Note added in proof: After this report was accepted for publication, a paper (*27*) concluded from a different assay that the EST1 protein is required for telomerase activity. Our results show that EST1 is neither an essential catalytic nor primer-binding component of telomerase.

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12. Whole cell extracts were prepared as follows: Cells (*24*) harvested in early log phase (optical density at 600 nm = 1) were resuspended in TMG buffer [10 mM tris-HCl (pH 8), 1.2 mM $MgCl_2$, 15% (v/v) glycerol, 0.1 mM EDTA, 0.1 mM EGTA, 1.5 mM dithiothreitol, 1 mM pefabloc, 1 μM pepstatin, 1 μM leupeptin, 130 μM bestatin, and RNasin (40 units/ml)] and disrupted in a Bead-Beater (Biospec Products). S-100 extracts were prepared by ultracentrifugation at 100,000*g* for 90 min at 4°C, divided into portions, and stored at −80°C; the protein concentration was 22 mg/ml for *S. castellii* and 17 mg/ml for *S. cerevisae* S-100 extracts. The S-100 supernatants were fractionated on a DEAE-agarose column (Biogel; Bio-Rad) that had been equilibrated with TMG buffer (with 10% glycerol). The column was washed with four volumes of TMG buffer containing 0.5 M sodium acetate for *S. castellii* and 0.6 M sodium acetate for *S. cerevisiae*. After elution with 1.8 column volumes of 0.65 or 0.75 M sodium acetate in TMG buffer for *S. castellii* and *S. cerevisiae*, respectively, eluted fractions were desalted and concentrated on Microcon-30 columns (Amicon). Protein concentrations were not measured after purification. It was anticipated that the same relative amounts were still present in the extracts because they were treated identically. *Saccharomyces cerevisiae* extracts were diluted ~100 times and *S. castellii* extracts ~10 times during the Microcon-30 column desalting. For telomerase assays, equal volumes of desalted DEAE fractions (usually 10 or 20 μl) and 2× reaction buffer were mixed and incubated at either 20° or 30°C for 30 min. Final concentrations for *S. castellii* were 50 mM tris-HCl (pH 8), 1 mM spermidine, 1 mM dithiothreitol, 100 mM potassium glutamate, 50 μM dCTP, 50 μM dTTP, 1.9 μM [α-^{32}P]dGTP (800 Ci/mmol), and 1 μM primer (*25*). Potassium glutamate and dCTP were omitted for *S. cerevisiae* reactions. Reaction products were resolved on denaturing 10% acrylamide gels containing 7 M urea as described (*14*). RNase treatment of extracts was performed with RNase A (6.3 μg/ml) at 25°C for 5 min, immediately before starting the reaction. RNase A was inhibited by incubating the extract with RNasin (2300 units/ml) at 25°C for 5 min before addition of RNase A (6.3 μg/ml) and incubation for another 5 min.
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25. All oligonucleotides were purified to a single species by separation on a denaturing gel. Markers were created by elongation of primers $(TGTGGG)_2$ and $(GGGTGTCT)_2$ with terminal deoxynucleotidyl transferase (Boehringer Mannheim) and [α-^{32}P]dGTP. The primary products were 13 and 17 nt, respectively.
26. The diploid *S. cerevisiae* strain TVL115 contains the *est1-*Δ*3::HIS3* mutation, in which ~80% of the *EST1* gene is deleted. The heterozygous diploid was sporulated and the tetrads dissected. The haploid deletion strain showed the expected senescence and correct genomic band on Southern (DNA) blot analysis (*11*) with an *EST1* riboprobe (*17*). The protein concentration of the *est1* S-100 extract was 16 mg/ml.
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30 December 1994; accepted 19 April 1995

Simultaneous Identification of Bacterial Virulence Genes by Negative Selection

Michael Hensel, Jacqueline E. Shea, Colin Gleeson, Michael D. Jones, Emma Dalton, David W. Holden*

An insertional mutagenesis system that uses transposons carrying unique DNA sequence tags was developed for the isolation of bacterial virulence genes. The tags from a mixed population of bacterial mutants representing the inoculum and bacteria recovered from infected hosts were detected by amplification, radiolabeling, and hybridization analysis. When applied to a murine model of typhoid fever caused by *Salmonella typhimurium*, mutants with attenuated virulence were revealed by use of tags that were present in the inoculum but not in bacteria recovered from infected mice. This approach resulted in the identification of new virulence genes, some of which are related to, but functionally distinct from, the *inv/spa* family of *S. typhimurium*.

Several different approaches have been used to exploit transposon mutagenesis for the isolation of bacterial virulence genes, including screens for the loss of specific virulence-associated factors (*1*), survival within macrophages (*2*), and penetration of epithelial cells (*3*). Although these screens have identified many bacterial genes required for virulence, they are restricted to certain stages of infection. Transposon mutants have also been tested individually for altered virulence in live animal models of infection (*4*), but comprehensive screening of bacterial genomes for virulence genes has not been possible because of the inability to identify mutants with attenuated virulence within pools of mutagenized bacteria and the impracticability of separately assessing the virulence of each of the several thousand mutants necessary to screen a bacterial genome. We have circumvented this problem by developing a transposon mutagenesis system, termed signature-tagged mutagenesis, in which each transposon mutant

M. Hensel, J. E. Shea, C. Gleeson, D. W. Holden, Department of Infectious Diseases and Bacteriology, Royal Postgraduate Medical School, Hammersmith Hospital, Du Cane Road, London W12 ONN, UK.
M. D. Jones and E. Dalton, Department of Virology, Royal Postgraduate Medical School, Hammersmith Hospital, Du Cane Road, London W12 ONN, UK.

*To whom correspondence should be addressed.

is tagged with a different DNA sequence. This permits the identification of bacteria recovered from hosts infected with a mixed population of mutants as well as the selection of mutants with attenuated virulence.

We used this system to identify virulence genes of *Salmonella typhimurium* in a murine model of typhoid fever (*5*) (Fig. 1). The pool of double-stranded DNA sequence tags comprised variable central regions flanked by arms of invariant sequences (Fig. 1A). The central region sequences were designed to prevent the occurrence of sites for the restriction enzymes Hind III, Kpn I, Pst I, and Sal I (which were used for ligation and before hybridization of tags and for subsequent cloning of DNA flanking the transposons) but were sufficiently variable to ensure that the same sequence should only occur once in 2×10^{17} molecules. The arms were designed so that amplification of the tags in polymerase chain reactions (PCRs) with primers P2 and P4 and radiolabeled deoxycytidine 5′-triphosphate (dCTP) would produce probes with 10 times more label in the central region than in each arm. Specific probes were generated by digestion of the radiolabeled PCR products with Hind III, which releases the arms from the central regions.

The double-stranded tags were ligated into the transposon mini-Tn*5* Km2 (*6*). These were transferred from *Escherichia coli* to *S. typhimurium* by conjugation, and a bank of 1510 exconjugants resulting from transposition events was stored in the wells of microtiter dishes. To ensure the suitability of DNA tags before use, we determined their efficiency of amplification and labeling in PCRs with pooled exconjugant DNAs as templates. After growth to stationary phase, the 96 *S. typhimurium* exconjugants of each microtiter dish were combined. DNA extracted from each pool of cells was used as a template for a PCR with primers P2 and P4, and the radiolabeled products were hybridized with colony blots of DNA from the corresponding microtiter dishes. Of the 1510 tags tested in this way, 1152 yielded clear signals on autoradiograms after overnight exposures of colony blots. These were reassembled into 12 96-well microtiter dishes.

To test whether the hybridization signals

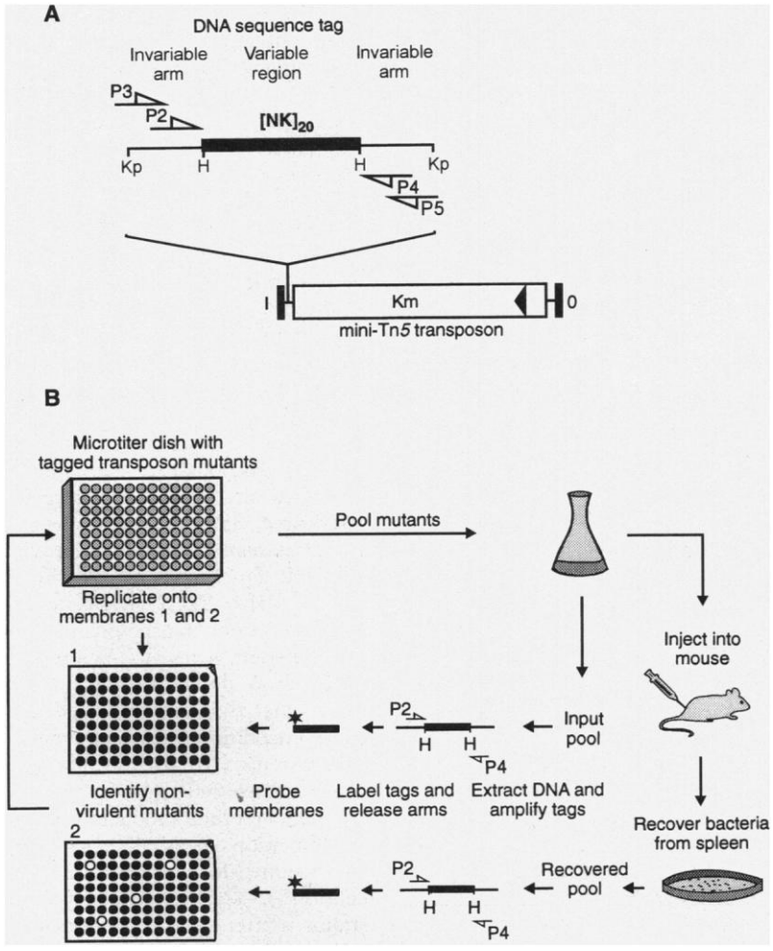

Fig. 1. (**A**) Design of transposon tags. A complex mixture of double-stranded DNA tags was generated by oligonucleotide synthesis and the PCR (*21*). Each tag comprises a different sequence of 40 bp ($[NK]_{20}$; N = A, C, G, or T; K = G or T) flanked by arms of 20 bp, which are common to all of the tags. The arms allow the sequence tags to be amplified in a PCR with the use of primers P3 and P5. The tags are digested with Kpn I (Kp) and ligated into the transposon mini-Tn*5*Km2, the I and O ends of which are indicated. The transposon is carried on a suicide vector (*22*) that is maintained as a plasmid in *E. coli* but is lost after transfer to *S. typhimurium* (*23*). Transposition events lead to the stable and near-random single-copy integration of the transposon, along with its unique tag, into the *S. typhimurium* genome. H, Hind III. (**B**) Virulence gene screen. A bank of *S. typhimurium* transposon-tagged mutants is arrayed in 96-well microtiter dishes. DNA colony blots representing individual members of the bank are prepared by replica plating from the microtiter dishes. The 96 mutants of each microtiter dish are pooled and an aliquot is removed for DNA extraction (input pool). The pooled bacterial cells are injected into mice (*24*), and after 3 days the spleens are removed and mutants that have reached and multiplied within this organ are recovered by plating of spleen homogenates onto laboratory medium (*25*). Approximately 10,000 colonies are combined and DNA is extracted (recovered pool). The tags within the input and recovered pools are amplified and radiolabeled in a PCR. The arms are released by digestion with Hind III (H) and the tags are used to probe replica colony blots from the microtiter dish. Colonies that hybridize to the probe from the input pool but not to the probe from the recovered pool represent mutants with attenuated virulence.

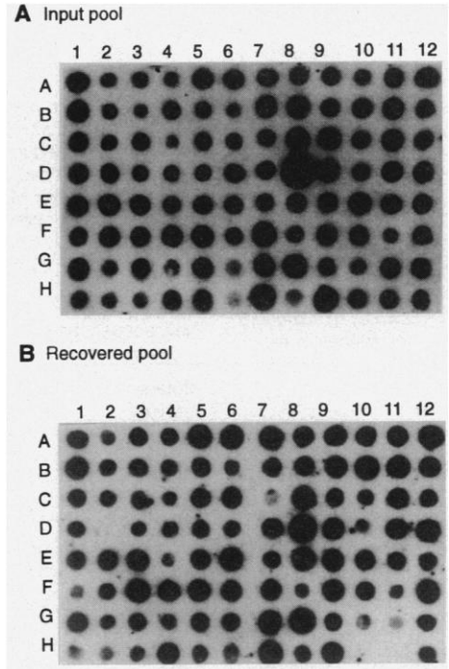

Fig. 2. Identification of virulence genes. DNA colony blot hybridization analysis (*26*) of 96 *S. typhimurium* exconjugants of a microtiter dish (A1 to H12) that were injected into a mouse. Replicate filters were hybridized with labeled amplified tags (*27*) from the input pool (**A**) or with labeled amplified tags from the recovered pool (**B**). Hybridization signals from colonies D2, H10, and H11 are present on the filter probed with the input pool but not on the filter probed with the recovered pool.

resulted from cross-hybridization between tags, amplified tags from a pool of 24 colonies were used to probe a colony blot of 48 colonies, which included the 24 used to generate the probe. There was no cross-hybridization to the 24 not used to generate the probe (7). DNA sequencing of 12 randomly selected tags showed that none shared more than 50% identity over the variable region, which indicates that the sequence dissimilarity between the tags is sufficient to prevent cross-hybridization. To confirm that integration of the tagged transposons into the *S. typhimurium* genome was random, total DNA from 36 mutants was digested with Eco RV and subjected to Southern (DNA) hybridization analysis with the use of the kanamycin resistance gene of the mini-Tn*5* transposon as a probe. In each case, the exconjugant had arisen as a result of a single integration of the transposon into a different site in the bacterial genome (7).

There are two factors that restrict the complexity of pools of different mutants for use as inocula in infection studies. First, as the complexity of the pool increases, so must the probability that some virulent mutants will not be present in sufficient numbers in the organs of an infected animal to produce enough labeled probe for hybridization analysis, leading to false identification of avirulent mutants. Second, in the hybridization analysis, the quantity of labeled tag for each transposon will be inversely proportional to the complexity of the tag pool, so there is a limit to the pool size above which hybridization signals will become too weak to be detected by autoradiography. We obtained strong and reproducible hybridization signals after overnight exposure of colony blots probed with pools of 96 mutants; but when the pool size was increased to 192, the intensity of hybridization signals was reduced, and hybridization patterns of colony blots probed with tags from different animals inoculated with the same pool became less reproducible.

Twelve pools of inoculum, each comprising 96 different sequence-tagged insertion mutants of a microtiter dish, were screened for attenuated virulence in BALB/c mice (Fig. 1B). Each pool was injected into two animals. Three days after intraperitoneal injection, mice were killed, and bacteria were recovered by plating of spleen homogenates onto laboratory medium. Approximately 10,000 colonies recovered from each mouse were pooled and DNA was extracted. The tags present in this DNA sample were amplified and labeled by the PCR, and colony blots were probed and compared with the hybridization pattern obtained with the use of tags amplified from the inoculum as a probe (Fig. 2). As a control, an avirulent *aro*A mutant of *S. typhimurium*, CL1509 (*aro*A::Tn*10*) (8), was transposon tagged and used as one of the 96 mutants in one of the inoculum pools. In total, 40 mutants were identified whose DNA hybridized to labeled tags from the inoculum but not to labeled tags from pooled bacteria recovered from the spleens of two animals. One of these was the *aro*A strain, as expected. DNA was extracted from 28 of the mutants, and regions flanking the transposons were cloned, sequenced, and subjected to DNA database searches (Table 1). Thirteen of the mutations were in previously identified *S. typhimurium* virulence genes (thereby validating the screening approach), six were in homologs of known genes of *S. typhimurium* and other bacteria, and nine were in sequences without similarity to entries in DNA databases. The LD_{50} (dose at which half the mice were killed) for mutants P3F4, P7G2, P9B7, and P11C3 was determined to be at least 100 times greater than that of the wild-type parental strain (Table 1).

Mutant P1F10 arose from insertion of a transposon into a homolog of *E. coli clpP*, which encodes an adenosine 5′-triphosphate (ATP)–dependent protease involved in selective intracellular protein degradation (9). Although *E. coli* $ClpP^-$ cells do not have an obvious phenotype (9), the attenuated virulence of the corresponding *S. typhimurium* mutant might provide a clue to ClpP function. Five of the mutants (P2D6, P3F4, P7G2, P9B7, and P11C3) originated from transposon insertions into four different genes (P2D6 and P11C3 resulted from different insertions into the same gene) that are structurally similar to genes encoding proteins of type III secretion systems (10). In *S. typhimurium*, the genes are referred to as the *inv/spa* family (11, 12), and their products are involved in the formation of specialized surface appendages required for entry into host epithelial cells (13). The virulence of *inv* mutant strains is attenuated if the inoculum is administered orally but not when it is given by the intraperitoneal route (11). The existence of a set of *inv*-related genes that are required for virulence after intraperitoneal injection suggests that *S. typhimurium* must synthesize another type of export apparatus after entry into the body in order to cause disease. Further characterization of these genes and the phenotypic effects of nonpolar mutations in them will help to elucidate their functions during the infection process.

Approximately 75 *S. typhimurium* virulence genes have been isolated to date (14, 15), representing half the total number estimated to be present in the *Salmonella* genome (16). The ratio of known to unknown sequences in Table 1 is in keeping with this

Table 1. Characterization of tagged genes. Transposons and flanking DNA regions were cloned from exconjugants with the use of the kanamycin resistance gene of the transposon to select for desired inserts in pUC18, after transformation of ligation reactions into *E. coli* DH5α. Approximately 300 to 600 bp of DNA flanking each transposon were sequenced (18), and deduced amino acid sequences were assembled with the Macvector 3.5 software package run on a Macintosh Power Mac 7100 computer. Sequences were compared with the European Molecular Biology Laboratory and Genbank DNA databases with the use of the BLAST and FASTA network service at the Human Genome Mapping Project Resource Centre, Hinxton, United Kingdom. Abbreviations: *Bsu*, *Bacillus subtilis*; *Eco*, *Escherichia coli*; *Sty*, *S. typhimurium*; and *Yen*, *Yersinia enterocolitica*. ND, not determined.

Known *S. typhimurium* virulence genes		New sequences similar* to known genes			New sequences with no similarity to database entries
Mutant strain	Gene	Mutant strain	Similarity to	LD_{50}†	Mutant strain
P4E3	*purD*	P1F10	*clpP (Eco)*	ND‡	P4F8
P5D2	*rfbB*	P2D6	*invA (Sty), lcrD (Yen)*	ND	P4G5
P5D10	*spvA*	P3F4	*invG (Sty)*	$>10^4$	P6G5
P6F1	*rfbD*	P7G2	*yscC (Yen), invG (Sty)*	$>10^3$	P7A3
P7G6	*rfbK*	P9B7	*fliQ (Bsu), invX (Eco)*	$>10^4$	P9B6
P9G2	*rfbK*	P11C3	*invA (Sty), lcrD (Yen)*	$>10^3$	P9G4
P9H12	*spvR*				P10E11
P10C12	*purL*				P11D10
P10D7	*spvD*				P11H11
P10D10	*spvA*				
P10H1	*purD*				
P10H9	*rfbM*				
P12D9	*ompR/envZ*				

*Similarities ranged from 30 to 80% identity at the amino acid level over a minimum of 60 deduced residues. †The virulence levels of individual mutant strains were investigated by first transferring the mutations to the naladixic acid–sensitive parent strain of *S. typhimurium* 12023 by phage P22–mediated transduction (19). Transductants were checked by restriction mapping before being injected intraperitoneally (10^2, 10^3, or 10^4 bacteria) into groups of BALB/c mice. The LD_{50} was determined by the method of Reed and Muench (20). The intraperitoneal LD_{50} for strain 12023 was six cells (7). ‡This mutant was analyzed by mixed infection experiments. Four mice were injected with inocula consisting of 5×10^4 cells of strain 12023 and 5×10^4 cells of mutant P1F10. Over 150 colonies recovered from the spleens of each of these animals were analyzed, and P1F10 accounted for 9.5 to 28% of these.

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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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)].

28. We thank K. Timmis for providing plasmid pUTmini-Tn*5*Km2 and *E. coli* strains CC118 λ*pir* and S17-1 λ*pir*; F. Heffron for strain CL1509 (*aroA*::Tn*10*); G. Dougan for phage P22; and H. Arst, N. Lowndes, and J. Holden for critical review. M.H. was supported by a grant from the European Community Human Capital and Mobility Programme. J.E.S. was supported by a grant from the Medical Research Council.

9 March 1995; accepted 16 May 1995

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

Alan J. Whitmarsh, Paul Shore, Andrew D. Sharrocks, Roger J. Davis*

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

A. J. Whitmarsh and R. J. Davis, Howard Hughes Medical Institute, and Program in Molecular Medicine, Department of Biochemistry and Molecular Biology, University of Massachusetts Medical School, Worcester, MA 01605, USA.
P. Shore and A. D. Sharrocks, Department of Biochemistry and Genetics, The Medical School, University of Newcastle-upon-Tyne, NE2 4HH, UK.

*To whom correspondence should be addressed.