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# Transient Activation of RAF-1, MEK, and ERK2 Coincides Kinetically with Ternary Complex Factor Phosphorylation and Immediate-Early Gene Promoter Activity In Vivo

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**We have investigated the early in vivo signaling events triggered by serum that lead to activation of the c-*fos* proto-oncogene in HeLa cells. Both RAF-1 and MEK kinase activities are fully induced within 3 min of serum treatment and quickly decrease thereafter, slightly preceding the activation and inactivation of p42$^{MAPK}$/ERK2. ERK2 activity correlates tightly with a transient phosphatase-sensitive modification of ternary complex factor (TCF), manifested by the slower electrophoretic mobility of TCF-containing protein-DNA complexes. These induced complexes in turn correlate with the activity of the c-*fos*, *egr-1*, and *junB* promoters. Phorbol ester treatment induces the same events but with slower and prolonged kinetics. Inhibition of serine/threonine phosphatase activities by okadaic acid treatment reverses the repression of the c-*fos* promoter either after induction or without induction. This corresponds to the presence of the induced complexes and of ERK2 activity, as well as to the activation of a number of other kinases. Inhibition of tyrosine phosphatase activities by sodium vanadate treatment delays but does not block ERK2 inactivation, TCF dephosphorylation, and c-*fos* repression. The tight linkage in vivo between the activity of MAP kinase, TCF phosphorylation, and immediate-early gene promoter activity is consistent with the notion that a stable ternary complex over the serum response element is a direct target for the MAP kinase signaling cascade. Furthermore, serine/threonine phosphatases are implicated in regulating the kinase cascade, as well as the state of TCF modification and c-*fos* promoter activity, in vivo.**

Treatment of quiescent cells with serum or individual growth factors leads to their entry into the cell cycle and to cell growth. These events are mediated by the activation of various signaling pathways linking the cell membrane to the nucleus and resulting in the activation of genes essential for cell proliferation. The best characterized example of these rapid response or immediate-early genes is the c-*fos* proto-oncogene (17). The c-*fos* promoter is fully active within minutes of stimulation and thereafter is rapidly inactivated (17). The brief, transient appearance of the Fos protein is further insured by the instability of its mRNA and of the protein itself (17). The rapid burst of expression of Fos appears to be one of the key events in the cellular transition from $G_0$ to $G_1$.

Within the c-*fos* promoter, the serum response element (SRE) is a target for many of these signaling pathways (62, 81, 82). Genomic footprinting studies show that the SRE is constitutively occupied in vivo and that no apparent alteration in its occupancy occurs during induction or the subsequent repression (30, 41). Although many proteins can bind to the SRE (81, 82), the pattern of DNA contacts observed in vivo is reproduced in vitro by the formation of a ternary complex between the SRE, a dimer of serum response factor (SRF), and ternary complex factor (TCF) (71, 73). Disruption of this ternary complex by mutating the appropriate contact points on the SRE significantly reduces activation of c-*fos* reporter genes by proliferative signals (25, 28, 48, 65, 73). Using such mutations, Graham and Gilman (25) argued that signaling pathways activated by serum (protein kinase C [PKC]-independent) target the SRF-SRE binary complex, while PKC-dependent signaling induced by phorbol 12-myristate 13-acetate (PMA) necessitates the full ternary complex.

Both in vivo and in vitro data suggest that the ternary complex assembled at the SRE is a direct target for signals generated by many agents (62, 81–83) and that, accordingly, SRF and TCF are hyperphosphorylated upon induction in human cells and mouse cells (34, 35, 63, 82, 87). This signal-induced modification has little detectable effect on the mobility of SRF-DNA complexes (35, 48, 63, 80, 87), while it leads to a striking change in the mobility of TCF-containing protein-DNA complexes that correlates with the activity of the c-*fos* promoter (35, 87). A similar modification of these complexes can be reproduced in vitro by treating TCF with either MAP kinase (23, 57) or an endogenous kinase partially copurifying with TCF (57, 87). The preponderant TCF activity in human cells is indistinguishable from the protein encoded by the Ets-related *elk-1* gene (36, 59), and expressing Elk-1 or fusion proteins containing the *elk-1* carboxy-terminal region in culture cells can transactivate appropriate reporter constructs when the signaling cascade is activated (32, 38, 49). Thus, TCF/Elk-1 appears to play a key role in SRE-dependent activation mediated by MAP kinase.

Many of the signals inducing c-*fos* activate tyrosine kinase receptors and/or PKC (62, 81–83). Ras clearly plays a role in activating the kinase/phosphatase cascade, transmitting the signals to the nucleus, partly via interactions with Raf-1 (7, 51). Raf-1 and MEKK (MAP and ERK kinase kinase), another kinase lying in a seemingly distinct pathway (22, 46), can activate MEK (MAP and ERK kinase), which then activates MAP kinase (mitogen activated protein kinase, also known as

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extracellular signal-regulated kinase or ERK) (7, 51). This scheme is based on biochemical data (7, 51), transfection experiments (7, 51), and yeast, nematode, and *Drosophila* genetics (53, 56) and shows a remarkable conservation, from yeast up to vertebrates (51, 53, 56). Since Elk-1 and TCF are in vitro substrates for MAP kinases (23, 35, 38, 49, 57, 61) and appear to mediate signal-dependent gene induction in vivo (83), our hypothesis is that a ternary complex assembled on the c-*fos* SRE in vivo represents an important nuclear target for this pathway.

To test this idea, we set out to investigate the early signaling events involved in the activation of the c-*fos* promoter in vivo. We have previously found that TCF phosphorylation after epidermal growth factor (EGF) stimulation of human astrocytoma and epithelial carcinoma cells correlates with the transcription of the c-*fos* gene (87). Therefore, TCF modification, as measured in band shift assays, can be used as a marker to determine whether activation of signaling pathways is directly linked to TCF phosphorylation and the transcription of c-*fos* and other immediate-early genes (IEGs). Using this approach, we analyzed extracts made from HeLa cells at multiple time points after serum induction. The phosphorylation status of TCF corresponds directly to MAP kinase activity, and both correlate with transcription of the c-*fos* gene. Two components of the MAP kinase signaling cascade, RAF-1 and MEK, show a rapid, transient spurt of activity slightly preceding that of MAP kinase. PMA induction yields a slower and more prolonged peak of activation of c-*fos*, MAP kinase, and TCF phosphorylation, reinforcing their tight temporal linkage. Okadaic acid (OA) blocks the subsequent down-regulation of these activities after serum induction or leads to their induction on its own, while sodium orthovanadate does not. This suggests that the inactivation of MAP kinase, along with TCF dephosphorylation and c-*fos* repression, is dependent upon serine/threonine phosphatase activities. Thus, induction of c-*fos* in vivo by different signaling pathways correlates kinetically with MAP kinase activation and TCF phosphorylation.

## MATERIALS AND METHODS

**Cell culture, inductions, and extract preparation.** HeLa cells were maintained in 90-mm-diameter dishes in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% fetal calf serum (Seromed). One day prior to the experiment, monolayers at about 80% confluence were washed twice with phosphate-buffered saline (PBS) and were then kept for 24 h in Dulbecco's modified Eagle's medium without serum. The appropriate inducers and inhibitors were added from concentrated stocks as required, since changing the medium alone was sufficient to induce TCF modification and IEG expression. Serum was added to 10% PMA (Sigma) in ethanol, was diluted to 1 μg/ml in Dulbecco's modified Eagle's medium, and was then added to 100 ng/ml. OA (Moana Biochemicals) in dimethyl sulfoxide (DMSO) was used at 1 μM. Sodium orthovanadate (Sigma) was prepared fresh as described below and was added from concentrated stock solutions. In no case did the addition of DMSO, ethanol, or pH 10 buffer lead to induction.

At the appropriate time points, the culture dishes were placed on ice, the medium was immediately removed, and the monolayers were washed twice with ice-cold PBS containing 10 mM NaF and 100 μM $Na_3VO_4$. Monolayers were scraped off in 500 μl of lysis buffer (10 mM Tris-HCl [pH 7.05], 50 mM NaCl, 50 mM NaF, 1% Triton X-100, 30 mM $Na_4P_2O_7$, 5 μM $ZnCl_2$, 100 μM $Na_3$ $VO_4$, 1 mM dithiothreitol, 2.8 μg of aprotinin per ml, 2.5 μg each of leupeptin and pepstatin per ml, 5 U of $\alpha_2$-macroglobulin per ml, 0.5 mM benzamidine, and 0.5 mM phenylmethylsulfonyl fluoride) (35). After vigorous vortexing for 45 s at 4°C, the lysates were cleared by centrifugation at 10,000 × *g* for 10 min at 4°C. Aliquots were immediately processed for RNA extraction, kinase assays, and band shifts as described below. The remainder of the extract was stored in aliquots at −70°C. Stock solutions (10 or 100 mM) of $Na_3VO_4$ were titrated to pH 9.5 to 10, boiled until colorless, and then retitrated to pH 9.5 to 10.

**Western blotting (immunoblotting).** Cell lysates (25 μg per lane) were separated by sodium dodecyl sulfate–7.5% polyacrylamide gel electrophoresis (SDS-PAGE) prior to electrophoretic transfer onto Hybond C Super (Amersham). The blots were blocked with 5% nonfat dry milk (Biorad) and were incubated with one of the following antisera: a RAF-1 rabbit polyclonal antiserum raised against a carboxy-terminal peptide of v-*raf*, a MEK-1-specific mouse monoclonal antibody (Transduction Labs/Affiniti, Nottingham, United Kingdom), either an ERK-1 and -2-specific rabbit polyclonal antiserum raised against an ERK peptide as described previously (11), a pan-ERK mouse monoclonal antibody (Transduction Labs/Affiniti), or an antibody fragment specifically recognizing phosphotyrosine residues (RC20; Transduction Labs/Affiniti). The blots were subsequently incubated for 1 h at room temperature with the appropriate anti-rabbit or anti-mouse horseradish peroxidase-conjugated antibodies prior to exposure to the ECL (Amersham) substrate. All Western blotting reagents were from Amersham.

**Immune-complex kinase assays.** Immunoprecipitations were carried out as previously described (6). RAF-1 and MEK-1 were precipitated from cell lysates by using the same antiserum or antibody used for the Western blots. Immune complexes were collected following incubation for 1 to 2 h at 4°C with protein A-Sepharose beads. The immune-complex kinase assays were carried out as described previously (46) by using kinase-compromised mutants of MEK-1 and ERK2 as substrates for RAF-1 and MEK-1, respectively.

**In-gel kinase assay.** Immediately after the clearing spin, an aliquot of extract was mixed with an equal volume of 20% glycerol, 125 mM Tris-HCl (pH 6.8), 5% SDS, 10 mM EDTA, and 0.01% bromphenol blue. Dithiothreitol was added to 100 mM, and the mixtures were heated at 85°C for 5 min before SDS-PAGE. If they were not used immediately, the mixes were stored at −20°C and were then denatured again prior to electrophoresis. The gels were polymerized with 0.2 mg of myelin basic protein (Sigma) per ml and, after electrophoresis, were denatured in 6 M guanidine-HCl, renatured, and assayed for kinase activity as described previously (11, 13, 35).

**Band shift assays.** Reaction mixtures (7.5 μl) contained the following components: 2.5 μg of poly(dI-dC) · (dI-dC), 250 ng of salmon sperm DNA, 5% glycerol, 66 mM NaCl, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.35% Triton X-100, 0.05% low-fat milk, 15 mM dithiothreitol, 10,000 to 15,000 cpm of $^{32}$P-labeled probe (0.2 ng/4 fmol), and ca. 10 μg of protein, depending upon the extracts. Within an experiment, equal amounts of extract protein were used in all reaction mixtures to allow direct comparisons between lanes. After 30 min at room temperature, the entire reaction mixture was loaded on a 4% polyacrylamide (acrylamide-bisacrylamide, 29:1) gel containing 0.5× Tris-borate-EDTA and was run at 1 mA/cm for 3 to 4 h. The gels were dried, and the complexes were visualized by autoradiography using intensifying screens. core$SRF_{90-244}$ was produced in HeLa cells (by using a recombinant vaccinia virus [33, 36]) or in bacteria (33). The SRE probe was prepared from a fragment subcloned in front of a G-free cassette plasmid (35, 36). After *Eco*RI and *Nar*I digestion, the ends were labeled by

a Klenow fill-in reaction mixture containing [α-$^{32}$P]dATP and cold dCTP, dGTP, and dTTP. The fragment was isolated from polyacrylamide gels by electroelution. The kinased collagenase TRE oligonucleotide (upper strand sequence AGCATGAGT CAGACAC) was kindly provided by F. Bange.

**Run-on analyses.** HeLa cells were treated as indicated in the appropriate figure legend, by using three plates per time point. Nuclei were isolated, and run-on transcription was performed as described previously (26, 87). Plasmid DNAs were linearized with an appropriate restriction enzyme, denatured, neutralized, and then applied in equimolar amounts to nitrocellulose (26) by using a slightly warped slot blotting apparatus, which gives rise to an unfortunate smearing of some of the points. The following DNAs were used: two fragments from the human c-*fos* gene, spanning either the first 973 bp of the transcription unit or 480 bp of exon 4, mouse *junB*, rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH), mouse IEF-4α, human *elk-1*, mouse c-*jun*, mouse *EGR-1*, human *ets-2*, and the cloning vector Bluescript (Stratagene).

**RNA isolation and analysis.** Aliquots of the whole-cell extracts were mixed with an equal volume of 0.2 M Tris-HCl (pH 7.5), 0.44 M NaCl, 2% SDS, and 25 mM EDTA. Proteinase K was added to 0.2 mg/ml, and the mix was incubated at 37°C for 30 to 60 min (26). After organic extraction, the RNA was collected by ethanol precipitation. Ten micrograms of total RNA was loaded per lane on formaldehyde-agarose gels. After electrophoresis, the gels were equilibrated in 25 mM sodium phosphate buffer (pH 6.8) and were blotted onto positively charged nylon (Zetabind; CUNO, Inc.) in the same buffer. After UV crosslinking and drying, the filters were wetted in water and were then hybridized as described previously (87). After hybridization, the filters were stripped for 30 min at room temperature in three changes of 30 mM NaOH–1% SDS, washed in 25 mM sodium phosphate buffer (pH 6.8) followed by 0.2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–1% SDS, and then reprobed. The riboprobes corresponded to either the first or the fourth exon of the human c-*fos* gene (the two give identical results) and the entire rat GAPDH cDNA.

## RESULTS

**Slower complexes containing modified TCF appear rapidly after serum stimulation of HeLa cells.** Serum stimulation of quiescent HeLa cells leads to a rapid, transient transcriptional activation of the c-*fos* gene and many other IEGs (17, 62, 81–83; see below). To investigate the effects of serum stimulation on endogenous SRF and TCF activities, we made extracts from HeLa monolayers at various times after the addition of serum to cells made quiescent by overnight serum starvation. To facilitate these analyses, an extraction protocol involving direct lysis of the monolayers on ice was adopted, thereby eliminating the more extensive manipulations required for nuclear extract preparation. SRF and TCF binding activities were visualized by incubating the nuclear extracts with a $^{32}$P-labeled c-*fos* SRE probe, followed by gel-electrophoretic separation of the protein-DNA complexes. Two major complexes are seen: one contains a dimer of SRF bound to the SRE, called complex I (cI), while the other involves a ternary complex between TCF, a dimer of SRF, and the SRE, labeled complex II (cII) (54, 70, 71, 73; see Fig. 4). Both of these complexes were present in the extracts prepared from serum-starved and serum-induced cells. Induction led to no apparent change in cI, whereas cII showed a slightly lower mobility and an apparent weak increase in intensity that was somewhat variable between experiments (33).

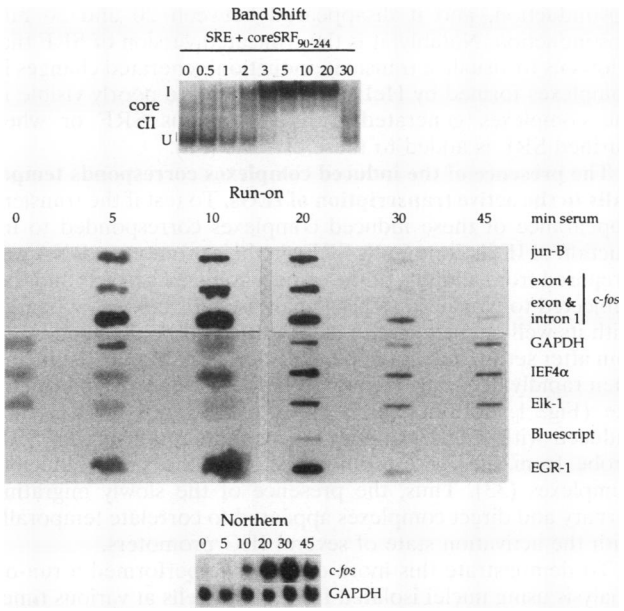


FIG. 1. The presence of TCF-containing complexes with slower mobilities correlates with the active transcription of IEGs. Serum was added to quiescent HeLa monolayers, which were then lysed at the times (in minutes) indicated above each lane (upper panel). The whole-cell extracts were then incubated under binding conditions with a $^{32}$P-labeled c-*fos* SRE probe, and the protein-DNA complexes were resolved by electrophoresis on 4% polyacrylamide gels. The region of the autoradiogram shown corresponds to the coreSRF-TCF-SRE ternary complexes. See Fig. 4 and 6 for their migration relative to the coreSRF-SRE binary complex cI. The middle panel shows the transcriptional status of genes at various times after serum induction of HeLa cells. Run-on transcripts were labeled in nuclei harvested at the indicated time points and were hybridized to filters containing equimolar amounts of the cloned genes shown. Two separate exposures (*junB* and c-*fos*, 15 h, and GAPDH to *egr-1*, 52 h) of the same experiment are shown to facilitate comparison. The exon and intron 1 DNA spans the first 973 bp of the human c-*fos* gene, while the exon 4 DNA contains 480 bp of the human c-*fos* sequence. The bottom panel is a Northern blot analysis of RNA isolated from the supernatants remaining after isolation of the nuclei for the run-on analysis. The same filter was initially hybridized with a c-*fos* riboprobe, and then with a GAPDH riboprobe.

Much more distinct differences could be visualized in ternary complexes when these extracts were complemented with a shortened version of SRF, coreSRF$_{90\ to\ 244}$ (Fig. 1, upper panel; also see Fig. 4; 36). This protein spans amino acids 90 to 244 of SRF and thus contains the domains necessary for dimerization, binding, and interaction with TCF (52, 54, 70). In extracts from unstimulated cells we saw a fast-migrating ternary complex, termed core cII uninduced, along with several weak, more slowly-migrating ternary complexes. After the addition of serum, the uninduced complex diminished. At the same time, the slower ternary complex core cII-I, which will be referred to as "induced," appeared. Core cII-I reached its maximum intensity 3 to 5 min postinduction and remained at this level for another 15 min (Fig. 1, upper panel), along with another, much weaker coreSRF-dependent ternary complex of greater mobility. By 30 min after serum induction, these induced complexes disappeared and the uninduced complex reappeared (Fig. 1, upper panel). Although these kinetics varied slightly among experiments, the transition to the induced complex was consistently apparent between 2 and 3 min

postinduction, and it disappeared between 20 and 30 min postinduction. Notably, it is the truncated version of SRF that allows us to visualize transient, induction-generated changes in complexes formed by HeLa TCFs, which are poorly visible in the complexes generated with endogenous SRF or when purified SRF is added to these extracts (33).

**The presence of the induced complexes corresponds temporally to the active transcription of IEGs.** To test if the transient appearance of these induced complexes corresponded to induction of IEGs containing SREs in their promoters, RNA was prepared from aliquots of the same whole-cell extracts and was subjected to Northern (RNA) analysis. c-*fos* mRNA appeared with its well-characterized kinetics. It could be detected by 10 min after serum induction, peaking between 20 and 30 min and then rapidly decaying, frequently through a shorter intermediate (Fig. 1, bottom panel; 17, 33). *egr-1* mRNA was also induced with similar kinetics, and band shifts using an SRE probe from the *egr-1* promoter showed the same inducible complexes (33). Thus, the presence of the slowly migrating ternary and direct complexes appeared to correlate temporally with the activation state of several IEG promoters.

To demonstrate this more directly, we performed a run-on analysis using nuclei isolated from HeLa cells at various times after serum stimulation. Basal transcription of the c-*fos* first exon was not observed in starved cells (Fig. 1, middle panel), suggesting that an attenuation mechanism is not operative in HeLa cells (45). However, 5 min after serum induction, the transcription of several genes, namely *junB*, *egr-1*, and c-*fos*, was already activated (Fig. 1, middle panel). In the case of c-*fos*, a stronger signal was seen with the first exon and intron than with exon 4. However, by 10 min postinduction, the two regions showed similar signals. This indicates that the region of the c-*fos* gene 3 to 4 kbp downstream of the RNA initiation site was not fully covered by RNA polymerase molecules 5 min after induction, thereby suggesting that the *fos* promoter could not have been activated long before this time. Consistent with this, the appearance of the induced ternary complex to a significant level occurred only between 2 and 3 min after induction (Fig. 1, upper panel). Similarly, transcription of the *junB* and *egr-1* genes was also stronger at the 10-min point. All three were then inactivated between 20 and 30 min after the addition of serum, when the induced ternary complex had disappeared. In contrast, the GAPDH, IEF4α, and *elk-1* genes were uninfluenced by serum induction at either the transcriptional level or the mRNA level (Fig. 1, lower panel; 33). This contradicts a suggestion that *Elk-1* expression is inducible (61) and in fact reflects the lack of *Elk-1* induction by growth factors in many different cell lines (33; also see Fig. 3B).

A number of other genes tested, including *fra-1*, c-*jun*, and *ets-2*, were activated with slower kinetics than those described above (33). Northern analysis of the mRNA prepared from the postnuclear supernatants illustrated the delayed appearance of the c-*fos* mRNA relative to its transcription (Fig. 1, lower panel). These data show that the appearance of the slowly-migrating, induced TCF complexes did indeed reflect the actual activation of the c-*fos* and *egr-1* promoters, and the decay of these complexes corresponded to the subsequent repression of c-*fos* and *egr-1* transcription.

**The induced ternary complexes arise from a phosphatase-sensitive modification of a factor immunologically related to Elk-1.** We have previously shown that TCF purified from chloroquine-extracted HeLa nuclei was indistinguishable from the protein encoded by the *elk-1* locus (36). Simultaneously, Dalton and Treisman (19) reported the cloning of SAP-1, another TCF that shared three regions of homology with Elk-1, including an amino-terminal ETS domain. Since both *elk-1* and *SAP-1* mRNAs are expressed in HeLa cells, in which the *elk-1* mRNA is considerably more abundant (33), distinct TCFs might be forming the ternary complexes observed in both uninduced and induced whole-cell extracts. To test this, we added Elk-1- and SAP-1a-specific antisera to the binding reaction mixtures. Four different Elk-1-specific antisera blocked the formation of the major uninduced and induced coreSRF-directed ternary complexes (33, 57). An antiserum reacting specifically with the SAP-1a protein did not affect these bands but did block a weak uninduced ternary complex and the weak, faster-migrating ternary complex also observed upon induction (35). Thus, the major species of TCF in HeLa whole-cell extracts is closely related to Elk-1, while SAP-1a represents a minor component of the TCF activity present in the HeLa whole-cell extracts. Interestingly, both TCFs form more-slowly-migrating complexes correlating with serum induction of HeLa cells (33, 35).

TCF cannot bind directly to the c-*fos* SRE. However, it can bind to the Ets-protein recognition site present in the *Drosophila* E74 promoter (39, 60, 87), and we have previously exploited this direct binding assay to characterize TCF modification after EGF induction in several human cell lines (87). As described above for the coreSRF-directed ternary complexes, distinct uninduced direct complexes containing Elk-1 and SAP-1a were shifted to the induced position upon serum treatment of HeLa cells (33). The cross-compatibility between the direct and ternary complexes (33) indicated that the same factors were involved in both complexes, which was confirmed by our ability to isolate direct binding activities from the ternary complexes (87).

The induced ternary complexes were sensitive to several different phosphatases (33). Treatment with bacterial alkaline phosphatase and the eukaryotic serine/threonine phosphatase PP-2A, but not PP-1α, chased the induced complexes down to the uninduced position without affecting the uninduced complexes. A $Mg^{2+}$-dependent activity in the extracts also targeted the induced but not the uninduced coreSRF-driven ternary complexes. This, together with our ability to shift ternary complexes from the uninduced to the induced position by kinase treatment of purified TCF and Elk-1 (35, 87), suggests that the induced complexes arise from the uninduced complexes via phosphorylation of TCF.

**The kinetics of RAF-1, MEK, and ERK2 activation correlate with those of the induced TCF-containing complexes.** The results shown above suggest that TCF phosphorylation played a role in shifting the uninduced complexes to the induced position. In addition, there is ample evidence that both TCF and Elk-1 are substrates for MAP kinase and that MAP kinase treatment can also generate the lower mobility characteristic of the induced complexes (35, 57). Therefore we investigated the kinetics of activation of components in the MAP kinase (or ERK) signaling pathway, namely RAF-1, MEK, and MAP kinase, in the whole-cell extracts analyzed above. RAF-1 and MEK were immunoprecipitated, and their kinase activities were measured by phosphorylation of kinase-compromised MEK and MAPK, respectively (Fig. 2A and B; 46). MAP kinase activity was measured by "in-gel" kinase assays (13), in which the whole-cell extract proteins were resolved by SDS-PAGE in a gel containing myelin basic protein (MBP). After renaturation, the activities of the MAP kinase species ERK1 and ERK2, as well as other MBP kinases, were visualized by the transfer of radioactive phosphate to MBP.

RAF-1 kinase was fully activated within 3 min after the addition of serum, while little activity was present 1 min after induction or in quiescent cells. RAF was rapidly turned off, with significantly decreased activity as soon as 10 min postin-

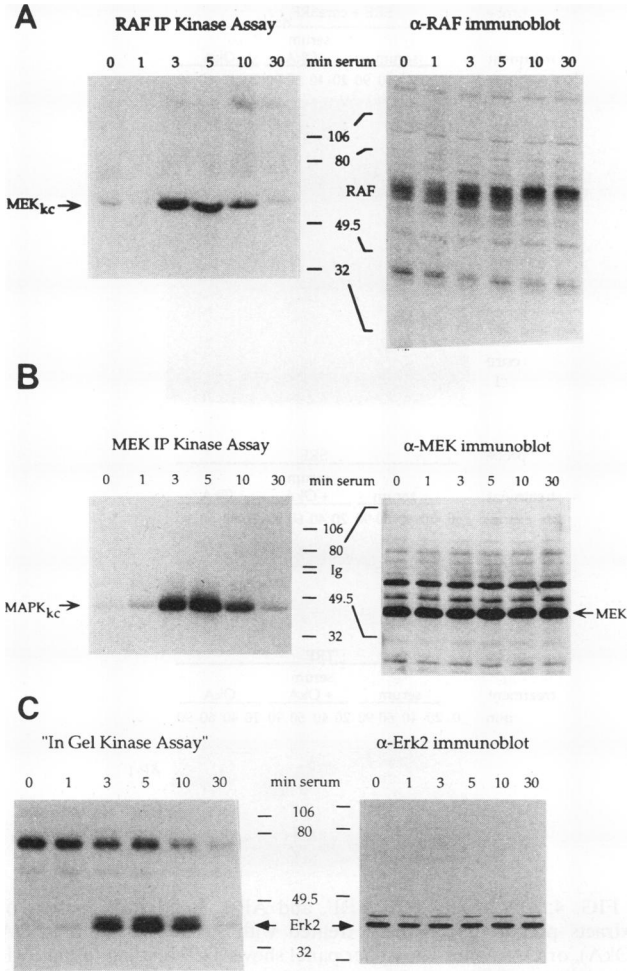


FIG. 2. Kinetics of activation of RAF-1, MEK, and ERK2 following serum induction of HeLa cells. (A) RAF-1 was immunoprecipitated from HeLa whole-cell extracts prepared at various times after serum induction. The immune precipitates were incubated with a kinase-compromised mutant of MEK (49.5-kDa $MEK_{kc}$) in the presence of [$\gamma$-$^{32}$P]ATP and were then subjected to SDS-PAGE. The autoradiogram of the dried gel is shown in the left panel. The somewhat distorted appearance of the labeled MEK band is due to the large amount of immunoglobulin electrophoresing just above it. The right panel shows a RAF-1 immunoblot. The position of RAF-1 (72 kDa) is indicated at the left. (B) MEK immunoprecipitates from serum-induced HeLa cell extracts were incubated with a kinase-compromised mutant of MAP kinase (42-kDa $MAP_{kc}$) and were analyzed as in the left side of panel A. Ig indicates the immunoglobulin light chain. The right panel shows a MEK immunoblot, and the labeled arrow denotes the 49.5-kDa MEK band. (C) The left panel shows an in-gel kinase assay of MAP kinase activity in whole-cell extracts of serum-induced cells. Protein (15 μg) was loaded in each lane of a gel where MBP was polymerized in the separating gel. After electrophoresis and renaturation, kinase activity was visualized by incubation in the presence of [$\gamma$-$^{32}$P]ATP. The autoradiogram of the dried gel is shown, as is the Western blot probed with an ERK2-specific antiserum. The positions of prestained markers are shown (in kilodaltons).

duction (Fig. 2A). In the Western blot, the appearance of intermediately phosphorylated RAF-1 corresponded to maximum activity (3- and 5-min lanes), while hyperphosphorylation of RAF-1 correlated with its inactivation after induction (Fig. 2A). MEK also showed full activation 3 min after the addition of serum, and a slight increase was already detectable at the 1-min point (Fig. 2B). MEK activity also decreased between 5 and 10 min postinduction but did not completely return to preinduction levels at the 30-min point. The MEK Western blot showed comparable levels of the enzyme at all time points and no obvious shift in mobility upon induction (Fig. 2B). The in-gel kinase assay (Fig. 2C) indicated that MAP kinase was also activated by 3 min after the addition of serum, with some increase apparent at the 5-min point. MAP kinase then decreased slightly by 10 min postinduction and decreased significantly by 30 min postinduction. These kinetics seem to show a slight lag relative to those of RAF-1 and MEK. The predominant species of MAP kinase in our HeLa extracts is ERK2, as is shown in Fig. 2C and with a pan-ERK antiserum in Fig. 5. We did not see a mobility shift in ERK2 upon induction in our gel system (Fig. 2C; 11, 34, 35). Thus, by 3 min after serum induction of HeLa cells, three components in the MAPK signaling pathway were activated, with ERK2 showing a slight lag in full activation and inactivation relative to RAF-1 and MEK. Moreover, the activation of this pathway showed a tight temporal correlation to the appearance of the induced TCF-containing complexes, thereby providing the first in vivo evidence linking them kinetically.

**PMA induction of ERK2, modified TCF, and c-*fos* transcription occur with slower kinetics.** The tumor promoter PMA also induces IEG transcription and cell proliferation in a variety of cell types via PKC activation, a pathway that has been argued to be distinct from serum in activating the SRE (25). Therefore, HeLa cells were treated with PMA, and whole-cell extracts were prepared at various times thereafter. The induced shift characteristic of TCF hyperphosphorylation appeared with somewhat slower kinetics than did those observed with serum, and it decayed much more slowly, with a strong signal still present 30 min postinduction (Fig. 3A). In-gel kinase assays using the same extracts showed activation of ERK2 in parallel with the induced shift, including sustained strong activity up to 30 min (34). Taken together, these data further confirm the temporal correlation between MAP kinase activation and TCF modification.

Since the 30-min time point still showed fully modified TCF, a longer PMA time course was performed. The kinetics of the appearance of the induced complexes were superimposable on those seen above (Fig. 3A, right panel) and showed that they were still detectable 80 min after induction, in contrast to their complete disappearance 30 min after the addition of serum (Fig. 1 and 6). However, a transition was obvious between 30 and 45 min, when there was a significant decrease in the slowest complexes, accompanied by a smear throughout the ternary complex region in the gel. Northern and run-on analyses demonstrated that activation and repression of the *fos* promoter were also delayed relative to the kinetics observed with serum (Fig. 3B) (33). c-*fos* mRNA was first seen at the 20-min point and disappeared between 60 and 80 min after induction. The c-*fos* gene was fully activated 15 min after PMA induction, while c-*jun* and *ets-2* showed a somewhat delayed activation and the controls (*Elk-1* and GAPDH) were unaltered (Fig. 3B). c-*fos* transcription was still strong 30 min after PMA induction, and weak *fos* promoter activity could still be detected at 60 min. Thus, the activity of the c-*fos* promoter coincided once again with a strong induced ternary complex and with ERK2 activation. The intermediate levels of the induced complex seen late after PMA induction, when c-*fos* is clearly inactive, may reflect continued low stimulation of intracellular signaling pathways by the phorbol ester.

**OA reverses c-*fos* repression and TCF dephosphorylation and induces activation of multiple kinase activities.** OA is a

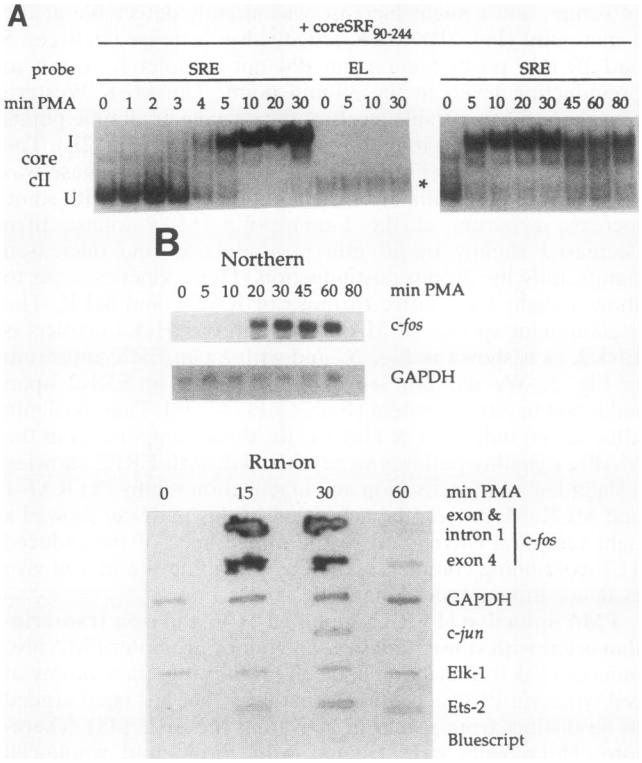


FIG. 3. PMA induces a slower and longer lasting activation of the c-*fos* promoter and TCF modification. (A) Band shift analysis of whole-cell extracts prepared after PMA induction for the indicated times. The reaction mixtures contained coreSRF$_{90-244}$ and either a $^{32}$P-labeled SRE probe or the mutant EL, which can bind SRF/coreSRF$_{90-244}$ but cannot form ternary complexes (36, 73). This is used to demonstrate that the core cII complexes arise from TCF binding. When ternary complex formation is blocked, however, a complex of unknown origin (indicated by the asterisk) that is not related to TCF is seen (33). The right panel shows a separate experiment analyzing longer times after PMA induction. (B) The upper panel shows a Northern blot analysis of RNA isolated from the same extracts shown in panel A. The GAPDH signal is particularly weak because of problems in stripping the filter after the c-*fos* hybrization. The lower panel shows the transcriptional state of the indicated genes after PMA induction, as measured by run-on analysis using isolated nuclei (see legend to Fig. 1).

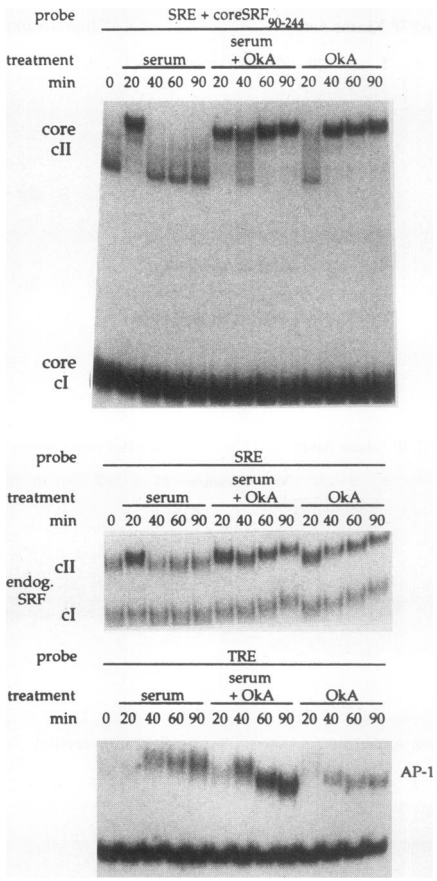


FIG. 4. OA affects TCF, SRF, and AP-1. Band shift analysis of extracts prepared from cells treated with serum, serum plus OA (OkA), or OA alone. The upper panel shows TCF binding to the SRE driven by coreSRF$_{90-244}$. The unbound probe cannot be seen because of the prolonged electrophoresis time required for optimal resolution. The middle panel shows the portion of the autoradiogram indicating the endogenous SRF-driven binary complexes (cI) and ternary complexes (cII). Note the changes in the mobility of both complexes cI and cII at later times after OA addition. The bottom panel shows the portion of a band shift gel portraying AP-1 binding activity on the collagenase TRE, as well as another complex (lower band) that is unaffected by induction.

potent inhibitor of serine/threonine phosphatases both in vitro and in vivo (29, 68). When added to cells in culture, OA slowly induces c-*fos* mRNA and leads to its stabilization, and it prolongs c-*fos* expression after induction (69, 87). To test if OA had similar effects in HeLa cells, starved cells were treated with serum alone, serum together with OA, or OA alone, and then extracts were prepared at various time points. OA treatment led to the prolonged presence of high levels of c-*fos* mRNA after serum induction, and, when used alone, led to the delayed appearance of c-*fos* mRNA at a level comparable to that seen when serum alone was used (33). Similar effects were observed for the mRNAs from a variety of other IEGs, notably the MAP kinase-specific phosphatase MKP-1 and *egr-1*, which contains multiple SREs in its promoter region (33).

Serum induction led to the transient appearance of the more slowly migrating, induced coreSRF ternary complexes in the extracts (Fig. 4, upper panel). The addition of OA during induction led to the persistence of these induced complexes, in correlation with the continued presence of c-*fos* mRNA. OA treatment alone led to the derepression of the c-*fos* promoter (33) and, correspondingly, to the appearance of the induced complexes between 20 and 40 min after addition (Fig. 4, upper panel). At the 60- and 90-min points, the effects of OA alone or after serum induction were identical. A progressive small decrease in the migration of both the coreSRF- and endogenous SRF-driven ternary complexes was visible, suggestive of an increased degree of phosphorylation of TCF (Fig. 4). In addition, the binary complex cI showed the same behavior, which may reflect the hyperphosphorylation of SRF (Fig. 4, middle panel). In this experiment, serum induction also led to increased ternary complex formation with endogenous SRF (cII in Fig. 4, middle panel).

As already shown in Fig. 2, serum induction led to a transient peak of ERK2 activation (Fig. 5) that correlated with the brief presence of the induced complexes indicative of modified TCF (Fig. 4). In extracts prepared from cells treated with serum plus OA, ERK2 showed prolonged activity that slightly increased at later time points, in correspondence with

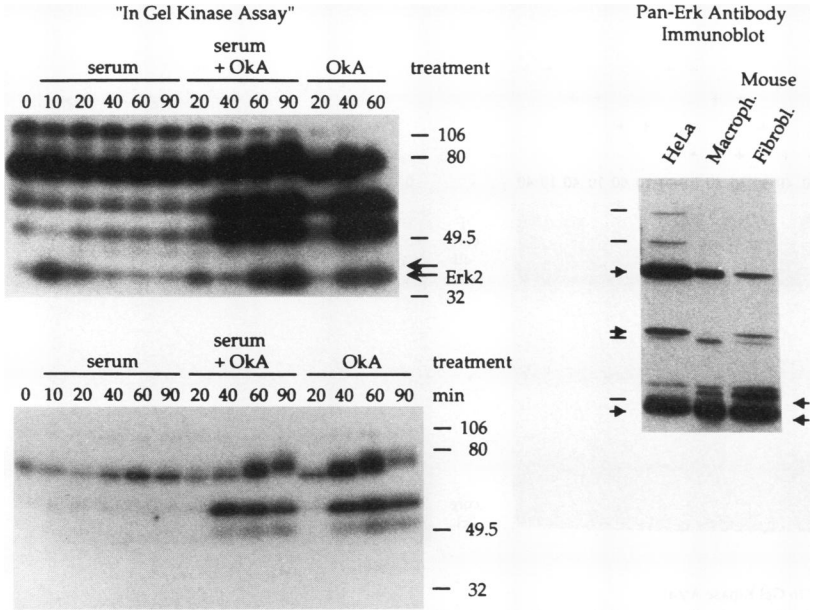


FIG. 5. OA induces several MBP kinases. The upper left panel shows an in-gel kinase assay of extracts from serum- and/or OA-induced cells. Extract protein (15 μg) from cells treated as indicated above the lanes was analyzed as described in the legend to Fig. 2. The lower left panel is a lighter exposure of the same gel. The positions of the prestained size standards (in kilodaltons) and the position of ERK2 are shown. The right panel shows a comparison of HeLa and mouse whole-cell extracts immunoblotted with an antibody recognizing different members of the ERK family. The arrows on the left indicate major species of ERKs seen in both HeLa and mouse extracts. However, mouse extracts appear to contain one species of ERK not present in HeLa extracts (upper arrow of doublet on the right side) that might represent ERK1.

the further decrease in the mobility of the induced complexes noted above. It is unclear whether the decrease in ERK2 at 40 min is significant (34), but it was reflected in a slight decrease in modified TCF (Fig. 4). The tight linkage between the presence of the induced complexes and ERK2 activity was further illustrated by the slower kinetics of ERK2 activation upon treatment with OA alone (Fig. 5). It is interesting that at time points when OA caused prolonged activation of ERK2, MKP-1 mRNA was present at high levels (33). The phosphatase activity encoded by this gene specifically inactivates MAP kinase in vitro and has been argued to do so in vivo (5, 40, 67, 76, 86), so OA apparently interferes with MKP-1-mediated inactivation of ERK2.

We also detected the OA-dependent activation of other MBP kinases, several of which cross-react with a pan-ERK antiserum (Fig. 5) and may correspond to other species of ERKs (7, 8, 44). The high-molecular-weight kinase, which is normally constitutively active, migrated more slowly with increased time of OA treatment. This was suggestive of hyperphosphorylation and seemed to correspond to increased activity, as evidenced by the shorter exposure shown in the lower panel of Fig. 5. OA also led to gradual hyperphosphorylation of Raf-1 (34). In contrast, no hyperphosphorylation of ERK2 or of the 50- to 60-kDa activities was apparent in our SDS-PAGE system (Fig. 5).

Since OA strongly up-regulated c-*fos* mRNA levels, we tested whether OA might also affect the Fos protein, as manifested by the binding of the Fos-containing transcription factor AP-1 (17, 78). Binding to an AP-1 recognition site, labeled TRE in Fig. 4, was readily visible 40 min after serum induction and then increased slightly (Fig. 4, bottom panel), consistent with the need for de novo synthesis of Fos. Little binding activity was seen before this time, suggesting that binding of c-Jun homodimers is not detected under our conditions. The combination of serum and OA led to increased levels of AP-1 and to its altered mobility, without affecting the other binding activity visible in the gel (Fig. 4). OA treatment alone also caused a weak induction of faster-migrating AP-1 relative to that induced by serum alone. This progressive increase in the mobility of AP-1 upon OA treatment was suggestive of modification of the proteins forming AP-1, most probably Jun and Fos. Both proteins are substrates for various kinases implicated in signal transduction (1, 15, 31, 58, 74, 77) and possibly activated by OA (3), and Jun is a target for a cellular Ser/Thr phosphatase (9, 74). So this effect is not surprising, and some evidence suggests that it reflects hyperphosphorylation of at least Fos (33).

**Vanadate treatment of HeLa cells has little effect on the induced complexes or on ERK2 activity.** A considerable number of regulatory events in signal transduction are mediated by tyrosine phosphorylation/dephosphorylation of the proteins involved, including ERK2 (5, 7, 18, 67, 76, 79, 86). To test the role tyrosine dephosphorylation plays in the repression of c-*fos* and the concomitant loss of the induced ternary complexes, we induced HeLa cells with serum in the presence of the tyrosine phosphatase inhibitor sodium orthovanadate. Surprisingly, vanadate, even at high concentrations, did not block the disappearance of the induced complexes but rather slightly delayed their disappearance (Fig. 6A, left panel). This is more obvious in the kinetic analysis, in which the induced complex was still weakly detectable 30 min after serum induction in the presence of vanadate (Fig. 6A, right panel). Vanadate also appeared to slightly delay repression of transcription, as c-*fos* mRNA was stronger 40 min after serum induction in the presence of vanadate than with serum alone. However, in both cases *fos* mRNA disappeared 60 min postinduction (33), indicating that vanadate did not block the events associated with c-*fos* repression. Vanadate treatment alone led to the

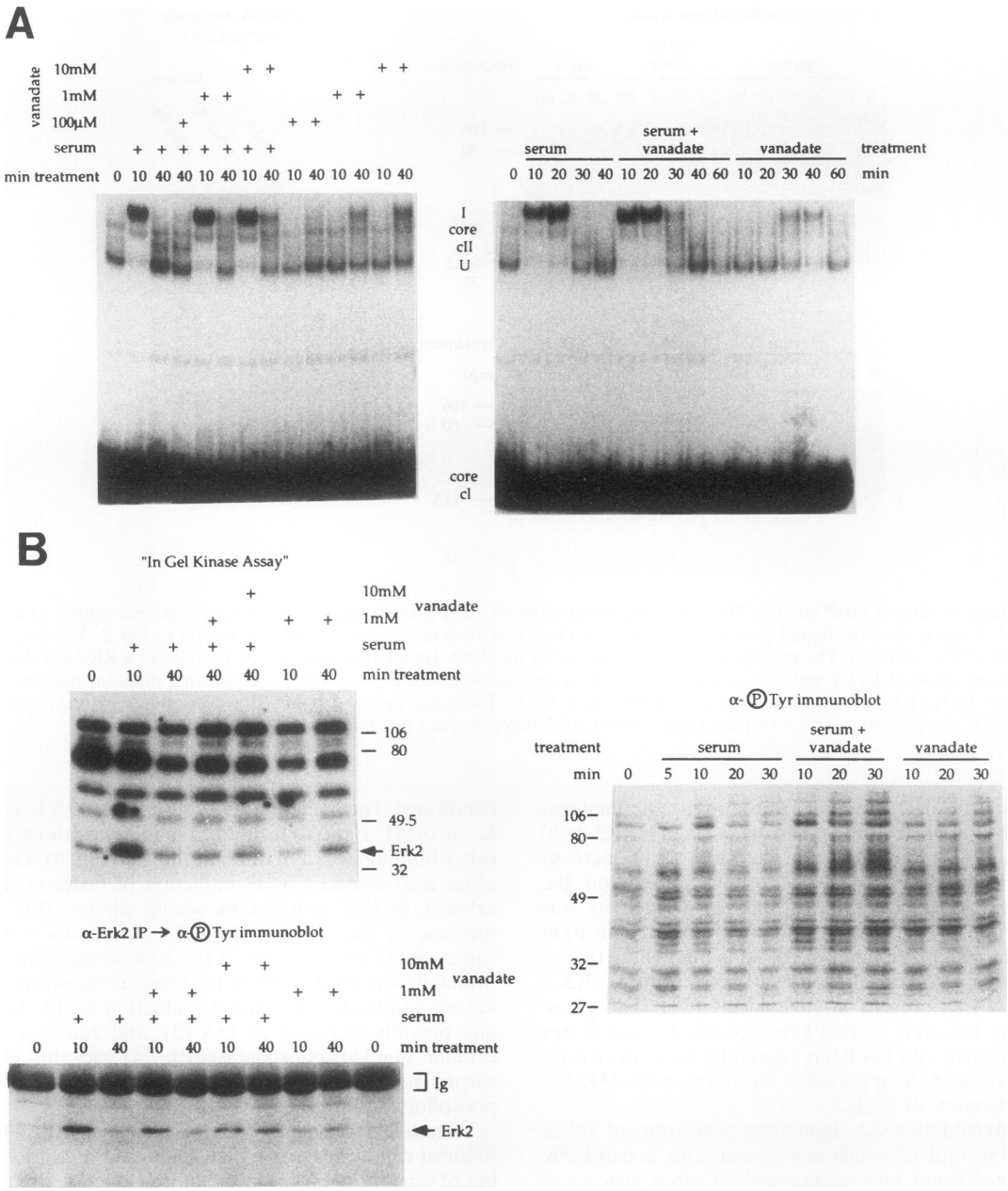


FIG. 6. Sodium orthovanadate does not block the decay of the induced complexes or block ERK2 inactivation. (A) Whole-cell extracts were prepared from HeLa cells treated with serum and/or sodium orthovanadate for the times indicated. Binding activity was analyzed by using coreSRF$_{90-244}$ and the $^{32}$P-labeled SRE probe. The left panel shows the effect of varying the vanadate concentration over a 100-fold range, and the right panel shows a more detailed kinetic analysis using 1 mM vanadate. (B) The upper left panel shows an in-gel kinase assay performed on the same extracts used in the binding reactions in panel A. In the lower left panel, ERK2 was immunoprecipitated from the extracts, and the Western blot of the protein gel was probed with an antibody fragment recognizing phosphorylated tyrosine. The right panel shows the antiphosphotyrosine immunoblot of whole-cell extract proteins. Marker sizes are given in kilodaltons, and the positions of ERK2 and immunoglobulin (Ig) are indicated.

transient appearance of a weak induced complex 30 to 40 min after addition but did not lead to the induction of c-*fos* mRNA (33).

Vanadate also did not block the decrease in ERK2 activity after serum induction (Fig. 6B; 34), in spite of the fact that the ERK2-specific phosphatase is inhibited by vanadate (5, 40, 67, 86). On its own, vanadate slightly induced ERK2 activity 30 to 40 min after addition; this is consistent with the weak induced ternary complexes seen at these times. To determine whether vanadate really did block tyrosine dephosphorylation of ERK2 and other cellular proteins, immunoprecipitates of ERK2 from the whole-cell extracts and aliquots of the extracts were run on SDS-PAGE, blotted, and probed with an antibody specific for phosphotyrosine (Fig. 6B). ERK2 and total cellular proteins

were clearly transiently phosphorylated on tyrosine after serum induction, and dephosphorylation was blocked by vanadate (Fig. 6B, especially the 40-min lanes, lower left panel). In spite of this, ERK2 activity was diminished to the same level seen without vanadate (upper panel). Vanadate treatment in the absence of serum still led to increased tyrosine phosphorylation, albeit less than the level described above, and to a weak activation of ERK2 (Fig. 6B). These data indicate that tyrosine phosphorylation alone is not sufficient for high levels of ERK2 activity and that c-*fos* repression, ERK2 inactivation, and dephosphorylation of TCF are not solely mediated by tyrosine phosphatases.

## DISCUSSION

**Serum induction and PMA induction of HeLa cells lead to the modification of TCFs.** We set out to characterize the early events occurring upon serum or PMA induction of serum-starved HeLa cells. A direct lysis protocol was particularly suited to this purpose, as it immediately arrests cellular activities and thus permitted us to visualize changes in DNA binding activities, kinase activities, and cellular RNA at each time point taken. We concentrated on SRF and TCF, proteins implicated in regulation of IEG induction via SREs (62, 81–83), by measuring their binding to the SRE via high-resolution band shift assays. Consistent with previous observations (63, 80, 82, 87), no changes could be detected in the electrophoretic behavior of the SRF-SRE binary complex upon induction or repression, in spite of the serum-activated phosphorylation of SRF by RSK in some cell lines (63). In contrast, the TCF-SRF-SRE ternary complexes, formed with either endogenous SRF or additional purified SRF, showed decreased mobility at times when c-*fos* and other IEGs were active transcriptionally. This change arose from the modification of TCF and could be more readily seen when ternary complexes were generated by using a shortened version of SRF, core$SRF_{90-244}$. A quantitative shift to more-slowly-migrating complexes rapidly occurred after serum or PMA induction that correlated with the induction of the c-*fos* and *egr-1* promoters. These induced complexes disappeared concomitantly with the subsequent inactivation of the promoters, apparently decaying into those seen prior to induction. Consistent with this, the induced complexes decayed more slowly or remained when repression was delayed or blocked completely.

Transfection experiments using HeLa cells emphasize the importance of the ternary complex in the activation of c-*fos* transcription. In the context of the full *fos* promoter, mutations affecting the binding of TCF abrogate serum or PMA inducibility (65, 72). Furthermore, the ternary complex is also implicated in mediating induction of the SRE-driven reporter constructs by activated Ras, Src, Mos, and Raf (28). These data, when taken together with the observations which we have obtained by using nontransfected HeLa cells, make a strong case that the appearance of the induced ternary complex reflects activation of signaling pathways targeting the SRE. There may be some cell-type variability, since in certain mouse cell lines serum-induced signals appear to bypass TCF and to target SRF directly (25).

We have concentrated on the modification of TCF because it has been observed when a variety of mitogens and cytokines were used to treat many different human and mouse cell lines (23, 33–35, 48, 49). In addition, modification of TCF is not seen as a result of activation of pathways that do not target the SRE, e.g., cyclic AMP/protein kinase A, or as a result of treatments not inducing the c-*fos* promoter (33, 49). Nevertheless, the activation of intracellular signaling cascades also targets factors interacting with the other regulatory elements in the c-*fos* promoter (33), and these multiple events are almost certainly required for the subsequent activation of transcription in vivo.

**The MAP kinase pathway is rapidly activated after serum induction.** Several enzymes implicated in the MAP kinase signaling cascade (7, 51, 53) are rapidly activated with similar kinetics upon serum stimulation. RAF-1 and MEK are strongly induced by 3 min and are then rapidly turned off, with a clear decrease in activity already visible between 5 and 10 min after induction. However, there appear to be subtle kinetic differences between the two. RAF-1 shows weak activity in the resting cells, no apparent increase 1 min after the addition of serum, and then a decrease to the preinduction level of activity by 30 min. This inactivation of RAF-1 correlates kinetically with the appearance of hyperphosphorylated RAF protein, providing further evidence that hyperphosphorylation is not a measure of RAF activation (43, 66). MEK shows no detectable activity prior to the addition of serum and a slight but reproducible activation 1 min thereafter. At later time points, MEK activity does not decrease to preinduction levels but rather shows a prolonged weak signal. RAF has been proposed to directly activate MEK, since MEK is an in vitro substrate for RAF-1 (37, 43). Our kinetic analysis is consistent with a simultaneous activation of the two. Ras activation is important in triggering these events (7, 51), and both MEK and RAF-1 can interact with Ras (7, 51). Activated MEK is found in this complex with Ras (50) and is also generated via MEKK, which appears to lie in another signaling cascade (22, 46). Given the heterogeneous composition of serum, it is possible that this reflects the interplay between multiple signaling pathways (10, 12, 13, 20–22, 27, 46, 85). Both MEK and RAF activation peak slightly before that of MAP kinase, which also shows a somewhat slower decay than do MEK and RAF. These kinetics are consistent with the hypothesis of MEK and RAF induction preceding that of MAP kinase in vivo and thus provide an important in vivo corroboration of the current view of the MAP kinase signaling pathway (7, 51).

We see the rapid activation of one major species of MAP kinase, $p42^{MAPK}$ or ERK2. Immunoblots using several different ERK-specific antisera indicate that, in agreement with Chen et al. (16), there is little ERK1 in HeLa cells, especially in comparison with mouse macrophage and fibroblast cell lines. However, our in-gel kinase assays show a second kinase activity slowly decreasing after induction and then being activated at later times. Notably, this kinase is strongly induced and hyperphosphorylated by OA treatment. We are unsure of the identity of this activity, although it appears to be recognized by the pan-ERK antibody. A second ERK-related kinase is also activated by OA, which does not affect its SDS-PAGE migration. This ca. 55-kDa activity may correspond to ERK3 (8, 44). Thus, the activation of other renaturable MBP kinases is observable under certain conditions but not immediately after serum or PMA induction.

**The phosphorylation state of TCF is tightly linked to ERK2 activation and IEG transcription.** Induced TCF-containing complexes are sensitive to phosphatase treatment, which leads to the reappearance of the faster-migrating uninduced complexes. This suggests that the induced complexes arise from phosphorylation of TCF species that form the uninduced complexes. Several other lines of evidence substantiate this interpretation. Both TCF and Elk-1 have been shown to be MAP kinase substrates in vitro (23, 35, 38, 49, 57, 61), and treatment of partially purified TCF from HeLa cells either with a copurifying endogenous kinase or with activated MAP kinase shifts both core cII and E74 complexes comigrating with the

uninduced complexes to the induced position (33, 57, 87). 2D gel analysis of the same kinased TCF fraction shows it migrating in a series of $^{32}$P-labeled diagonal spots (57, 87). Similarly, the induced complexes comigrate with those generated upon treatment of Elk-1, produced by coupled in vitro transcription/translation, with activated ERK2 (35).

Also compelling is the striking correlation between the activation of ERK2 and the appearance of the more slowly-migrating TCF-containing complexes. In fact, the degree of ERK2 activation parallels the amount of the induced complex observed in both the serum and PMA kinetics shown in Fig. 1 and 3 (34). Similarly, using an immune-complex MAP kinase assay on fractionated HeLa cell homogenates, Chen et al. (16) demonstrated a rapid activation of nuclear MAP kinase upon serum induction and a slightly slower activation by PMA. Taken together, these data are consistent with the hypothesis that MAP kinase directly modifies TCF in vivo. In agreement with this, transfection studies document a MAP kinase-dependent induction of transactivation by Elk-1 or of fusion proteins containing the Elk-1 carboxy-terminal domain (32, 38, 49). It is unclear whether the induced, SAP-1a-like complexes also arise from MAP kinase modification, although their appearance in mouse cells can be uncoupled from MAP kinase activation. That is, when MAP kinase activation is suppressed, the complexes containing the SAP-1a-like TCF, but not Elk-1, are still modified upon induction (35).

The induction of ERK2 and the appearance of the complexes indicative of phosphorylated TCF show a tight temporal correlation with the onset of c-*fos*, *egr-1*, and *junB* transcription. The SRE is a DNA regulatory element common to many IEGs (62, 81). Thus, it seems reasonable to postulate that induction of the c-*fos* and other IEG promoters could arise from activated MAP kinase phosphorylating TCF bound in a stable ternary complex with SRF on the SRE, consistent with the hypothesis formulated above. In contrast, the decline in MAP kinase activity precedes the decay of the induced complexes correlated with the repression of IEG transcription. One possible explanation for the rapid inactivation of MAP kinase in our cell extracts is the corresponding appearance of the mRNA for the dual specificity phosphatase MKP-1. Its rapid induction by mitogens (14), along with the ability of the encoded protein to specifically inactivate MAP kinase in vitro and block MAP kinase activation in transfected cells (5, 67, 76, 86), is consistent with it down-regulating MAP kinase in vivo. On the other hand, the phosphatase responsible for reversing the induction-related phosphorylation of TCF and, by implication, for inactivating the c-*fos* promoter, is not yet characterized. As discussed below, an OA-sensitive activity is implicated in this process. We have evidence for a TCF phosphatase activity in HeLa whole-cell extracts, and a similar activity in mouse extracts is seemingly specific for the induced complexes containing the Elk-1-related but not the SAP-1a-like TCF (35).

**Serine/threonine phosphatases but not tyrosine phosphatases are implicated in kinase and IEG down-regulation after mitogen induction.** The strong correlation between c-*fos* transcriptional activity and the presence of phosphorylated TCF is further supported by the results of OA treatment. The addition of OA blocked postinduction repression of the c-*fos* promoter, which was accompanied by the persistence of the induced complexes. On its own, OA led to derepression of c-*fos* transcription and to the appearance of the induced complexes (Fig. 4; 33, 69, 87). In contrast, vanadate inhibition of tyrosine phosphatases had little effect. These data imply an important role for serine/threonine phosphatases sensitive to OA, such as PP-1α and PP-2A (68), in maintaining the inactivity of the c-*fos* promoter prior to induction and in down-regulating c-*fos* after induction. This could occur by the phosphatase targeting TCF directly or indirectly at some other point in this complex system (3, 29, 75). For example, Fos-mediated repression occurs via the SRE and requires phosphorylation of the Fos carboxy-terminal domain (24, 42, 47, 55, 64, 72, 84). Although OA has little effect on protein synthesis (69), it might specifically affect Fos, or it might influence autorepression by affecting the degree of Fos phosphorylation (1, 15, 77). Consistent with this is the altered mobility of the AP-1 shift upon OA treatment (Fig. 4; 78). Similarly, the SRF-SRE binary complex was affected by OA, possibly reflecting increased phosphorylation (63).

The effects of OA and vanadate treatment alone suggest the existence of a kinase/phosphatase equilibrium that is shifted by inhibiting serine/threonine phosphatase but not tyrosine phosphatase activities (29, 75). This leads to the activation of MAP kinase and of several other MBP-specific kinases, the apparent hyperphosphorylation of one of the kinases, as well as of TCF, SRF, and AP-1, and the activation of IEGs, such as c-*fos* and MKP-1, together with the stabilization of their mRNAs. We can specifically address the effects on MAP kinase. Its increased activity would explain TCF hyperphosphorylation and thereby c-*fos* induction. MAP kinase might also lead to the hyperphosphorylation of AP-1 (15, 74), as well as the ca. 80-kDa MBP kinase. The superinduction of MKP-1 mRNA when MAP kinase is highly active suggests some regulation of MKP-1 translation or activity via serine/threonine phosphatases. Since vanadate inhibits MKP-1 activity (5, 40, 67, 86), the down-regulation of MAP kinase in the presence of vanadate hints at the existence of another, vanadate-resistant phosphatase that is capable of inactivating MAP kinase. These results indicate an important role for Ser/Thr phosphorylation, along with Tyr phosphorylation, in MAP kinase activation and inactivation in HeLa cells.

We have shown the activation of the MAP kinase signaling pathway by serum treatment of untransfected HeLa cells. Similarly, PMA also activates MAP kinase via PKC (2, 4, 18, 79), and both lead to the phosphorylation of TCF and the concomitant activation of SRE-driven IEGs. Thus, we demonstrate the convergence of distinct signaling pathways in vivo on MAP kinase and TCF phosphorylation, which is consistent with the hypothesis of a stable ternary complex assembled on the SRE being a direct target of the mitogen-activated signaling cascade. The phosphorylation state of TCF in this complex would then reflect and possibly determine the activity of the promoter, and how this is accomplished mechanistically will be a topic of considerable interest.

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