Mitogenic stimulation of resting T cells causes rapid phosphorylation of the transcription factor LSF and increased DNA-binding activity

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The mammalian transcription factor LSF (CP2/LBP-Ic) binds cellular promoters modulated by cell growth signals. We demonstrate here that LSF-DNA-binding activity is strikingly regulated by induction of cell growth in human peripheral T lymphocytes. Within 15 min of mitogenic stimulation of these cells, the level of LSF-DNA-binding activity increased by a factor of five. The level of LSF protein in the nucleus remained constant throughout this interval. However, a rapid decrease in the electrophoretic mobility of LSF, attributable to phosphorylation, correlated with the increase in DNA-binding activity. pp44 (ERK1) phosphorylated LSF in vitro on the same residue that was phosphorylated in vivo, specifically at amino acid position 291, as indicated by mutant analysis. As direct verification of the causal relationship between phosphorylation and DNA-binding activity, treatment in vitro of LSF with phosphatase both increased the electrophoretic mobility of the protein and decreased LSF-DNA-binding activity. This modulation of LSF-DNA-binding activity as T cells progress from a resting to a replicating state reveals that LSF activity is regulated during cell growth and suggests that LSF regulates growth-responsive promoters.

[Key Words: LSF; MAP kinase; mitogens; phosphorylation; DNA-binding; T lymphocytes]

To obtain proper progression into the cell cycle, growth stimulation of resting cells requires precisely regulated patterns of transcription. Primary human peripheral T cells represent a particularly useful model system for cell growth regulation. These cells, when isolated, are in G0 and only enter the cell cycle when presented with antigen or mitogens. On antigen stimulation of T cells in vivo, a specific set of ordered events occurs within minutes to up to 10 days, eventually leading to cell division and immunologic function (Crabtree 1989; Rao 1991). This biological progression can be mimicked in vitro by stimulating cells with phorbol esters, ionophores, lectins, or antibodies to the T-cell receptor. Stimulation of T cells either with antigen or chemical mitogens results in expression of c-fos and c-myc within 15 and 30 min, respectively [Granelli-Piperno et al. 1986; Reed et al. 1986, 1987; Crabtree 1989]. Following expression of these immediate early/early genes, the T cells express IL-2 and IL-2 receptor at 2 hr, replicate DNA at 16–20 hr, and finally divide at 24–48 hr post-stimulation [Crabtree 1989].

Signal transduction cascades induced by mitogenic stimuli directly drive the modification of preexisting pools of cellular proteins, including transcription factors [Hunter and Karin 1992; Montminy 1993]. These mitogen-induced post-translational modifications, often phosphorylation events, subsequently regulate cell-growth-dependent transcription. In particular, extracellular response kinases (ERK), a subfamily of the mitogen-activated protein kinases (MAP kinases), have been shown to phosphorylate a variety of transcription factors that have been implicated in controlling the expression of genes essential for cell proliferation [Davis 1993; Hill and Treisman 1995; Hunter 1995; Vojtek and Cooper 1995]. These factors include c-jun [Pulverer et al. 1991], Elk-1 [Gille et al. 1992; Marais et al. 1993], and STATs [Boulton et al. 1995; David et al. 1995; Wen et al. 1995]. All of these factors are involved in activating early response genes.

In general, phosphorylation has been shown to modulate multiple functions of transcription factors: DNA-binding activity, nuclear localization (Ghosh and Baltimore 1990; Moll et al. 1991; Fu 1992, Fu and Zhang 1993; Shuai et al. 1993), protein:protein interactions [Chel-
lappan et al. 1991; Shirodkar et al. 1992), and transactivation activity [Pulverer et al. 1991; Marais et al. 1993; for review, see Hunter and Karin 1992]. When DNA-binding activity is targeted, the affinity of the protein/DNA interaction is generally decreased by phosphorylation, as in Oct-1 [Roberts et al. 1991; Segil et al. 1991] and c-Jun [Lin et al. 1992]. For a small number of transcription factors, however, phosphorylation can increase the specific affinity of the factor for its recognition sequence in DNA. This group of proteins includes serum response factor (SRF), when phosphorylated at a ribosomal protein S6 kinase (RSK)-dependent site (Rivera et al. 1993), the plant transcription factor GBF1 [Klimczak et al. 1992], and ATF-2 [Abdel-Hafiz et al. 1992]. Finally, phosphorylation can alter the rate of binding of a protein to DNA, but not the overall affinity (e.g., phosphorylation of SRF by casein kinase II; Janknecht et al. 1992; Marais et al. 1992).

The mammalian transcription factor LSF was identified initially as a 63-kD protein that specifically binds to and stimulates transcription from the simian virus 40 (SV40) major late promoter [Huang et al. 1990]. Analysis of the LSF clone indicates that it is identical to the human α-globin transcription factor CP2 [Lim et al. 1992] and to LBP1c/UBP-1, which binds multiple sites within the human immunodeficiency virus (HIV) long terminal repeat (LTR) [Jones et al. 1988; Wu et al. 1988; Malim et al. 1989; Kato et al. 1991; Yoon et al. 1994]. LSF also binds with high affinity to sites within the c-fos promoter immediately downstream of the serum response element (SRE) [R. Misra, H.-C. Huang, M. Greenberg, and U. Hansen, unpub.] and within the ornithine decarboxylase (ODC) promoter [J. Volker, A.P. Butler, and U. Hansen, unpub.] in a region responsive to mitogens [Mar et al. 1995]. Because of the well-characterized regulation of the c-fos promoter at the G0/G1 boundary, including in human peripheral T cells [Greenberg and Ziff 1984; Granelli-Piperno et al. 1986; Crabtree 1989], the mitogenic induction of ODC transcription [Kahana and Nathans 1984; Feinstein et al. 1985; Greenberg et al. 1985; Olson and Spizz 1986; Katz and Kahana 1987; Abrahamsen and Morris 1990] and the coupling of SV40 late gene expression to cell growth, we have investigated whether LSF–DNA-binding activity is cell growth-regulated. We present evidence that LSF is rapidly phosphorylated on mitogenic stimulation of resting T cells, that MAP kinase, specifically pp44 (ERK1), phosphorylates LSF in vitro on the same serine residue that is phosphorylated in vivo, and that the DNA-binding activity of LSF is enhanced by phosphorylation.

Results

Regulation of LSF–DNA-binding activity during the G0/G1 transition

To investigate the biological regulation of LSF, immunological reagents were developed. Rabbit polyclonal antisera were raised against two peptides (Shirra et al. 1994). As proof that these antisera were directed against the defined LSF–DNA-binding activity, immunoaffinity purified antibodies (α-pepLSF) were assayed in a gel mobility-shift experiment for their ability to interact with the LSF–DNA complex. α-pepLSF-1 completely supershifted the LSF–DNA complex formed from HeLa nuclear extract (Fig. 1A, lanes 1,2). We note that the level of antibody–LSF–DNA complex was elevated as compared with that of the nonsupershifted species, presumably attributable to stabilization of the LSF–DNA interaction by this antibody. In contrast, α-pepLSF-2 neither supershifted nor disrupted the LSF–DNA complex (Fig. 1A, lane 3), although it did react against denatured, purified LSF by Western blot analysis [data not shown]. In addition to the α-pepLSF-1 antisera, two other LSF antipeptide antisera directed against either peptide 1 [α-pepLSF-1-2] or peptide 2 (α-pepLSF-2-2) completely supershifted the HeLa cell LSF–DNA complexes [data not shown]. Therefore, all detectable proteins that interact specifically with LSF–binding sites in these HeLa extracts contain the product of the identified LSF (CP2/LBP1c) gene. Similar data were obtained for crude

Figure 1. LSF antipeptide antibody characterization. (A) Gel mobility-shift analysis of LSF–DNA–antibody ternary complexes from HeLa cell nuclear extract. DNA-binding reactions contained HeLa cell nuclear extract, LSF–280 DNA [Huang et al. 1990], and either no antibody [lane 1], α-pepLSF-1-1 [lane 2], or α-pepLSF-2-1 [lane 3]. Positions of LSF–DNA and supershifted LSF–DNA [LSF–DNA–antibody] complexes are indicated. Free DNA is shown at the bottom of the gel. The remaining two bands reflect nonspecific binding to DNA, as shown by competition analyses [data not shown]. (B) Immunoprecipitation analysis of radio-labeled LSF from HeLa cells. HeLa cells were labeled with [35S]methionine as described in Materials and Methods, and extracts immunoprecipitated with either α-pepLSF-1-1 [lane 1] or α-pepLSF-2-1 [lane 2]. Samples were separated by SDS-PAGE and an autoradiograph of the gel developed. The positions of molecular weight markers and the broad position of the LSF bands are indicated.
nuclear extracts from human peripheral T cells and Balb/c-3T3 (clone A31) mouse fibroblasts (data not shown). The specificity of the α-pepLSF1-1 antiserum for LSF was addressed by immunoprecipitation analysis [Fig. 1B]. α-pepLSF2-1 antiserum was used as a negative control, because of its inability to recognize native LSF. α-pepLSF1-1 [Fig. 1B, lane 1] immunoprecipitated a group of bands from HeLa nuclear extract labeled with [35S]methionine migrating at approximately the mobility expected for LSF, which were not immunoprecipitated by α-pepLSF2-1 [Fig. 1B, lane 2]. As shown below [Fig. 3A], these multiple bands represent differentially phosphorylated forms of the same LSF gene product.

Based both on the presence of LSF–DNA-binding sites within the human c-fos promoter and the human ODC promoter, and on the rapid induction of expression from these promoters as cells exit G0 [see Introduction], we monitored LSF–DNA-binding activity on progression of resting T cells into the cell cycle. LSF–DNA-binding activities within nuclear extracts from unstimulated G0 cells and from early G1 cells at varying times following mitogen treatment were compared by a gel mobility-shift assay [Fig. 2A]. The high affinity LSF-280 site within the SV40 late promoter [Huang et al. 1990], which is nearly identical to the LSF–DNA-binding site within the c-fos promoter, was used for these assays. Extremely low levels of LSF–DNA-binding activity were detected in unstimulated peripheral T cells [Fig. 2A, inset, lane 1]. However, LSF–DNA-binding activity increased rapidly to an average of 4.5-fold above the G0 level within 15 min of mitogenic stimulation [Fig. 2A]. The level of LSF–DNA-binding activity remained fivefold higher than the level in resting cells for at least 3 hr.

To determine whether these striking alterations in DNA-binding activity were attributable to changes in the levels of the modification of the LSF protein within the nucleus, these nuclear extracts were examined by Western blot analysis [Fig. 2B]. The resulting data indicated, first, that the level of LSF protein in the nucleus was unchanged as T cells progressed into the cell cycle [Fig. 2B, cf. lane 2 with lanes 3–5]. Second, extracts containing higher levels of LSF–DNA-binding activity contained only a slower migrating form of the LSF protein [Fig. 2B, lanes 3–5]. Finally, and most strikingly, a quantitative shift in the mobility of LSF was observable within 5 min of mitogenic stimulation [Fig. 2B, lane 3], suggesting that the protein is modified rapidly. Serum stimulation of quiescent NIH 3T3 fibroblasts also resulted in a quantitative shift in the mobility of LSF within 5 min of serum addition [data not shown]. Consistent with the modification of a preexisting pool of LSF, the shift in mobility of the protein after mitogenic stimulation was also observed in the presence of the protein translation inhibitor cycloheximide [data not shown]. Similar data were obtained whether or not additional steps were taken to remove contaminating B cells, neutrophils, macrophages, or previously activated T cells from the population of cells before analysis [data not shown]. Therefore, the rapid increase in LSF–DNA-binding activity occurs following mitogenic stimulation of T cells, and is not attributable to an increase in protein level but rather to modification of the preexisting pool of LSF.
LSF is a phosphoprotein

Mitogenic stimulation of all cell types, including T cells, results in the induction of signal transduction cascades and the concomitant change in the phosphorylation state of many target proteins. We initially examined whether LSF is normally phosphorylated in cycling cells, to determine whether the electrophoretic mobility shift following mitogenic stimulation could be explained by an induced phosphorylation event. Western blot analysis of LSF from HeLa nuclear extract revealed at least three bands migrating where LSF was anticipated to migrate [Fig. 3A, lanes 1,6]. Treatment of HeLa nuclear extract with phosphatase in vitro caused the three prominent LSF bands to shift into one band migrating faster than all of the others [Fig. 3A, cf. lane 1 with lanes 2–5]. In the presence of phosphatase inhibitors, the mobilities of the LSF bands remained unchanged in the presence of increasing amounts of phosphatase [Fig. 3A, lanes 6–10]. Furthermore, phosphatase treatment of nuclear extracts prepared from mitogenically stimulated human T cells also increased the mobility of the slower migrating, induced form of LSF to that of a faster migrating species [data not shown]. These data demonstrate that the multiple species of LSF that are visualized on one-dimensional gel electrophoresis are largely, if not totally, attributable to differential phosphorylation.

To definitively demonstrate that the mobility change in LSF on mitogenic stimulation was attributable to rapid phosphorylation, human peripheral T cells were labeled in vivo with $^{32}$P orthophosphate before stimulation. Extracts from equivalent numbers of unstimulated and mitogenically stimulated cells were then immunoprecipitated with a monoclonal antibody against LSF, $\alpha$-LSFmAb11, and the radio-labeled, immunoprecipitated proteins were displayed by SDS-PAGE [Fig. 3B]. The antibody precipitated one band not present in control immunoprecipitations [Fig. 3B, cf. lanes 2 and 3] that migrated in the expected position for LSF [Fig. 3, lane 1]. The level of LSF phosphorylation increased sixfold

![Figure 3](image-url)

**Figure 3.** LSF is phosphorylated. (A) HeLa cell nuclear extract was treated with 1 to 4 units of potato acid phosphatase, as indicated, in the absence [lanes 1–5] or the presence [lanes 6–10] of 2 mM ammonium molybdate for 30 min at 37°C. Extracts were analyzed by Western blots as described in Materials and Methods. The region of migration of LSF species is indicated by the bracket. Note that the upper band was not reproducibly observed, depending on the batch of purified antibody, and therefore does not appear to represent LSF, but rather a nonspecifically reacting protein. (B) LSF is phosphorylated in vivo within 30 min of mitogen treatment of T cells. Biosynthetically $^{32}$P-labeled LSF was immunoprecipitated with $\alpha$-LSFmAb11 [lanes 3,4] or without antibody [lane 2] and the pellets were analyzed by 10% SDS-PAGE. (Lane 1) In vitro-translated LSF marker (Shirra et al. 1994); (lanes 2,3) immunoprecipitates from unstimulated cell extract; (lane 4) immunoprecipitate from extract of cells stimulated for 30 min. (C) Phosphoamino acid analysis of LSF from unstimulated cells. Biosynthetically $^{32}$P-labeled LSF was immunoprecipitated with $\alpha$-LSFmAb11 from unstimulated cells and processed for phosphoamino acid analysis as described in Materials and Methods. The positions of migration of the marker phosphoamino acids [(SER) phosphoserine; (THR) phosphothreonine; and (TYR) phosphotyrosine] are marked by arrows. (D) Phosphoamino acid analysis of LSF from stimulated cells. Biosynthetically $^{32}$P-labeled LSF was immunoprecipitated with $\alpha$-LSFmAb11 from cells stimulated for 30 min and processed for phosphoamino acid analysis as described in Materials and Methods. The markers are indicated as in C.
within 30 min of mitogenic stimulation [Fig. 3, lanes 3,4]. Phosphorylation of LSF occurred on serine residue(s), as shown by phosphoamino acid analyses (Fig. 3D). We note that although LSF is phosphorylated on serine residue(s) in the unstimulated cell extracts [Fig. 3C], the level of radio-labeled phosphoserine was always increased on mitogenic stimulation.

The rapid phosphorylation of LSF on serine residue(s) in response to mitogens suggested potential serine kinases that might phosphorylate LSF in vivo. In particular, we hypothesized that LSF may be a substrate of the MAP kinase family, specifically pp44/42 (ERK1/2) [Chen et al. 1992; Davis 1993, 1994; Hill and Treisman 1995], based on the following facts. [1] The rapid kinetics of LSF modification in the nucleus after stimulation of quiescent cells mirrors the spatial and temporal activation of pp44/42 [ERK1/2]; (2) pp44/42 [ERK1/2] are serine/threonine kinases, corresponding with phosphorylation of LSF on serine residue(s) in vivo; and (3) LSF contains six serine-proline dipeptide (SP) sequences, which are similar to known phosphorylation motifs for pp44/42 [ERK1/2]. To test this hypothesis, purified, recombinant, histidine-tagged LSF [His-LSF] was incubated with pp44 [ERK1] immune complexes from either unstimulated or stimulated T cells in the presence of [γ-32P]ATP. There was minimal phosphorylation of LSF on incubation with control immune complexes precipitated without pp44 [ERK1] antibody from either unstimulated or stimulated cell extracts, or with immune complexes precipitated with pp44 [ERK1] antibody from unstimulated cell extracts [Fig. 4A, lanes 2-4]. In contrast, LSF was phosphorylated effectively by pp44 [ERK1] immune complexes derived from cells stimulated for 5 min [Fig. 4A, lane 5]. Quantitation revealed a 12-fold increase in the phosphorylation of His-LSF by pp44 [ERK1] in stimulated cell extracts compared with phosphorylation from unstimulated cell extracts. Phosphoamino acid analysis indicated that pp44 [ERK1] phosphorylated His-LSF on serine residue(s) [Fig. 4B]. We note that the antibody to pp44 [ERK1] also crossreacts with pp42 [ERK2], so it is likely that both kinases are present in the immune complex assays.

Phosphorylation by ERK1 in vitro mimics the rapid growth-induced phosphorylation of LSF in vivo

Phosphotryptic peptide maps derived from phosphorylated LSF suggested that the same peptide was phosphorylated both in vitro by ERK1 and in vivo on mitogen stimulation (data not shown). Only three tryptic peptides in LSF contain SP motifs. The size and charge of the largest peptide, derived from residues 264–339 and containing four SP motifs [Shirra et al. 1994], was most consistent with the migration of the peptide visualized on the phosphotryptic peptide maps. To determine whether this central 76-amino-acid region of LSF is in fact targeted by growth-stimulated signal transduction cascades, the serine residues at positions 278, 289, 291, and 309 were mutated to alanine residues either singly or in combination. Using growth-regulated NIH 3T3 fibroblasts, we tested whether any of these substitution mutations would abrogate the electrophoretic mobility-shift of LSF following mitogenic stimulation [Fig. 5A]. As expected, serum stimulation of quiescent fibroblasts resulted in a decrease in the electrophoretic mobility of wild-type hemagglutinin-tagged LSF [HA–LSF; Fig. 5A, lanes 3,4], although unlike the endogenous LSF, only a portion of the introduced LSF became modified. In contrast, the mobility of the mutant HA–LSF, in which all four serine residues were changed to alanine residues, was not affected by serum stimulation [Fig. 5A, lanes 5,6], indicating that one or more of these serine residues is targeted by serum stimulation. LSF mutants containing alanine at positions 278, 289, or 309 still responded to serum stimulation by a shift in the electrophoretic mobility [Fig. 5A, lanes 7–10,13,14]. However, the HA–LSF mutant containing a serine to alanine substitution at position 291 did not [Fig. 5A, lanes 11,12], demonstrating that phosphorylation of serine 291 is critical for the growth-regulated electrophoretic mobility shift of LSF.

Equal amounts of these same substitution mutants, in a His–LSF context [Fig. 5C], were also tested in vitro as

Figure 4. LSF is phosphorylated by ERK in vitro. [A] Immune complexes isolated from unstimulated [lanes 2,4] or stimulated [lanes 3,5] T cells in the presence [lanes 4,5] or absence [lanes 2,3] of antibody against pp44 [ERK1], α-ERK1, were incubated with His–LSF and [γ-32P]ATP and the reactions separated by 10% SDS-PAGE. [Lane 1] In vitro-translated LSF marker [Shirra et al. 1994]. The bracket indicates the region of migration of LSF species. Quantitation was performed on a PhosphorImager using ImageQuant software. [B] Phosphoamino acid analysis of LSF phosphorylated by pp44 [ERK1] in vitro. His–LSF phosphorylated by an ERK immunoprecipitate derived from T cells 5 min after mitogen treatment was processed for phosphoamino acid analysis as described in Materials and Methods. The migrations of marker phosphoamino acids are indicated as in Fig. 3C.
Figure 5. In vivo and in vitro phosphorylation of serine to alanine substitution mutants of LSF. (A) Western blot analysis of nuclear extracts from NIH-3T3 fibroblasts transfected with the indicated HA-LSF expression constructs. Transfected cells were growth-arrested and nuclear extracts prepared from equal numbers of either unstimulated cells (odd-numbered lanes) or cells stimulated for 30 minutes with 20% FCS (even-numbered lanes). Extracts were subjected to 10% SDS-PAGE, blotted to a nitrocellulose membrane, and probed with 12CA5 antibody specific to the HA tag. The region of migration of HA-LSF is indicated by the bracket. (Lanes 1,2) Mock transfected cells. (B) Purified, activated GST-ERK1 was incubated with [γ-32P]ATP and purified His-LSF derivatives or myelin basic protein (MBP) as substrates, and the reaction products were separated by 7.5% SDS-PAGE. (Lane 1) In vitro-translated LSF marker (Shirra et al. 1994); (lane 2) 1 μg MBP; (lanes 3,9) control with no substrate; (lanes 4,10) wild-type His-LSF; (lanes 5,11) His-LSF quadruple substitution mutant: S278A, S289A, S291A, and S309A; (lane 6) His-LSF S278A; (lane 7) His-LSF S289A; (lane 8) His-LSF S291A; and (lane 12) His-LSF S309A. The positions of migration of His-LSF, MBP, and GST-ERK1 are marked. (C) Two micrograms each of the same preparations of His-LSF proteins used in (B) were electrophoresed through a 7.5% SDS-PAGE and stained with coomassie blue. His-LSF protein derivatives are as labeled.

substrates in a pp44 [ERK1] kinase assay. Activated GST-pp44 [ERK1], purified from 293 cells, efficiently phosphorylated wild-type His-LSF in vitro, in addition to its autophosphorylation [Fig. 5B, lanes 3,4,9,10]. However, there was minimal phosphorylation by pp44 [ERK1] of His-LSF that was quadruply mutated at the four central serine residues [Fig. 5B, lanes 5,11]. As with in vivo modification, His-LSF mutants containing serine to alanine substitutions at positions 278, 289, or 309 were still phosphorylated [Fig. 5B, lanes 6,7,12], but there was a marked decrease in the amount of phosphorylation by pp44 [ERK1] on the His-LSF mutant with a serine to alanine change at position 291 [Fig. 5B, lane 8]. The amino acid sequence around this phosphorylation site is as follows: 295 YVNNPSPGFN295 (the serine residue at position 291 is in bold). These in vivo and in vitro data demonstrate that the central SP motifs of LSF are the target of growth stimulation in vivo and of pp44 [ERK1] in vitro and that the serine residue at position 291 is crucial for complete phosphorylation of the protein in both cases. These data therefore implicate pp44/42 [ERK1/2] as the kinases that mediate LSF modification in vivo.

Dephosphorylation of LSF diminishes its DNA-binding activity

The kinetics of LSF phosphorylation in stimulated T cells are similar to the kinetics of increased LSF-DNA-binding activity, but not identical (Fig. 2, cf. B with A), suggesting that LSF may be the target of multiple modes of regulation on mitogenic stimulation. Therefore, to determine whether there is a causal relationship between phosphorylation and enhancement in DNA-binding activity, HeLa nuclear extract was treated in vitro with phosphatase, followed by gel mobility-shift analysis with the LSF-280 DNA-binding site. In phosphatase-treated extracts, the level of LSF-DNA-binding activity dropped sharply, as compared with mock-treated extracts [Fig. 6A, lanes 1,2]. However, in extracts that were treated not only with phosphatase but also with phosphatase inhibitors, the level of LSF-DNA-binding activity changed minimally (Fig. 6A, lanes 3,4). These experiments suggest that phosphorylation of LSF, at least at particular sites, directly increases its DNA-binding activity.

To verify that effects of phosphatase on LSF-DNA-binding activity were specifically attributable to dephosphorylation of LSF and not to effects of the phosphatase preparation on other proteins contained in the nuclear extracts, a highly purified preparation of LSF from HeLa cells was treated with phosphatase and the same sample was assayed both for the modification state of LSF and for DNA-binding activity. Analysis of the protein by Western blotting demonstrated three LSF bands in the nontreated sample [Fig. 6C, lane 1]. Treatment with phosphatase caused a shift from the upper two bands to a faster migrating species [Fig. 6C, lane 2]. As antici-
was treated with calf intestinal phosphatase (lanes 2, 4) in the
lation of LSF. (A) Phosphatase treatment of HeLa nuclear ex-
stances (lanes 1, 2) or the presence (lanes 3, 4) of phosphatase
hibitors. Samples were analyzed in a gel mobility-shift assay
as described in Materials and Methods. LSF-DNA complexes
are the slowest migrating complexes in the gel. Free DNA is
shown at the bottom of the gel. (B) Phosphatase treatment of
purified LSF reduces its DNA-binding activity. A purified prepa-
ration of LSF from HeLa cells was incubated in the absence (lane
1) or the presence (lane 2) of calf intestinal phosphatase.
Samples were subjected to gel mobility-shift analysis as de-
scribed in Materials and Methods. (C) Dephosphorylation of pu-
rified LSF increases its electrophoretic mobility. The same
samples of purified LSF and purified dephosphorylated LSF used
for B were subjected to Western blot analysis as described in
Materials and Methods. [Lane 1] No phosphatase; [lane 2] treated
with calf intestinal phosphatase.

Figure 6. Reduction in DNA-binding activity on dephosphory-
lation of LSF. (A) Phosphatase treatment of HeLa nuclear ex-
tracts reduces LSF-DNA-binding activity. HeLa nuclear extract
was treated with calf intestinal phosphatase (lanes 2, 4) in the
absence (lanes 1, 2) or the presence (lanes 3, 4) of phosphatase
inhibitors. Samples were analyzed in a gel mobility-shift assay
as described in Materials and Methods. LSF-DNA complexes
are the slowest migrating complexes in the gel. Free DNA is
shown at the bottom of the gel. (B) Phosphatase treatment of
purified LSF reduces its DNA-binding activity. A purified prepa-
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1) or the presence (lane 2) of calf intestinal phosphatase.
Samples were subjected to gel mobility-shift analysis as de-
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for B were subjected to Western blot analysis as described in
Materials and Methods. [Lane 1] No phosphatase; [lane 2] treated
with calf intestinal phosphatase.

Discussion

Transcription factors are crucial targets of mitogenic sig-
nal transduction cascades (Hunter and Karin 1992; Davis
1993; Hill and Treisman 1995; Hunter 1995). Here we
have shown that the mammalian transcription factor
LSF is one such target. LSF is a phosphoprotein that is
phosphorylated rapidly on serine residues in human T
cells following mitogenic stimulation. Our data strongly
suggest that LSF is a target of the MAP kinase pp44/42
(ERK1/2) in vivo. The DNA-binding activity of LSF is
concomitantly stimulated in vivo, by approximately
fivetold within 30 min of stimulation. In vitro phospha-
tase experiments support the relevance of the in vivo
correlation between phosphorylation and enhancement
of DNA-binding activity. These data suggest a link be-
tween a major mitogenic signaling pathway and phos-
phorylation of LSF and support the hypothesis that LSF
has a biological role in growth stimulation of resting
cells, and in particular, of human peripheral T cells.

Enhanced LSF–DNA-binding activity results from
direct phosphorylation of the LSF gene product

Our data indicate that the increase in LSF–DNA-binding
activity is directly attributable to the phosphorylation of the
LSF (CP2/LBP-1c) gene product. First, rapid phospha-
Tylation of LSF in vivo as a result of mitogenic stimu-
lation is accompanied by a dramatic increase in LSF–
DNA-binding activity. Second, dephosphorylation of ei-
ther highly purified HeLa LSF or LSF from HeLa nuclear
extract diminishes its DNA-binding activity. Further-
more, the majority of the LSF–DNA-binding activity in
cells is attributable to a homomultimer of LSF (Shirra
et al. 1994), arguing against the possibility that dramatic
alterations of LSF–DNA-binding activity would be attri-
butable to modifications of a partner protein.

Phosphorylation of proteins has been shown to induce
conformational changes (Kapiloff et al. 1991; Manak
and Prywes 1991) or overcome internal inhibitory interac-
tions (Abdel-Hafiz et al. 1992), either of which could lead
to higher DNA-binding activity. The site of phosphory-
ation of LSF lies immediately carboxy-terminal of the
presumed DNA-binding surface (Uv et al. 1994; Shirra
1995) consistent with either of these models. It is also
possible that the rapid phosphorylation of LSF seen on
mitogenic stimulation might modulate additional LSF
activities. For example, phosphorylation of c-Jun at mul-
tiple sites attributable to a signal transduction cascade
increases both the DNA-binding activity of the factor
and its transactivation potential (Pulverer et al. 1991; Lin
et al. 1992; Hibi et al. 1993; Dérijard et al. 1994; Minden
et al. 1994).

Phosphorylation sites in LSF

Based on phosphotryptic peptide analyses, we hypothe-
sized that the central 76-amino-acid peptide containing
four serine–proline motifs in LSF was the target of a
growth-induced signal transduction cascade. The muta-
genesis data indicated that serum stimulation of quies-
cent cells resulted in a decreased electrophoretic mobility
of wild-type LSF or mutant proteins containing a ser-
ine to alanine substitution at positions 278, 289, or 309.
However, serum stimulation of quiescent cells containing
either mutant LSF with serine to alanine changes at

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all four positions or mutant LSF with a serine to alanine change at only position 291 did not alter the mobility of the introduced proteins. Furthermore, mutation of all four serine-proline motifs in this region to alanine-proline completely abolished phosphorylation in vitro by pp44 (ERK1), indicating that this peptide is the sole target of this kinase. Although the single serine to alanine substitution at position 291 decreased markedly the amount of phosphorylation by pp44 (ERK1), residual phosphorylation was clearly higher than in the quadruply mutated LSF. Taken together, our data indicate that serine 291 is the major target of phosphorylation by ERK1/2 and is critical for the growth-induced modification of LSF in cells.

Phosphorylation of LSF by MAP kinase

Several lines of evidence strongly suggest that either pp44/42 (ERK1/2) or another member of the MAP kinase family with a similar substrate specificity and activation profile is involved in phosphorylating LSF after mitogenic stimulation in vivo. First, the spatial and temporal activity of pp44/42 (ERK1/2) correlates with the stimulation of LSF-DNA-binding activity by phosphorylation (Chen et al. 1992; Davis 1993; Hill and Treisman 1995). Biochemical fractionations and immunofluorescence (Q. Zhu and U. Hansen, unpubl.) indicate that LSF is a nuclear protein. On mitogen induction, pp44/42 (ERK1/2) is rapidly phosphorylated and translocated to the nucleus (Chen et al. 1992; Davis 1993; Hill and Treisman 1995). The rapidity of LSF phosphorylation, within 5 min of mitogen stimulation, correlates well with the temporal peak in pp44/42 (ERK1/2) activity. Second, LSF is phosphorylated on serine 291 both by pp44 (ERK1) in vitro and on mitogenic stimulation in vivo. Third, stimulation of T cells with PMA alone was sufficient to phosphorylate LSF within 5 min. However, stimulation with PHA and ionomycin, either in combination or individually, did not affect the mobility of LSF with such rapid kinetics (data not shown). The pp44/42 (ERK1/ERK2), but not the Jun, kinases are activated by phorbol esters alone in T lymphocytes (Su et al. 1994).

Despite this preponderance of evidence, our data do not unequivocally prove that the pp44/42 (ERK1/2) are the kinases that phosphorylate LSF in vivo. Both Jun kinase and p38 kinase can also phosphorylate LSF on serine 291 in vitro (J. Volker and U. Hansen, unpubl.). Therefore, further experiments, currently underway, are required to test directly the involvement of the ras/raf/ERK signal transduction pathway in the biological induction of LSF.

Potential biological role for LSF in growth stimulation of T cells

A biological role for LSF in T cells is suggested both by its rapid phosphorylation on mitogenic stimulation, presumably by pp44/42 (ERK1/2), and by the profile of LSF-DNA-binding activity, peaking within 15 min, and remaining high for at least another 3 hr. These data, coupled with the intriguing locations of DNA-binding sites for LSF within the c-fos and ODC promoters, which are induced as immediate early/early events in T cells (see Introduction), lead us to hypothesize that LSF contributes to the regulation of such promoters during the G0 to G1 transition. The LSF-DNA-binding sites within the c-fos and ODC promoters are both conserved in their sequence and in their locations from mouse to human. Deletion analysis of the ODC promoter revealed that a 90-bp region of the promoter containing the LSF-DNA-binding site was responsible for serum and TPA induction of the ODC gene (Mar et al. 1995). Collectively, these observations suggest that LSF is the downstream target of a MAP kinase signal transduction cascade, that LSF contributes to the regulation of immediate early and/or early genes, and that LSF therefore has an important role as a cell growth regulator.

Materials and methods

Cell culture

Human peripheral T cells were isolated by layering human donor blood (from the Dana-Farber Cancer Institute Blood Component Lab) onto Ficoll-paque cushions as described by the manufacturer (Pharmacia). Cells were washed in RPMI 1640 medium (GIBCO) supplemented with 2.5% heat-inactivated fetal calf serum (FCS; Bio-Whittaker) and separated from contaminating lymphocytes by culturing on plastic dishes in RPMI 1640 supplemented with 10% heat-inactivated FCS at 37°C in 5% CO2 for 2 hr. Nonadherent cells were cultured further at a concentration of 1 x 10^7 cells/ml and stimulated at indicated times with 2 µg/ml of phytohemagglutinin (PHA; Murex Diagnostics), 0.12 µM phorbol myristate acetate (PMA; Sigma), and 0.12 µg/ml of ionomycin (CalBiochem).

NIH-3T3 fibroblasts were cultured in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% calf serum (CS, IRF Biosciences) at 37°C in 10% CO2. For transfection, 3 x 10^6 cells were treated with 12 µg of total DNA and 35 µl of lipofectamine (GIBCO) as described by the manufacturer. Eighteen hr after transfection, cells were starved for 48 hr in DMEM supplemented with 0.5% CS.

Nuclear extracts

Nuclear extracts were prepared as described (Andrews and Faller 1991), with the following modifications: 4 x 10^7 (HeLa), 1 x 10^8 (T), or 3 x 10^6 (NIH 3T3) cells were swelled on ice in hypotonic buffer and lysed in a Dounce homogenizer (HeLa) or lysed by vortexing for 10 sec (T and NIH-3T3). Nuclei were resuspended in a high salt buffer [Andrews and Faller 1991] with the following additions: 2 mM Na2PO4, 2 mM Na2HPO4, 1 mM NaF, and 50 mM β-glycerophosphate, incubated on ice for 20 min, and pelleted. The supernatant was frozen in liquid nitrogen and stored at -70°C. Final protein concentrations were between 2 and 4 mg/ml in a total volume of 50 µl of extract.

Antibody production

Antipeptide antibodies to LSF were generated against the synthetic peptides NH2-CLPLADEVIETGLVQD-COOH (pepl, amino acids 7-21), and NH2-CDETLTYLNQGQSYEIR-COOH (pep2, amino acids 83-98) (Shirra et al. 1994). Two rabbits were immunized with each peptide conjugated to chicken serum al-
bumin, using sulfo-MBS (Pierce) as a cross-linking reagent. Antibodies against pep1 were affinity purified as described [Harlow and Lane 1988]. For α-pepLSF1-1, whole sera from immunized rabbits were precipitated, dialyzed, and affinity purified against pep1. Antibody against pep2, α-pepLSF2-1, was IgG purified [Harlow and Lane 1988]. α-LSFmAb11 monoclonal antibody supernatant was generated against recombinant, purified, His-LSF as described [Harlow and Lane 1988].

Western blot analysis

Proteins were separated by 7.5% or 10% SDS-PAGE for 12 hr at 25 mA at 4°C and transferred to a nitrocellulose membrane (Schleicher & Schuell) for 4 hr at 100 V at 4°C. For immunoblots using LSF antibodies, nitrocellulose membranes were blocked for 1 hr in 3% gelatin in Tris-buffered saline (TBS): 150 mM NaCl, 10 mM Tris-HCl [pH 8.0], incubated with a 1/1000 dilution of α-pepLSF1-1 in TBS plus 0.05% Tween 20 [TBST] for 12 hr, washed three times in TBST, incubated with anti-rabbit antibody conjugated to horse radish peroxidase [Bio-Rad], and visualized by chemiluminescence as described by the supplier [DuPont NEN]. For HA tag protein blots, nitrocellulose membranes were blocked in 5% dried milk in TBS plus 0.25% Tween 20, incubated with a 1/1000 dilution of 12CA5 antibody [BAbCol], and visualized as described above.

Gen mobility-shift assays

For highly purified HeLa LSF, 1 ng of protein was pre-incubated at room temperature for 10 min in 1.2% NP-40, 127 mM KCl, 8 mM Tris-HCl [pH 8.0], 20 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid] [pH 7.9], 21.3% glycerol, 0.18 mM EDTA, 2% polyvinyl alcohol, 0.86 mM dithiothreitol (DTT), and 0.1-1 µg of poly[d(I-C)]-poly[d(I-C)] in a final volume of 15 µl. Subsequently, 16 moles of 32P-labeled oligonucleotide containing the LSF-280-binding site (Huang et al. 1990) was added, and the reactions were incubated for an additional 30 min. Samples were electrophoresed through a 5% polyacrylamide gel for 2 hr at 11 V/cm in buffer containing 44.5 mM Tris base, 44.5 mM boric acid, and 1 mM EDTA.

For T cell or HeLa extracts, 7.5 µg or 5 µg of extract, respectively, was pre-incubated as indicated above, except that 1 µg of poly[d(I-C)]-poly[d(I-C)] was added per reaction. Subsequent steps were described as above.

Phosphatase assays

For HeLa nuclear extract used in gel mobility-shift assays, phosphatase reactions were performed as follows: 12.5 µg of HeLa nuclear extract was incubated for 30 min at 37°C in a buffer containing 10 mM Tris-HCl [pH 8.0], 2 µg/ml of aprotinin, 0.5 µg/ml of leupeptin, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and phosphatase inhibitors, where indicated, at final concentrations of 50 mM NaF, 0.2 mM Na3VO4, and 50 mM β-glycerophosphate, in a final reaction volume of 25 µl. Reactions were incubated with or without one unit of calf intestinal alkaline phosphatase conjugated to agarose beads [Sigma]. Ten microliters of the reaction (equivalent to 5 µl of extract) was subjected to gel mobility-shift analysis as described above, except that the final reaction volume was 30 µl.

For HeLa nuclear extract used in Western analysis, phosphatase reactions were performed as follows: 55 µg of HeLa nuclear extract was incubated for 30 min at 37°C in a buffer containing 10 mM Pipes [piperazine-N,N′-bis(2-ethanesulfonic acid) [pH 6.0], 2 µg/ml of aprotinin, 0.5 µg/ml of leupeptin, 0.5 mM PMSF, and 2 mM ammonium molybdate, where indicated, as a phosphatase inhibitor. Reactions were incubated with or without 1 to 4 units of potato acid phosphatase [Sigma] in a final reaction volume of 50 µl. Forty-five microliters of the reaction was subjected to SDS-PAGE and Western blotting analysis as described.

For purified LSF, phosphatase reactions were performed as follows: -4 ng of highly purified LSF was incubated for 30 min at 37°C in a buffer containing 50 mM Tris-HCl [pH 8.0], 12 mM HEPES [pH 7.9], 0.9 mM MgCl2, 15% glycerol, 250 mM NaCl, 0.12 mM EDTA, 0.3 mM DTT, 1% NP-40, 0.2 µg/ml of aprotinin, 0.5 µg/ml of leupeptin, 0.5 mM PMSF, and 4 units of calf intestinal alkaline phosphatase conjugated to agarose beads [Sigma], where indicated. Final reaction volumes were 50 µl. Four microliters of the reaction was subjected to gel mobility-shift analysis and 40 µl of the reaction were separated by 10% SDS-PAGE and immunoblotted as described.

In vivo labeling, immunoprecipitation reactions, and phosphoamino acid analysis

For [35S]methionine labeling, a 100-mm plate of HeLa cells (gift of R. Weinberg, Massachusetts Institute of Technology, Boston) was incubated for 4 hr in DMEM containing [35S]methionine at a final concentration of 100 µCi/ml. Extracts were prepared by lysis in a buffer containing 1% NP-40, 50 mM HEPES [pH 7.5], 300 mM NaCl, 1 mM EGTA, 1.5 mM MgCl2, 0.2 µg/ml of aprotinin, 0.5 µg/ml of leupeptin, and 0.5 mM PMSF, and diluted in the same buffer lacking NaCl to bring the extracts to a final salt concentration of 150 mM NaCl. Lysates were clarified for 3 hr by incubating with 3 µl of pre-immune serum and 50 µl of 50% protein A-Sepharose (CL4B, Pharmacia. One milliliter of the precleared supernatants (~1 x 106 cpm) was incubated for 1 hr with 3 µl of α-pepLSF1-1 or α-pepLSF2-1 antiserum with 30 µl of 50% protein A-Sepharose. Precipitates were washed with a buffer containing 20 mM HEPES [pH 7.5], 150 mM NaCl, and 0.1% NP-40, and separated by 10% SDS-PAGE as described.

For 32P i labeling and phosphoamino acid analysis, quiescent T cells were starved in phosphate-free RPMI containing 10% dialyzed, heat-inactivated FCS for 30 min at 37°C. Cells were pelleted, resuspended to a final cell concentration of 1 x 106 cells/ml in 10 ml of phosphate-free RPMI containing 10% dialyzed, heat-inactivated FCS, 20 mM HEPES [pH 7.0], and 25 µCi of [32P]orthophosphate (8000 Ci/mmol, NEN) and incubated for 3 hr at 37°C. Cells were subsequently stimulated for 30 min as described above. Equal numbers of unstimulated and stimulated cells were washed twice with a buffer containing 150 mM NaCl and 20 mM HEPES [pH 7.0], resuspended in 500 µl of lysis buffer [10 mM HEPES [pH 7.6], 100 mM NaCl, 1% Triton X-100, 2 mM Na2VO4, 50 mM β-glycerophosphate, 1 mM NaF, 0.1 mg/ml of aprotinin, 0.1 mg/ml of leupeptin, and 0.18 mM PMSF] and incubated on ice for 30 min. Lysates were centrifuged at 4°C for 20 min at 16000g to pellet cell debris. The supernatant was combined with 500 µl of lysis buffer, 300 µl of α-LSFmAb11 supernatant, 1 µg of goat anti-mouse IgA + IgG + IgM ([H + L], [Kirkgaard and Perry]), and 50 µl of a 50% slurry of protein A-Sepharose and incubated at 4°C for 60 min with rocking. Immunoprecipitates were washed and were separated by 10% SDS-PAGE. The gels were dried, fixed, and autoradiographed. Gel slices containing LSF were excised from the dried gel and prepared for phosphoamino acid analysis exactly as described [Boyle et al. 1991]. Samples were autoradiographed, the positions of the phosphoamino acid standards were determined with 2% ninhydrin [Sigma].

MAP kinase assays

For in vitro pp42 (ERK1) immune complex assays, 1 x 106 human peripheral T cells, either unstimulated or stimulated for 5
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min, were lysed in RIPA buffer (140 mM NaCl, 2.7 mM KCl, 8 mM Na2HPO4, 1.5 mM KH2PO4, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.5% Triton X-100, 0.2 mM PMSF, 2 mM Na3VO4, 1 mM NaF, and 50 mM β-glycerophosphate). Extracts were incubated with α-ERK 1 (c-16) antibody (Santa Cruz Biotech) and protein A-Sepharose as recommended by the manufacturer. Immune complexes containing pp44 [ERK1] were washed three times in RIPA buffer, two times in 20 mM HEPES (pH 7.2), 10 mM MgCl2, and then resuspended in 20 µl of kinase buffer containing: 100 mM MgCl2, 20 mM HEPES (pH 7.2), 0.1 mg of BSA, 0.1 mg/ml of aprotinin, 0.1 mg/ml of leupeptin, 0.2 mM PMSF, 2 mM Na3VO4, 1 mM NaF, and 50 mM β-glycerophosphate, 1 µg of His–LSF, and 10 µCi of [γ-32P]ATP, followed by incubation at 30°C for 20 min (Chung et al. 1991; Chen et al. 1992, 1993). Samples were separated by 10% SDS-PAGE and autoradiographed.

For in vitro glutathione S-transferase (GST)-pp44 [ERK1] assays, 200 ng of GST-ERK1, purified and activated as described (Tanaka et al. 1995), was incubated with 2 µg of His–LSF substrate in 20 µl of kinase buffer containing: 20 mM HEPES (pH 7.2), 20 mM MgCl2, 2 mM DTT, 100 µM ATP, and 10 µCi [γ-32P]ATP, followed by incubation at 30°C for 20 min. Samples were separated by 7.5% SDS-PAGE and autoradiographed.

**Constructs**

Site directed mutants of LSF under the control of a cytomegalovirus (CMV) promoter (Q. Zhu and U. Hansen, in prep.) were obtained by PCR using mutagen oligonucleotides. The mutations were made with the following codon changes: $278A = TCT to TCA, and $309A = TCA to GCA. The mutations were confirmed by sequencing the region that was generated by PCR. The mutant LSF regions were subcloned by fragment exchange into a His-tagged LSF bacterial expression vector (Qiagen) and into a pJ3H vector (Sells and Chernoff 1995), encoding an HA-tagged LSF.

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**References**

Abdel-Hafiz, H.A.M., L.E. Heasley, J.M. Kyriakis, J. Avruch, D.J. Kroll, G.L. Johnson, and J.P. Hoeffler. 1992. Activating transcription factor-2 DNA-binding activity is stimulated by phosphorylation catalyzed by p42 and p54 microtubule-associated protein kinases. *Mol. Endocrinol.* 6: 2079–2089.

Abrahamsen, M.S. and D.R. Morris. 1990. Cell type-specific mechanisms of regulating expression of the ornithine decarboxylase gene after growth stimulation. *Mol. Cell. Biol.* 10: 5525–5528.

Andrews, N.C. and D.V. Faller. 1991. A rapid micropreparation technique for extraction of DNA binding proteins from limiting numbers of mammalian cells. *Nucleic Acids Res.* 19: 2499.

Boulton, T.G., Z. Zhong, Z. Wen, J.E. Darnell Jr., N. Stahl, and G.D. Yancopoulos. 1995. STAT3 activation by cytokines utilizing gp130 and related transducers involves a secondary modification requiring an H7-sensitive kinase. *Proc. Natl. Acad. Sci.* 19: 6915–6919.

Boyle, W.J., P. Van Der Geer, and T. Hunter. 1991. Phosphopeptide mapping and phosphoamino acid analysis by two-dimensional separation of thin-layer cellulose plates. *Methods Enzymol.* 201: 110–149.

Chellappan, S.P., S. Hiebert, M. Mudryj, J.M. Horowitz, and J.R. Nevins. 1991. The E2F transcription factor is a cellular target for the RB protein. *Cell* 65: 1053–1061.

Chen, R.-H., C. Sarnecki, and J. Blenis. 1992. Nuclear localization and regulation of erk- and rsk-encoded protein kinases. *Mol. Cell. Biol.* 12: 915–927.

Chen, R.-H., C. Abate, and J. Blenis. 1993. Phosphorylation of the c-Fos transrepression domain by mitogen-activated protein kinase and 90-kDa ribosomal S6 kinase. *Proc. Natl. Acad. Sci.* 90: 10952–10956.

Chung, I., R.-H. Chen, and J. Blenis. 1991. Coordinate regulation of pp90src and a distinct protein-serine/threonine kinase activity that phosphorylates recombinant pp90src in vitro. *Mol. Cell. Biol.* 11: 1868–1874.

Crabtree, G.R. 1989. Contingent genetic regulatory events in T lymphocyte activation. *Science* 243: 355–361.

David, M., E. Petriconi III, C. Benjamin, R. Pine, M.J. Weber, and A.C. Larner. 1995. Requirement for MAP kinase [ERK2] activity in interferon α- and interferon β-stimulated gene expression through STAT proteins. *Science* 269: 1721–1723.

Davis, R.J. 1993. The mitogen-activated protein kinase signal transduction pathway. *J. Biol. Chem.* 268: 14553–14556.

Fu, X.-Y. and J.-J. Zhang. 1993. Transcription factor p91 interferonα stimulates c-fos transcription in NK cells: Kinetics, mitogen requirements, and effects of cyclosporin A. *J. Exp. Med.* 163: 922–937.
LSF phosphorylation increases binding to DNA

Greenberg, M.E., I.A. Greene, and E.B. Ziff. 1985. Nerve growth factor and epidermal growth factor induce rapid transient changes in proto-oncogene transcription in PC12 cells. J. Biol. Chem. 260: 14101–14110.

Greenberg, M.E. and E.B. Ziff. 1984. Stimulation of 3T3 cells induces transcription of the c-fos proto-oncogene. Nature 311: 433–438.

Harlow, E. and D. Lane. 1988. Antibodies: A laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Hibi, M., A. Lin, T. Smeal, A. Minden, and M. Karin. 1993. Identification of an oncoprotein- and UV-responsive protein kinase that binds and potentiates the c-Jun activation domain. Genes & Dev. 7: 2135–2148.

Hill, C.S. and R. Treisman. 1995. Transcriptional regulation by extracellular signals: Mechanisms and specificity. Cell 80: 199–211.

Huang, H.-C., R. Sundseth, and U. Hansen. 1990. Transcription factor LSF binds two variant bipartite sites within the SV40 late promoter. Genes & Dev. 4: 287–298.

Hunter, T. 1995. Protein kinases and phosphatases: The Yin and yang of protein phosphorylation and signaling. Cell 80: 225–236.

Hunter, T. and M. Karin. 1992. The regulation of transcription by phosphorylation. Cell 70: 375–387.

Janknecht, R., R.A. Hipskind, T. Houthaeve, A. Nordheim, and H.G. Stunnenberg. 1992. Identification of multiple SRF N-terminal phosphorylation sites affecting DNA binding properties. EMBO J. 11: 1045–1054.

Jones, K.A., P.A. Luciw, and N. Duche. 1988. Structural arrangements of transcription control domains within the 5′-untranslated leader regions of the HIV-1 and HIV-2 promot- ers. Genes & Dev. 2: 1101–1114.

Kahana, C. and D. Nathans. 1984. Isolation of cloned cDNA encoding mammalian ornithine decarboxylase. Proc. Natl. Acad. Sci. 81: 3645–3649.

Kapiloff, M.S., Y. Farkash, M. Wegner, and M.G. Rosenfeld. 1991. Variable effects of phosphorylation of Pit-1 dictated by the DNA response elements. Science 253: 786–789.

Kato, H., M. Horikoshi, and R.G. Roeder. 1991. Repression of HIV-1 transcription by a cellular protein. Science 251: 1476–1479.

Katz, A. and C. Kahana. 1987. Transcriptional activation of mammalian ornithine decarboxylase during stimulated growth. Mol. Cell. Biol. 7: 2641–2643.

Klimczak, L.J., U. Schindler, and A.R. Cashmore. 1992. DNA-binding activity of the Arabidopsis G-box binding factor GBFI is stimulated by phosphorylation by calsekinase II from broccoli. Plant Cell 4: 87–98.

Lim, L.C., S.L. Swendeman, and M. Sheffery. 1992. Molecular cloning of the α-globin transcription factor CP2. Mol. Cell. Biol. 12: 828–835.

Lin, A., J. Frost, T. Deng, T. Smeal, N. Al-Alawi, U. Kikkawa, T. Hunter, D. Brenner, and M. Karin. 1992. Casein kinase II is a negative regulator of c-Jun DNA binding and AP-1 activity. Cell 70: 777–789.

Malin, M.H., R. Fenrick, D.W. Ballard, J. Hauber, E. Böhlein, and B.R. Cullen. 1989. Functional characterization of a complex protein-DNA-binding domain located within the human immunodeficiency virus type 1 long terminal repeat leader region. J. Virol. 63: 3213–3219.

Manak, J.R. and R. Prywe. 1991. Mutation of serum response factor phosphorylation sites and the mechanism by which its DNA-binding activity is increased by calsekinase II. Mol. Cell. Biol. 11: 3652–3659.

Mar, P.K., A.P. Kumar, D.-C. Kang, B. Zhao, L.A. Martinez, R.L. Montgomery, L. Anderson, and A.P. Butler. 1995. Characterization of novel phorbol ester- and serum-responsive sequences of the rat ornithine decarboxylase gene promoter. Mol. Carcinogen. 14: 240–250.

Marais, R.M., J.J. Hsuan, C. McGuigan, J. Wynne, and R. Treisman. 1992. Casein kinase II phosphorylation increases the rate of serum response factor-binding site exchange. EMBO J. 11: 97–105.

Marais, R., J. Wynne, and R. Treisman. 1993. The SRF accessory protein Elk-1 contains a growth factor-regulated transcriptional activation domain. Cell 73: 381–393.

Minden, A., A. Lin, T. Smeal, B. Dériard, M. Cobb, R. Davis, and M. Karin. 1994. c-Jun N-terminal phosphorylation correlates with activation of the JNK subgroup but not the ERK subgroup of mitogen-activated protein kinases. Mol. Cell. Biol. 14: 6683–6688.

Moll, T., G. Tebb, U. Surana, H. Robitsch, and K. Nasmyth. 1991. The role of phosphorylation and the CDC28 protein kinase in cell cycle-regulated nuclear import of the S. cerevisiae transcription factor SWI5. Cell 66: 743–758.

Montminy, M. 1993. Trying on a new pair of SH2s. Science 261: 1694–1695.

Olson, E.N. and G. Spiz. 1986. Mitogens and protein synthesis inhibitors induce ornithine decarboxylase gene transcription through separate mechanisms in the BC1,H1 muscle cell line. Mol. Cell. Biol. 6: 2792–2799.

Pulverer, B.J., J.M. Kyriakis, J. Avruch, E. Nikolakaki, and J.R. Woodgett. 1991. Phosphorylation of c-jun mediated by MAP kinases. Nature 353: 670–674.

Rao, A. 1991. Signaling mechanisms in T cells. Crit. Rev. Immunol. 10: 495–519.

Reed, I.C., J.D. Alpers, P.C. Nowell, and R.C. Hoover. 1986. Sequential expression of protooncogenes during lectin-stimulated mitogenesis of normal human lymphocytes. Proc. Natl. Acad. Sci. 83: 3982–3986.

Reed, J.C., J.D. Alpers, P.A. Scherle, R.G. Hoover, P.C. Nowell, and M.B. Prystowsky. 1987. Proto-oncogene expression in cloned T lymphocytes: Mitogens and growth factors induce different patterns of expression. Oncogene 1: 223–228.

Rivera, V.M., C.K. Miranti, R.P. Misra, D.D. Ginty, R.-H. Chen, J. Blenis, and M.E. Greenberg. 1993. A growth factor-induced kinase phosphorylates the serum response factor at a site that regulates its DNA-binding activity. Mol. Cell. Biol. 13: 6260–6273.

Roberts, S.B., N. Segil, and N. Heintz. 1991. Differential phosphorylation of the transcription factor Oct1 during the cell cycle. Science 253: 1022–1026.

Segil, N., S.B. Roberts, and N. Heintz. 1991. Mitotic phosphorylation of the Oct-1 homeodomain and regulation of Oct-1 DNA binding activity. Science 254: 1814–1816.

Sells, M.A. and J. Chernoff. 1995. Epitope-tag vectors for eukaryotic protein production. Gene 152: 187–189.

Shirodkar, S., M. Ewen, J.A. DeCaprio, J. Morgan, D.M. Livingston, and T. Chitten. 1992. The transcription factor E2F interacts with the retinoblastoma product and a p107-cyclin A complex in a cell cycle-regulated manner. Cell 68: 157–166.

Shirra, M.K., Q. Zhu, H.-C. Huang, D. Pallas, and U. Hansen. 1994. One exon of the human LSF gene includes conserved regions involved in novel DNA-binding and dimerization motifs. Mol. Cell. Biol. 14: 5076–5087.

Shirra, M.K. 1995. “Characterization of DNA-binding and oligomerization properties of the mammalian transcription factor LSF.” Ph.D. thesis, Harvard University, Cambridge, MA.

Shuai, K., G.R. Stark, I.M. Kerr, and J.E. Darnell, Jr. 1993. A single phosphotyrosine residue of Star91 required for gene activation by interferon-γ. Science 261: 1744–1746.
Su, B., E. Jacinto, M. Hibi, T. Kallunki, M. Karin, and Y. Ben-Neriah. 1994. JNK is involved in signal integration during costimulation of T lymphocytes. *Cell* 77: 727–736.

Tanaka, M., R. Gupta, and B.J. Mayer. 1995. Differential inhibition of signaling pathways by dominant-negative SH2/SH3 adapter proteins. *Mol. Cell. Biol.* 15: 6829–6837.

Uv, A.E., C.R.L. Thompson, and S.J. Bray. 1994. The *Drosophila* tissue-specific factor grainyhead contains novel DNA-binding and dimerization domains which are conserved in the human protein CP2. *Mol. Cell. Biol.* 14: 4020–4031.

Vojtek, A.B. and J.A. Cooper. 1995. Rho family members: Activators of MAP kinase cascades. *Cell* 82: 527–529.

Wen, Z., Z. Zhong, and J.E. Darnell Jr. 1995. Maximal activation of transcription by Stat1 and Stat3 requires both tyrosine and serine phosphorylation. *Cell* 82: 241–250.

Wu, F.K., J.A. Garcia, D. Harrich, and R.B. Gaynor. 1988. Puriﬁcation of the human immunodeﬁciency virus type 1 enhancer and TAR binding proteins EBP-1 and UBP-1. *EMBO J.* 7: 2117–2129.

Yoon, J.-B., G. Li, and R.G. Roeder. 1994. Characterization of a family of related cellular transcription factors which can modulate human immunodeﬁciency virus type 1 transcription in vitro. *Mol. Cell. Biol.* 14: 1776–1785.
Mitogenic stimulation of resting T cells causes rapid phosphorylation of the transcription factor LSF and increased DNA-binding activity.

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