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Comparative analysis of the ternary complex factors Elk-1, SAP-1a and SAP-2 (ERP/NET)

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A transcription factor ternary complex composed of Serum Response Factor (SRF) and Ternary Complex Factor (TCF) mediates the response of the c-*fos* Serum Response Element (SRE) to growth factors and mitogens. Three Ets domain proteins, Elk-1, SAP-1 and ERP/NET, have been reported to have the properties of TCF. Here we compare Elk-1 and SAP-1a with the human ERP/NET homologue SAP-2. All three TCF RNAs are ubiquitously expressed at similar relative levels. All three proteins contain conserved regions that interact with SRF and the c-*fos* SRE with comparable efficiency, but *in vitro* complex formation by SAP-2 is strongly inhibited by its C-terminal sequences. Similarly, only Elk-1 and SAP-1a efficiently bind the c-*fos* SRE *in vivo*; ternary complex formation by SAP-2 is weak and is substantially unaffected by serum stimulation or v-ras co-expression. All three TCFs contain C-terminal transcriptional activation domains that are phosphorylated following growth factor stimulation. Activation requires conserved S/T-P motifs found in all the TCF family members. Each TCF activation domain can be phosphorylated *in vitro* by partially purifed ERK2, and ERK activation *in vivo* is sufficient to potentiate transcriptional activation.

Key words: ERK/Ets domain/serum response factor/ternary complex factor/transcription

Introduction

The MAP kinases (MAPKs) are proline-directed protein kinases whose activity can be regulated by extracellular signals including growth factors, mitogens and cellular stresses such as UV light. In mammalian cells, the best understood MAP kinases are p42ERK2 and p44ERK1, which are activated by growth factors via an evolutionarily conserved signalling pathway involving the formation of ras.GTP, recruitment of the c-raf protein kinase to the membrane, and activation of MEKs, the ERK-activating kinases (Marshall, 1994). Other MAPK subfamilies such as the JNKs/SAPKs and p38/RK/MPK2 are regulated independently of the ERKs (Deng and Karin, 1994; Han *et al.*, 1994; Minden *et al.*, 1994; Rouse *et al.*, 1994; Sanchez *et al.*, 1994; Yan *et al.*, 1994). The targets of MAPKs include a number of transcription factors, and it is presumed that it is by phosphorylation of these factors that extracellular stimuli regulate the transcription of cellular immediate–early genes such as c-*fos* and c-*jun*.

One regulatory target for the ERKs is c-*fos* Serum Response Element (SRE). The c-*fos* SRE binds a transcription factor ternary complex comprised of the transcription factors SRF (Serum Response Factor; Norman *et al.*, 1988) and TCF (Ternary Complex Factor; Shaw *et al.*, 1989). TCF binds an Ets motif, CAGGAT, adjacent to the SRF binding site, but cannot do so independently of SRF (Shaw *et al.*, 1989). Genomic footprinting studies show that the ternary complex is present at the SRE even in unstimulated cells, suggesting that SRE activation involves regulation of transcriptional activation rather than DNA binding (Herrera *et al.*, 1989; König *et al.*, 1989). TCF binding is required for an efficient response of the c-*fos* promoter to activators of the ERKs: c-*fos* promoter mutants that can bind SRF, but which cannot form the ternary complex, cannot respond to stimuli such as v-raf and TPA (König *et al.*, 1989; Graham and Gilman, 1991; Hill *et al.*, 1994; Kortenjann *et al.*, 1994), but remain responsive to uncharacterized signals elicited by whole serum (Graham and Gilman, 1991; Hill *et al.*, 1994; Johannsen and Prywes, 1994; C.Hill, J.Wynne and R.Treisman, unpublished data).

Three Ets domain proteins with DNA-binding properties characteristic of the TCFs have been identified: Elk-1, SAP-1 and ERP/NET. These proteins contain three regions of substantial sequence homology, termed boxes A, B and C (Hipskind *et al.*, 1991; Dalton and Treisman, 1992; Giovane *et al.*, 1994; Lopez *et al.*, 1994). Boxes A and B mediate DNA binding and cooperative interaction with SRF at the SRE (for a review, see Treisman, 1994; also Shore and Sharrocks, 1994). The C-terminal region, including box C, contains several conserved potential MAPK phosphorylation sites. Both HeLa cell TCF and recombinant Elk-1 can be phosphorylated at many of these sites by ERK2 *in vitro*, and the same sites become phosphorylated *in vivo* in response to ERK activation (Gille *et al.*, 1992, 1995; Janknecht *et al.*, 1993; Marais *et al.*, 1993). Phosphorylation of Elk-1 at specific C-terminal sites potentiates transcriptional activation and, under certain conditions, the DNA-binding activity of the protein (Gille *et al.*, 1992, 1995; Hill *et al.*, 1993; Janknecht *et al.*, 1993; Marais *et al.*, 1993; Nebreda *et al.*, 1993; Kortenjann *et al.*, 1994).

Recent studies have suggested that the different TCF proteins may possess different regulatory properties. For example, inducible phosphorylation of the SAP-1/SRF ternary complex, as assessed by its reduced mobility in gel mobility-shift assays, occurs independently of ERK activation in some cell lines (Hipskind *et al.*, 1994), and transcriptional activation by ERP/NET can be induced by v-ras, c-mos and v-src, but not by serum, v-raf or ERK1 (Giovane *et al.*, 1994). We therefore undertook a comparative analysis of DNA binding and transcriptional activation by Elk-1, SAP-1a and SAP-2, the human homologue of ERP/NET. Our results indicate that SAP-2 does not bind DNA or form ternary complexes as

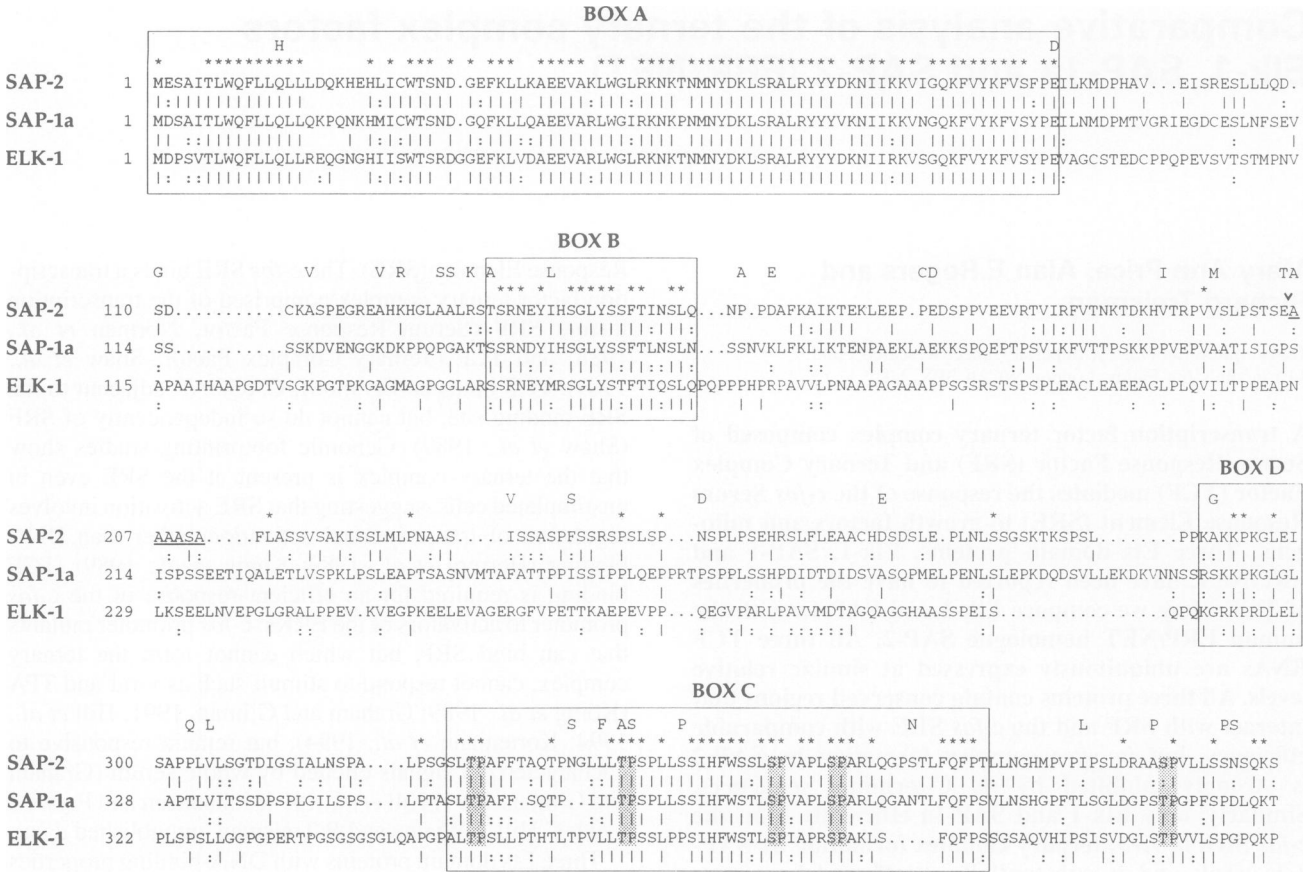

Fig. 1. Alignment of Elk-1, SAP-1a and SAP-2 sequences. ERP/NET residues that differ from SAP-2 are shown above the SAP-2 sequence. Identities are indicated by the vertical lines and similarities by colons; symbols below the Elk-1 sequence indicate its relationship to SAP-2. Asterisks indicate positions where all three human proteins are identical. The four homology regions previously noted are boxed (Dalton and Treisman, 1992; Lopez *et al.*, 1994); S/T-P motifs conserved among all three proteins are shaded. The homology to the Krüppel repression domain in SAP-2 is underlined.

efficiently as Elk-1 or SAP-1a, suggesting that it may have different target sites or that its binding is regulated differently from the other two proteins. We also demonstrate that ERK activation is sufficient to potentiate transcriptional activation by the C-terminal regions of all three proteins.

Results

Isolation of cDNAs encoding SAP-2, a human ERP/NET homologue

During screening of a human placenta cDNA library with a SAP-1 cDNA probe, we recovered cDNAs encoding a related protein, SAP-2, which is the human homologue of the mouse protein ERP/NET (Dalton and Treisman, 1992; Giovane *et al.*, 1994; Lopez *et al.*, 1994). SAP-2 contains three characteristic conserved regions shared by the Elk-1 and SAP-1 TCFs (Dalton and Treisman, 1992; Figure 1) and exhibits 91% identity to ERP/NET. In general, sequence differences occur outside the conserved regions; however, not all the S/T-P motifs in the C-terminal homology region are conserved among the entire family (Figure 1; see Discussion). SAP-2 contains a short additional conserved region termed box D (residues 290–299; Lopez *et al.*, 1994), but a short homology to the Krüppel repression domain (Licht *et al.*, 1990) is conserved only between SAP-2 and ERP/NET (Giovane *et al.*, 1994).

Expression of TCF RNAs

To compare the expression of TCF RNAs in different human tissues, we used an RNase protection assay. The SAP-1 probe spans the point at which the SAP-1a sequence diverges from that of a presumed splice variant, SAP-1b, which truncates the C box (Dalton and Treisman, 1992); analogous probes were used for the other TCF RNAs. Elk-1, SAP-1a and SAP-2 RNAs were detected in each of the wide variety of tissues tested, at similar levels relative to each other and to a GAPDH control; in contrast, SAP-1b RNA was present above the detection limit only in brain, HeLa and HT1080 cells (Figure 2). No cleavage products characteristic of Elk-1 or SAP-2 variants analogous to SAP-1b were detectable. We conclude that there is little differential expression of the different TCFs, at least at the RNA level.

DNA binding and ternary complex formation by SAP-2 in vitro

The presence of homology regions A and B in SAP-2 suggests that it should form a ternary complex with SRF, as do Elk-1 and SAP-1a. To allow comparison of DNA binding and ternary complex formation by the three TCF family members, we produced the intact proteins or truncation derivatives by cell-free translation. Gel mobility-shift assays were performed in the presence or absence of SRF(133–265), an SRF fragment sufficient for DNA

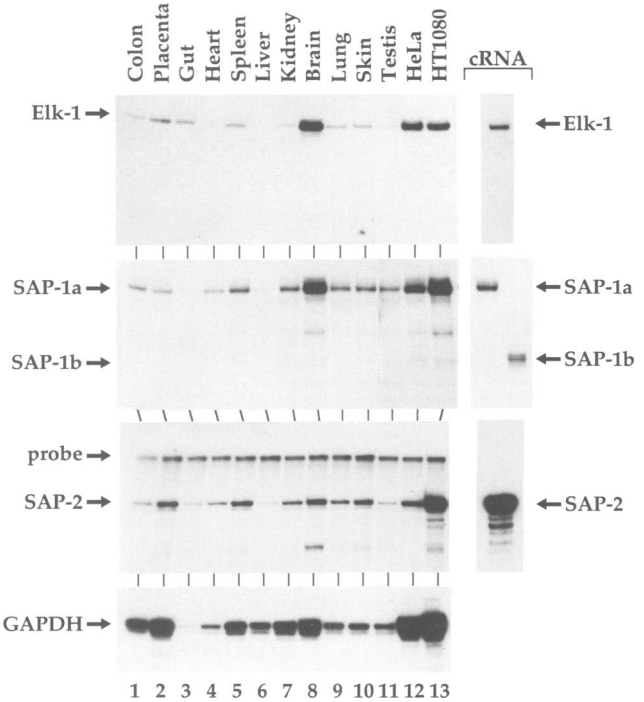

Fig. 2. RNase protection analysis of the different TCF RNAs. Sources of RNA are indicated at the top. Top panel, Elk-1 RNA; second panel, SAP-1 RNAs; third panel, SAP-2 RNA; bottom panel, GAPDH RNA. The probe RNAs, which encompass a region including the N-terminus of the C box in each case, were complementary to the following parts of the three RNAs: Elk-1, codons 317–378; SAP-1a, codons 315–377; SAP-2, codons 283–352. The products of control digestions of TCF complementary RNA/probe hybrids are shown at the right. Digestion products of SAP-1 and SAP-2 hybrids not seen in these control reactions may represent other TCF splice variants, but we have not investigated this further.

binding and ternary complex formation. As probes, we used either the c-*fos* SRE or probe G, a synthetic sequence containing high-affinity Ets and SRF consensus sequences, to which Elk-1 can bind autonomously (see Treisman *et al.*, 1992).

None of the three proteins bound autonomously to the c-*fos* SRE, but weak autonomous binding of Elk-1 and SAP-1a, but not SAP-2, was detectable with probe G (Figure 3, lanes 1, 3 and 5). Elk-1 formed ternary complexes with SRF(133–265) on either probe more efficiently than SAP-1a; however, no ternary complex formation by intact SAP-2 or ERP/NET was detectable, even with the high-affinity probe (Figure 3, lanes 9, 11 and 13; Figure 4A, lanes 1–3; data not shown). C-terminal truncation of each TCF increased its DNA binding or ternary complex factor activity: strikingly, ternary complex formation by SAP-2 truncations was comparable with that of analogous derivatives of the other proteins (Figure 3A and B; lanes 10, 12, 14 and 15), while their autonomous binding activity was if anything greater (Figure 3B, lanes 2, 4, 6 and 7). Similar results were obtained with other probes, including the *Xenopus laevis* c-*fos* SRE and a second high-affinity synthetic site, probe L (data not shown; Treisman *et al.*, 1992).

The results above show that the SAP-2 boxes A and B are capable of interaction with DNA and SRF, but that in the context of the intact SAP-2 protein these interactions are inhibited. To map the sequences responsible for inhibition, we tested the activity of a set of SAP-2 deletion mutants. Deletion of the conserved C box resulted in low, but detectable ternary complex formation (Figure 4A, lane 4); however, ternary complex formation progressively increased upon removal of further sequences between amino acids 253 and 222 (Figure 4A, lanes 7–9). Analysis of an internal deletion series showed that only a mutant resulting from the deletion of all sequences between amino acids 158 and 399, including the C box, formed ternary complexes efficiently (Figure 4B). Similar results were obtained when autonomous binding of SAP-2 derivatives to probe G was examined (data not shown).

To test whether the inhibitory effect of the SAP-2 C-terminal sequences reflects specific interaction with the SAP-2 N-terminal sequences, we examined SAP-2/Elk-1 chimeras. Substitution of the SAP-2 Ets domain (box A), or both boxes A and B, by the corresponding Elk-1 sequences generated proteins which, like SAP-2, failed to form ternary complexes (Figure 4C, lanes 5 and 6); conversely, substitution of the SAP-2 C-terminal sequences by those of Elk-1 generated proteins which were competent to form ternary complexes (Figure 4C, lanes 2 and 3). Similar results were obtained when autonomous binding to the G probe was analysed (data not shown). The inhibitory effect of SAP-2 C-terminal sequences is therefore not dependent on specific SAP-2 N-terminal sequences.

SAP-2 binds the c-fos SRE poorly in vivo

We used fusion proteins containing the constitutively active VP16 transcriptional activation domain to compare binding of the TCFs with the c-*fos* SRE *in vivo*. Transcriptional activation of SRE-controlled reporter genes by such fusion proteins requires intact SRF and TCF binding sites, and can thus be taken as a measure of ternary complex formation *in vivo* (Dalton and Treisman, 1992; Hill *et al.*, 1994). C-terminally VP16-tagged Elk-1 and SAP-1a derivatives efficiently activated the wild-type c-*fos* SRE (WT SRE) in serum-deprived NIH3T3 cells, while SREs lacking the Ets motif (SRE.L) or the SRF site (SRE.M) were activated less efficiently (Figure 5). In contrast, activation by the SAP-2 fusion protein was barely above background levels, although control immunoblotting experiments showed that expression of SAP-2.VP16 was comparable with that of Elk.VP16 and several-fold greater than expression of SAP-1a.VP16 (data not shown). Similar results were obtained in HeLa cells, and with N-terminally VP16-tagged proteins (data not shown). Thus, in the absence of growth factor stimulation, it appears that only Elk-1 and SAP-1a can efficiently bind the c-*fos* SRE.

We next investigated whether growth factor stimulation would affect ternary complex formation by SAP-2, a possibility suggested by the observation that ERK2-phosphorylated Elk-1 exhibits increased ternary complex activity under certain DNA-binding conditions (Gille *et al.*, 1992). NIH3T3 fibroblasts were transfected with Elk-1, SAP-1a or SAP-2 expression plasmids with or without a co-transfected v-ras expression plasmid, and extracts from serum-deprived and serum-stimulated cells tested for ternary complex activity. Serum- or v-ras-induced phosphorylation of Elk-1 reduces the mobility of the Elk-1/SRF ternary complex in the gel mobility-shift assay (Figure 6, lanes 4–6; Marais *et al.*, 1993); increased

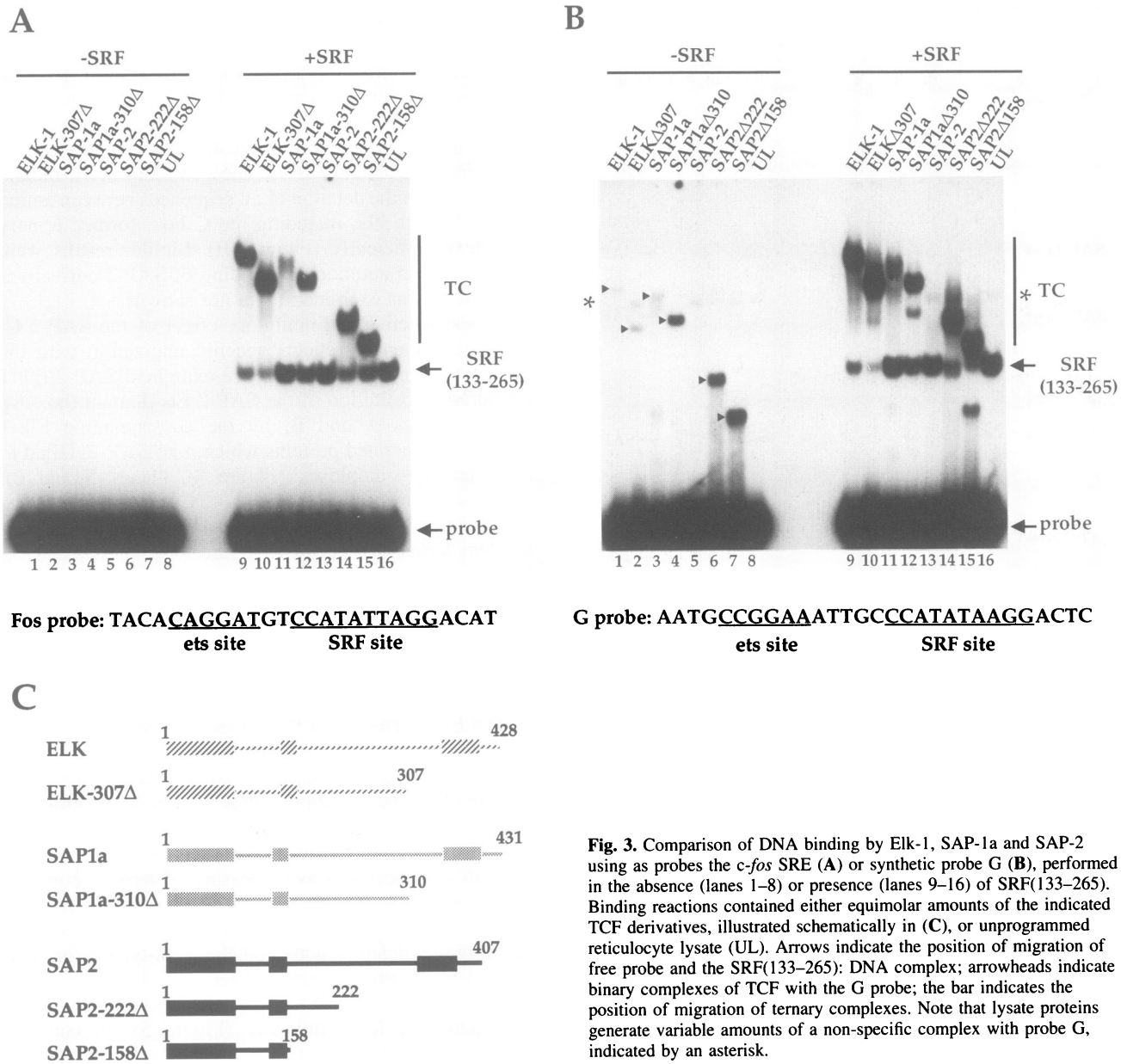

Fig. 3. Comparison of DNA binding by Elk-1, SAP-1a and SAP-2 using as probes the c-*fos* SRE (**A**) or synthetic probe G (**B**), performed in the absence (lanes 1–8) or presence (lanes 9–16) of SRF(133–265). Binding reactions contained either equimolar amounts of the indicated TCF derivatives, illustrated schematically in (**C**), or unprogrammed reticulocyte lysate (UL). Arrows indicate the position of migration of free probe and the SRF(133–265): DNA complex; arrowheads indicate binary complexes of TCF with the G probe; the bar indicates the position of migration of ternary complexes. Note that lysate proteins generate variable amounts of a non-specific complex with probe G, indicated by an asterisk.

ternary complex yield in the presence of v-ras is due to overproduction of Elk-1 (see Discussion). Similar results were obtained with SAP-1a, which forms a ternary complex of similar mobility to that formed by NIH3T3 cell TCF (Figure 6, compare lanes 1–3 and 7–9). In contrast, extracts from uninduced or serum-induced cells expressing SAP-2 generated no detectable ternary complex in addition to the endogenous complex. However, upon co-expression of v-ras, where SAP-2 is overproduced, weak ternary complex formation was detectable (Figure 6, lanes 10–12). Control immunoblotting experiments using epitope-tagged derivatives of Elk-1 and SAP-2 showed that these proteins were expressed at comparable levels, and several-fold more efficiently than SAP-1a (data not shown). These results suggest that Elk-1 and SAP-1a, but not SAP-2, can efficiently form ternary complexes with SRF at the c-*fos* SRE in the absence of growth factor signals *in vivo*, and that neither serum nor v-ras stimulation is sufficient to allow efficient ternary complex formation by SAP-2.

Transcriptional activation by the TCF C-termini

We previously developed an assay for activity of the Elk-1/SRF ternary complex using altered specificity derivatives of Elk-1 and SRF (Hill *et al.*, 1993). We constructed analogous altered-specificity derivatives of SAP-1a and SAP-2, and found that while the SAP-1a derivative bound DNA and regulated transcription in this assay, the SAP-2 derivative does neither (data not shown). To compare the ability of the TCFs to regulate transcription, we therefore constructed fusion proteins in which their respective C-termini are linked to the LexA repressor (Figure 7A) and tested their ability to activate a LexA operator-controlled reporter gene, which is unable to respond to serum stimulation or v-ras (Marais *et al.*, 1993). In the presence of the SAP-1a fusion protein NLex.SAP-1C, regulated activity of the reporter gene in response to serum, phorbol esters and CSF1 stimulation was comparable with that brought about by NLex.ELKC (Figure 7B; data not shown). In contrast, neither

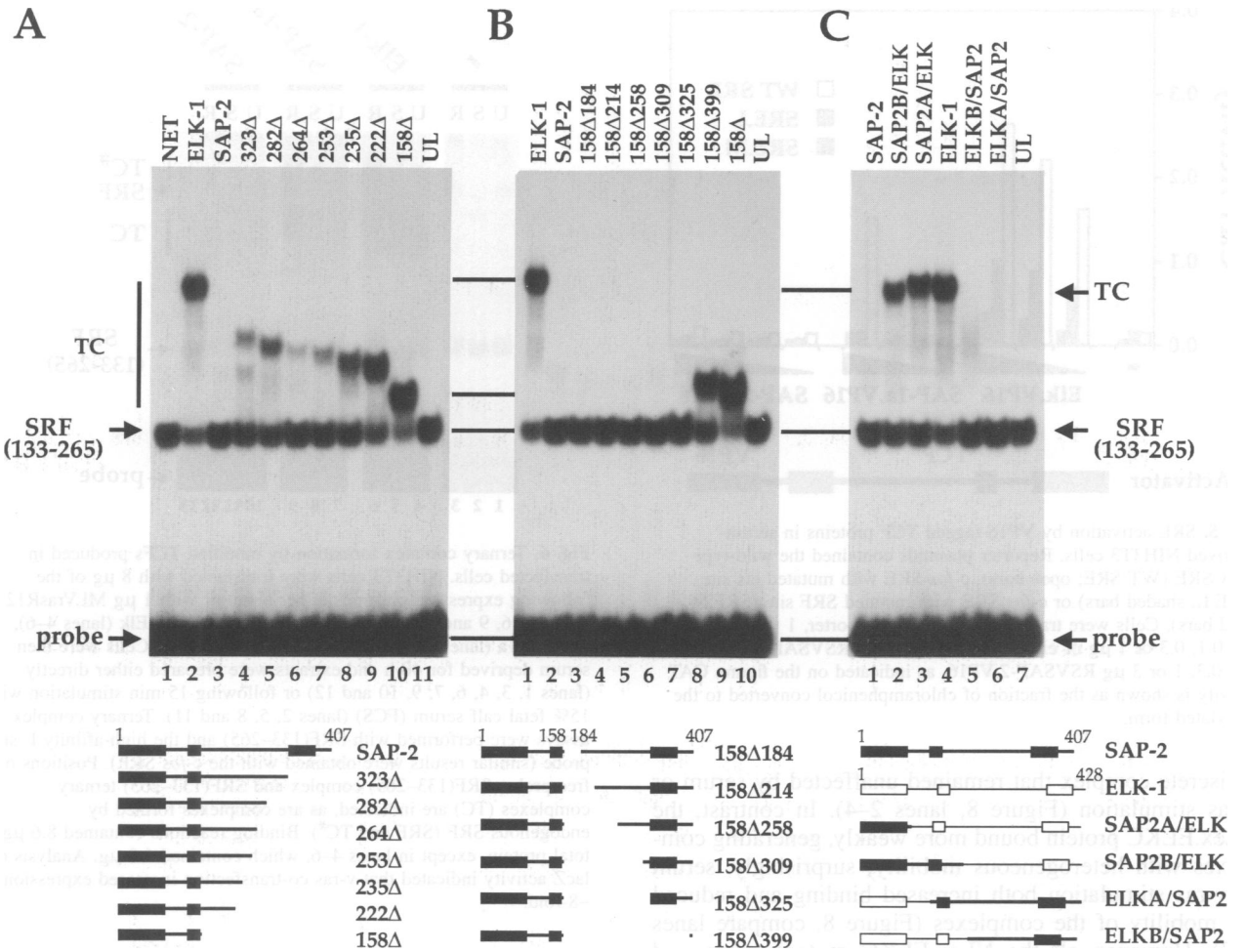

Fig. 4. SAP-2 C-terminal sequences inhibit DNA binding. TCF derivatives were incubated with SRF(133–265) and a c-*fos* SRE oligonucleotide probe. (**A**) C-terminal SAP-2 deletions: lane 1, NET; lane 2, Elk-1; lane 3, SAP-2; lanes 4–10, SAP-2 C-terminal deletion mutants as indicated below; lane 11, unprogrammed reticulocyte lysate. (**B**) Internal SAP-2 deletions series: lane 1, Elk-1; lane 2, SAP-2; lanes 3–8, SAP-2 internal deletions as indicated below; lane 9, SAP2-158Δ; lane 10, unprogrammed reticulocyte lysate. (**C**) Binding by SAP-2/Elk-1 chimeras: lane 1, SAP-2; lane 2, SAP2B/ELK; lane 3, SAP2A/ELK; lane 4, Elk-1; lane 5, ELKB/SAP2; lane 6, ELKA/SAP2; lane 7, unprogrammed reticulocyte lysate; the proteins are shown schematically below.

NLex.SAP-2C nor two additional SAP-2 derivatives containing residues 282–407 or residues 325–407 of SAP-2 conferred serum regulation on the reporter (Figure 7B; data not shown). However, fusion proteins derived from all the TCFs could activate transcription of the reporter in cells expressing v-ras (Figure 7B; see also Figure 10B).

Serum-regulated transcriptional activation by NLex.-ElkC is dependent on the integrity of several C-terminal S-T/P phosphoacceptor sites, which are conserved in the other TCFs; of these sites, Elk-1 serine 383 is especially important (Marais *et al.*, 1993). We therefore tested the effects of analogous mutations in the NLex.SAP-1C and NLex.SAP-2C fusion proteins (Figure 7B). Mutation to alanine of SAP-1a serine 381 or SAP-2 serine 357 (corresponding to Elk-1 residue 383) severely reduced transcriptional activation by the fusion proteins, although the SAP-1a S381A mutation was less detrimental than the Elk-1 S383A mutation. Substitution of SAP-1a residues S387, T420 and S425 by alanine also reduced activation, but less effectively, while an S381A/S387A double mutant was almost completely inactive, similar to its Elk-1 counterpart (Figure 7B). The C box exhibits substantial sequence conservation in addition to the conserved S/T-P motifs, including a conserved string of hydrophobic amino acids (see Figure 1). Deletion of Elk-1 residues F378 and W379, or their individual substitution by alanine, abolished the ability of NLex.ELKC to regulate transcription in response to serum stimulation (Figure 7B). Taken together, these data show that the C-terminus of each TCF can regulate transcription in response to activation of intracellular signalling pathways, and that this requires phosphorylation sites and sequence motifs conserved among the three proteins.

DNA binding by LexA–TCF fusion proteins

To test whether the failure of the various LexA–TCF fusion proteins to activate transcription resulted from their inability to bind DNA, we analysed cell extracts by gel mobility-shift assay, using a high-affinity LexA operator probe. [In our previous study (Marais *et al.*, 1993), we were unable to detect LexA DNA-binding activity with a lower affinity probe.] The intact LexA protein generated

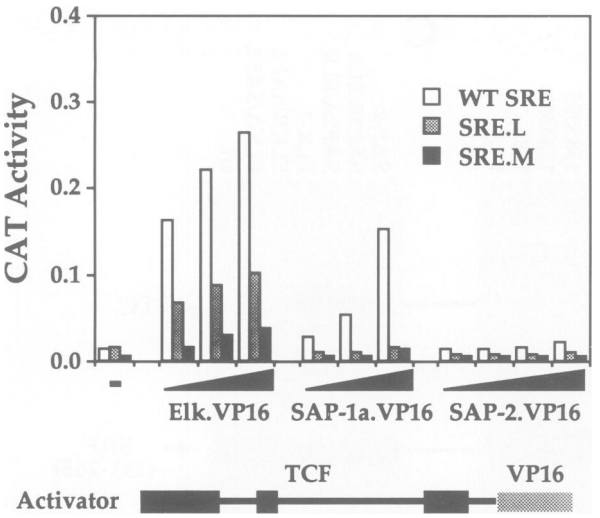

Fig. 5. SRE activation by VP16-tagged TCF proteins in serum-deprived NIH3T3 cells. Reporter plasmids contained the wild-type c-*fos* SRE (WT SRE; open bars), c-*fos* SRE with mutated ets site (SRE.L; shaded bars) or c-*fos* SRE with mutated SRF site (SRE.M, solid bars). Cells were transfected with 4 μg reporter, 1 μg MLVlacZ, and 0.1, 0.3 or 1 μg of either RSVElk.VP16 or RSVSAP-1a.VP16, or 0.1, 0.3, 1 or 3 μg RSVSAP-2.VP16, as indicated on the figure. CAT activity is shown as the fraction of chloramphenicol converted to the acetylated form.

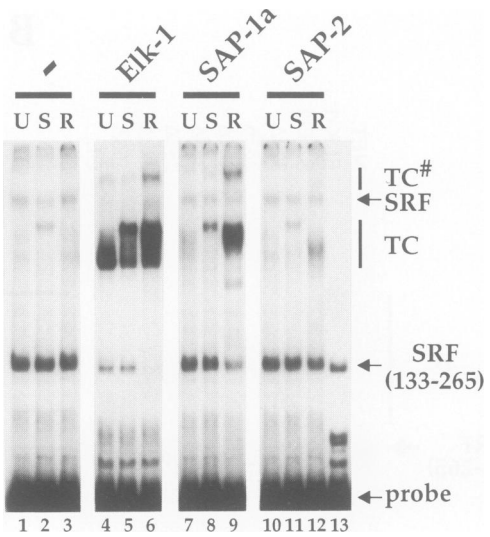

Fig. 6. Ternary complex formation by modified TCFs produced in transfected cells. NIH3T3 cells were transfected with 8 μg of the following expression plasmids either alone or with 1 μg MLVrasR12 (lanes 3, 6, 9 and 12): MLV128β (lanes 1–3), MLV.Elk (lanes 4–6), EF.SAP-1a (lanes 7–9) or EF.SAP-2 (lanes 10–12). Cells were then serum deprived for 40 h and extracts were prepared either directly (lanes 1, 3, 4, 6, 7, 9, 10 and 12) or following 15 min stimulation with 15% fetal calf serum (FCS) (lanes 2, 5, 8 and 11). Ternary complex assays were performed with SRF(133–265) and the high-affinity L site probe (similar results were obtained with the c-*fos* SRE). Positions of free probe, SRF(133–265) complex and SRF(133–265) ternary complexes (TC) are indicated, as are complexes formed by endogenous SRF (SRF and TC#). Binding reactions contained 8.6 μg total protein, except in lanes 4–6, which contained 1.3 μg. Analysis of lacZ activity indicated that v-ras co-transfection increased expression ~8-fold.

a discrete complex that remained unaffected by serum or v-ras stimulation (Figure 8, lanes 2–4). In contrast, the NLex.ELKC protein bound more weakly, generating complexes with heterogeneous mobility; surprisingly, serum or v-ras stimulation both increased binding and reduced the mobility of the complexes (Figure 8, compare lanes 5–7). However, all the NLex.ELKC mutants examined retained this regulated binding, including those that abolish transcription such as S383A, S389A, S383/389A and T417A/S422A (Figure 8, lanes 14–19; data not shown). NLex.SAP-1C was not produced at sufficient levels to generate detectable DNA-binding activity, except in the presence of co-transfected v-ras, which induces its over-expression (Figure 8, lanes 8–10; see also Figure 9B); however, the SAP-1a S381A mutation, which abolished transcriptional activation, did not affect DNA binding (data not shown). Finally, both NLex.SAP-2C and its mutant derivative S357A generated LexA operator binding activity in serum-deprived cells, which decreased upon serum stimulation or co-transfection with v-ras (Figure 8, lanes 11–13; data not shown). We conclude that the failure of the mutant fusion proteins to activate transcription is not caused by defective DNA binding (see Discussion).

Regulated phosphorylation of the TCF C-terminal regions

Growth factor-regulated transcriptional activation by Elk-1 coincides with the phosphorylation of several conserved S/T-P motifs by ERK2. To test whether the other TCFs are substrates for ERK2, we partially purified the enzyme from TPA-stimulated U937 cells (Adams and Parker, 1991) and tested its ability to phosphorylate GST–TCF fusion proteins containing the C-terminal regions present in the LexA–TCF fusion proteins. Each fusion protein was efficiently phosphorylated, resulting in a significant reduction of its mobility on SDS–PAGE (Figure 9A). We exploited this property to ask whether serum or v-ras stimulation of transfected cells induces modification of the LexA–TCF fusion proteins, using fractionation of transfected cell extracts by SDS–PAGE and immunoblot with a LexA antibody. In serum-deprived cells, NLex.-ELKC and NLex.SAP-1C were detected as polypeptides of uniform mobility, while NLex.SAP-2C migrated heterogeneously, suggesting that it is constitutively modified (Figure 9B, lanes 5, 8 and 11). Constitutive modification of SAP-2 probably occurs between residues 222 and 282, because NLex.SAP-2(282–407) migrates as a single species (data not shown). Stimulation by addition of serum or co-transfection with v-ras generated modified low-mobility forms of all the fusion proteins, but had no effect on intact LexA protein (Figure 9B, lanes 2–13).

Taken together with the *in vitro* phosphorylation studies, the above data suggest that the SAP-1a and SAP-2 C-terminal regions are phosphorylated in response to serum or v-ras stimulation. To test this more directly, we treated the cell extracts with alkaline phosphatase. The serum- and v-ras-induced modifications of all the fusions were sensitive to phosphatase treatment, but were preserved when phosphatase inhibitors were included in the reaction (NLex.SAP-2C, Figure 9B, lanes 19–23; NLex.SAP-1C, data not shown). Treatment of NLex.SAP-2C extracts from serum-deprived cells caused only partial conversion of the protein to its most rapidly migrating form; this conversion could also be blocked by phosphatase inhibitors (Figure 9B, lanes 14–18), but it remains possible that the

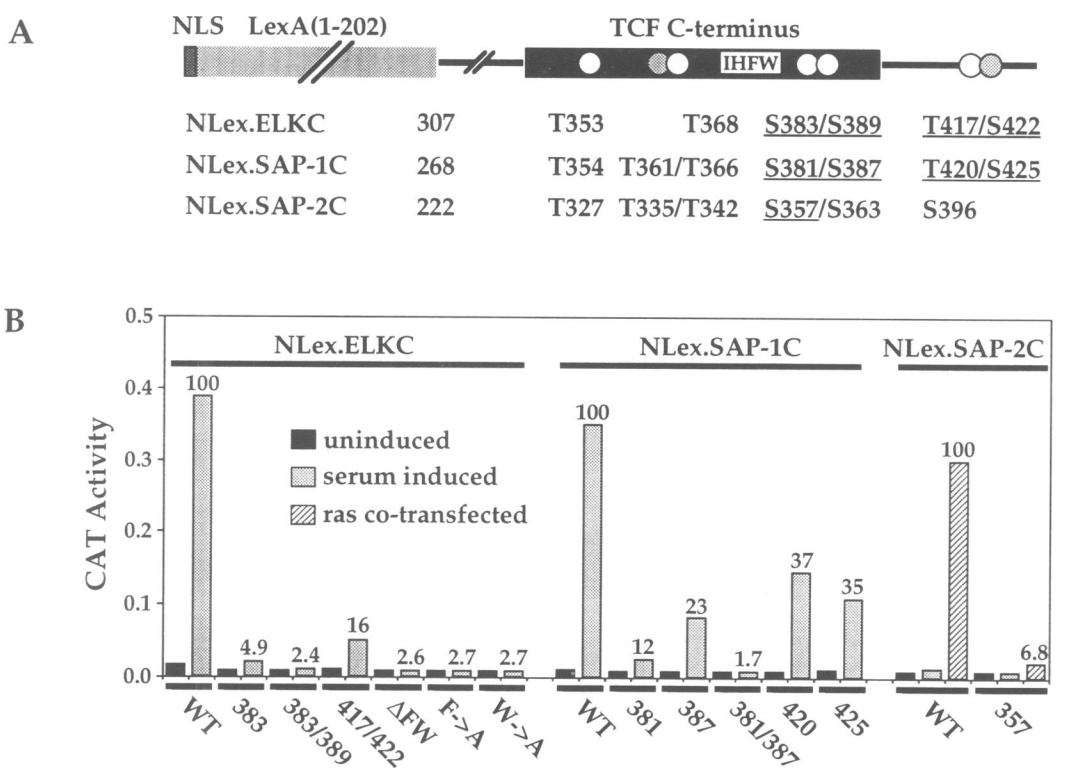

Fig. 7. Transcriptional activation by the TCF C-termini. (**A**) LexA–TCF fusion proteins. Dark shading, SV40 NLS; light shading, complete LexA coding region (residues 1–202); solid rectangles, TCF homology regions, with conserved hydrophobic sequence shown; open or shaded circles, conserved S/T-P motifs present in all (open) or only two (shaded) of the proteins with residue number and identity indicated below. (**B**) NIH3T3 cells were transfected with 4 μg 2LexOP.tkCAT and 1 μg MLVlacZ, with either 0.25 μg MLVNLex.ELKC derivatives, 1 μg MLVNLex.SAP-1C derivatives or 3 μg MLVNLex.SAP-2C derivatives, and 0.3 μg MLVrasR12 as indicated. CAT activity was determined and expressed as fraction of chloramphenicol acetylated: solid bars, serum-deprived cells; shaded bars, serum-stimulated cells; hatched bars, v-ras co-transfected cells. A representative experiment is shown; numbers above the bars indicate the average relative to wild-type determined from several independent experiments.

modifications in serum-deprived cells are not exclusively phosphorylations.

ERK activation potentiates transcriptional activation by each TCF C-terminus

Considered in the light of our previous results with Elk-1, the results presented above are consistent with the idea that transcriptional activation by the SAP-1a and SAP-2 C-termini is potentiated by regulated phosphorylation of specific conserved S/T-P motifs, probably by the ERKs. To determine whether ERK activation is sufficient for activation of the different TCF C-termini *in vivo*, we tested whether constitutive activation or inappropriate expression of cellular signalling molecules, previously shown to cause ERK activation, could potentiate transcriptional activation by the LexA–TCF fusion proteins. We tested a number of molecules that act either as ERK kinase kinases or higher up the pathway, including an activated raf derivative, ΔNraf (Howe *et al.*, 1992), c-mos (Nebreda *et al.*, 1993), MEKK (Lange-Carter *et al.*, 1993), v-src and v-ras. We also directly activated the ERKs intracellularly by expression of a constitutively activated MAPKK (or MEK) derivative, MAPKK(217E/221E) (Cowley *et al.*, 1994), and evaluated activation by an ERK2 mutant, ERK2(D319N), whose *Drosophila* counterpart is weakly activated by background signal in the ras/raf/ERK pathway in the absence of receptor activation (Brunner *et al.*, 1994; Bott *et al.*, 1994; E.Hafen, personal communication). To verify that these proteins did not cause general activation of cellular signalling pathways, we also tested their effect on transcriptional activation by a LexA–Jun fusion protein, which is independently controlled by the JNK/SAPK pathway (Minden *et al.*, 1994; Sanchez *et al.*, 1994; Yan *et al.*, 1994). The results are shown in Figure 10.

All the activators tested stimulated transcriptional activation by the Elk-1 C-terminus in the absence of growth factor stimulation; most importantly, expression of MAPKK(217E/221E) efficiently activated Elk-1, and even expression of ERK2(D319N) allowed activation, albeit weakly (Figure 10A and B, upper panels). In contrast, activation of transcription by the LexA–Jun fusion protein was stimulated by only MEKK and v-src, and efficient ERK activators such as v-ras, c-mos, ΔNraf and MAPKK(217E/221E) had no effect (Figure 10A, lower panel). Both the SAP-1a and SAP-2 C-terminal regions behaved similarly to that of Elk-1, although as noted above transcriptional activation by NLex.SAP-2C was less efficient (Figure 10B). Activation by each TCF C-terminus in these experiments was dependent on the integrity of the S-P motif corresponding to Elk-1 serine 383 (Figure 10B, compare shaded and solid bars); a more comprehensive survey of the Elk-1 and SAP-1a mutants showed that activation by these signalling molecules requires the same S/T-P motifs as are required for growth factor regulation (data not shown). Analysis of cell extracts

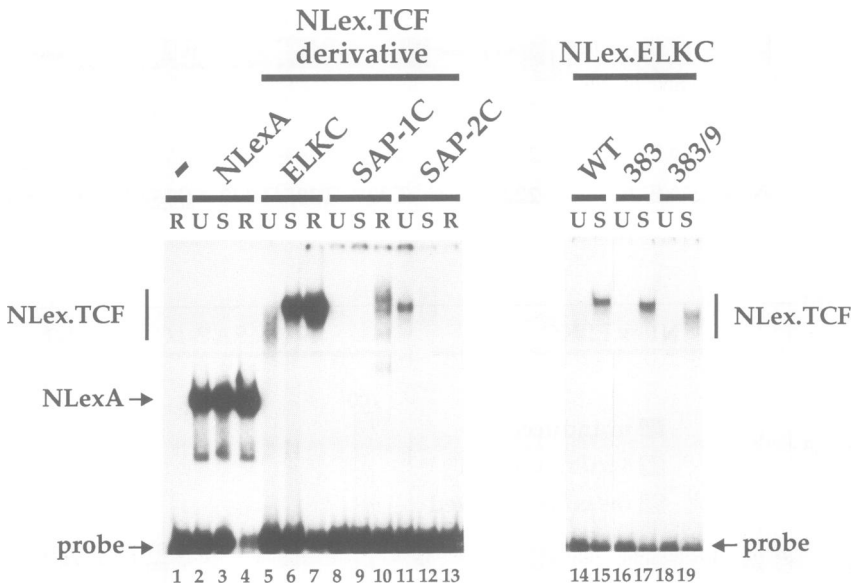

Fig. 8. Transcriptional activation by LexA–TCF does not reflect regulation of DNA-binding activity. NIH 3T3 cells transfected with 8 μg MLV128β (lane 1), 8 μg MLVNLexA (lanes 2–4), 8 μg MLVNLex.ELKC (lanes 5–7, 14 and 15), 8 μg EFNLex.SAP-1C (lanes 8–10), 8 μg EFNLex.SAP-2C (lanes 11–13), 8 μg MLVNLex.ElkS383A (lanes 16 and 17), 8 μg MLV.NLex.ElkS383A/S389A (lanes 18 and 19) and 0.3 μg MLVrasR12 (lanes 1, 4, 7, 10 and 13), and serum deprived for 40 h. Extracts were prepared either directly (lanes 1, 2, 4, 5, 7, 8, 10, 11, 13, 14, 16 and 18) or following 15 min stimulation with 15% FCS (lanes 3, 6, 9, 12, 15, 17 and 19), and analysed by gel mobility-shift assay using the LexA operator probe.

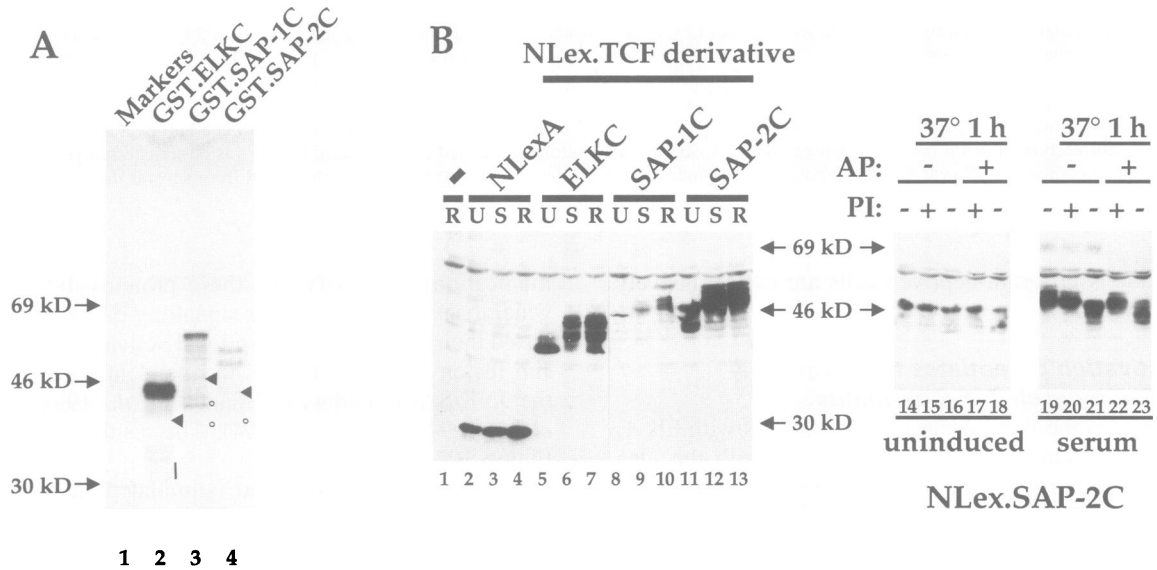

Fig. 9. Phosphorylation of TCF C-termini. (**A**) Phosphorylation of purified GST–TCF fusion proteins by partially purified ERK2. Reaction products were fractionated by SDS–PAGE following addition of a 5-fold excess of unlabelled protein. Lane 1, molecular markers; lane 2, GST.ElkC; lane 3, GST.SAP-1C; lane 4, GST.SAP-2C. The GST.ElkC reaction contained about twice as much substrate as the other reactions. The positions where the unphosphorylated fusion proteins migrate are marked by an arrowhead; open circles and the vertical line indicate the positions of major unphosphorylated breakdown products. (**B**) Phosphorylation of the TCF C-termini *in vivo*. NIH3T3 cells were transfected with 8 μg of the following plasmids either alone or with 0.3 μg MLVrasR12 (lanes 1, 4, 7, 10 and 13): MLV128β (lane 1), MLV.NLexA (lanes 2–4), MLVNLex.ElkC (lanes 5–7), EFNLex.SAP-1C (lanes 8–10) or EFNLex.SAP-2C (lanes 11–23). Following serum deprivation for 40 h, extracts were prepared either directly (lanes 1, 2, 4, 5, 7, 8, 10, 11 and 13–18) or following 15 min stimulation with 15% FCS (lanes 3, 6, 9, 12 and 19–23). Forty micrograms were analysed by SDS–PAGE and immunoblotting with LexA antiserum. Extracts shown in lanes 14–23 were first treated with alkaline phosphatase (AP) and/or phosphatase inhibitors (PI), as indicated in the figure.

by immunoblotting using the LexA antibody demonstrated that co-expression of any of the activator molecules induced modification of each TCF C-terminus, although ΔNraf and ERK2(D319N) were less effective (Figure 10C). Activated MAPKK was also sufficient to induce modification of each of the fusion proteins (data not shown). However, although serum and PMA were as effective as v-ras in inducing phosphorylation of the SAP-2 C-terminus, as judged by this assay, they did not effectively potentiate transcriptional activation (Figure 10, lanes 2–4; see Discussion). Nevertheless, these results demonstrate that activation of the ERKs is sufficient to potentiate modification and transcriptional activation by each of the TCFs.

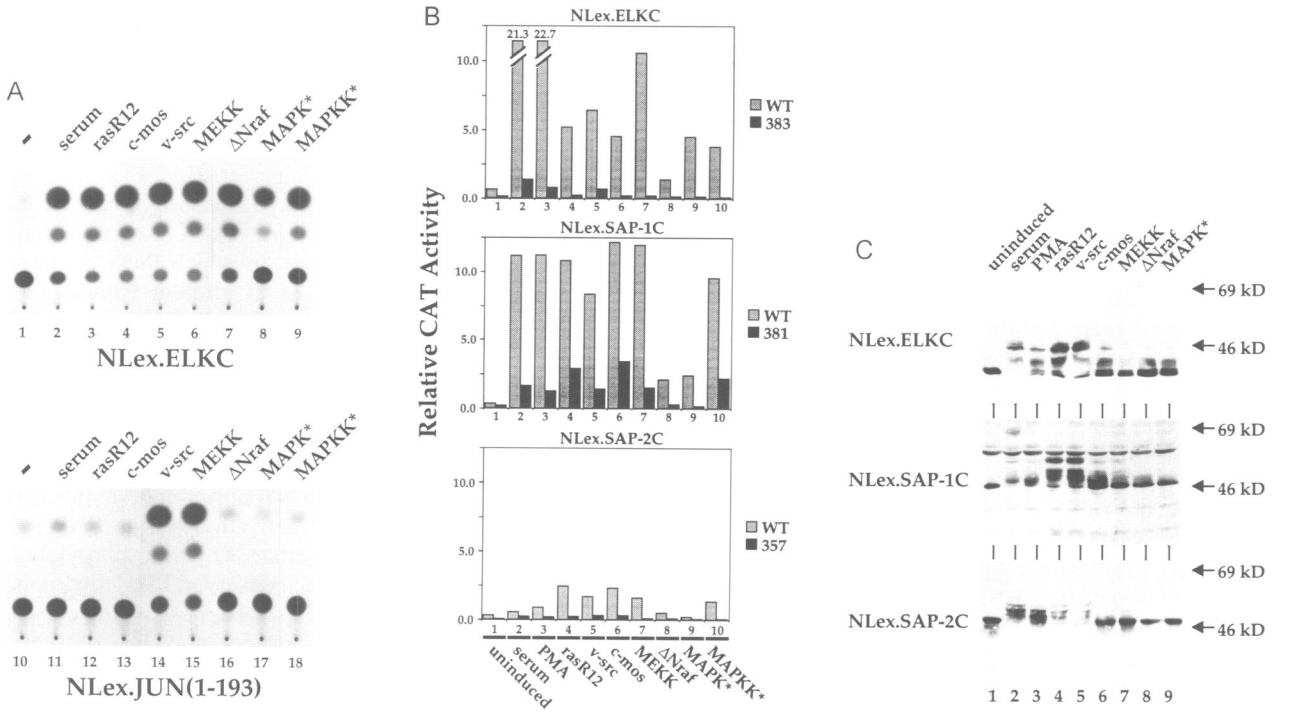

Fig. 10. Activation of the ERKs is sufficient to activate the TCF C-termini. (**A**) Transcriptional activation by the Elk-1 C-terminal and c-Jun N-terminal activation domains. NIH3T3 cells were transfected with 4 μg 2LexOP.tkCAT, 1 μg MLVlacZ (all lanes) and either 0.25 μg NLex.ELKC (lanes 1–9) or 0.3 μg NLexJun(1–193) (lanes 10–18). Expression vectors encoding the signalling molecules shown at the top of each panel were co-transfected as follows: 0.3 μg MLVrasR12 (lanes 3 and 12), 1 μg MLVmos (lanes 4 and 13), 1 μg EFv-src (lanes 5 and 14), 1 μg EFMEKK (lanes 6 and 15), 3 μg EXΔN-raf (lanes 7 and 16), 3 μg EFERK2(D319N) (MAPK*; lanes 8 and 17), or 3 μg EXMAPKK (217E/221E) (MAPKK*; lanes 9 and 18). Cells were serum deprived for 40 h and extracts prepared either directly or following 8 h stimulation with 15% FCS as indicated. A representative experiment is shown. (**B**) Comparison of the response of the three TCFs to activators of the ERKs. CAT activity normalized to activity of the co-transfected lacZ plasmid is shown for a representative experiment. NIH3T3 cells were transfected as for (A), with 1 μg of each activator plasmid except for MLVras (0.3 μg). Top panel, NLex.ElkC (shaded bars) and NLex.ElkC(S383A) (solid bars); middle panel, NLex.SAP-1C (shaded bars) and NLex.SAP-1C(S381A) (solid bars); bottom panel, NLex.SAP-2C (shaded bars) and NLex.SAP-2C(S357A) (solid bars). Induction with PMA (50 ng/ml) was for 8 h. (**C**) Modification of TCF C-termini. NIH3T3 cells were transfected with 8 μg of MLVNLex.ElkC (top panel), EFNLex.SAP-1C (middle panel) or EFNLex.SAP-2C (lower panel), together with activator plasmids as indicated. Following 40 h serum deprivation, extracts were prepared either directly, or after PMA or serum stimulation as indicated, and fractionated by SDS–PAGE; fusion proteins were detected by immunoblotting with LexA antiserum.

Discussion

We have compared the structure, expression and properties of three members of the TCF family of Ets domain proteins, Elk-1, SAP-1a and SAP-2, the human homologue of the recently reported mouse ERP/NET protein. All three proteins can form ternary complexes with SRF, but the inefficient ternary complex formation by SAP-2 may reflect additional regulatory constraints. All three proteins contain a conserved C-terminal region that activates transcription upon phosphorylation induced by activation of the ERK pathway.

Structure and expression of the TCFs

We previously identified three regions of homology between the TCFs SAP-1a and Elk-1, which are also conserved in SAP-2 and its murine homologue ERP/NET (Figure 1; Dalton and Treisman, 1992; Giovane *et al.*, 1994; Lopez *et al.*, 1994). The two N-terminal regions, box A (the Ets domain) and the hydrophilic box B, which in Elk-1 and SAP-1a are required for DNA binding and ternary complex formation with SRF (Dalton and Treisman, 1992; Janknecht and Nordheim, 1992; Treisman *et al.*, 1992; Hill *et al.*, 1993; Shore and Sharrocks, 1994), are well conserved. However, in the C-terminal homology region, the T-P motif at SAP-2 residue 342 is conserved in Elk-1 and SAP-1a, but not present in ERP/NET, while the S-P motif at Elk-1/SAP-1a positions 422/425 is absent from SAP-2, but present in ERP/NET; whether these differences are significant remains unclear.

Using an RNase protection assay, we found that the three TCF RNAs are expressed to some extent in all tissues and cell lines tested, generally at a similar level relative to each other and to a GAPDH RNA control. The SAP-1b RNA, which potentially encodes a non-regulated variant of SAP-1 that lacks the C box (Dalton and Treisman, 1992), was present at barely detectable levels relative to SAP-1a RNA. Other studies have suggested that expression of the TCF family RNAs is to some degree tissue specific (Rao *et al.*, 1989; Giovane *et al.*, 1994; Lopez *et al.*, 1994); it is likely that the discrepancy with previous results arises from the greater sensitivity of the RNase protection assay. Recent data show substantial variation in TCF expression between cell lines at the protein level (Hipskind *et al.*, 1994; Pingoud *et al.*, 1994).

DNA binding and ternary complex formation

Our data suggest that while SAP-1a and Elk-1 form ternary complexes at the c-*fos* SRE with comparable efficiency, ternary complex formation by SAP-2 occurs inefficiently *in vitro*. The mouse SAP-2 homologue

ERP/NET was also found to bind DNA weakly *in vitro* (Giovane *et al.*, 1994; Lopez *et al.*, 1994) and to form ternary complexes with SRF weakly (Giovane *et al.*, 1994) or not at all (Lopez *et al.*, 1994). We find that ERP/NET and SAP-2 behave similarly in our *in vitro* binding assays. Efficient DNA binding and ternary complex formation by SAP-2 is prevented by its C-terminal sequences: a truncated protein comprising the SAP-2 A and B boxes alone binds as well as corresponding truncated derivatives of SAP-1a and Elk-1. Experiments with chimeric TCF proteins showed that inhibition of DNA binding does not depend on specific interactions between the SAP-2 N- and C-terminal sequences. Consistent with this observation, although functional 'altered-specificity' mutants of Elk-1 and SAP-1a can be generated by substitution of their Ets domains by the DNA-binding domain of LexA, the analogous derivative of SAP-2 fails to form ternary complexes efficiently at an appropriate site (M.A. Price and R.Treisman, unpublished observations). We found that removal of SAP-2 C-terminal sequences, including the C box, allows DNA binding and ternary complex formation, but that maximal binding occurs only upon removal of all sequences C-terminal to SAP-2 residue 222. This result contrasts with those obtained with ERP/NET where efficient DNA binding required removal only of the C box region (Giovane *et al.*, 1994; Lopez *et al.*, 1994).

We used TCF–VP16 fusion proteins to test whether the different TCF proteins were capable of interacting with the c-*fos* SRE *in vivo*. We found that the SAP-1a and Elk-1, but not the SAP-2, fusion proteins efficiently activated the SRE in this assay. Taken together with our *in vitro* data, these observations strongly suggest that SAP-2 is not capable of interacting with the c-*fos* SRE *in vivo*, at least in unstimulated cells. However, expression of ERP/NET antisense RNA was reported to increase transcriptional activity of the c-*fos* SRE (Giovane *et al.*, 1994), although it remains unclear whether this effect is direct, as reporters lacking a TCF binding site were not examined.

One possible explanation for the apparently weak DNA-binding activity of SAP-2 is that it is subject to regulatory constraints not reproduced in our experiments. For example, DNA binding or ternary complex formation might depend on induced modification of the SAP-2 inhibitory region. Indeed, MAP kinase-regulated ternary complex formation by the Elk-1 protein can be observed under certain binding conditions (Gille *et al.*, 1992). However, our results indicate that if this regulation does occur, it must depend on signalling pathways distinct from those activated by serum or v-ras which cause modification of the SAP-2 C-terminus, but do not increase ternary complex formation by SAP-2. An alternative view is that SAP-2 may recognize different target sites from the other TCFs. This does not seem likely, however, since, like Elk-1 and SAP-1a, a SAP-2 truncation mutant comprising its A and B boxes can form ternary complexes on probes containing SRF and Ets binding motifs in a variety of spacings and orientations. Finally, it is possible that SAP-2 must interact with other proteins in order to bind DNA efficiently. Future experiments will investigate these possibilities.

Transcriptional activation by the SAP-1a and SAP-2 C-terminal regions

The Elk-1 C-terminus contains a transcriptional activation domain, whose activity is potentiated by phosphorylation of multiple conserved S/T-P motifs (Hill *et al.*, 1993; Janknecht *et al.*, 1993; Marais *et al.*, 1993; Kortenjann *et al.*, 1994; Gille *et al.*, 1995). Here we have shown that the C-termini of SAP-1a and SAP-2 can also activate transcription, using an assay in which these regions are linked to the full-length LexA protein (Marais *et al.*, 1993). Alanine substitutions at SAP-1a serines 381, 387 and 425, and threonine 420, and at SAP-2 serine 357, reduce transcriptional activation. The relative effects of these mutations were similar to the analogous mutations in Elk-1, except for the SAP-1a S381A mutation, which was less detrimental. Considered together with previous studies of Elk-1, the data strongly suggest, but do not prove, that transcriptional activation by the SAP-1a and SAP-2 C-termini is potentiated by phosphorylation at these sites. Mutations at the conserved Elk-1 hydrophobic amino acids F378 and W379, like those in the conserved S/T-P motifs, lead to an almost complete loss of transcriptional activation. These mutations do not prevent extensive phosphorylation of the C box, and it is likely that they alter a conserved structure involved in activation.

Transcriptional activation by the SAP-1a and Elk-1 C-termini is associated with their phosphorylation; both activation and phosphorylation can be brought about either by extracellular stimuli such as serum, phorbol esters or CSF1 (in NIH3T3 cells expressing the CSF1 receptor; unpublished results) or by intracellular activators of the ras/raf/ERK pathway. In contrast, activation by the SAP-2 C-terminus appears relatively weak and can be brought about by intracellular activation of the ERKs, but not by extracellular stimulation by serum or PMA, even though phosphorylation of the C-terminal region occurs in all cases. Serum stimulation also fails to potentiate transcriptional activation by the intact ERP/NET protein (Giovane *et al.*, 1994). All agents that potentiated transcriptional activation by the TCF C-termini induced their phosphorylation. However, we could observe transcriptional activation even in situations in which much of the TCF fusion protein appeared unmodified, e.g. with ΔNraf and ERK2(D319N). It is possible that the transcription activation results reflect a relatively small amount of stoichiometrically modified fusion protein produced in such cases. Alternatively, substoichiometric modification may be sufficient for transcription activation, although this is hard to reconcile with the severe effect of alanine mutations at several different phosphorylation sites. Finally, the partial modification of the TCF C-termini observed by immunoblot may merely reflect downregulation of the signalling pathway by the time the samples are analysed. We are currently investigating these possibilities.

We found that the LexA–TCF fusion proteins possess unusual DNA-binding properties. Using a sensitive gel mobility-shift assay, we showed that the NLex.ELKC and NLex.SAP-1C fusion proteins exhibit increased DNA-binding activity upon phosphorylation, while the DNA-binding activity of NLex.SAP-2C fusions decreases upon phosphorylation. However, the various mutant proteins behave similarly to the wild-type protein in DNA-binding assays; therefore, their failure to activate transcription

cannot be ascribed to their failure to bind DNA. We suggest that the changes in DNA-binding activity by the various fusion proteins reflect alterations in the conformation of the TCF C-terminal regions that occur upon phosphorylation. A previous study showed that LexA fusions often bind poorly compared with LexA itself (Golemis and Brent, 1992), but we do not know of other examples of inducible binding by a lexA fusion protein.

It is unclear why the SAP-2 C-terminus fails to activate transcription in response to serum or PMA stimulation, even though these agents induce its phosphorylation and do not affect its stability. It is possible that the serum-induced modifications of the SAP-2 C-terminal region differ from those induced by the intracellular activators. Alternatively, the serum- or PMA-induced decrease in the DNA-binding activity of the NLex.SAP-2C fusion protein might outweigh any increase in its transcriptional activity induced by phosphorylation. In contrast, intracellular activators of the ras/raf/ERK pathway, which stimulate protein expression in NIH3T3 cells by all expression vectors that we have tested, may cause overproduction of the NLex.SAP-2C fusion protein to an extent sufficient to compensate for weaker DNA binding by the phosphorylated protein.

ERK activation is sufficient for transcription activation by TCF C-termini

Previous studies have demonstrated that activation of the ras/ERK pathway correlates with transcriptional activation by Elk-1 (Janknecht *et al.*, 1993; Marais *et al.*, 1993; Nebreda *et al.*, 1993; Kortenjann *et al.*, 1994) and that ERK2 is necessary for TCF-dependent activation of the c-*fos* promoter *in vivo* (Kortenjann *et al.*, 1994). We used a variety of activated derivatives of intracellular signalling molecules to test whether the TCFs differ in their ability to respond to ERK activation *in vivo*. The different TCFs behaved identically in this assay: in particular an activated form of ERK kinase, MAPKK(217E/221E) (Cowley *et al.*, 1994), efficiently potentiated transcriptional activation. We also found that ERK2(D319N), an ERK2 derivative which is weakly responsive to stimulus-independent noise in the ras/raf/ERK pathway (Brunner *et al.*, 1994; Bott *et al.*, 1994; E.Hafen, personal communication), is sufficient to activate each TCF, albeit weakly. Taken together with our demonstration that the C-termini of each of the TCFs can be phosphorylated *in vitro* by partially purified ERK2, these results indicate that ERK2 activation is sufficient to potentiate transcriptional activation by each of the TCF C-termini. In contrast, transcriptional activation by the N-terminal activation domain of c-Jun was potentiated by overexpressed MEKK and by v-src, but not affected by activated ras, raf, MAPKK or ERK2, confirming recent reports concerning the differential regulation of the ras/raf/ERK and JNK/SAPK signalling pathways (Minden *et al.*, 1994; Sanchez *et al.*, 1994; Yan *et al.*, 1994).

Our data indicate that ERK-independent signalling is not necessary for transcriptional activation by SAP-1. In contrast, a recent study suggested that activation of SAP-1a is ERK-independent: in BAC-1 cells, low-mobility 'activated' SAP-1a ternary complexes are induced under conditions where ERK activation is weak and the corresponding Elk-1 ternary complexes are not inducible (Hipskind *et al.*, 1994). We think that this apparent disagreement arises from use of the gel mobility-shift assay as a measure of TCF activation. Three observations suggest that such low-mobility complexes need not reflect efficient phosphorylation of SAP-1a: (i) only partial phosphorylation of Elk-1 is sufficient to generate the low-mobility 'activated' ternary complex in gel mobility-shift assays; (ii) even inactive Elk-1 point mutants generate such complexes (see Hill *et al.*, 1993; Marais *et al.*, 1993); (iii) the most slowly migrating Elk-1 ternary complex persists for up to 2 h following serum stimulation, after c-*fos* transcription has ceased (F.Cruzalegui, J.Wynne and R.Treisman, unpublished; see Hill *et al.*, 1993).

In conclusion, the TCFs appear to constitute a family of transcription factors involved in the regulation of transcription in response to activation of the ERK pathway. In the assays we have used, the different family members appear to function interchangeably, although one member of the family, SAP-2 (ERP/NET), has DNA-binding properties that suggest it may have target sites distinct from the others. It will be interesting to see whether the TCFs respond differentially to the recently characterized JNK/SAPK (Minden *et al.*, 1994; Sanchez *et al.*, 1994; Yan *et al.*, 1994) and p38/HOG1 (Han *et al.*, 1994; Rouse *et al.*, 1994) signalling pathways.

Materials and methods

Plasmids and oligonucleotides

All plasmids were constructed by standard methods and verified by DNA sequencing where appropriate. Full plasmid and oligonucleotide details are available on request.

Expression vectors. The following expression plasmids were used: for *in vitro* translation, T7βplink (Dalton and Treisman, 1992); for transient expression in mammalian cells, MLVβplink (Dalton and Treisman, 1992), pEFBOS (Mizushima and Nagata, 1990), pEF-BOS derivatives containing the β globin 5′ untranslated region (Bardwell and Treisman, 1994), and RSVplink, a derivative of MLVβplink with the RSV 5′ LTR sequences in place of the MLV and β globin promoter sequences, (R.Treisman, unpublished). EFBOS-derived vectors were essential to achieve detectable levels of some SAP-1 and SAP-2 derviatives. Expression vectors used are specified in the figure legends.

TCFs and deletion derivatives. Expression plasmids encoding intact Elk-1 and SAP-1 reading frames were as described previously (Dalton and Treisman, 1992; Treisman *et al.*, 1992; Marais *et al.*, 1993), but containing the authentic SAP-1a sequence (Genbank accession number M85165), lacking UTR sequences 3′ to nucleotide 1519. MLV.SAP-2 and T7.SAP-2 contain SAP-2 codons 1–407, inserted as an *Nco*I (initiation codon)–*Xba*I site (3′UTR); an E to D mutation at residue 2 was introduced during construction.

SAP2-xxxΔ C-terminal deletions encode residues 1 to xxx except for SAP2-323Δ, SAP2-282Δ, SAP2-222Δ and SAP2-158Δ, which were produced by *in vitro* translation using templates generated by appropriate cleavage of T7SAP-2. Elk-307Δ and SAP1–310Δ were described previously (Dalton and Treisman, 1992; Marais *et al.*, 1993). SAP2-xxxΔyyy are internal deletion mutants that lack codons between xxx and yyy. SAP2A/ELK comprises (SAP-2 residues 1–94)(Elk-1 residues 107–428). SAP2B/ELK comprises (SAP-2 residues 1–197)(Elk-1 residues 206–428). ELKA/SAP2 comprises (Elk-1 residues 1–135)(SAP-2 residues 116–407). ELKB/SAP2 comprises (Elk-1 residues 1–205)(SAP-2 residues 197–407).

VP16 derivatives. Elk-1.VP16 encodes (Elk-1 codons 1–405)–QIRTRD–(VP16 codons 410–490). SAP-1a.VP16 encodes (SAP-1a codons 1–402)–SGSAQVQIRTRD–(VP16 codons 410–490). SAP-2.VP16 encodes (SAP-2 codons 1–398)–IRTRD–(VP16 codons 410–490).

TCF C-terminal fusion proteins. NLex.ELKC (Marais *et al.*, 1993) encodes MA–(SV40 NLS)–E–(LexA codons 3–202)–AAAHV–(Elk-1 residues 307–428). NLex.SAP-1C encodes MA–(SV40 NLS)–E–(LexA codons 3–202)–AAAHV–(SAP-1a residues 268–431). NLex.SAP-2C

encodes MA–(SV40 NLS)–E–(LexA codons 3–202)–AMVDGS-(SAP-2 residues 222–407). NLex.Jun(1–194) encodes MA–(SV40 NLS)–E–(LexA codons 3–202)–AMVDGSPGIS–(Jun codons 1–194)–EFLEID.

Point mutant derivatives were constructed by polymerase chain reaction (PCR) using appropriate primers. The mutant constructs contain the same sequences as the wild-type, except for NLex.ELKC F378A, W379A and ΔFW, where the residues AMVDGS are inserted between the LexA and Elk-1 sequences.

GST.ELKC was described previously (Marais *et al.*, 1993). GST.SAP-1C and GST.SAP-2C are analogous, containing Elk-1 residues 307–428, SAP-1a residues 268–431 or SAP-2 residues 222–407, respectively.

Reporter genes. Reporter plasmids are derivatives of pBLCAT2ΔBam containing two copies of either the c-*fos* SRE (SRE.tkCAT), mutant derivatives in which the Ets motif is mutated to a LexA half-operator (SRE.L.tkCAT) or the SRF site is changed to an MCM1 site (SRE.M.tkCAT), or two copies of a LexA operator (2LexOP.tkCAT) (Hill *et al.*, 1993, 1994; Marais *et al.*, 1993).

Activator plasmids. The following plasmids are as described: EXVrafΔN (Leevers and Marshall, 1992), EXVMAPKK(217E/221E) (Cowley *et al.*, 1994), MLVrasR12 (Marais *et al.*, 1993), MLVmos (Nebreda *et al.*, 1993), EFsrc (Wotton *et al.*, 1993). EFMEKK comprises the MEKK coding sequence inserted into EF-BOS as a *Sca*I–*Xho*I fragment from pBSSK(MEKK) (Lange-Carter *et al.*, 1993). For ERK2(D319N), mutagenesis of EXVERK2 (Leevers and Marshall, 1992) was carried out using appropriate oligonucleotides followed by recloning into pEF-BOS.

RNase protection probes. The probe RNAs encompass the following parts of the three RNAs: Elk-1, codons 317–378; SAP-1a, codons 315–377; SAP-2, codons 283–352. TCF DNA fragments were isolated by PCR and inserted into T7plink: SAP-1, *Bam*HI–(SAP-1a nucleotides 1092–1279)–*Eco*RI; Elk-1, *Bam*HI–(Elk-1 nucleotides 1265–1449)–*Eco*RI; plasmids were linearized using *Hin*dIII and transcribed using SP6 polymerase; SAP-2, *Xho*I–(SAP-2 nucleotides 1136–1344)–*Eco*RI; the plasmid was linearized using *Xho*I and transcribed by T7 polymerase. GAPDH nucleotides 888–988 were transcribed from a PCR fragment by T7 polymerase.

Cell culture, transfections and extract preparation

Maintenance and DEAE–dextran transfection of NIH 3T3 cells, and preparation of extracts for CAT assays and gel mobility-shift analysis were as described previously (Hill *et al.*, 1993, 1994; Marais *et al.*, 1993). Amounts of transfected plasmid DNA are specified in the figure legends. In general, the activity of a co-tranfected lacZ reference plasmid varied <2-fold between any transfected sample. In NIH3T3 cells, many of the activated signalling molecules lead to activation of the expression vectors and consequent overproduction of co-transfected TCF or LexA–TCF fusion proteins. This occurs with all SV40-, RSV-, MLV-, CMV- and EF-derived expression vectors that we have tested. To allow for this, we have this normalized CAT activity in these experiments to the lacZ activity from an appropriate reference plasmid. Whole-cell extracts for immunoblotting and gel mobility-shift assays were prepared as described (Marais *et al.*, 1993). For phosphatase treatment, cell extracts were prepared with E buffer containing 20 mM Tris–HCl (pH 8.8) in place of HEPES (Marais *et al.*, 1993). Phosphatase reactions (30 μl) contained buffer E (20 μl) with 10 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 0.1% NP40, 20 U alkaline phosphatase (Boehringer) and 40 μg extract protein. Inhibitors were K_2HPO_4 (10 mM), L-cystein (1 mM), EGTA (20 mM) and okadaic acid (0.1 μg/ml).

Gel mobility-shift assays

Cell-free translation was by the coupled transcription/translation method (Promega TNT), using 0.5–1 μg supercoiled or linearized plasmid in a 10–15 μl reaction; protein quantitation was by [^{35}S]methionine incorporation as measured using the PhosphorImager. Gel mobility-shift assays were performed as previously described (Marais *et al.*, 1993). Binding reactions contained 15 fmol oligonucleotide probe or 0.2 ng PCR-generated probe and purified SRF(133–265) (Marais *et al.*, 1993) and cell extract as specified in the figure legends.

Binding probes were synthesized by PCR (Treisman *et al.*, 1992) with the following plasmids and primers: LexA probe, 2LexOP.tkCAT plasmid, M13 forward and pBLCAT.R primers (Treisman *et al.*, 1992); L site probe, plasmid 1.3-27 (Treisman *et al.*, 1992), KS and T7 primers.

Other methods

Standard methods were used for the screening of a placental cDNA library (Clontech) with SAP-1 cDNA clone 18 (Dalton and Treisman, 1992) as probe, washing at 0.5× SSC at 60°C. The SAP-2 cDNA sequence assembly used the Intelligenetics suite of programs. Kinase reactions were as described (Marais *et al.*, 1993). RNA preparation, RNase protection mapping, SDS–PAGE and immunoblotting were by standard methods.

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