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c-*fos* transcriptional activation and repression correlate temporally with the phosphorylation status of TCF

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EGF-induction of human astrocytoma and A431 cells leads to c-*fos* transcriptional activation and then repression. This could be correlated with changes in the DNA binding characteristics of the c-*fos* regulatory protein ternary complex factor (TCF) present in nuclear extracts from these cells. Band shifts showed the appearance of induction-related slowly migrating protein-DNA complexes, detected as ternary complexes on the c-*fos* SRE using a truncated SRF molecule and by direct binding to the *Drosophila* E74 Ets-protein recognition sequence. By several criteria both types of complexes represented TCF. The appearance of the slow ternary and direct complexes correlated with c-*fos* transcriptional activation, and their disappearance coincided with the ensuing c-*fos* shut-off. Blocking c-*fos* transcriptional repression with the phosphatase inhibitor okadaic acid led to their continued presence. They were sensitive to protein phosphatase 2A but not 1α, and similar slow complexes were formed by partially purified p62^{TCF} phosphorylated by a copurifying kinase activity. Thus the phosphorylation state of TCF correlated strongly with c-*fos* promoter activity. Since ternary complex formation mediated by full-sized SRF was only slightly affected under comparable conditions, we propose a model for c-*fos* regulation involving modification of constitutively bound TCF.

Key words: c-*fos* regulation/protein phosphatase/signal transduction/SRE/ternary complex factor

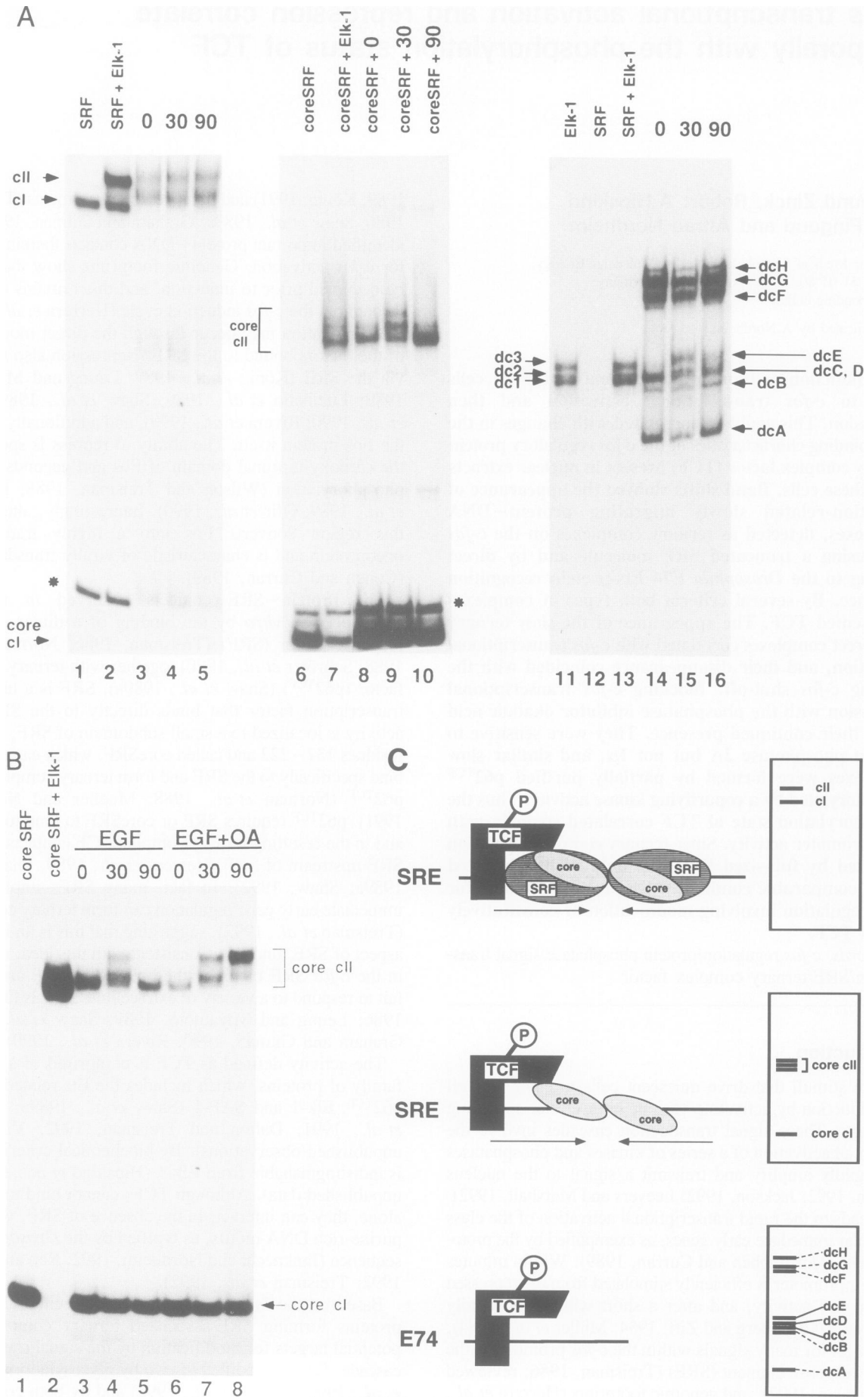
Introduction

Growth stimuli that drive quiescent cells to enter the cell cycle function by activating various intracellular signalling pathways. These signal transduction cascades involve the sequential activation of a series of kinases and phosphatases that rapidly amplify and transmit a signal to the nucleus (Cohen, 1992; Jackson, 1992; Leevers and Marshall, 1992). This leads to the rapid transcriptional activation of the class of cellular immediate early genes, as exemplified by the proto-oncogene c-*fos* (Cohen and Curran, 1989). Within minutes the c-*fos* promoter is efficiently stimulated from the repressed state to full activity, and after a short while it is rapidly inactivated (Greenberg and Ziff, 1984; Müller *et al.*, 1984). The target for many signals within the c-*fos* promoter is the serum response element (SRE) (Treisman, 1986; reviewed in Treisman, 1992), and genomic footprints (Herrera *et al.*, 1989; König, 1991) and transfection experiments (Treisman, 1986; Shaw *et al.*, 1989a; Graham and Gilman, 1991) have identified important protein-DNA contacts therein essential for c-*fos* activation. Genomic footprints show the SRE to be occupied prior to induction, and this remains unaltered throughout the c-*fos* induction cycle (Herrera *et al.*, 1989). Thus activation may occur through the direct modification of the factors bound to the SRE. Repression also functions via the SRE (König *et al.*, 1989; Leung and Miyamoto, 1989; Lucibello *et al.*, 1989; Shaw *et al.*, 1989b; Gius *et al.*, 1990; Rivera *et al.*, 1990), and additionally involves the Fos protein itself. The ability to repress is specific for the carboxy-terminal domain of Fos and depends upon its phosphorylation (Wilson and Treisman, 1988; Lucibello *et al.*, 1989; Ofir *et al.*, 1990). Interestingly, alteration of this region converts Fos into a highly transforming oncoprotein and is characteristic of virally transduced Fos (Cohen and Curran, 1989).

The protein-SRE contacts observed *in vivo* are reproduced *in vitro* by the binding of a dimer of serum response factor (SRF) (Treisman, 1986; Norman *et al.*, 1988; Schröter *et al.*, 1990) together with ternary complex factor (p62^{TCF}) (Shaw *et al.*, 1989a). SRF is a ubiquitous transcription factor that binds directly to the SRE. This activity is localized to a small subdomain of SRF, spanning residues 132-222 and called coreSRF, which can dimerize, bind specifically to the SRE and form ternary complexes with p62^{TCF} (Norman *et al.*, 1988; Mueller and Nordheim, 1991). p62^{TCF} requires SRF or coreSRF to bind to the SRE and in the resulting ternary complex TCF contacts the c-*fos* SRE upstream of SRF (Herrera *et al.*, 1989; Shaw *et al.*, 1989a; Shaw, 1992). In fact, many SREs implicated in immediate early gene regulation can form ternary complexes (Treisman *et al.*, 1992), suggesting that this is an important aspect of SRE function. Consistent with this idea, mutations in the c-*fos* SRE blocking the binding of SRF and p62^{TCF} fail to respond to a variety of extracellular signals (Treisman, 1986; Leung and Miyamoto, 1989; Shaw *et al.*, 1989a; Graham and Gilman, 1990; Rivera *et al.*, 1990).

The activity defined as TCF is comprised of a complex family of proteins, which includes the Ets-related proteins p62^{TCF}, Elk-1 and SAP-1 (Shaw *et al.*, 1989a; Hipskind *et al.*, 1991; Dalton and Treisman, 1992; V.Pingoud, unpublished observations). By biochemical criteria p62^{TCF} is indistinguishable from Elk-1 (Hipskind *et al.*, 1991; our unpublished data). Although TCFs cannot bind to the SRE alone, they can interact, in the absence of SRF, with other purine-rich DNA motifs, as typified by the *Drosophila* E74 sequence (Janknecht and Nordheim, 1992; Rao and Reddy, 1992; Treisman *et al.*, 1992).

Based on the *in vivo* and *in vitro* data cited above, the proteins forming SRE-associated ternary complexes are potential targets for modification by the signal transduction cascade. SRF is modified *in vivo* by glycosylation (Schröter *et al.*, 1990; Reason *et al.*, 1992) and by both constitutive


A
SRF
SRF + Elk-1
0 30 90
coreSRF
coreSRF + Elk-1
coreSRF + 0
coreSRF + 30
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Elk-1
SRF
SRF + Elk-1
0 30 90
cII
cI
core cII
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dc3
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1 2 3 4 5
6 7 8 9 10
11 12 13 14 15 16
B
core SRF
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0 30 90 0 30 90
core cII
core cI
1 2 3 4 5 6 7 8
C
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core cI
E74
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and induced phosphorylation (Prywes *et al.*, 1988; Manak *et al.*, 1990; Schalasta and Doppler, 1990; Misra *et al.*, 1991; Janknecht *et al.*, 1992; Marais *et al.*, 1992). TCF appeared to be phosphorylated in nuclear extracts derived from induced Swiss 3T3 cells, and treatment with MAP kinase (MAPK, Boulton *et al.*, 1991) could reproduce this effect *in vitro* (Gille *et al.*, 1992).

To investigate the possibility that the activated signal transduction cascade directly targets the c-*fos* promoter by modifying transcription factors interacting with the SRE, we analyzed nuclear extracts from cells at various times after mitogen stimulation with regard to their DNA binding characteristics. A very rapid change in TCF binding was observed which directly correlated with the kinetics of c-*fos* transcriptional activation. The ensuing shut-off of the c-*fos* promoter correlated tightly with the loss of the induced TCF complexes, and both events were blocked by the phosphatase inhibitor okadaic acid. We demonstrate that $p62^{TCF}$ is a direct target for multiple phosphorylation by an intracellular protein kinase that copurifies with $p62^{TCF}$ in our biochemical preparations. The modification introduced on TCF *in vitro* and *in vivo*, probably phosphorylation, is sensitive to protein phosphatase (PP) 2A but not PP-1α.

Results

Mitogenic activation leads to alterations in the mobility of TCF–DNA complexes

The ternary complex between the SRE, a dimer of SRF and TCF, which is implicated in c-*fos* transcriptional regulation, can be visualized in gel retardation assays using a ^{32}P-labelled SRE probe (Figure 1A; diagrammed in Figure 1C, upper). To determine whether the binding of these proteins was affected by mitogens inducing c-*fos* transcription, binding activity was assayed in nuclear extracts prepared from resting and EGF-stimulated human astrocytoma cells. No significant differences were observed during induction in either the SRF–SRE binary complex (cI) or the ternary complex which additionally contains TCF (cII; Figure 1A, lanes 3–5). The identity of these complexes was shown by their cross reaction with SRF- and Elk-1-specific antisera (Hipskind *et al.*, 1991; see below, and data not shown) and they resemble those generated by the recombinant human (rh) protein rhSRF and the TCF homologue rhElk-1 (lanes 1 and 2). Induction-related differences could be seen with the more rapidly migrating ternary complexes. These were generated by adding a truncated derivative of SRF (coreSRF) that was fully capable of forming binary (core cI) and ternary complexes (core cII) (Figure 1A, lanes 8–10; diagrammed in Figure 1C, middle). The core ternary complexes appeared as a major and minor band in starved cell extracts (0 min, lane 8) and in extracts from cells when c-*fos* transcription was again turned off (90 min, lane 10 and below). An additional slower complex appears in the extract made 30 min after EGF addition (lane 9), when *fos* is actively transcribed. This entire range of complexes with coreSRF was also formed by rhElk-1 produced by overexpression in logarithmically growing HeLa cells (lane 7).

Upon incubation with the E74 DNA sequence many *d*irect *c*omplexes (dcs) were generated by astrocytoma nuclear extracts, labelled dcA to dcH (Figure 1A, lanes 14–16; diagrammed in Figure 1C, lower). Some of these complexes were formed by TCF, namely dcC, dcD and dcE (discussed below, Figure 3A and 3B). Complex dcE was only observed in nuclear extracts from cells induced for 30 min with EGF (compare lanes 9 and 15). Complexes similar to dcC–dcE were also generated by rhElk-1 (dc1–dc3) and appeared to arise from differentially phosphorylated species of rhElk-1 (Figure 1A, lane 11; see also Figure 5). SRF does not bind to the E74 probe and also does not affect the direct binding of Elk-1 (lanes 12 and 13). We note that the E74 direct complexes dcC–dcE and dc1–dc3 display similar heterogeneity to ternary complexes formed by the induced extracts with coreSRF and the SRE.

Figure 1B shows more clearly the slowly migrating core cII ternary complex formed with the extracts induced for 30 min upon coreSRF addition, but not with the 0 min or 90 min samples (lanes 3–5). Simultaneous treatment of the cells with both EGF and the phosphatase inhibitor okadaic acid leads to a prolonged presence of the slow ternary complex (lanes 6–8) and continued *fos* transcription (see below). Thus changes could be detected in TCF binding activity in nuclear extracts prepared from induced cells and, as will be shown below, these changes correlated with c-*fos* transcriptional activity. These differences could not be seen in our assay system with ternary complexes generated with endogenous SRF. However, these differences can be visualized either by core cII-type ternary complex formation on the SRE, as mediated by the truncated coreSRF, or by direct binding to the E74 binding site.

Reversible changes in TCF DNA binding correlate temporally with c-fos promoter activity

We wanted to investigate in more detail the possible correlation of these slower TCF-dependent complexes with the transcriptional activity of the c-*fos* gene. Therefore

Fig. 1. Extracts from mitogenically activated astrocytoma cells display altered mobility of TCF–DNA complexes. (**A**) Gel retardation analyses of complexes formed by nuclear extracts from resting cells (0 min) and those induced with EGF for the indicated times (30 or 90 min). The probes were either ^{32}P-labelled c-*fos* SRE (lanes 1–10) or E74 (lanes 11–16) DNA. Control reactions contained rhElk-1 (lane 11), rhSRF (lanes 1 and 12), rhSRF + rhElk-1 (lanes 2 and 13), coreSRF (lane 6) and coreSRF + rhElk-1 (lane 7). Extracts were also supplemented with coreSRF (spanning residues 90–244) (lanes 8–10). The binary complexes (cI, core cI), direct complexes (dc1–dc3 and dcA–dcH) and ternary complexes (cII, core cII) are defined in the text. The core cII region resolves into a set of differently migrating bands, the slowest of which is found with recombinant Elk-1 and only in 30 min extracts. Similarly the E74 direct complex dcE is only seen with the 30 min extract and it corresponds to complex dc3 generated with recombinant Elk-1. The asterisk marks an unidentified band generated with some protein samples. (**B**) Serum-starved astrocytoma cells were induced with EGF for 0, 30 or 90 min in the absence (lanes 3–5) or presence (lanes 6–8) of the phosphatase inhibitor okadaic acid. The binding reactions contained the SRE probe and a smaller coreSRF, spanning residues 132–222, produced by coupled *in vitro* transcription/translation. Lane 1 contains coreSRF alone, while lane 2 also includes rhElk-1. Lighter exposures of lane 2 show a pattern of core cII complexes which resembles that of extracts from 30 min EGF-treated cells (lane 4), except that the latter group consistently runs slightly more slowly. (**C**) Schematic drawing of the three different types of assay conditions used for the analysis of TCF DNA binding above. Binding to the SRE was analyzed in the absence (upper) or presence (middle) of added coreSRF. Ternary complex formation was therefore either solely dependent on endogenous SRF (upper) or competed for by coreSRF (middle). The lowest panel depicts direct binding to the E74 probe. The appearance of the corresponding complexes and their designations is schematized on the right.

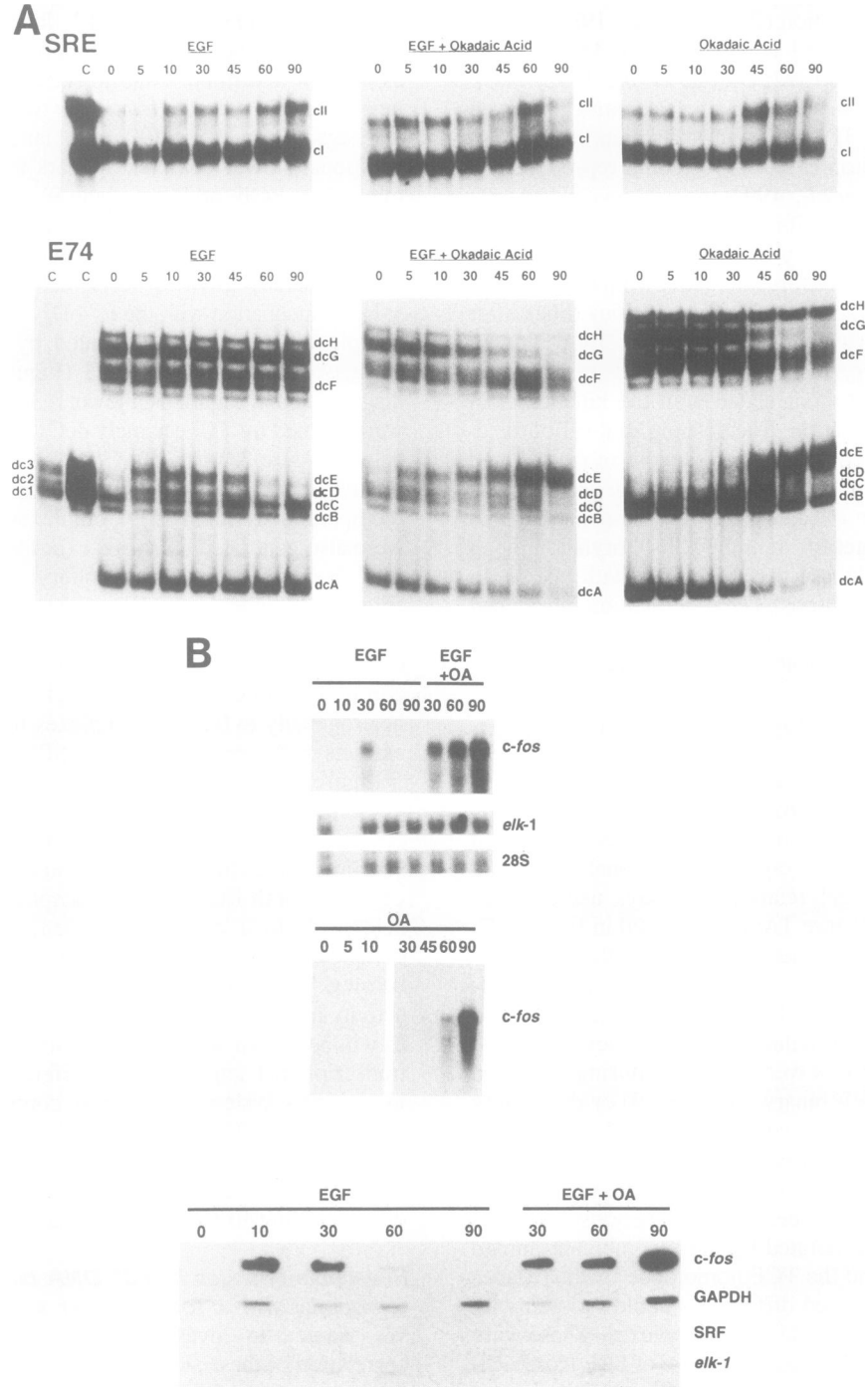

Fig. 2. Reversible changes in TCF DNA binding activity correlate temporally with c-*fos* promoter activity. (**A**) SRE and E74 binding activities in nuclear extracts from resting and induced cells: mitogenic stimulation leads to the transient presence of complexes with the E74 DNA probe. Nuclear extracts were prepared from starved cells (0) or those induced for the times (in minutes) shown above the lanes. Induction was by EGF (left column), EGF + okadaic acid (middle column) or okadaic acid alone (right column). Gel retardation analyses using the SRE and the E74 probe are shown in the upper and lower panels, respectively. In the upper panel, binary complexes (cI, SRE−SRF) and ternary complexes (cII, SRE−SRF−TCF) are generated in control lanes (C) using rhSRF and rhElk-1. The upper left panel shows the largest difference in cI and cII we have observed over the induction cycle; the upper middle panel represents the amount of variation typically found. In the lower panels the lanes designated C contained rhElk-1. The complex designation is described in the text. (**B**) c-*fos* transcriptional response to EGF and okadaic acid. The upper and middle panels show northern analyses of cytoplasmic RNA remaining after nuclear isolation for run-on analysis or extract preparation respectively. Both were hybridized to probes from c-*fos*, Elk-1 and GAPDH (not shown). The RNA in the 10 min lane in the upper panel was lost during purification; in other experiments no *fos* signal is seen at this time point. Controls for the middle Northern blot showed equal amounts of RNA in all lanes (data not shown). The lower panel presents the transcriptional status of the indicated genes at the time points shown. Run-on transcripts were labelled in astrocytoma nuclei prepared from cells treated as indicated. The transcripts were hybridized to a filter containing equimolar amounts of the indicated cloned genes.

nuclear extracts were prepared from astrocytoma cells at multiple points throughout the course of EGF induction. These extracts were tested for both ternary complex formation mediated by endogenous SRF on the SRE and direct binding to the E74 DNA sequence. Again, only small variations were seen in complexes cI and cII upon EGF

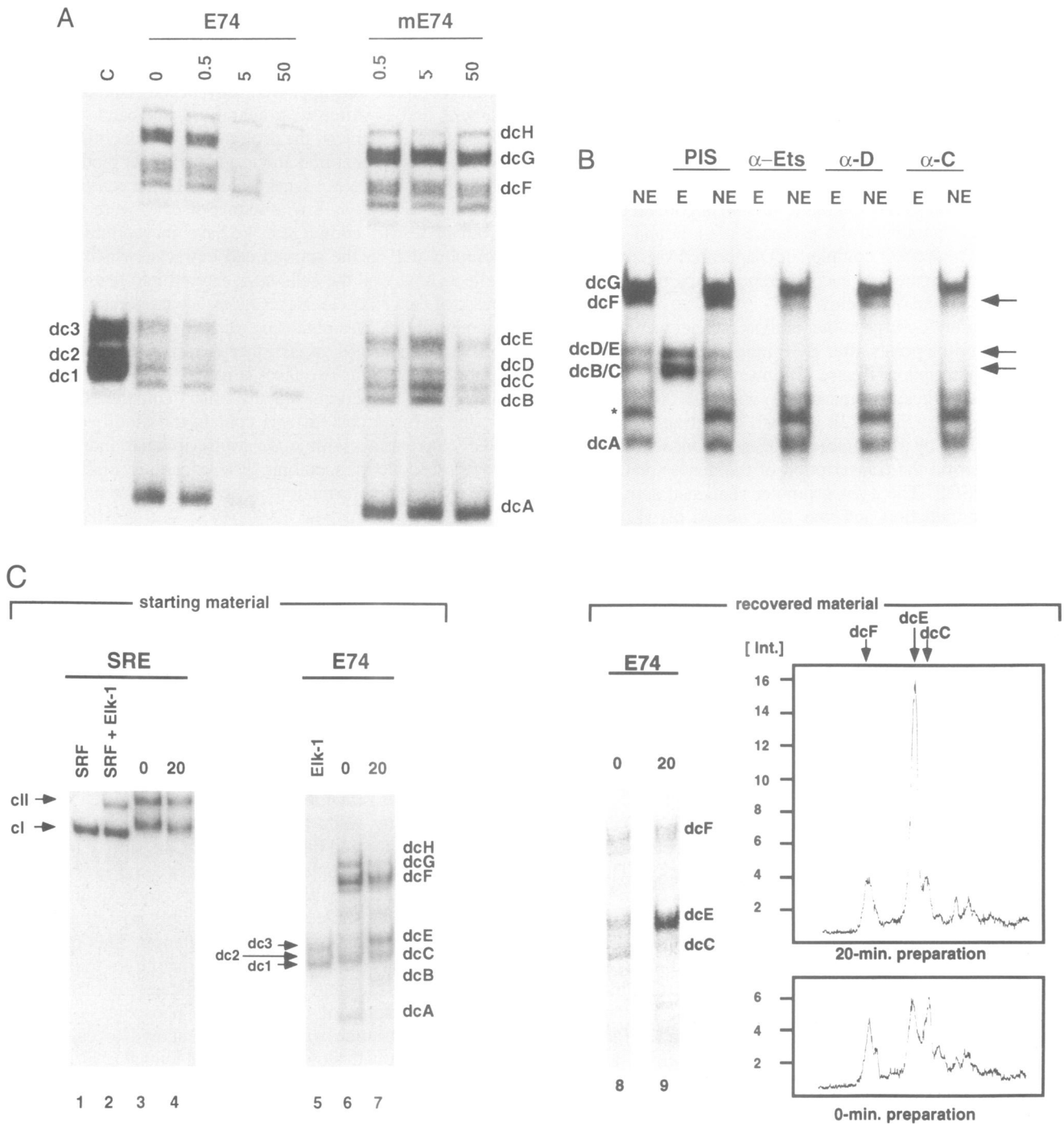

Fig. 3. Identification of TCF activity in E74 direct complexes. (**A**) Specificity of complexes dcA to dcH for the E74 sequence. Direct binding was visualized by gel retardation analyses. Competition experiments contained the indicated amount, in ng, of either the unlabelled E74 binding site oligonucleotide (..CCGGAAGT.., upper strand) or the mutated derivative mE74 (..CCCCAAGT.., upper strand), which cannot bind Elk-1. Lane C shows the complexes formed by rhElk-1. Reactions contained 500 pg labelled E74 probe. (**B**) Antibody reactivity of direct complexes dcA to dcG. Binding reactions contained either rhElk-1 (E) or induced astrocytoma nuclear extract (NE). The complexes sensitive to the various antisera are indicated by arrows. The polyclonal antisera (α-Ets, α-D and α-C) are specific for the Elk-1 domains spanning aa 1−82, 83−307 and 307−428, respectively. The asterisk indicates an unidentified band not seen reproducibly. This gel was electrophoresed for only half of the normal time, thus explaining its lower resolution. (**C**) Direct complex binding activities can be isolated from SRE−SRF−TCF ternary complexes. Proteins were isolated from ternary complexes (cII) formed with starved or induced (20 min with EGF) A431 nuclear extracts on a preparative scale. Binding activity in the extracts, labelled starting material, was tested as above using both SRE (lanes 1−4) and E74 (lanes 5−7) probes. After preparative isolation of ternary complexes, the proteins were renatured and tested by direct binding with the E74 probe (lanes 8 and 9). The signal was visualized and quantified (right panels) by PhosphorImager analysis. The weak signals arising below the position of dcB are probably due to some degradation during the preparative procedure, as they were not reproducibly observed.

induction (Figure 2A, upper left), at variance with other published observations (Malik *et al.*, 1991; Gille *et al.*, 1992). We have seen no more than small increases in cI or cII activity that might correlate with *fos* transcriptional induction (see also Figure 1A), because considerable levels of TCF activity were already present in extracts prepared from starved astrocytoma cells, as well as starved A431 and Swiss 3T3 cell extracts (data not shown). In contrast, the

extracts from the EGF-induced cells showed a significant increase in the direct E74 complex dcE, as well as an intermediate complex dcD, at 5 min and this level of binding remained until 45 min (Figure 2A, lower left). At time points of 60 and 90 min post-induction these complexes declined rapidly again. Notably the other direct complexes varied little during the EGF induction. When these extracts were tested for coreSRF-mediated ternary complex formation, the presence of the slowly migrating version of core cII described in Figure 1 paralleled the presence of dcD and dcE (not shown). The weaker complex dcD appeared variably in our extracts (cf. Figure 1A) and may represent partially modified TCF (see discussion below).

To measure c-*fos* promoter activity, nuclei were prepared at several time points after EGF induction and analyzed by run-on transcription (Figure 2B, lower). The mRNA present in the post-nuclear supernatant was also visualized on Northern blots (Figure 2B, upper). c-*fos* transcription was fully induced by 10 min after EGF addition, while this had little effect on the transcription of the genes for GAPDH, Elk-1 or SRF. The c-*fos* promoter remained active for 30 min after induction and was then turned off (Figure 2B, lower). In this and other experiments, c-*fos* mRNA appeared between 10 and 30 min, remained high at 45 min and was then degraded, while the Elk-1 mRNA and 28S rRNA signals remained constant (Figure 2B, upper). These kinetics of c-*fos* promoter activity after EGF induction correlate with the appearance of the binding activities forming slowly migrating core cII or direct complexes dcD and dcE in nuclear extracts. Furthermore, c-*fos* repression coincided with their disappearance.

Identity of protein components generating dcD/dcE and ternary complex factors

Nuclear extract-derived direct complexes dcC, dcD and dcE comigrated with the rhElk-1-derived direct complexes dc1, dc2 and dc3, which indicated their similarity. This was confirmed by competition experiments and Elk-1-specific antisera (Figure 3). All complexes, except for dcB and dcH, were competed by an increasing excess of unlabelled E74 binding site, while a mutant E74 site did not compete (Figure 3A; for rhElk-1 see Janknecht and Nordheim, 1992). The E74 binding site also competed for the formation of endogenous SRF-dependent ternary complexes (data not shown). Complexes dcC to dcE were recognized, together with dcF, by several Elk-1-specific antisera (Figure 3B, lanes NE), as were the rhElk-1 direct complexes (lanes E). The specificity of the antisera for Elk-1 was established by Western analyses and immunoprecipitation (R.Zinck and A.Nordheim, in preparation). These antisera also inhibited ternary complex formation with coreSRF on the SRE probe (data not shown). Although the other direct complexes are specific for the E74 DNA sequence, they were unaffected by the various Elk-1 antisera. None of the direct complexes react with antisera against SRF or MAPK (not shown).

We then set out to establish that the factors generating direct complexes dcD and dcE were those forming ternary complexes with full-length SRF on the SRE. For this purpose preparative scale band shift reactions were set up using nuclear extracts from human A431 cells that had been either starved or induced with EGF for 20 min, since A431 cells yielded significantly larger quantities of binding activity. Nuclear extracts from these cells showed binding that was indistinguishable from astrocytoma extracts, namely comparable amounts of SRF-dependent cII under both starved and induced conditions (Figure 3C, lanes 3 and 4) while complex dcE appeared only in the induced extracts (lanes 6 and 7). After the preparative scale band shift of the 0 and 20 min extracts, the proteins present in complexes cII were isolated, renatured and tested for direct binding to the E74 probe (Figure 3C, lanes 8 and 9). Direct complexes dcC and dcE, as well as low amounts of dcF, were recovered from the ternary complexes. We have not reproducibly seen complex dcE in the starved cell extracts, which probably reflects how well the cells have entered into the resting, G_0 phase of the cell cycle. Nevertheless a considerably increased amount of dcE was obtained from cII formed with induced cell extracts (lane 9), as is further shown by the quantification of the signals in the two lanes (Figure 3C, right panel). This provides direct evidence that the ternary complexes generated by the induced cell extracts contain the modified form of TCF correlating with c-*fos* transcriptional induction. In contrast, no corresponding differences are observable in ternary complex formation between uninduced and induced extracts.

The observation that complexes dcC, dcD, dcE and to a lesser extent dcF could be competed with an unlabelled SRE–SRF binary complex (data not shown), along with the data described above, argues that the same proteins generated both these E74 direct complexes and SRE ternary complexes. Consistent with this, the protein components present in the direct complexes dc1 and dc3 generated by rhElk-1, after isolation from a preparative E74 band shift, could form cII upon addition of SRF and coreSRF (not shown).

Okadaic acid prevents c-fos repression and concomitantly leads to hypermodification of TCF

The appearance and disappearance of the slowly migrating core cII ternary complex and the direct complexes dcD and dcE in conjunction with *fos* promoter activity indicated that TCF was being subjected to a reversible post-translational modification, most likely phosphorylation. This raised the possibility that post-induction repression of c-*fos* transcription is linked to the activity of phosphatases intracellularly. Indeed, treatment of cells with the phosphatase inhibitor okadaic acid alone was previously shown to cause c-*fos* activation but with slower kinetics (Schönthal *et al.*, 1991a). We therefore prepared nuclear extracts from cells induced with EGF in the presence of okadaic acid at various time points (Figures 1B and 2A). Only little variation was seen in ternary complex formation on the SRE probe with the endogenous SRF (Figure 2A, upper middle), and the kinetics of appearance of direct complexes dcD and dcE was the same as with EGF alone (Figure 2A, lower middle). However, direct complexes dcD and dcE did not diminish 60 min post-induction but remained; in fact, dcE showed a progressive increase in intensity throughout the induction. The same extracts were tested with coreSRF, and the appearance of the slowly migrating core cII ternary complex corresponded to the presence of dcD and dcE (Figure 1B and data not shown). Inclusion of okadaic acid in the medium blocked the inactivation of the c-*fos* promoter after EGF induction, as shown by the specific accumulation of *fos* mRNA and the *fos* signal at 60 and 90 min in the run-on analysis (Figure 2B). Thus the continued presence of these complexes

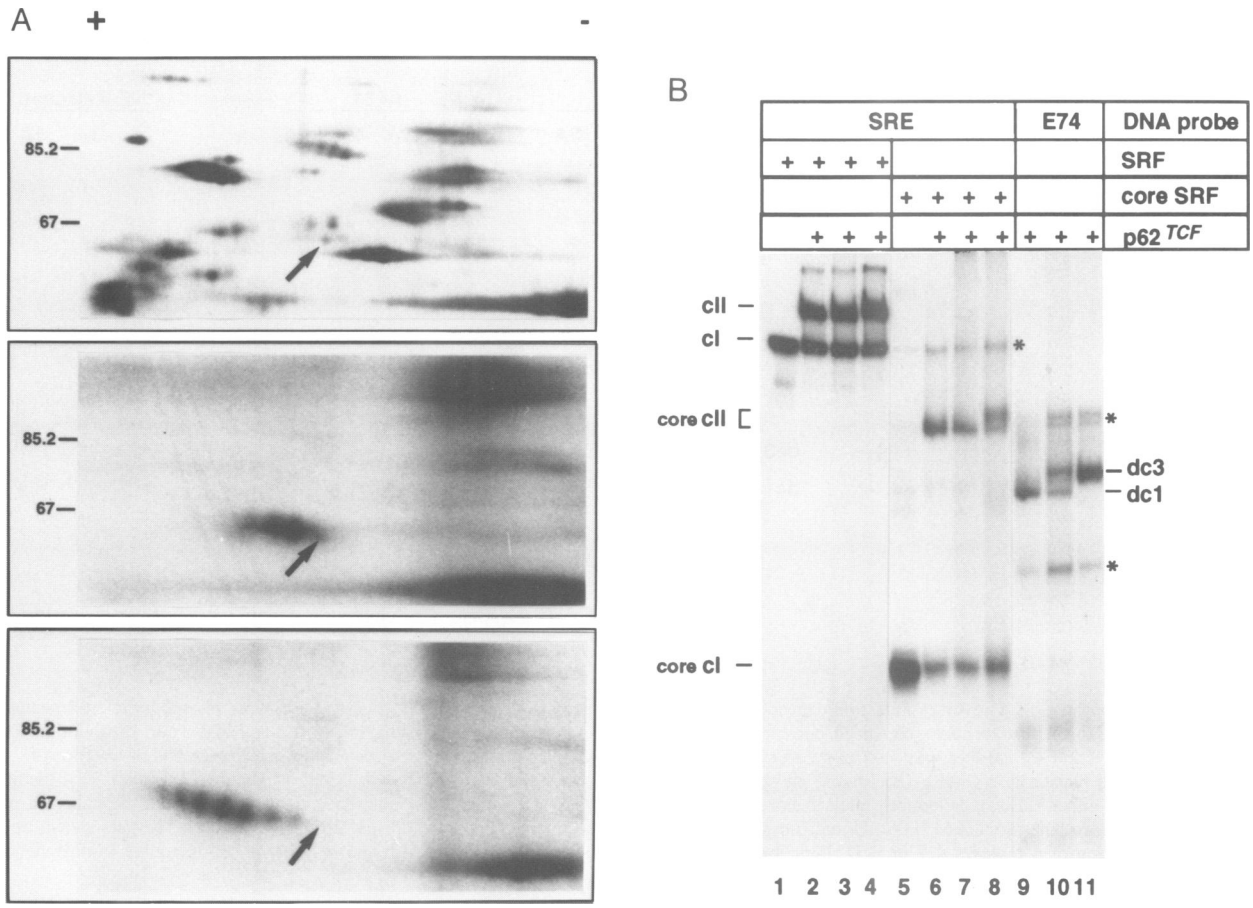

Fig. 4. Phosphorylation, 2D-gel analysis and DNA−protein complex formation of HeLa cell p62^{TCF}. Partially purified p62^{TCF} containing a copurifying endogenous kinase activity was incubated with different amounts of ATP and analyzed by 2D-gel electrophoresis and gel retardation. (**A**) 2D analysis of the protein preparation after incubation with 10 μM cold ATP and 50 nM [γ-^{32}P]ATP, shown as the silver stained gel (upper panel) or the corresponding autoradiogram (middle panel). The lower panel shows the autoradiogram of a 2D analysis of reaction products generated upon incubation with 100 μM cold ATP and 50 nM [γ-^{32}P]ATP. The anode and cathode for the first dimension are shown, as are two markers used in the second dimension. The arrow indicates the spot corresponding to hypophosphorylated p62^{TCF} (see text). (**B**) Gel retardation analysis of DNA−protein complexes obtained with the p62^{TCF} reaction products generated and analyzed in part A. Three types of DNA binding studies were performed according to the scheme shown in Figure 1C, using SRE or E74 binding sites as indicated. The SRE binding reactions contained added rhSRF (lanes 1−4) or core SRF containing aa 90−244 (lanes 5−8). The partially purified p62^{TCF} samples used were preincubated without ATP (lanes 2, 6 and 9) or as described above. Lanes 3, 7 and 10 correspond to p62^{TCF} shown in the middle panel of part A, and lanes 4, 8 and 11 correspond to the lower panel. The bands obtained are labelled according to Figure 1. The asterisks denote unidentified bands in lanes 5−11.

correlated with the prolonged activity of the c-*fos* promoter, and this was dependent upon okadaic acid. Since okadaic acid is a phosphatase inhibitor we reasoned that the slowly migrating complexes are probably the result of phosphorylation of TCF and a subsequent phosphatase activity causes their disappearance parallel to c-*fos* repression. Inhibition of this phosphatase activity by okadaic acid blocked *fos* post-induction repression and this corresponded to the continued presence of the complexes. Interestingly okadaic acid also led to the gradual disappearance of other direct complexes, namely dcA, dcG and dcH, during the time course with (Figure 2A, lower middle) or without (lower right) EGF induction. Since they do not react with the Elk-1 antisera (Figure 3B), it is unclear whether they are related to the induced complexes.

Cells were also treated with okadaic acid alone and nuclear extracts prepared at the same time points as above. As above, complex dcE progressively increased starting at 30 min, i.e. with delayed kinetics (Figure 2A, lower right). This corresponded to the slower appearance of c-*fos* mRNA (Figure 2B), suggesting that *fos* transcription is slowly activated in the presence of okadaic acid alone, as has been noted previously (Schönthal *et al.*, 1991a). Similarly a clear increase in the slowly migrating core cII complexes correlating with induction after long okadaic acid treatment was seen when coreSRF and SRE probe were added to the extracts (data not shown). This increase is also sometimes apparent in ternary complex formation with endogenous SRF (Figure 2A, upper). Identical results have been obtained in nuclear extracts prepared from A431 cells treated with okadaic acid alone or EGF together with okadaic acid, including the increase of endogenous cII after prolonged okadaic acid treatment (data not shown). These results confirm that okadaic acid abrogated c-*fos* transcriptional repression in both the induced and uninduced states, as previously shown by Schönthal *et al.* (1991a,b), and this coincided with the presence of induced complexes. This implicates a phosphatase activity in the process of c-*fos* transcriptional repression. Consistent with this, phosphatase treatment of induced extracts eliminated the more slowly migrating core cII as well as dcD and dcE activities (see below).

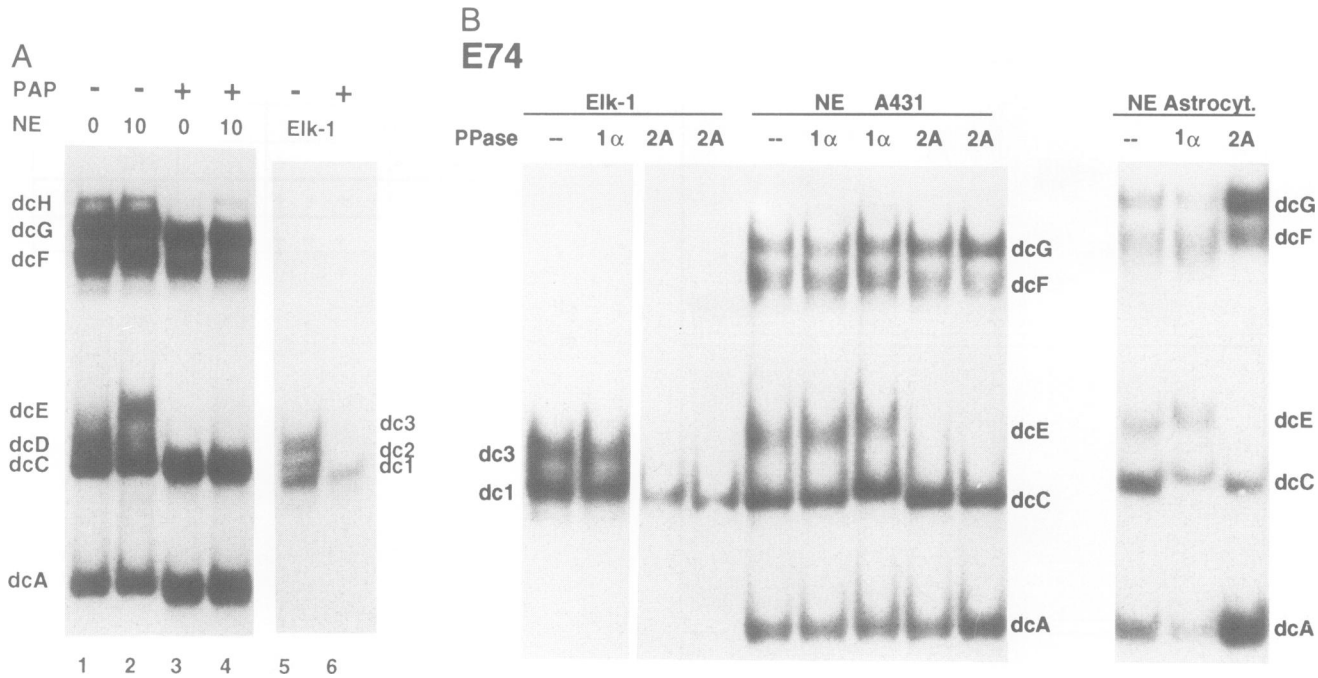

Fig. 5. Protein phosphatase 2A reverses induction-dependent TCF phosphorylation. (**A**) Phosphatase digestion identifies phosphorylation-dependent direct complexes. E74 binding reactions contained nuclear extracts from astrocytoma cells before induction (0; lanes 1 and 3) or 10 min after EGF addition (10; lanes 2 and 4). rhElk-1 was also analyzed (lanes 5 and 6). All samples were pretreated identically in the presence or absence of dialyzed potato acid phosphatase (PAP). (**B**) Protein phosphatase 2A, but not 1α, removes induction-associated TCF phosphorylation. E74 binding reactions contained either rhElk-1 or induced nuclear extracts (NE) from A431 or astrocytoma cells subsequent to treatment with the indicated protein phosphatase. Duplicate lanes contained 0.06 μU and 0.6 μU phosphatase, respectively. Otherwise 0.6 μU enzyme was used. The gels shown do not give a clear resolution of complexes dc2 or dcD. Reactions with rhElk-1 contained much less protein and may be more susceptible to degradation during the phosphatase reaction.

Phosphorylation of purified p62^{TCF} by a copurifying cellular kinase affects the migration of TCF-containing DNA–protein complexes

We have also partially purified TCF from logarithmically growing HeLa cells, a procedure involving selective nuclear extraction and two column chromatography steps followed by two-dimensional electrophoretic gel separation (V.Pingoud, in preparation). The silver stained proteins could be renatured and tested for TCF activity, and the arrow in Figure 4A points to the p62^{TCF} protein as originally described (Shaw *et al.*, 1989a; Schröter *et al.*, 1990; V.Pingoud, in preparation). The same spot was also recognized by an Elk-1-specific antiserum in immuno-western analysis (data not shown). During this purification we noticed that a kinase activity copurified with p62^{TCF} through two chromatography steps, and that it separated from p62^{TCF} upon gel filtration chromatography, where this kinase activity eluted at the same position as MAPK (V.Pingoud, in preparation). This endogenous kinase activity could directly label p62^{TCF} when supplied with [γ-^{32}P]ATP, as shown in the middle panel of Figure 4A. This auto-radiogram was obtained by exposing the stained gel shown in the upper panel and also demonstrates that none of the other proteins electrophoresing around p62^{TCF} were modified by the kinase activity. Phosphorylation occurred on multiple sites, and after incubation at a higher ATP concentration as many as seven spots could be distinguished (Figure 4A, lower panel). Clearly the p62^{TCF} in these HeLa extracts is hypophosphorylated and thus serves as a direct substrate for the copurifying kinase activity.

The partially purified p62^{TCF} generated ternary complexes with rhSRF and the SRE probe (Figure 4B, lane 2). In addition, it yielded a rapidly migrating ternary complex with coreSRF (lane 6) and a rapidly migrating direct complex, labelled dc1, with the E74 probe (lane 9). Aliquots from the kinased p62^{TCF} displayed in the panel in Figure 4A showed in addition the slowly migrating core cII ternary complex (Figure 4B, lane 8) and the slowly migrating direct complex dc3 (lane 11). Increased phosphorylation had no effect on ternary complex formation with full-sized SRF (compare lanes 2, 3 and 4). Thus the endogenous kinase activity reproduces the same alterations in both core cII formation and direct E74 binding observed for TCF activities in nuclear extracts of mitogen-stimulated cells and this correlated with increased phosphorylation of p62^{TCF}. The alterations of TCF DNA binding activity seen during the time course of mitogen-stimulated c-*fos* activation are therefore most probably due to phosphorylation.

Protein phosphatase 2A reverses induction-dependent TCF phosphorylation

To substantiate further the relationship between TCF phosphorylation and the induced complexes seen in mitogen-stimulated cell extracts, purified phosphatases were incubated with the extracts. Potato acid phosphatase caused the elimination of complexes dcD and dcE, as well as rhElk-1-derived complexes dc2 and dc3 (Figure 5A). No major changes were seen with the other direct complexes (Figure 5A), nor did this treatment alter the amount of ternary complex formation in extracts prepared before induction or 30 min thereafter (data not shown).

Several eukaryotic protein phosphatase activities have been characterized and cloned (Cohen, 1992). Two were tested for their ability to reverse the mitogen-stimulated TCF

phosphorylation described here, namely PP-1α and PP-2A (Figure 5B). Treatment with purified PP-2A removed the induced complex dcE in nuclear extracts prepared from EGF-treated astrocytoma and A431 cells (Figure 5B), as well as the induced slowly migrating core cII complex (data not shown). PP-2A also removed the slowly migrating, Elk-1-derived complex dc3. Okadaic acid, which inhibits PP-2A activity (Takai and Mieskes, 1991), also blocked its effect in our reactions (data not shown). No effect was observed by treatment with PP-1α (Figure 5B). PP-2A treatment of purified HeLa $p62^{TCF}$ phosphorylated by the intrinsic kinase did not affect ternary complex formation with full-length rhSRF while it clearly dephosphorylated the protein (data not shown).

Discussion

Induction of resting cells leads to alterations in TCF–DNA complexes

Our aim was to determine whether the factors binding to the c-*fos* SRE were indeed modified by mitogenic signals that activate *fos* transcription through this element and if c-*fos* induction could be directly correlated with such modifications. Therefore nuclear extracts were prepared from cells during the time in which the c-*fos* promoter is induced and subsequently repressed. The binding of the relevant proteins, namely SRF and TCF, was visualized in gel retardation assays using the *fos* SRE as the probe. No significant changes were seen in either the SRF–SRE binary complex cI or the TCF–SRF–SRE ternary complex cII upon EGF induction. However, the addition of a truncated SRF molecule, which contained the protein domains essential for dimerization, DNA binding and ternary complex formation, yielded better separation of the TCF-dependent ternary complexes on the SRE. This revealed altered TCF-dependent complexes correlating with the activity of the c-*fos* promoter. Likewise, new complexes could also be detected when TCF was assayed for direct binding to the purine-rich Ets-protein binding site present in the *Drosophila* E74 promoter. Furthermore, these complexes disappeared upon c-*fos* repression and blocking repression led to their continued presence.

We have seen few differences in the amount of cII formation throughout the period of c-*fos* activation and then repression, when TCF is clearly undergoing reversible modification. This might reflect poor resolution in the gel retardation assays or masking of the effects of TCF modification on complex mobility by full-sized SRF. Others have seen an increase in ternary complex formation related to TCF phosphorylation (Malik *et al.*, 1991; Gille *et al.*, 1992). These differences probably lie in the experimental conditions used, since we see abundant cII activity in starved cell extracts and even with phosphatase-treated TCF. Furthermore, no differences in ternary complexes were seen in uninduced and induced A431 extracts under conditions where the induction-related complex dcE was significantly increased in the nuclear extracts from the EGF-induced cells.

Phosphorylation and dephosphorylation of TCF correlate temporally with c-fos promoter activity

In all three cell types used for making extracts, the presence and absence of the 'induced' complexes correlated with the transcriptional activity of the c-*fos* promoter. Several lines of evidence suggest that these 'induced' complexes contain phosphorylated TCF. They comigrated with $p62^{TCF}$ complexes that resulted from multiple phosphorylation events on $p62^{TCF}$, and the level of phosphorylation affected the gel migration of the complexes. Both the induced extract and phospho-$p62^{TCF}$ complexes were sensitive to phosphatase treatment, and PP-2A but not PP-1α removed them. This is in contrast to the transcription factor CREB (Hagiwara *et al.*, 1992), which appears to be a specific substrate for PP-1α.

An endogenous kinase phosphorylated $p62^{TCF}$ but no other proteins of a similar size and isoelectric point. It coeluted with $p44^{MAPK}$, and treatment of $p62^{TCF}$ with these enzymes, or with $p42^{MAPK}$, gave rise to the same set of complexes (data not shown). Initial attempts to determine whether the endogenous kinase activity is immunologically related to MAPK have been unsuccessful, and it is not yet clear if the copurification of $p62^{TCF}$ and the kinase activity is biologically relevant or coincidental. Nevertheless these data show that both the endogenous kinase and MAPK modify $p62^{TCF}$, thereby giving rise to the 'induced' complexes observed in extracts from mitogen-stimulated cells.

We conclude that the appearance of these complexes results from the phosphorylation of TCF, which implicates it as a significant target for the incoming signal generated by EGF. c-*fos* activation correlates temporally with the phosphorylation of TCF and may be a consequence of this modification. Consistent with this, the loss of the induced complexes, presumably by dephosphorylation since it can also be reproduced by treatment with PP-2A, correlates with the post-induction repression of the c-*fos* promoter. Blocking phosphatase activity with okadaic acid leads to uninterrupted *fos* transcription and to the continued presence of the 'induced' complexes. The increased intensity of the 'induced' complexes seen after prolonged okadaic acid treatment may represent the disruption of a kinase/phosphatase equilibrium normally maintaining an intermediate level of phosphorylation on the target proteins.

A role for reversible in situ modification of TCF in c-fos regulation

We have observed constant levels of ternary complex formation over the c-*fos* SRE by endogenous SRF and TCF throughout the *fos* induction cycle, when TCF is clearly undergoing reversible modification. This is consistent with genomic footprinting, which indicated no changes in the occupancy of the SRE during the same period while changes were clearly occurring elsewhere in the c-*fos* promoter (Herrera *et al.*, 1989; König, 1991). It suggests a model (Figure 6) where SRF and TCF are constitutively bound to the SRE, and it is this ternary complex that is the target for the signalling events activating *fos* transcription. Consistent with this, MAPK, which currently is the furthest known downstream component in the signal-activated kinase cascade (Leevers and Marshall, 1992), can modify TCF (Gille *et al.*, 1992; our unpublished data). Phosphorylation of TCF would not affect the efficiency of ternary complex formation but rather the interaction of this complex with other elements of the *fos* promoter and possibly the transcriptional machinery itself. Accordingly dephosphorylation of TCF, possibly by PP-2A, would play a pivotal role in the inactivation of transcription. Okadaic acid would abrogate repression by maintaining 'induced' TCF in the

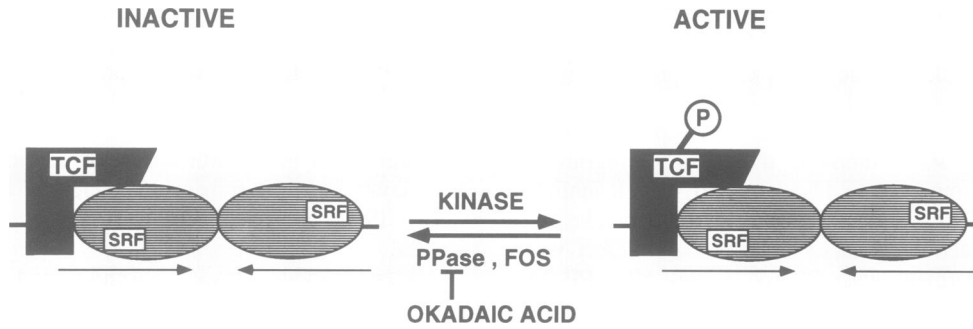

Fig. 6. Model for induction-associated TCF phosphorylation during transcriptional stimulation of the c-*fos* promoter *in vivo*. The diagram shows SRF and TCF constitutively bound to the SRE in the repressed as well as the induced state of the c-*fos* gene. Upon induction TCF becomes phosphorylated, an event postulated to involve TCF directly or indirectly in transcriptional activation. This is reversed by phosphatase 2A-like activities as an integral event during c-*fos* shut-off and c-*fos* basal level repression.

phosphorylated form or by disrupting a kinase/phosphatase equilibrium which would lead to TCF phosphorylation and *fos* activation.

Fos protein autoregulates its promoter through the SRE (König *et al.*, 1989; Lucibello *et al.*, 1989). This is mediated by the COOH-terminal domain and requires the phosphorylation of this region (Wilson and Treisman, 1988; Gius *et al.*, 1990; Ofir *et al.*, 1990). It is possible that Fos plays a role in directing the dephosphorylation of TCF, and okadaic acid may influence Fos synthesis or modification.

We have previously shown that the Ets-related protein Elk-1 is indistinguishable from p62^{TCF} (Hipskind *et al.*, 1991). Here we show that recombinant Elk-1, overexpressed in HeLa cells, behaves similarly to TCF activity in induced astrocytoma and A431 nuclear extracts, and that Elk-1-specific antisera react with the extract TCF activities. Thus our results implicate a transcription factor, belonging to the *ets* proto-oncogene family (MacLeod *et al.*, 1992), as a direct nuclear target of the mitogen-activated signal transduction cascade when associated with the *fos* promoter (Herrera *et al.*, 1989; König, 1991; Gille *et al.*, 1992) (Figure 6). The concept of promoter-bound regulatory factors as targets for sequentially activated kinases (Leevers and Marshall, 1992) and phosphatases (Cohen and Cohen, 1989; Cohen, 1992) could apply to rapidly and transiently induced genes in general, and emphasizes the importance of the reversible modification of transcription factors in the regulation of genes controlling cell proliferation.

Materials and methods

Cell culture and nuclear salt extract preparation

Astrocytoma cells (U87MG, ATCC no. HTB14) were grown to near confluence in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 4 mM glutamine, penicillin (100 u/ml) and streptomycin (100 μg/ml) streptomycin. Serum starvation was done for 16 h in RPMI in the absence of FCS, followed by treatment with recombinant hEGF (100 ng/ml) or okadaic acid (500 nM) or a combination of the two for the indicated lengths of time. A431 cells were grown and starved in DMEM medium supplemented as described above.

Nuclear salt extracts were prepared as described by Andrews and Faller (1991) with the following variations: PBS was supplemented with 2 mM Na_3VO_4, 10 mM NaF and protease inhibitors (see Buffer D below). Additionally 20 mM β-glycerophosphate, 10 mM *p*-nitrophenyl phosphate (pNPP) and 400 nM okadaic acid were added to Buffers A and C. RNA was prepared from the post-nuclear supernatants as described below. The presence of the phosphatase inhibitors throughout the extract preparation was necessary to maintain the 'induced' band shift activity.

Gel retardation analysis

Probes were prepared from subcloned SRE (Hipskind *et al.*, 1991) and E74 (Janknecht and Nordheim, 1992) sequences by labelling isolated fragments with [α-^{32}P]dATP and Klenow enzyme. Gel retardation experiments were carried out by mixing ~10 μg of protein with ^{32}P-labelled SRE or E74 probe in a total volume of 10 μl containing 10 mM Tris−HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 0.05% non-fat dry milk, 5% glycerol, 10 mM dithiothreitol and 0.5 μg (SRE) or 2 μg (E74) sheared salmon sperm DNA. In reactions including coreSRF 2 μg poly(dI-dC) was used in place of salmon sperm DNA. The binding reactions were incubated for 30 min at room temperature. 2.5 μl loading buffer (20% glycerol and 0.1% bromophenol blue) were added and then the samples were loaded on a 5% native polyacrylamide gel (29:1 acrylamide:bisacrylamide; 0.5×TBE). The gels were prerun for 30 min at 10 V/cm and after loading electrophoresed for a further 5 h. In Figure 4B oligonucleotide probes were used and the gel was electrophoresed for 4 h. Dried gels were autoradiographed with intensifying screens. Recombinant SRF and Elk-1 were produced by transient transfection of HeLa cells with the appropriate expression vector (Janknecht and Nordheim, 1992). Two versions of coreSRF were used. SRF_{90-244} was produced using recombinant vaccinia virus (Hipskind *et al.*, 1991) and $SRF_{132-222}$ was generated by *in vitro* transcription/translation as described by Mueller and Nordheim (1991).

For the preparative retardation gel the reaction was scaled up 10-fold using the SRE probe and A431 cell nuclear extract prepared from cells starved or induced with EGF for 20 min. The proteins in cII complexes were excised and eluted by diffusion at 37°C into 300 μl of buffer containing 50 mM Tris−HCl, pH 8.0, 0.1% SDS, 0.1 mM EDTA, 5 mM DTT, 150 mM NaCl and 0.1 mg/ml BSA. The eluates were precipitated and redissolved in 20 μl of 20 mM HEPES, pH 7.9, 6 M guanidinium−HCl, 100 mM KCl, 0.1% NP40, 0.2 mM EDTA, 2 mM $MgCl_2$. After desalting on a G25-Sephadex spin column, proteins were renatured by incubation on ice for 3 h and then tested for binding to the E74 probe. The resulting gel retardation was analyzed using a PhosphorImager.

Phosphatase digestion

Potato acid phosphatase (PAP) was dialyzed against 20 mM PIPES, pH 6.0, 0.5 mM PMSF, 0.2 mM TPCK and other protease inhibitors. Nuclear extracts or rhElk-1 protein were treated with PAP in a 15 μl reaction at 30°C for 30 min, with 0.02 units PAP/μg protein.

Digestions with PP-1α or PP-2A (generously provided by Dr G.Mieskes, Göttingen) (Takai and Mieskes, 1991) were also performed for 30 min at 30°C in a 15 μl reaction containing 12 μl buffer (20 mM Tris−HCl, pH 7.4, 0.5 mM DTE, 0.1 mM EGTA and 0.5 mM benzamidine), 2 μl of nuclear extract and 0.06 mU or 0.6 mU of the respective phosphatases. The control reactions were incubated under the same conditions without phosphatase.

Partial purification of p62^{TCF} and its in vitro phosphorylation

Full details of the preparation protocol of p62^{TCF} will be described elsewhere (V.Pingoud, in preparation). Briefly, HeLa cell nuclei were extracted with chloroquine according to Schröter *et al.* (1987), precipitated with $(NH_4)_2SO_4$ and dialyzed against Buffer D (20% glycerol, 20 mM HEPES, pH 8.0, 2 mM $MgCl_2$, 0.2 mM EDTA, 0.1% NP40, 10 mM DTT, 5 mM NaF, 0.2 mM Na_3VO_4, 0.5 mM PMSF, 0.5 μg/ml leupeptin, 2 μg/ml aprotinin, 0.5 μg/ml pepstatin, 1 U/ml α_2-macroglobulin and 0.5

mM benzamidine) plus 50mM NaCl. Proteins were bound to a DNA–cellulose column (Sigma) in 100 mM NaCl–Buffer D and eluted with 400 mM NaCl–Buffer D. $p62^{TCF}$-containing fractions were identified by gel retardation analysis, pooled and bound to a Fractogel EMD TMAE-650 column (Merck, Darmstadt). The $p62^{TCF}$ activity eluted between 80 and 300 mM NaCl in Buffer D, as did a protein kinase activity. These fractions were analyzed by two-dimensional gel electrophoresis (see below).

For phosphorylation by the copurifying kinase activity the fractions were brought to kinase reaction conditions (20 mM MOPS, pH 7.2, 20 mM $MgCl_2$, 30 mM β-glycerophosphate, 2 mM DTT, 5 mM EGTA, 0.5 mM Na_3VO_4, 10 mM pNPP) and incubated in the presence of 10 μM or 100 μM ATP plus 50 nM [γ-^{32}P]ATP at 37° C for 30 min. 200 μl of this reaction mixture, corresponding to 3×10^8 cells, were precipitated with 50 μl 100% TCA.

Two-dimensional gel electrophoresis of proteins

The first dimension, non-equilibrium pH-gradient gel electrophoresis (NEPHGE), was performed according to Eckerskorn *et al.* (1988) with modifications and will be described elsewhere (V.Pingoud, in preparation). The SDS–PAGE second dimension was followed by silver staining. The gels were dried and the phosphoproteins visualized by autoradiography.

Antibodies

GST fusion proteins containing three different domains of Elk-1 were expressed in *Escherichia coli* BL21. Proteins were purified from SDS gels and used to immunize rabbits. Either crude serum or IgG fractions enriched by Na_2SO_4 precipitation were used (R.Zinck and A.Nordheim, in preparation). The polyclonal sera were specific for the Elk-1 Ets domain (α-Ets, aa 1–82), a central region of Elk-1 (α-D, aa 83–307) or the Elk-1 carboxy-terminus (α-C, aa 307–428). The specificity of the antisera was established by Western blotting and immunoprecipitation. Each antiserum recognizes its epitopes, as shown by testing with various Elk deletion mutants, and did not show cross-reactivity with other cellular proteins. In addition, two of the antisera could also detect enriched TCF from HeLa cells after immunoblotting of a second dimension gel. All antisera precipitated *in vitro* translated [^{35}S]Elk-1, while α-Ets also precipitated Ets-1, SAP-1a and SAP-1b.

RNA preparations, Northern blot hybridization and nuclear run-on analysis

For RNA preparation 400 μl of post-nuclear supernatants were spun for 10 min at 10 000 *g*. The cleared supernatants were mixed with 2 μl RNasin, 400 μl 2×proteinase K buffer (200 mM Tris–HCl, pH 7.0, 440 mM NaCl, 2% SDS and 25 mM EDTA) and 8 μl proteinase K (20 mg/ml) (Greenberg, 1989). Samples were incubated for at least 30 min at 37°C and purified by organic extraction and ethanol precipitation. 10 μg of total RNA were loaded per lane on formaldehyde–agarose gels. Details of the Northern analysis and the probes used have been described previously (Herrera *et al.*, 1989; Stewart *et al.*, 1990). Nuclear run-on analysis was performed as described by Greenberg (1989) and Stewart *et al.* (1990).

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