estimate. Using the approach described here, it should be feasible to screen the entire *S. typhimurium* genome for virulence genes with the use of a small number of mice, and so provide a basis for a more comprehensive understanding of *S. typhimurium* pathogenicity. Signature-tagged mutagenesis should find general applicability to other animal and plant pathogens that can undergo transposon or other forms of insertional mutagenesis (*17*).

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21. The variable oligonucleotide pool RT1(5′-CTAGGTACCTACAACCTCAAGCTT-[NK]$_{20}$-AAGCTTGGTTAGAATGGGTACCATG-3′), and primers P2 (5′-TACCTACAACCTCAAGCT-3′), P3 (5′-CATGGTACCCATTCTAAC-3′), P4 (5′-TACCCATTCTAACCAAGC-3′) and P5 (5′-CTAGGTACCTACAACCTC-3′) were synthesized on a oligonucleotide synthesizer (Applied Biosystems, model 394). Double-stranded DNA tags were prepared from RT1 in a 100-μl volume PCR containing 1.5 mM $MgCl_2$, 50 mM KCl, and 10 mM tris-Cl (pH 8.0) with 200 pg of RT1 as target; 250 μM each of dATP, dCTP, dGTP, and dTTP; 100 pM of primers P3 and P5; and 2.5 U of Amplitaq (Perkin-Elmer Cetus). Thermal cycling conditions were 30 cycles of 95°C for 30 s, 50°C for 45 s, and 72°C for 10 s. The PCR product was gel-purified and digested with Kpn I before ligation into pUTmini-Tn*5*Km2 (*6*).
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23. The products of ligations between pUT mini-Tn*5*Km2 and the double-stranded tag DNA were used to transform *E. coli* strain CC118. Approximately 10,300 transformants were pooled, and plasmid DNA extracted from the pool was used to transform *E. coli* S-17 λ*pir* (*6*). For mating experiments, a pool of approximately 40,000 ampicillin-resistant *E. coli* S-17 λ*pir* transformants and a spontaneous nalidixic acid–resistant (nal^r) mutant of *S. typhimurium* NCTC strain 12023 were cultured separately to an optical density $(OD)_{580}$ of 1.0. Aliquots of each culture (0.4 ml) were mated on Millipore membranes (*6*).
24. *S. typhimurium* exconjugants grown in the wells of microtiter plates were pooled and diluted in sterile saline. Groups of two female BALB/c mice (weight 20 to 25 g) were injected intraperitoneally with 0.2 ml of bacterial suspension containing approximately 5 × 10^5 colony-forming units per milliliter.
25. Half of each spleen was homogenized in 1 ml of sterile saline in a microfuge tube. Cellular debris was allowed to settle and 1 ml of saline containing cells still in suspension was removed to a fresh tube and centrifuged for 2 min in a microfuge. The supernatant was aspirated and the pellet resuspended in 1 ml of sterile distilled water. A dilution series was made in sterile distilled water and 100 μl of each dilution was plated onto LB agar containing kanamycin (50 μg ml^{-1}). Bacteria were recovered from plates containing between 1000 and 4000 colonies, and a total of over 10,000 colonies recovered from each spleen were pooled for DNA extraction.
26. A metal replicator was used to transfer exconjugants from microtiter dishes to Hybond N nylon filters (Amersham, UK) that had been placed on the surface of LB agar containing kanamycin (50 μg ml^{-1}). After overnight incubation, bacterial DNA was liberated according to the nylon manufacturers' instructions and fixed to the filters by exposure to ultraviolet (UV) light from a Stratalinker (Stratagene).
27. DNA extracted from bacterial pools was used as a template for two rounds of PCR to generate labeled probes. The first PCR was done in 100-μl reactions containing 20 mM tris-Cl (pH 8.3); 50 mM KCl; 2 mM $MgCl_2$; 0.01% Tween 20; 200 μM each of dATP, dCTP, dGTP, and dTTP; 2.5 U of Amplitaq polymerase (Perkin-Elmer Cetus); 770 ng each of primers P2 and P4; and 5 μg of target DNA. After an initial denaturation of 4 min at 95°C, thermal cycling consisted of 20 cycles of 45 s at 50°C, 10 s at 72°C, and 30 s at 95°C. PCR products were extracted with chloroform–isoamyl alcohol (24:1) and precipitated with ethanol. DNA was resuspended in 10 μl of TE buffer [10 mM tris-Cl (pH 8.0) snd 1 mM EDTA], and the PCR products were purified by electrophoresis through a 1.6% Seaplaque (FMC Bioproducts) gel in TAE buffer [40 mM tris-acetate (pH 8.0) and 1 mM EDTA]. Gel slices containing fragments of approximately 80 base pairs (bp) were excised and used for the second PCR. This reaction was carried out in a 20-μl total volume and contained 20 mM tris-Cl (pH 8.3); 50 mM KCl; 2 mM $MgCl_2$; 0.01% Tween 20; 50 μM each of dATP, dTTP, and dGTP; 10 μl of [^{32}P]dCTP (3000 Ci/mmol, Amersham); 150 ng each of primers P2 and P4; approximately 10 ng of target DNA (1 to 2 μl of 1.6% Seaplaque agarose containing the first-round PCR product); and 0.5 U of Amplitaq polymerase. The reaction was overlayed with 20 μl of mineral oil and thermal cycling was done as described above. Filter hybridizations to ^{32}P-labeled probes were done under stringent conditions as described [D. W. Holden, J. W. Kronstad, S. A. Leong, *EMBO J.* **8**, 1927 (1989)].

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Integration of MAP Kinase Signal Transduction Pathways at the Serum Response Element

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The ternary complex factor (TCF) subfamily of ETS-domain transcription factors bind with serum response factor (SRF) to the serum response element (SRE) and mediate increased gene expression. The TCF protein Elk-1 is phosphorylated by the JNK and ERK groups of mitogen-activated protein (MAP) kinases causing increased DNA binding, ternary complex formation, and transcriptional activation. Activated SRE-dependent gene expression is induced by JNK in cells treated with interleukin-1 and by ERK after treatment with phorbol ester. The Elk-1 transcription factor therefore integrates MAP kinase signaling pathways in vivo to coordinate biological responses to different extracellular stimuli.

The SRE mediates increased immediate-early gene expression (for example, c-*fos*) in cells treated with growth factors or cytokines or subjected to environmental stress (*1*). The SRF binds to the SRE along with a TCF (*2*). The TCF proteins belong to a subgroup of the ETS-domain family (*3*) that includes Elk-1, SAP-1, and NET-1/ERP/SAP-2 (*4*). Phosphorylation of TCF is associated with activated, SRE-dependent gene expression (*5*, *6*). The ERK group of MAP kinases phosphorylate Elk-1 and cause both increased ternary complex formation (*7*, *8*) and activation of the Elk-1 COOH-terminal transcriptional activation domain (*5*, *6*, *9*). Thus, the SRE is a target of the ERK signal transduction pathway. However, the ERK pathway is not the only means by which SRE-dependent gene expression is increased. For example, the SRE located within the c-*fos* promoter mediates increased c-Fos expression in response to

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the exposure of cells to environmental stress (*10*). These effects of environmental stress [for example, ultraviolet (UV) radiation and exposure to anisomycin], however, are not mediated by the ERK signal transduction pathway (*10*). Furthermore, colony-stimulating factor can induce changes in ternary complex formation by a mechanism that is independent of the ERK signal transduction pathway (*11*). Therefore, at least one ERK-independent signal transduction pathway causes increased SRE-dependent gene expression. Signal transduction pathways that mediate ERK-independent activation of NFκB, STAT, and c-Jun transcription factors have been identified (*12*, *13*). However, mechanisms that can account for the ERK-independent activation of SRE-dependent gene expression have not been defined.

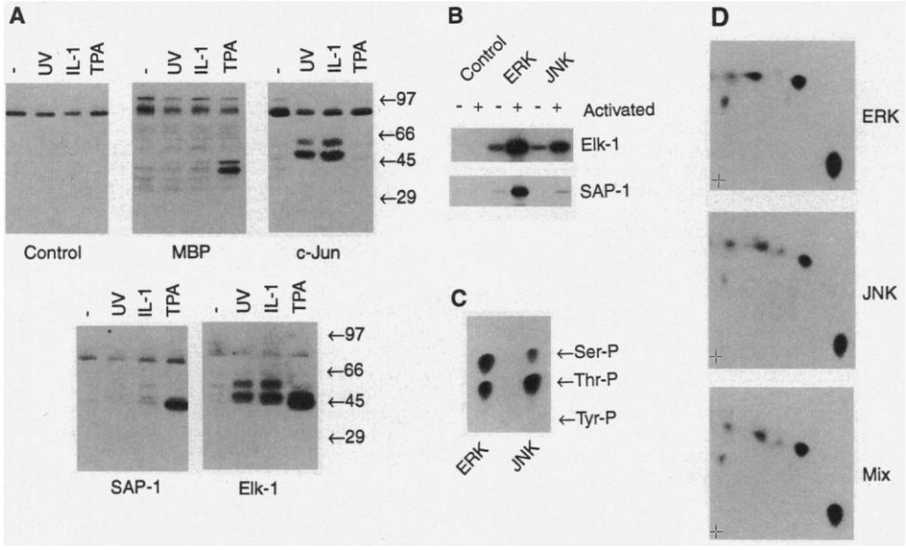

Fig. 1. TCF phosphorylation by JNK and ERK MAP kinases. (**A**) In-gel protein kinase assays of lysates prepared from CHO cells (*25*) treated without and with IL-1, UV radiation, and TPA were done with MBP, c-Jun, SAP-1, and Elk-1 as substrates (*28*). Control experiments were done without substrate. Molecular size standards (in kilodaltons) are indicated on the right. (**B**) The phosphorylation of Elk-1 (upper panel) and SAP-1 (lower panel) was examined in an immune complex protein kinase assay with epitope-tagged MAP kinases, including EGF-stimulated ERK2 and IL-1–stimulated JNK1 (*28*). Control experiments were done with immunoprecipitates of mock-transfected cells that do not express epitope-tagged MAP kinase. (**C**) The phosphorylated Elk-1 was examined by phosphoamino acid analysis (*28*). (**D**) Elk-1 tryptic phosphopeptides were examined by two-dimensional mapping (*28*). A map of Elk-1 phosphorylated by ERK2 and JNK1 is shown. A map of a 1:1 mixture of Elk-1 phosphorylated by ERK2 and JNK1 is also presented.

To examine signaling pathways that regulate SRE-mediated gene expression, we investigated the phosphorylation of the TCF proteins Elk-1 and SAP-1. Protein kinases present in extracts of cells treated with phorbol ester (TPA), UV radiation, or the proinflammatory cytokine interleukin-1 (IL-1) were examined after SDS–polyacrylamide gel electrophoresis (SDS-PAGE) through use of an in-gel assay with the substrate polymerized in the gel (Fig. 1A). Treatment with TPA caused increased phosphorylation of myelin basic protein (MBP), Elk-1, and SAP-1 by two protein kinases of 42 and 44 kD. Treatment with IL-1 or exposure to UV radiation caused increased phosphorylation of c-Jun and Elk-1 by two distinct protein kinases of 46 and 55 kD. A low level of SAP-1 phosphorylation by the UV- and IL-1–activated 46- and 55-kD protein kinases was also observed (Fig. 1A). The substrate specificity and pattern of activation indicates a possible role for the ERK (42 and 44 kD) and JNK (46 and 55 kD) groups of MAP kinases (*12*). To test this hypothesis, we examined the phosphorylation of Elk-1 and SAP-1 by using immunopurified MAP kinases. The ERK2 protein kinase phosphorylated both Elk-1 and SAP-1 (Fig. 1B). In contrast, the JNK1 protein kinase caused Elk-1 phosphorylation, but only a small amount of SAP-1 phosphorylation (Fig. 1B). Previous studies have identified c-Jun (*12*) and ATF2 (*14*) as JNK substrates. Comparison of Elk-1 with c-Jun and ATF2 indicates that JNK causes a similar amount of phosphorylation of each of these substrates (*15*). These data confirm previous studies that demonstrate a role for the ERK signal transduction pathway in the regulation of Elk-1 (*5*–*9*) and indicate that the ERK pathway

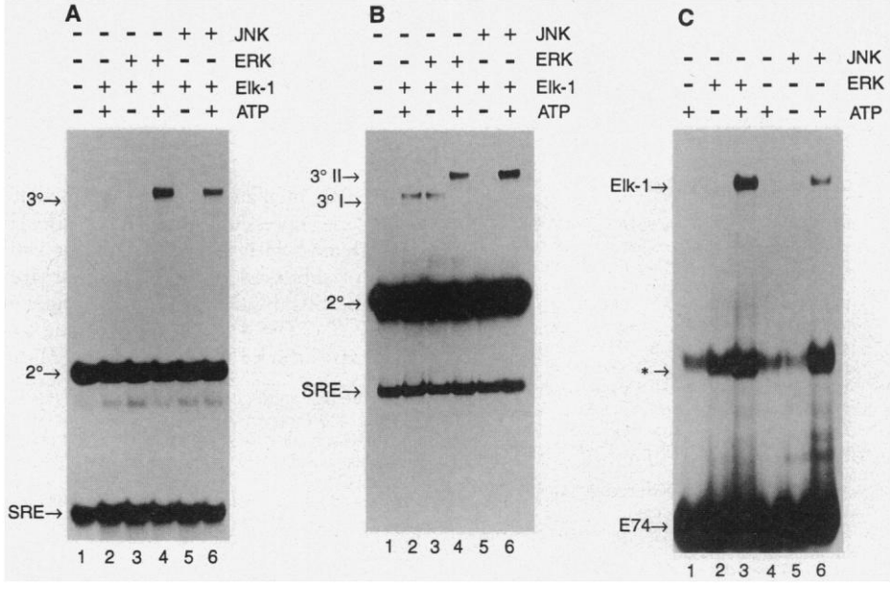

Fig. 2. Phosphorylation of Elk-1 by JNK and ERK causes increased ternary complex formation, decreased ternary complex electrophoretic mobility, and increased DNA-binding activity. Elk-1 was phosphorylated with ERK2 and JNK1 in vitro (*30*). (**A**) Ternary complex formation by Elk-1, SRF, and the SRE was measured by EMSA with a double-stranded oligonucleotide probe (*30*). The migration of the SRE probe, the secondary SRF-SRE complex (2°), and the ternary complex (3°) is indicated. Conditions of the Elk-1 preincubation are indicated above each lane. (**B**) Ternary complex formation was also examined with a 134-bp fragment of the c-*fos* promoter containing the SRE (*30*). The migration of the SRE probe, 2° complex, and the two ternary complexes (3° I and 3° II) are indicated. (**C**) The DNA-binding activity of Elk-1 was examined by EMSA with the ^{32}P-labeled double-stranded E74 probe that contains an *ets*-like binding site (*30*). The migration of the E74 probe and the E74–Elk-1 complex is indicated. An additional complex (marked with an asterisk) probably represents a complex of E74 with a proteolytic fragment of Elk-1.

may also regulate SAP-1. In addition, these data demonstrate that TCFs may be physiological targets of the JNK signal transduction pathway (Fig. 1).

Phosphoamino acid analysis of Elk-1 phosphorylated by ERK and JNK demonstrated the presence of both phosphoserine and phosphothreonine (Fig. 1C), but the phosphorylation of Elk-1 by these MAP kinases is not identical. Equal amounts of phosphoserine and phosphothreonine were detected in Elk-1 phosphorylated by ERK. In contrast, a larger amount of phosphothreonine than phosphoserine was detected in Elk-1 phosphorylated by JNK. The tryptic phosphopeptide maps of Elk-1 phosphorylated by JNK and ERK are similar, which indicates that these protein kinases phosphorylate the same group of sites (Fig. 1D). These MAP kinase phosphorylation sites have been identified and include critical regulatory sites, such as Ser-383 (5, 6, 8, 9). The major difference between Elk-1 phosphorylated by JNK and ERK is the relative amount of phosphorylation of these sites (Fig. 1D).

Phosphorylation of TCF/Elk-1 by ERK causes an increase in ternary complex formation with SRF at the SRE (*7*, *8*) and decreased ternary complex electrophoretic mobility (5, 6). To test whether JNK also regulates ternary complex formation, we compared the effects of phosphorylation of Elk-1 by JNK and ERK. Ternary complex formation by phosphorylated and nonphosphorylated Elk-1 was examined by electrophoretic mobility–shift analysis (EMSA) with core^{SRF} (*7*) and a ^{32}P-labeled DNA probe. Experiments with an SRE oligonucleotide probe (*16*) demonstrated that the phosphorylation of Elk-1 by JNK or ERK caused a large increase in ternary complex formation (Fig. 2A, lanes 4 and 6). In contrast, experiments with a 134–base pair (bp) fragment derived from the c-*fos* promoter as an SRE probe (5) demonstrated ternary complex formation (3° I) in the absence of TCF phosphorylation (Fig. 2B, lane 2). However, both JNK and ERK phosphorylation of Elk-1 caused a large decrease in the electrophoretic mobility of the ternary complex (3° II) (Fig. 2B, lanes 4 and 6). These data demonstrate that JNK and ERK phosphorylation of Elk-1 cause similar changes in ternary complex formation at the SRE. Because Elk-1 binds to the SRE in an SRF-dependent manner (*2*), we used a different assay to directly examine the effect of phosphorylation on the DNA-binding properties of Elk-1. This assay used a DNA probe that contains the *Drosophila* E74 *ets*-like site that binds Elk-1 in the absence of SRF (*17*). Phosphorylation of Elk-1 by JNK or ERK induced binding of Elk-1 to the E74 probe (Fig. 2C, lanes 3 and 6). Thus, JNK and ERK induce increased Elk-1 DNA-binding activity.

The phosphorylation of Elk-1 by JNK and ERK suggests that activators of both MAP kinase signal transduction pathways would cause increased SRE-dependent gene expression. We therefore compared the effects of TPA and IL-1 on gene expression in cotransfection experiments with the reporter plasmid pSRE-Luc. Although TPA caused activation of the ERK group of MAP kinases (Fig. 1A) and IL-1 caused activation of the JNK group of MAP kinases (Fig. 1A), both TPA and IL-1 caused a similar increase in reporter gene expression (Fig. 3A). This effect of TPA and IL-1 was blocked by a dominant-negative Elk-1 derivative (Fig. 3A) indicating that the increased reporter gene expression was TCF-dependent. The effect of TPA can be accounted for by the ERK signal transduction pathway (5–9). Control experiments were done to confirm that the actions of IL-1 were mediated by the JNK signal transduction pathway. The protein kinase MKK4 (also termed SEK1/JNKK) phosphorylates and activates JNK (*18*, *19*). Dominant-negative MKK4 inhibited IL-1–stimulated reporter gene expression but caused only a small decrease in the effects of TPA (Fig. 3B). These data demonstrate that the ef-

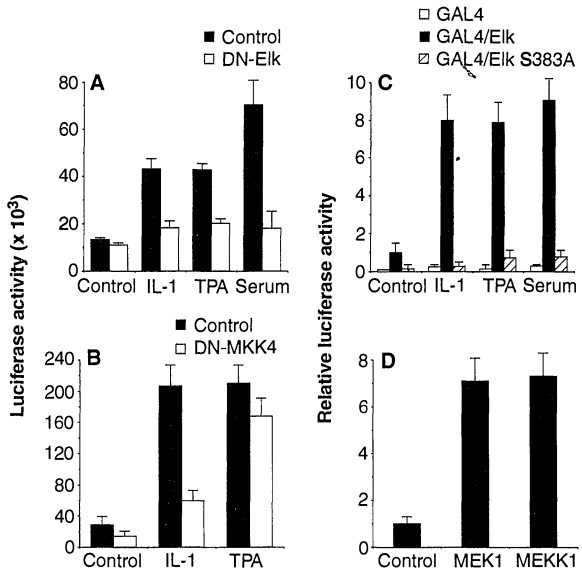

Fig. 3. Activation of TCF/Elk-1–dependent gene expression by the JNK and ERK signal transduction pathways. (**A**) The effect of treatment with IL-1 or TPA on the luciferase activity detected in extracts of CHO cells transfected with an SRE-luciferase reporter plasmid was examined (*31*). Control experiments were done by expression of dominant-negative Elk-1 (DN-Elk) that has a deletion of the COOH-terminal activation domain (*26*). Transfection efficiency was monitored with a β-galactosidase expression vector. The data are presented as the ratio of luciferase (light units) to β-galactosidase optical density (OD) units measured in the cell extracts (mean ± SD, $n = 3$). (**B**) The effect of dominant-negative MKK4 (DN-MKK4) on GAL4/Elk-dependent reporter gene expression was examined in cells treated with IL-1 or TPA (*26*, *31*). (**C**) The effect of treatment with IL-1, TPA, or serum on reporter gene expression in cells cotransfected with the reporter plasmid pG5E1bLuc and an expression vector that encodes the GAL4 DNA-binding domain (Control), wild-type GAL4/Elk-1 fusion protein, or mutated GAL4/Elk-1 (Ser-383 replaced with Ala) was examined (*26*, *31*). (**D**) The effect of constitutively activated MEK1 (*26*) and MEKK1 (*26*) on reporter gene expression was examined with cells cotransfected with an SRE-luciferase reporter plasmid (*31*). The cells were transfected with 10 ng of MEKK1 or 500 ng of MEK1 expression vector.

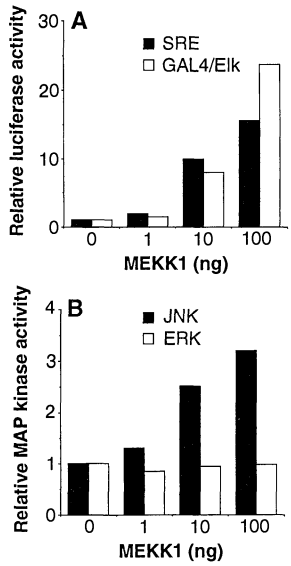

Fig. 4. Activation of the JNK signal transduction pathway increases SRE-mediated gene expression. CHO cells were transfected with increasing amounts of a constitutively activated MEKK1 expression vector (*25*, *26*). (**A**) The effect of MEKK1 on the expression of luciferase by an SRE-luciferase reporter plasmid was examined. The effect of MEKK1 on the transcriptional activity of Elk-1 was examined in cotransfection assays with an expression vector encoding a GAL4/Elk-1 fusion protein and a reporter plasmid (pG5E1bLuc) with GAL4 DNA-binding sites cloned upstream of the luciferase gene (*31*). Transfection efficiency was monitored with a β-galactosidase expression vector. The data are presented as the ratio of luciferase activity (light units) to β-galactosidase (OD units) measured in the cell extracts and are normalized to the activity detected in control cells transfected without MEKK1. (**B**) The effect of expression of constitutively activated MEKK1 on the activity of the endogenous JNK and ERK protein kinases was examined (*32*). The data are presented as the relative protein kinase activity (control cells transfected without MEKK1 = 1).

fects of IL-1 are independent of the ERK pathway and are consistent with a role for the JNK signal transduction pathway.

The Elk-1 activation domain is regulated by phosphorylation (*5*, *6*, *9*). To examine the function of the Elk-1 activation domain in cells treated with TPA or IL-1, we performed cotransfection assays with an expression vector that encodes the Elk-1 activation domain (residues 307 to 428) fused to the GAL4 DNA-binding domain (Fig. 3C). TPA and IL-1 caused increased GAL4-dependent reporter gene expression in cells transfected with GAL4/Elk-1 (Fig. 3C). In contrast, deletion of the Elk-1 sequence from the GAL4/Elk-1 fusion protein or replacement of the MAP kinase phosphorylation site Ser-383 (*5*, *6*, *8*, *9*) with Ala blocked the increased reporter gene expression (Fig. 3C). These observations are consistent with a role for both the ERK and JNK signal transduction pathways in the regulation of Elk-1 transcriptional activity by phosphorylation.

Because TPA and IL-1 may activate multiple signal transduction pathways, we performed further studies to obtain more direct evidence for the role of the ERK and JNK MAP kinase signal transduction pathways in vivo. Expression of activated MEK1 and MEKK1 causes constitutive activation of the ERK (*20*, *21*) and JNK (*22*, *23*) groups of MAP kinases, respectively. We therefore examined the effect of activated MEK1 and MEKK1 on SRE-dependent gene expression. Both MEK1 and MEKK1 caused increased reporter gene expression (Fig. 3D). Control experiments were done to examine the effects of MEKK1 on MAP kinase activity. Dose-response analysis demonstrated that transfection with increasing amounts of the MEKK1 expression vector caused activation of SRE- and GAL4/Elk-1–dependent reporter gene expression (Fig. 4A). Measurement of MAP kinase activity demonstrated that JNK, but not ERK, was activated by MEKK1 (Fig. 4B). Together, these data demonstrate that JNK activation in vivo is associated with increased SRE-dependent gene expression.

A major target of the JNK signal transduction pathway is the AP-1 transcription factor that is composed of dimeric complexes formed from members of the Fos and Jun bZIP subfamilies (*12*). Phosphorylation of c-Jun by JNK causes the activation of AP-1 (*12*). In addition, the activation of AP-1 also results from increased expression of Fos and Jun proteins (*10*). Increased expression of c-Jun may result from the autoregulation of the c-*jun* promoter at the proximal AP-1 site (*24*). TCF phosphorylation by JNK may contribute to increased c-Fos expression by activating the c-*fos* promoter at the SRE (Figs. 3 and 4). Thus, the JNK signal transduction pathway activates AP-1 by multiple mechanisms, including the phosphorylation of preexisting TCF and c-Jun transcription factors.

This study establishes that the SRE is a site of integration of signal transduction pathways (Fig. 5). Phosphorylation of Elk-1 by the ERK and JNK groups of MAP kinases causes increased ternary complex formation at the SRE and increased transcriptional activity. The ERK signal transduction pathway mediates the effects of protein kinase C and receptor tyrosine kinases (*12*). In contrast, the JNK signal transduction pathway mediates the effects of proinflammatory cytokines and environmental stress (*12*). The phosphorylation of Elk-1 by JNK and ERK therefore represents one mechanism that can account for the integration of these signal transduction pathways in vivo to yield coordinated biological responses to extracellular stimuli.

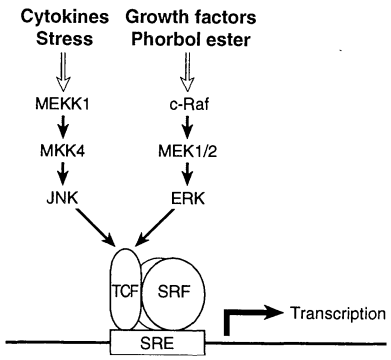

Fig. 5. SRE-dependent gene expression is activated by the ERK and JNK signal transduction pathways. ERK and JNK are two groups of MAP kinases that are activated by functionally independent signal transduction pathways (*12*). The ERK signal transduction pathway is activated by growth factors (for example, EGF and TPA), whereas the JNK pathway is activated by environmental stress (for example, UV radiation) and proinflammatory cytokines (for example, IL-1 and tumor necrosis factor). The TCF Elk-1 is a substrate for both ERK and JNK MAP kinases. This phosphorylation causes increased ternary complex formation at the SRE and increased transcriptional activity. Thus, the SRE is a site of functional integration of independent MAP kinase signal transduction pathways.

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25. CHO cells were maintained in Ham's F12 medium supplemented with fetal calf serum (5%) (Gibco-BRL). COS cells were maintained in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum (Gibco-BRL). Plasmid DNA (*26*) was transfected into cells by the lipofectamine method (Gibco-BRL) and harvested after 48 hours of incubation. The cells were treated with IL-1α (10 ng/ml) (Genzyme), 10 nM TPA (Sigma), 10% fetal calf serum, or 40 J/m^2 UV-C after incubation in serum-free Ham's F12 medium for 12 hours.
26. A constitutively activated MEKK1 fragment (residues 1 to 672) was isolated from mouse heart mRNA by reverse transcriptase–polymerase chain reaction (RT-PCR) and was cloned in the expression vector pCMV5 [S. Andersson, D. L. Davis, H. Dahlbäck, H. Jörnvall, D. W. Russell, *J. Biol. Chem.* **264**, 8222 (1989)]. The sequence of mouse heart MEKK1 has been deposited in GenBank (accession number U23470). The mammalian expression vectors for GAL4-ElkC (*5*), epitope-tagged Flag-JNK1 (*27*), Flag-MKK4 (*18*), and activated MEK1 (*20*) have been described. The epitope-tagged HA-ERK2 expression vector was provided by M. Weber (University of Virginia). The Elk-1 MAP kinase phosphorylation site Ser-383 was replaced with Ala through use of mutagenic primers and the overlapping PCR method [S. N. Ho, H. D. Hunt, R. M. Horton, J. K. Pullen, L. R. Pease, *Gene* **77**, 51 (1989)]. The dominant-negative Elk-1 expression vector encodes residues 1 to 168 of Elk-1 and was constructed by insertion of a Kpn I–Xba I fragment of pAS96 (*16*) into the expression vector pCMV5. The dominant-negative MKK4 expression vector (*18*) was constructed by replacement of the sites of MKK4-activating phosphorylation (Ser-257 and Thr-261) with Ala residues through use of the overlapping PCR method.
27. B. Dérijard *et al.*, *Cell* **76**, 1025 (1994).
28. The cells were solubilized with lysis buffer [20 mM tris (pH 7.4), 1% Triton X-100, 10% glycerol, 137 mM NaCl, 2 mM EDTA, 25 mM β-glycerophosphate, 1 mM sodium orthovanadate, 2 mM pyrophosphate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 mM dithiothreitol (DTT), leupeptin (10 μg/ml)] after treatment with UV radiation (60 min), TPA (15 min), IL-1 (15 min), and epidermal growth factor (EGF) (15 min).

The extracts were centrifuged at 100,000*g* for 15 min at 4°C. Immune complex kinase assays were done with extracts prepared from cells expressing epitope-tagged ERK2 (HA) or JNK1 (Flag). The epitope-tagged protein kinases were immunoprecipitated by incubation for 2 hours at 4°C with the M2 Flag monoclonal antibody (IBI-Kodak) or HA polyclonal antibody (BAbCo) bound to protein G–Sepharose (Pharmacia-LKB Biotechnology). The immunoprecipitates were washed twice with lysis buffer and twice with kinase buffer [25 mM Hepes (pH 7.4), 25 mM β-glycerophosphate, 25 mM $MgCl_2$, 0.5 mM DTT, 0.1 mM sodium orthovanadate]. The kinase assays were initiated by the addition of 1 μg of substrate proteins (His6–Elk-1 or His6–SAP-1) (*29*) and 50 μM [γ-^{32}P]ATP (10 Ci/mmol) in a final volume of 40 μl. The reactions were terminated after 30 min at 25°C by the addition of Laemmli sample buffer. The phosphorylation of the substrate proteins was examined after SDS-PAGE by autoradiography. In-gel kinase assays were performed with 0.25 mg/ml of substrate protein (MBP, GST-ElkC, GST-SAPC, or GST-Jun) (*29*) polymerized in the gel as described (*27*). Phosphoamino acid analysis and phosphopeptide mapping of His6–Elk-1 phosphorylated by JNK1 was done as described (*27*). The horizontal dimension of the peptide maps was electrophoresis and the vertical dimension was chromatography.

29. Bacterial expression of GST-Jun was done as described (*27*). We constructed expression plasmids for GST-ElkC (Elk-1 residues 307 to 428) and GST-SAPC (SAP-1 residues 287 to 431) by subcloning PCR fragments of Elk-1 and SAP-1 in the Bam HI and Eco RI sites of pGEX-3X (Pharmacia-LKB Biotechnology). Glutathione-S-transferase (GST) fusion proteins were purified by glutathione affinity chromatography [D. B. Smith and K. S. Johnson, *Gene* **67**, 31 (1988)]. The full-length Elk-1 protein (residues 1 to 428) fused to a COOH-terminal hexahistidine tag (His6–Elk-1) was expressed in bacteria with the plasmid pQE6/16Elk (*8*). The full-length SAP-1 protein (residues 1 to 431) fused to a COOH-terminal hexahistidine tag (His6–SAP-1) was expressed in bacteria with a plasmid constructed by the sequential ligation of three separate SAP-1 PCR fragments containing unique introduced restriction sites into the vector pET21d (Novagen). Hexahistidine fusion proteins were purifed by nickel chelate affinity chromatography as described by the manufacturer (Novagen).
30. The DNA-binding activity of Elk-1 was examined by EMSA with ^{32}P-labeled DNA probes. The E74 and SRE double-stranded oligonucleotide probes have been described (*16*). A 134–base pair (bp) PCR fragment of the c-*fos* promoter containing the SRE was prepared (*5*). Phosphorylation of His6–Elk-1 was done with recombinant ERK2 and JNK1 MAP kinases activated in vitro by the MAP kinase kinases MEK1 and MKK4, respectively. Epitope-tagged MKK4 was isolated from UV-irradiated transfected COS cells with the M2 monoclonal antibody (*18*). MEK1 was isolated from EGF-treated COS cells with the rabbit antibody 2880 [M. Wartmann and R. J. Davis, *J. Biol. Chem.* **269**, 6695 (1994)]. Immune complex kinase assays were done with the MEK1 and MKK4 MAP kinase kinases and 6 μg of bacterially expressed ERK2 and JNK1 (*18*) in kinase buffer (*28*) supplemented with 200 μM ATP for 20 min at 22°C. The phosphorylated and activated MAP kinases (ERK2 and JNK1) were incubated with 0.5 μg of His6–Elk-1 in 20 μl of kinase buffer (*28*) supplemented with 100 μM ATP for 20 min at 22°C. DNA-binding assays were done with 133 mM KCl and without salmon sperm DNA [A. D. Sharrocks, H. Gille, P. E. Shaw, *Mol. Cell. Biol.* **13**, 123 (1993)] with 50 ng of Elk-1 for binding to the E74 probe and 10 ng of Elk-1 for ternary complex analysis at the SRE. Ternary complex formation assays were done with core^{SRF} in the binding assays (*7*). Control experiments were done without ATP. Protein-DNA complexes were analyzed by PAGE (5% gel) in 1× tris-borate EDTA and visualized by autoradiography.
31. SRE-dependent gene expression was monitored in cotransfection assays (*25*) with the reporter plasmid pSRE-Luc, which contains two copies of the c-*fos* SRE cloned upstream of a minimal promoter element and the firefly luciferase gene [A. Seth *et al.*, *J. Biol. Chem.* **267**, 24796 (1992)]. The activity of GAL4/ElkC (*5*) was measured in cotransfection assays with the reporter plasmid pG5E1bLuc [A. Seth *et al.*, *J. Biol. Chem.* **267**, 24796 (1992)]. This reporter plasmid contains five GAL4 sites cloned upstream of a minimal promoter element and the firefly luciferase gene. Transfection efficiency was monitored with a control plasmid that expresses β-galactosidase (pCH110; Pharmacia-LKB Biotechnology). The luciferase and β-galactosidase activity detected in cell extracts was measured [S. Gupta, A. Seth, R. J. Davis, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 3216 (1993)].
32. JNK activity was measured by an immune complex kinase assay with a rabbit polyclonal antibody to JNK (*14*) and the substrate c-Jun. ERK activity was measured with the substrate MBP (*22*).
33. We thank N. Ahn, P. Shaw, R. Treisman, and M. Weber for providing reagents; B. Dérijard and I.-H. Wu for preparing the MKK4 vector; T. Barrett and R. Liddell for DNA sequence analysis; members of the Davis and Sharrocks laboratories for critical discussions; and M. Roberts for secretarial assistance. R.J.D. is an Investigator of the Howard Hughes Medical Institute. Supported by grants from the National Cancer Institute (R.J.D.), the North of England Cancer Research Campaign (A.D.S.), and the Royal Society (A.D.S.).

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Inactivation of the Mouse Huntington's Disease Gene Homolog *Hdh*

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Huntington's disease (HD) is a dominant neurodegenerative disorder caused by expansion of a CAG repeat in the gene encoding huntingtin, a protein of unknown function. To distinguish between "loss of function" and "gain of function" models of HD, the murine *HD* homolog *Hdh* was inactivated by gene targeting. Mice heterozygous for *Hdh* inactivation were phenotypically normal, whereas homozygosity resulted in embryonic death. Homozygotes displayed abnormal gastrulation at embryonic day 7.5 and were resorbing by day 8.5. Thus, huntingtin is critical early in embryonic development, before the emergence of the nervous system. That *Hdh* inactivation does not mimic adult HD neuropathology suggests that the human disease involves a gain of function.

Huntington's disease is a dominant neurodegenerative disorder (*1*) with a characteristic pattern of neuronal loss (*2*) and consequent chorea, psychiatric alterations, and intellectual decline. HD results when one copy of the gene encoding huntingtin, an ~350 kD cytoplasmic protein found in fetal and adult peripheral tissues and nervous system (*3–5*), contains an expanded stretch of CAG trinucleotides. Although the CAG repeat is normally a Mendelian polymorphism (11 to 34 units), the HD expanded repeat (37 to more than 100 units) is unstable through meiotic transmission and its length is correlated with disease severity (*3*).

The HD defect probably acts at the protein level, as the HD CAG repeat is translated, altering huntingtin by elongating a polyglutamine segment near the NH_2-terminus (*4*, *5*). One possibility is that elongating the polyglutamine stretch reduces huntingtin's normal activity, but individuals with one copy of the normal gene inactivated by translocation do not develop HD despite a 50% reduction (*6*). However, a huntingtin loss of function could still cause HD if the abnormal protein also produces "dominant negative" inhibition of its normal counterpart. An alternative is that the polyglutamine segment confers a new property (gain of function) that may be unrelated to huntingtin's normal activity.

To choose between these models, we generated an inactivating mutation of the mouse *Hdh* gene (*7*) by targeted disruption. If HD involves a dominant loss of function, we would expect mice heterozygous for *Hdh* inactivation to be phenotypically normal (like their human counterparts with *HD* gene translocations) and mice homozygous for huntingtin inactivation to mature and manifest HD-like neuropathology. Alternatively, if HD involves a dominant gain of function, neither heterozygous nor homozygous inactivation of *Hdh* would produce HD-like neuropathology, but the latter might produce a completely different phenotype

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