DNA fragment clearly show slower mobility than those in which the SRE is located towards the ends (Fig. 2B, compare 2° complexes with probes Sm and EV to those with Ml and BH), which is indicative of a protein-induced increase in DNA flexibility. The location of the minimum of the graph of the circular permutation function indicates that the position of the centre of DNA bending is 81 bp from the 5′-end of the DNA fragment. An additional probe in which the relative position of the SRE is between those in probes EV and Sm shows further decreased mobility and confirms this conclusion (data not shown). This position coincides with the centre of the CArG box in the c-*fos* SRE (Fig. 2C). Moreover, the apparent bend angle, α, induced by core^{SRF} was calculated to be 72° using the empirical equation $\mu M/\mu E = \cos(\alpha/2)$, where μM and μE are the relative mobilities of the core^{SRF} complexes with DNA fragments in which the CArG box is located either in the middle (M) or at the end (E) of the DNA fragment (37).

DNA bending in the ternary complex

Core^{SRF} forms ternary complexes with the SRE and a second protein p62^{TCF}/Elk-1 (4,11,42). Elk-1 recognizes a purine-rich sequence located upstream from the CArG box (2,11) (Fig. 1C). This binding site is centred on a GGA triplet which is similar to the ets motifs found in sites which are bound by other proteins containing ETS DNA-binding domains (reviewed in 43,44). The formation of this higher order complex containing Elk-1 in addition to SRF may further alter the DNA structure and thus potentially contribute to the transcriptional activation process. We therefore used circular permutation analysis to study the effect of ternary complex formation on DNA bending at the c-*fos* SRE.

Core^{SRF} and a truncated Elk-1 derivative, Elk1-168 were bound to the circularly permuted DNA fragments shown in Figure 2A. Ternary complexes containing Elk1-168, core^{SRF} and the SRE exhibited differential migration through polyacrylamide gels (Fig. 2B and C). Ternary complexes in which the SRE is located towards the centre of the DNA fragment clearly show slower mobility than those in which the SRE is located towards the ends (Fig. 2B, compare 3° complexes with probes Sm and EV to those with Ml and BH), which is indicative of protein-induced DNA bending or alternatively an increase in DNA flexibility. It is unlikely that this change in relative mobility is due to the increase in complex size, as there is no significant correlation between the magnitude of DNA bending and the molecular masses of the complexes (38). In agreement with the results obtained on the binary core^{SRF}:SRE complex, the position of the DNA bend is again located at the centre of the CArG box (Fig. 2C). The apparent angle of DNA bending within the ternary complex is 65° in a 5% polyacrylamide gel. These results indicate that SRF-induced DNA flexibility is maintained in the ternary complex and that the location of the centre of DNA bending in the binary (core^{SRF}:SRE) and ternary (core^{SRF}:Elk1-168:SRE) complexes are identical. However, the magnitude of core^{SRF}-induced DNA flexibility is apparently reduced during ternary complex formation with Elk-1–168.

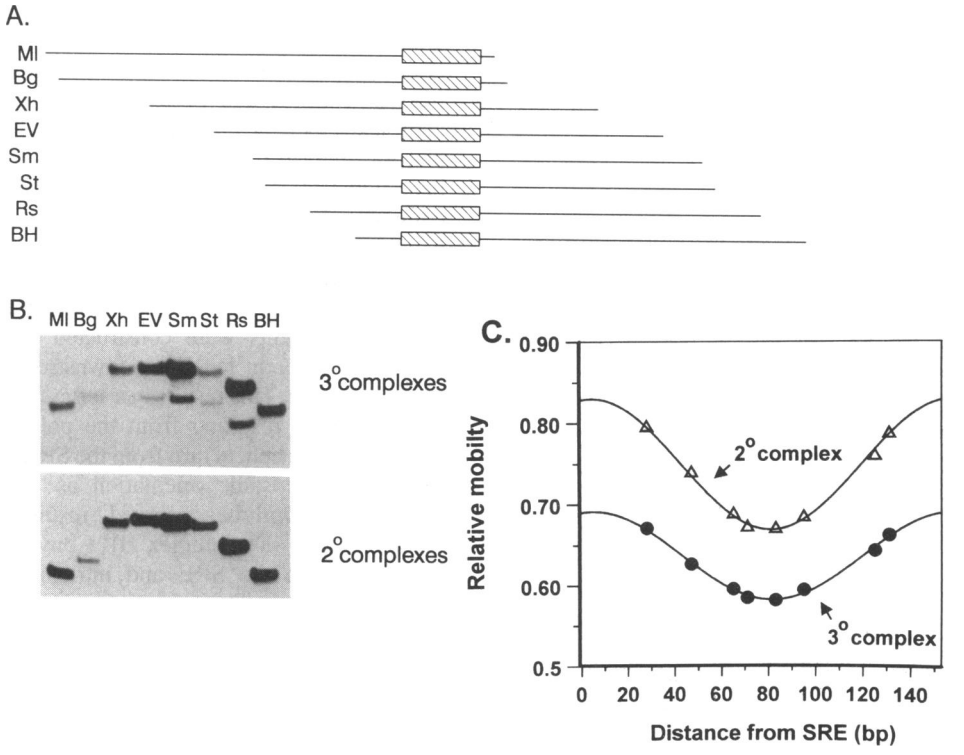

Figure 2. Circular permutaion analysis of complexes which form with the c-*fos* SRE. (**A**) Diagrammatic representation of the probes generated by restriction digestion of pAS76. All probes are of identical length but contain different circular permutations of the same DNA sequence. The SRE is indicated as a hatched box. Probes were generated by digestion with *Mlu*I (Ml), *Bgl*II (Bg), *Xho*I (Xh), *Eco*RV (EV), *Sma*I (Sm), *Stu*I (St), *Rsa*I (Rs) and *Bam*HI (BH). (**B**) Core^{SRF} or core^{SRF} and Elk1-168 were incubated with each of the circularly permuted probes and analysed by electrophoresis through a 5% non-denaturing polyacrylamide gel. The resulting binary (2°) complexes between core^{SRF} and the SRE (lower panel) or ternary (3°) complexes between core^{SRF}, Elk1-168 and the SRE (upper panel) are shown. The second faster migrating complex visible in some tracks in the upper panel represent residual binary complex which has not been bound by Elk1-168. (**C**) The relative mobilities of the binary (2°) and ternary (3°) complexes were normalized for differences in probe mobilities and plotted as a function of the position of the centre of the SRE from the 5′-end of the probe. The points (open triangles, binary complex; closed circles, ternary complex) are connected by the best fit of a cosine function.

The effect of MAP kinase phosphorylation of Elk-1 on DNA bending at the c-*fos* SRE

The data presented above indicate that truncated versions of Elk-1 do not significantly perturb the degree of DNA flexibility in the c-*fos* SRE upon ternary complex formation. It is possible that full-length Elk-1 exerts a different effect on the degree of DNA flexibility in the ternary complex. Moreover, phosphorylation of both p62^{TCF}, purified from nuclear extracts (16), and recombinant Elk-1, purified from *E.coli*, by MAP kinases p44/ERK1 (21) and p42/ERK2 (our unpublished data) stimulates their binding to the binary SRF:SRE complex. In addition, phosphorylation has been suggested to induce a conformational change in the ternary complex of Elk-1/p62^{TCF} with SRF and the c-*fos* SRE (17,18). This putative conformational change may be exhibited through an effect on either the DNA and/or either of the two proteins in the ternary complex.

The circular permutation assay was used to investigate the potential effect of the binding of full-length Elk-1 and its subsequent phosphorylation on the conformation of the DNA within the ternary complex. Recombinant Elk-1 purified from *E.coli* (and hence non-phosphorylated) was phosphorylated using purified MAP kinase (p42/ERK2). Phosphorylated and non-phosphorylated Elk-1 was bound to binary SRF:SRE complexes formed on circularly permuted fragments in which the location of the SRE was varied (Fig. 2A). The mobilities of the ternary complexes containing phosphorylated Elk-1 were less than those of the ternary complexes containing non-phosphorylated Elk-1 on each of the binding sites tested (Fig. 3A; compare lanes 1 and 2, 3 and 4, 5 and 6). Graphical representation of the relative mobilities of the complexes demonstrates that complexes containing either phosphorylated or unphosphorylated Elk-1 bend the SRE and the location of the bend centre is at the centre of the CArG box in both cases (Fig. 3B). Apparent bend angles of the DNA within ternary complexes were calculated as 46° in the presence of unphosphorylated Elk-1 and 55° in the presence of phosphorylated Elk-1. This indicates that phosphorylation of Elk-1 increases the DNA flexibility within the ternary complex. Both apparent bend angles in the ternary complexes are lower than the apparent angle of DNA bending within the binary SRF:SRE complex, which was calculated as 60°. All these angles are lower than those calculated from Figure 2, as this experiment was carried out in a 4% polyacrylamide gel. In general, an increase in gel percentage increases the apparent degree of bending using the circular permutaion assay (31). However, the relative increase in protein-induced DNA flexibility upon Elk-1 phosphorylation is similar on a 5% gel (data not shown), indicating that this effect is independent of gel concentration.

These data indicate that phosphorylation of Elk-1 by MAP kinase appears to affect the conformation of the DNA in the ternary Elk-1:SRF:SRE complex. However, phosphorylation of

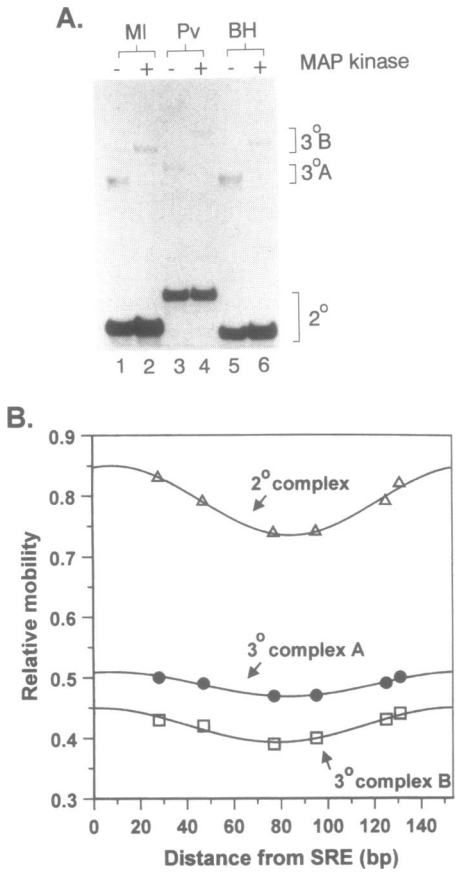

Figure 3. Circular permutation analysis of ternary complexes containing non-phosphorylated or MAP kinase phosphorylated Elk-1. (**A**) Core^{SRF} and Elk1-428 were incubated with each of the circularly permuted probes and analysed by electrophoresis through a 4% non-denaturing polyacrylamide gel. The positions of resulting binary (2°) complexes between core^{SRF} and the SRE or ternary (3°A and 3°B) complexes between core^{SRF}, Elk1-428 and the SRE are indicated. The DNA probes were generated by digestion of DNA amplified by PCR from pAS76 with *Mlu*I (Ml), *Pvu*II (Pv) or *Bam*HI (BH). The presence (+) or absence (–) of MAP kinase in pre-incubations of Elk1-428 is indicated above each lane. (**B**) The relative mobilities of the binary (2°) and ternary (3°A and 3°B) complexes were normalized for differences in probe mobilities and plotted as a function of the position of the centre of the SRE from the 5′-end of the probe. The points (open triangles, binary complex; closed circles, ternary complex A; open squares, ternary complex B) are connected by the best fit of a cosine function.

Elk-1 induces a change in the mobility of the ternary complex which is not dependent on the position of the SRE and therefore not due to a change in DNA conformation (Fig. 3A). A similar effect is observed when circularly permuted fragments derived entirely from c-*fos* promoter sequences are used (our unpublished data). This result implies that the commonly observed MAP kinase-induced mobility change in the ternary complex is only partially due to an increase in DNA flexibility. Instead, this alteration in mobility is due mainly to a direct change in one or both of the protein components of the ternary complex.

Phasing analysis of protein-induced DNA bending at the c-*fos* SRE

Circular permutation analysis alone cannot establish unequivocally whether protein binding induces DNA bending or merely increases DNA flexibility. Moreover, the shape of the protein:DNA complexes may also influence complex mobility in circular permutation assays. We therefore used phasing analysis (45) to confirm that proteins within the binary and ternary complexes with the c-*fos* SRE induce DNA bending. In addition, phasing analysis determines the direction of the protein-induced bend with respect to an intrinsic DNA bend. Binary and ternary complexes may bend DNA to equivalent degrees and in the same location, but in opposite directions. Such a scenario has been demonstrated by others, where Jun/Jun and Fos/Jun dimers both bend the same site, but in the opposite direction (25).

DNA fragments were constructed in which the SRE was located at different locations downstream from two poly[A:T] tracts (Fig. 4A). This results in a series of fragments in which the intrinsic bend, resulting from the poly[A:T] tracts, is phased throughout one helical turn from the SRE. If DNA bending at the SRE is in the same orientation as the intrinsic bend, then cooperativity will be observed, resulting in a slow moving complex (Fig. 4D; complex II). Conversely, if the protein-induced bend in the SRE and intrinsic bend are in opposite orientations, then the effect on the intrinsic bend will be neutralized, thus resulting in a faster moving complex (Fig. 4D, complex III).

Core^{SRF} and full-length MAP kinase-phosphorylated Elk-1 were utilized in the phasing experiments. The mobility of the free DNA fragments in polyacrylamide gels varied depending on the location of the SRE (Fig. 4C). Such changes reflect intrinsic bends within the inserted fragment and have been observed by others with different binding sites (23,26,45). Gel retardation demonstrated that the mobility of the binary core^{SRF}:SRE complexes and ternary Elk-1:core^{SRF}:SRE complexes varied depending on the spacing between the centre of the CArG box and the centre of the intrinsic bend (Fig. 4B). However, the mobilities of the ternary complexes showed less variation than those exhibited by the binary complexes. The relative mobilities of each free and each complexed DNA was calculated by normalization against the average mobility of either the total free or total complexed DNAs respectively. The ratio of relative protein:DNA complex mobility/relative free DNA mobility was then plotted as a function of the distance between the intrinsic and protein-induced bend centres (Fig. 4C). The minimum of the resulting curve for the binary complex shows that maximal cooperativity (as in complex II, Fig. 4D) is observed when the two bend centres are separated by 72 bp. This corresponds to 6.9 helical turns (assuming 10.5 bp/helical turn), indicating that the intrinsic and protein-induced bends are in phase. As a poly[A:T] tract bends DNA towards the minor groove, then the overall bend induced in the binary complex is towards the minor groove. In contrast, maximum cooperativity is observed for the ternary complex at a spacing of 68 bp (corresponding to 6.48 helical turns), indicating that the direction of bending is not towards the minor groove and is instead apparently towards the major groove. However, in contrast to the binary complex, the low degree of cooperativity and lack of clear helical phasing with the intrinsic bend observed in the ternary complex is consistent with a significantly lower degree of protein-induced directional bending (38).

Maximal cooperativity is observed when the SRE is in phase with the poly[A:T] tracts in the case of the binary but out of phase in the ternary complex. This indicates that the direction of DNA bending is apparently altered by the addition of Elk-1 to the core^{SRF}:SRE binary complex. However, the magnitude of the

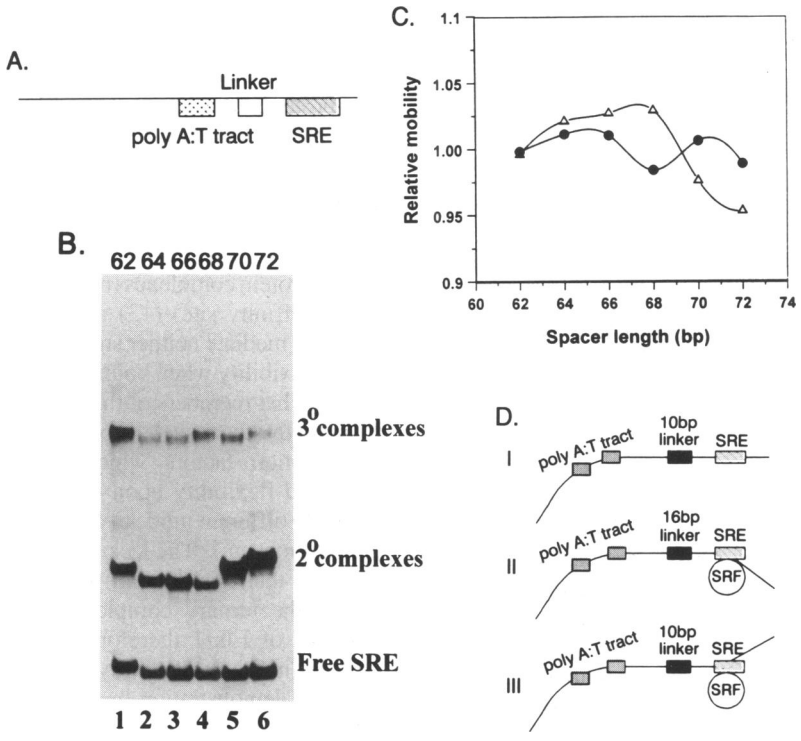

Figure 4. Phasing analysis of DNA bending in complexes which form with the c-*fos* SRE. (**A**) Diagrammatic representation of the phasing probes. The sequence giving rise to the intrinsic bend (crossed box) is composed of two helically phased hexameric [A:T] tracts. The linker (open box) varies between 10 and 20 bp, giving rise to a spacing of between 62 and 72 bp between the centre of the first hexameric [A:T] tract and the centre of the CArG box. The SRE is indicated by a hatched box. (**B**) Core^{SRF} or core^{SRF} and bacterially expressed Elk1 were incubated with each of the phasing probes and analysed by electrophoresis through a 5% non-denaturing polyacrylamide gel. Elk-1 was phosphorylated using MAP kinase (p41/ERK2). The resulting binary (2°) complexes between core^{SRF} and the SRE (lower complex) or ternary (3°) complexes between core^{SRF}, Elk1-428 and the SRE (upper complex) are shown. The mobilities of the free probes are also indicated. The different linkers in each probe resulted in spacer lengths between the centre of the first hexameric poly[A:T] tract and the centre of the CArG box of 62 (lane 1), 64 (lane 2), 66 (lane 3), 68 (lane 4), 70 (lane 5) and 72 bp (lane 6). (**C**) The relative mobilities of binary (2°) and ternary (3°) complexes were normalized for differences in probe mobilities and plotted as a function of the distance between the centre of the SRE from the intrinsic DNA bend. Open triangles indicate binary complexes, whereas closed circles indicate ternary complexes. (**D**) Diagrammatic representation of the structure of the complexes containing an intrinsic DNA bend (I) and an SRF-induced bend in phase (II) or out of phase (III) with the intrinsic DNA bend.

directional bend is clearly reduced in the ternary complex. This reduction in directional bending is consistent with the change in the apparent bend angle between binary and ternary complexes observed in the circular permutation analysis (Fig. 3). Moreover, the rather low degree of cooperativity observed in the phasing analysis suggests that, especially in the case of the ternary complex, the bend angles calculated from the circular permutation assay may be overestimated and rather reflect an induction of DNA flexibility upon complex formation (38).

The ETS DNA-binding domain does not induce SRE bending

Circular permutation analysis and phasing analysis indicate that the binding of Elk-1 to binary SRF:SRE complexes alters the protein-induced directional bending at the c-*fos* SRE and suggest that a change in the extent of DNA flexibility is induced by Elk-1. This suggests that autonomous binding of Elk-1 to the c-*fos* SRE might induce changes in DNA bending at the c-*fos* SRE. However, this could not be tested directly, as autonomous binding of Elk-1 to the SRE is not detectable (12; our unpublished data), although it can efficiently bind other sites *in vitro* (12,15). Moreover, the ETS DNA-binding domain of Elk-1 (Elk-93) efficiently binds to the *Drosophila* E74 site (13), but binding to the c-*fos* SRE is barely detectable even at 1000-fold greater concentrations (our unpublished data). However, the amino acid sequence of the ETS DNA-binding domain of Elk-1 is very similar to that of the related TCF SAP-1 (SAP-92). In order to study the effect of autonomous TCF binding to the SRE we therefore utilized SAP-92, since truncated versions of SAP-1 can bind autonomously to the SRE (8). Circular permutation analysis demonstrates that complexes containing SAP-92 have virtually identical mobilities (Fig. 5), indicating that SAP-92 does not introduce a significant conformational change in the SRE. This data is further substantiated by phasing analysis, which demonstrates that upon SAP-92 binding to the c-*fos* SRE no phase-dependent enhancement/interference with an intrinsic DNA bend occurs (data not shown). Furthermore, circular permutation analysis demonstrates that Elk-93 does not significantly bend the c-*fos* SRE (only visible by phosphorimaging; data not shown). These data are also consistent with the observation that the ETS DNA-binding domain of Elk-1 does not induce bending or flexibility in a high affinity binding site (13).

DISCUSSION

Representative members from several families of eukaryotic transcription factors bend DNA upon binding. These include the

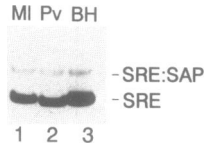

Figure 5. Circular permutation analysis of complexes containing the isolated ETS domain of SAP-1. SAP-92 (encoding the minimal DNA-binding domain of SAP-1, amino acids 1–92) was incubated with each of the circularly permuted probes containing the c-*fos* SRE and analysed by electrophoresis through a 5% non-denaturing polyacrylamide gel. The DNA probes were generated by digestion of DNA amplified by PCR from pAS76 with *Mlu*I (Ml), *Pvu*II (Pv) or *Bam*HI (BH). The location of the free SRE and SAP:SRE complex are indicated.

bZip proteins Fos/Jun (25,38) and CREB/CREM (28), the POU proteins Oct1/Oct2/Oct6/Pit1 (23), the helix–loop–helix proteins Myc/Max (24), the zinc finger protein YY1 (27) and the helix–turn–helix type protein Myb (26). In the case of Fos/Jun, Myc/Max, Myb and Oct1 it has been demonstrated that truncated proteins, which retain their minimal DNA-binding domains, are still able to induce DNA bending. In this study we demonstrate that the purified minimal DNA-binding domain of SRF ($core^{SRF}$), a representative member of the MADS box family of transcription factors, bends DNA. This is consistent with previous results which show that an SRF activity in nuclear extracts bends DNA and that a proteolytically stable fragment of this nuclear activity retains this property (29). In addition, we have demonstrated that bending occurs at the centre of the CArG box. The overall bend angle of 72° determined in a 5% gel is of a similar magnitude to the degree of bending exerted by other DNA-binding proteins. However, it should be noted that absolute values are difficult to obtain using circular permutation analysis, due to the dependence of DNA bending upon several parameters. One such parameter is gel concentration (31). Indeed, the apparent DNA bend angle for $core^{SRF}$ was calculated to be only 60° in a 4% gel. Bend angles are therefore quoted as apparent, because the circular permutation assay measures the extent of DNA flexibility, rather than fixed bend angles. Protein-induced increases in DNA flexibility do not necessarily correlate with the introduction of directional bends into DNA. Indeed, several members of the bZIP family induce DNA flexibility but vary greatly in their abilities to introduce a directional bend into the DNA (38). The relatively low degree of cooperativity shown in the phasing experiments indicates that although SRF induces a large degree of DNA flexibility, the magnitude of the directional bend is probably overestimated by the latter assay. It remains to be seen whether other members of the MADS box family induce DNA bending or DNA flexibility. However, in contrast to SRF, the minimal DNA-binding domains of two representative members of the ETS family of transcription factors, SAP-1 and Elk-1 (13), induce neither significant DNA bending nor increased flexibility.

Phasing analysis confirmed that SRF induces DNA bending and that the overall direction of SRF-induced DNA bending is towards the minor groove. However, as SRF has a core binding motif of 10 bp and interacts as a dimer, it is possible that this bend direction is due to a combined effect of SRF bending the DNA towards the major groove at each side of its binding site. A similar phenomenon has been reported for CAP, which inserts an α-helix into the major groove on each side of its binding site, causing a narrowing of the major groove and an overall bend towards the minor groove at the centre of the site (46). Indeed, in the case of SRF it has previously been suggested that DNA binding is mediated by one α-helix from each monomer (36) and methylation protection/interference studies indicate that contacts occur in the major groove (reviewed in 47). X-ray crystallographic studies on SRF:SRE co-crystals will help to confirm these results.

Elk-1 and the related protein SAP-1 belong to a subgroup of the ETS family of transcription factors known as the TCFs. Many members of the ETS family participate in multi-component nucleoprotein complexes (reviewed in 43). The isolated Elk-1 (on a high affinity site) (13) and SAP-1 (on the c-*fos* SRE) ETS domains mediate neither significant DNA bending nor increased DNA flexibility when bound autonomously to DNA. It therefore appears that members of the ETS family of transcription factors, along with others such as NF1/CTF1 (23), are part of a group of transcription factors which neither bend DNA nor induce increased flexibility upon binding. This might reflect a fundamentally different mode of DNA binding in which minimal DNA distortion occurs. The lack of DNA bending by the ETS domain suggests that this domain plays no role in distorting the DNA within the ternary complex containing Elk-1. The B and C domains of Elk-1 therefore appear to be sufficient to induce changes in DNA flexibility of the c-*fos* SRE. This is consistent with the demonstration that a derivative of Elk-1 which has its ETS domain replaced by a heterologous DNA-binding domain induces a phosphorylation-dependent conformational change in the ternary complex with the c-*fos* SRE (22).

A ternary complex comprised of Elk-1, SRF and the SRE forms within the c-*fos* promoter. It is through this complex that extracellular signals are transduced via the MAP kinase signal transduction pathway (16–18,20). However, the binding of Elk-1 to the binary SRF:SRE complex causes a change in the SRF-induced directional DNA bend and apparently induces changes in the extent of DNA flexibility. It is therefore possible that the binding of Elk-1 to the ternary complex transmits its activation signal, at least in part, through the DNA. Indeed, it has recently been demonstrated that binding of mercury to the MerR repressor causes a marked decrease in the directional DNA bending observed at its binding site (48). This correlates with a transition of MerR from a transcriptional repressor to an activator. As SRF has been shown to act as a repressor of the c-*fos* SRE in the absence of serum stimulation (49), it is tempting to speculate that recruitment of Elk-1 contributes to such a repressor to activator transition in SRF.

Phosphorylation of Elk-1/$p62^{TCF}$ by MAP kinase (p42/ERK2) correlates with increased ternary complex formation at the c-*fos* SRE (16,21; our unpublished data). Changes in ternary complex mobility are also observed upon Elk-1/$p62^{TCF}$ phosphorylation (17,18). Such mobility changes may be a result of a conformational change within one or more components of the ternary complex. In this study it has been clearly demonstrated that the change in ternary complex mobility induced by the phosphorylation of Elk-1 is only partially due to an increase in DNA flexibility. However, a large phosphorylation-induced change in mobility is observed which is independent of the DNA binding site location. This strongly implies that the change in mobility is due mostly to an alteration in the protein components of the ternary complex. The formation of a lower mobility complex would be consistent with a large conformational change in either Elk-1, SRF or both components. Alternatively, this lower mobility complex might reflect the assembly of a higher order

complex. Further studies are required to distinguish between these possibilities.

The function of SRF-dependent DNA bending is unclear. One hypothesis is that DNA bending serves to orient regulatory elements into a favourable location. Such repositioning of promoter elements and their bound proteins could facilitate crosstalk between transcription factors within the promoter. Indeed, it has been suggested that the transcription factor YY1 plays such a role in the c-*fos* promoter (27). In this case YY1 binds between the SRE and the TATA box and is thought to be important in the juxtapositioning of upstream activating elements with the basal transcription complex. In the case of SRF both directional DNA bending and increased flexibility could facilitate its interactions with other transcription factors, including Elk-1. A second potential target for such interactions could be a complex which forms on the upstream SIF element (50,51). An alternative, but not mutually exclusive, role for DNA bending would be in providing recognition specificity for SRF by indirect readout. In this scenario SRF would select sites based on both the presence of specific residues (flanking GG residues) and a central A:T-rich core which would be susceptible to DNA bending. Indeed, poly[A:T] tracts give rise to intrinsic DNA bending (reviewed in 52). Selection of binding sites from random oligonucleotide pools supports this hypothesis. The flanking G bases are almost completely conserved in the selected sites, whereas the central hexameric region is A:T-rich, with only the central two bases showing a strong preference for an individual base at a given position (6).

In summary, we have demonstrated that the MADS DNA-binding domain of SRF induces bending within the c-*fos* SRE. However, although SRF-induced DNA flexibility is apparantly maintained after the recruitment of Elk-1 to form a ternary complex, the magnitude of the directional bend is altered in this higher order complex. Phosphorylation of Elk-1 appears to induce an increase in DNA flexibility. Such increases in flexibility explain, in part, the commonly observed mobility change in the ternary complex after MAP kinase phosphorylation.

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REFERENCES

1 Shaw,P.E., Hipskind,R.A., Schröter,H. and Nordheim,A. (1989) In Eckstein,F. and Lilley,D.M.J. (eds), *Nucleic Acids and Molecular Biology*. Springer-Verlag, Berlin, Germany, Vol. 3, pp. 120–132.
2 Treisman,R. (1992) *Trends Biochem. Sci.*, **17**, 423–426.
3 Shaw,P.E., Schröter,H. and Nordheim,A. (1989) *Cell*, **56**, 563–572.
4 Prywes,R. and Roeder,R.G. (1987) *Mol. Cell. Biol.*, **7**, 3482–3489.
5 Herrera,R.E., Shaw,P.E. and Nordheim,A. (1989) *Nature*, **340**, 68–70.
6 Pollock,R. and Treisman,R. (1990) *Nucleic Acids Res.*, **18**, 6197–6204.
7 Rao,V.N., Huebner,K., Isobe,M., ar-Rushidi,A., Croce,C.M. and Reddy,E.S.P. (1989) *Science*, **244**, 66–70.
8 Dalton,S. and Treisman,R. (1992) *Cell*, **68**, 597–612.
9 Lopez,M., Oettgen,P., Akbarali,Y., Dendorfer,U. and Liberman,T.A. (1994) *Mol. Cell. Biol.*, **14**, 3292–3309.
10 Giovane,A., Pintzas,A., Maira,S.-M., Sobieszcuk,P. and Wasylyk,B. (1994) *Genes Dev.*, **8**, 1502–1513.
11 Hipskind,R.A., Rao,V.N., Mueller,C.G.F., Reddy,E.S.P. and NordheimA. (1991) *Nature*, **354**, 351–354.
12 Janknecht,R. and Nordheim,A. (1992) *Nucleic Acids Res.*, **20**, 3317–3324.
13 Shore,P., Bisset,L., Lakey,J., Waltho,J., Virden,R. and Sharrocks,A.D. (1995) *J. Biol. Chem.*, **270**, 5805–5811.
14 Rao,V.N. and Reddy,E.S.P. (1992) *Oncogene*, **7**, 2335–2340.
15 Shore,P. and Sharrocks,A.D. (1994) *Mol. Cell. Biol.*, **14**, 3283–3291.
16 Gille,H., Sharrocks,A.D. and Shaw,P.E. (1992) *Nature*, **358**, 415–417.
17 Marais,R., Wynne,J. and Treisman,R. (1993) *Cell*, **73**, 381–393.
18 Zinck,R., Hipskind,R.A., Pingoud,V. and Nordheim,A. (1993) *EMBO J.*, **12**, 2377–2387.
19 Janknecht,R., Ernst,W.H., Pingoud,V. and Nordheim,A. (1993) *EMBO J.*, **12**, 5097–5104.
20 Kortenjann,M., Thomae,O. and Shaw,P.E. (1994) *Mol. Cell. Biol.*, **14**, 4815–4824.
21 Gille,H., Kortenjann,M., Thomae,O., Moomaw,C., Slaughter,C., Cobb,M.H. and Shaw,P.E. (1995) *EMBO J.*, **14**, 951–962.
22 Hill,C.S., Marais,R., John,S., Wynne,J., Dalton,S. and Treisman,R. (1993) *Cell*, **73**, 395–406.
23 Verrijzer,C.P., van Oosterhout,J.A.W.M., van Wederen,W.W. and van der Vliet,P.C. (1991) *EMBO J.*, **10**, 3007–3014.
24 Fisher,D.E., Parent,L.A. and Sharp,P.A. (1992) *Proc. Natl. Acad. Sci. USA*, **89**, 11779–11783.
25 Kerppola,T.K. and Curran,T. (1991) *Science*, **254**, 1210–1214.
26 Saikumur,P., Gabriel,J.L. and Reddy,E.P. (1994) *Oncogene*, **9**, 1279–1287.
27 Natesan,S. and Gilman,M.Z. (1993) *Genes Dev.*, **7**, 2497–2509.
28 de Groot,R.P., Delmas,V. and Sassone-Corsi,P. (1994) *Oncogene*, **9**, 463–468.
29 Gustafson,T.A., Taylor,A. and Kedes,L. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 2162–2166.
30 Kim,J., Zweib,C., Wu,C. and Adhya,S. (1989) *Gene*, **85**, 15–23.
31 Drak,J. and Crothers,D.M. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 3074–3078.
32 Sharrocks,A.D. and Shaw,P.E. (1992) *Nucleic Acids Res.*, **20**, 1147.
33 Sharrocks,A.D., von Hesler,F. and Shaw,P.E. (1993) *Nucleic Acids Res.*, **21**, 215–221.
34 Smith,D.B., and Johnson,K.S. (1988) *Gene*, **67**, 31–40.
35 Sambrook,J., Fritsch,E.F. and Maniatis,T. (1990) *Molecular Cloning: A Laboratory Manual*, 2nd Edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
36 Sharrocks,A.D., Gille,H. and Shaw,P.E. (1993b) *Mol. Cell. Biol.*, **13**, 123–132.
37 Thompson,J.F. and Landy,A. (1988) *Nucleic Acids Res.*, **16**, 9687–9705.
38 Kerppola,T.K. and Curran,T. (1993) *Mol. Cell. Biol.* **13**, 5479–5489.
39 Mueller,C.G.F. and Nordheim,A. (1991) *EMBO J.*, **10**, 4219–4229.
40 Shaw,P.E. (1992) *EMBO J.*, **11**, 3011–3019.
41 Wu,H.-M. and Crothers,D.M. (1984) *Nature*, **308**, 509–513.
42 Treisman,R., Marais,R. and Wynne,J. (1992) *EMBO J.*, **11**, 4631–4640.
43 Janknecht,R. and Nordheim,A. (1993) *Biochim. Biophys. Acta*, **1155**, 346–356.
44 Wasylyk,B., Hahn,S.L. and Giovane,A. (1993) *Eur. J. Biochem.*, **211**, 7–18.
45 Zinkel,S.S. and Crothers,D.M. (1987) *Nature*, **328**, 178–181.
46 Schultz,S.C., Shields,G.C. and Steitz,T.A. (1991) *Science*, **253**, 1001–1007.
47 Treisman,R., (1990) *Semin. Cancer Biol.*, **1**, 47–58.
48 Ansari,A.Z., Bradner,J.E. and O'Halloran,V. (1995) *Nature*, **374**, 371–374.
49 Shaw,P.E., Frasch,S. and Nordheim,A. (1989) *EMBO J.*, **8**, 2567–2574.
50 Wagner,B.J., Hayes,T.E., Hoban,C.J. and Cochran,B.H. (1990) *EMBO J.*, **9**, 4477–4484.
51 Sadowski,H.B., Shuai,K., Darnell,J.E.,Jr and Gilman,M.Z. (1993) *Science*, **261**, 1739–1744.
52 Crothers,D.M., Haran,T.E. and Nadeau,J.G. (1990) *J. Biol. Chem.*, **265**, 7093–7096.