50 μM deoxyguanosine triphosphate (dGTP), dTTP, and dATP (each), and 10 μM dCTP, 200 μM GTP, uridine triphosphate (UTP), ATP, and CTP (each), 1 mM ATP, and ATP regenerating system [6 mM creatinine phosphate and creatinine kinase (10 U/ml)]; incubations were 3 hours.
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The Primary Structure of MEK, a Protein Kinase That Phosphorylates the *ERK* Gene Product

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Mitogen-activated protein (MAP) kinases, also known as extracellular signal–regulated kinases (ERKs), are thought to act at an integration point for multiple biochemical signals because they are activated by a wide variety of extracellular signals, rapidly phosphorylated on threonine and tyrosine, and highly conserved. A critical protein kinase lies upstream of MAP kinase and stimulates the enzymatic activity of MAP kinase. The structure of this protein kinase, denoted MEK1, for MAP kinase or ERK kinase, was elucidated from a complementary DNA sequence and shown to be a protein of 393 amino acids (43,500 daltons) that is related most closely in size and sequence to the product encoded by the *Schizosaccharomyces pombe byr1* gene. The *MEK* gene was highly expressed in murine brain, and the product expressed in bacteria phosphorylated the *ERK* gene product.

Peptide growth factors elicit a burst of intracellular protein phosphorylation in treated cells, most of which occurs on serine and threonine residues. Because tyrosine-specific growth factor receptor kinases initiate these phosphorylation events, they must regulate serine-threonine–specific protein kinases or phosphatases. The MAP kinases encoded by the *ERK* genes appear to be critical components in the conversion of tyrosine phosphorylation to signals that result in serine-threonine phosphorylation, because they are phosphorylated on tyrosine and threonine but phosphorylate downstream components on serine and threonine (*1*). Two protein kinases function in sequence upstream of MAP kinase (*2–6*). One is a 45-kD protein, MEK, which phosphorylates MAP kinase on the threonine and tyrosine regulatory sites and activates its serine-threonine kinase activity (*2–4, 6, 7*). MEK is in turn reported to be phosphorylated and activated by the proto-oncogene product c-Raf (*5*). The biochemical mechanisms for a number of steps in this signal transduction pathway remain to be elucidated. We have determined the primary structure of MEK, a threonine-tyrosine kinase activator of MAP kinases.

We purified MEK to near homogeneity from phorbol ester–stimulated murine T cell hybridomas (*2*). A single, autophosphorylating 45-kD band was visualized by SDS–polyacrylamide gel electrophoresis. The amino acid sequences of tryptic peptides from this protein band show it to be a protein kinase related to the *byr1* gene product from *Schizosaccharomyces pombe*. Degenerate oligonucleotides were synthesized encoding part of two distinct peptides and were used in a polymerase chain reaction (PCR) with a sample of a murine pre–B cell cDNA library in lambda gt10 phage as a template (*8*). A 350-bp PCR product was obtained, and sequence analysis showed that it encoded the tryptic peptides used in the PCR primer design and confirmed its similarity to Byr1. This fragment was then used to probe 1×10^6 phage plaques from the same cDNA library, and 39 putative positive clones were identified.

The five clones with the greatest hybridization signal intensity were selected for further analysis. One of these clones, 4-3, was chosen for sequence analysis, which revealed an insert of 2150 bp. The GenBank accession number for the DNA sequence is L02526. The starting methionine, which is a favorable initiation codon based on the Kozak rule (*9*), is at nucleotide 41. This clone can encode a protein of 393 amino acids, corresponding to a molecular size of about 43.5 kD. This is similar to the size of the enzyme purified from T cell hybridomas and that of the MAP kinase activators purified from murine 3T3 cells and *Xenopus* oocytes (*2, 3, 6*). Furthermore, the predicted protein sequence of clone 4-3 includes 101 of the 102 amino acids present in the six tryptic peptides from the purified T cell protein (*2*). The one mismatch (amino acid 374 in peptide 6) was resolved upon reevaluation of the peptide sequencing data and appears to be Trp^{374}, as encoded by the cDNA clone.

It is possible that clone 4-3 is missing 5′ untranslated sequence, as the size of the mRNA detected by Northern analysis (Fig. 1) was approximately 2.5 kb. Northern analysis of duplicate RNA gels done in the absence of ethidium bromide, however, resulted in the hybridization to a 2.2-kb transcript. The tissue distribution of MEK mRNA was determined with clone 4-3 as a probe for hybridization with a blot of RNA from various mouse organs. The amount of MEK expression was highest in brain.

To confirm that this cDNA encodes a kinase that phosphorylates the *ERK* gene product, a bacterially produced glutathione-S-transferase–MEK fusion protein was assayed for catalytic activity. The MEK fusion protein phosphorylated a catalytically inactive form of ERK1 in vitro predominantly on tyrosine and, to a lesser extent, on threonine (Fig. 2). A preparation of MEK purified from murine cells stimulated with phorbol ester and diluted to yield the same amount of activity phosphorylated similar proportions of tyrosine and threonine. This assay can be regarded only as a qualitative measure of MEK activity because the enzyme from bacteria presumably has not been activated by upstream components present in animal cells. Moreover, the phosphopeptides recovered after proteolytic digestion of ERK1(K63M) phosphorylated by mammalian MEK and recombinant MEK were identical (*10*). The mechanism for substrate recognition by MEK remains to be determined, however,

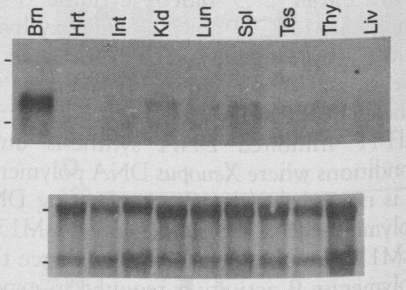

Fig. 1. Tissue distribution of MEK expression. Total RNA (10 μg) from various C57/Bl mouse tissues were Northern blotted and probed with oligonucleotide-labeled full-length MEK cDNA (*25*). Brn, brain; Hrt, heart; Int, intestine; Kid, kidney; Lun, lung; Spl, spleen; Tes, testis; Thy, thymus; and Liv, liver. Methylene blue–staining of the ribosomal RNA in each lane is shown to illustrate relative RNA concentrations.

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as it appears to resemble more closely enzymes with serine-threonine specificity than those with tyrosine specificity (*11*).

We compared the sequence of MEK to the *S. pombe byr1* gene product, the most closely related protein in terms of size and sequence, as expected from the previous tryptic peptide data (*12*) (Fig. 3). There is also a high degree of similarity within the domains of the *Saccharomyces cerevisiae PBS2* and *STE7* gene products that correspond to the 393 amino acids of MEK (*10, 13*). Genetic and biochemical information has indicated that *byr1* and *STE7* are likely to lie immediately upstream of the yeast homologs of *ERK*—*spk1* and *FUS3* or *KSS1* (*14*). *PBS2* was isolated on a multicopy plasmid that conferred resistance to polymyxin B. Polymyxin B is an inhibitor of protein kinase C (PKC); thus *PBS2* has the potential to act downstream of the *PKC1* gene (*15*), which encodes a protein similar to PKC in *S. cerevisiae*. To our knowledge, this possibility has not been tested.

Because MEK-like gene products appear to be critical for the regulation of downstream protein kinases that in turn may influence nuclear events, the characterization of the upstream regulators of MEK is of interest. The signal transduction pathways in fission yeast, budding yeast, and animal cells have certain similarities. Guanosine triphosphatase (GTPase) protein components are proximal to signal initiation in *S. pombe* (Ras1) (*16*), *S. cerevisiae* (Ste4) (*17*), and vertebrates (Ras) (*18, 19*). The protein kinases encoded by *byr2* in *S. pombe* and *STE11* in *S. cerevisiae* transduce signals from the GTPase protein to Byr1 (*20*) and Ste7 (*21*), respectively. In animal cells the events are less clear. Presumably there is a *byr2*-like gene product that could phosphorylate and activate MEK. The serine-threonine protein kinase encoded by c-*raf* has this capacity in vitro (*5*), and cells transformed by v-*raf* yield constitutively activated MAP kinases. The activated Raf oncoprotein does not activate MAP kinases in all cells tested (*22*); thus the in vivo role of Raf is currently unclear. The rapid activation of MEK and MAP kinases by phorbol ester indicates that PKC may function in this pathway. In animal cells the generation of diacylglycerol in response to activation of Ras (*23*) could stimulate PKC and lead to activation of MEK. In PC12 cells a dominant negative interfering mutant of Ras blocks activation of MAP kinases by growth factors and phorbol esters, suggesting that PKC acts upstream of Ras (*19*). In contrast, in rat-1 cells the interfering Ras mutant blocks activation of MAP kinase by PDGF but not by phorbol ester (*24*), which would indicate that PKC acts downstream of Ras or in a parallel pathway. Caution is required in the interpretation of these experiments, because oncogenically mutated and overexpressed proteins are constitutively active and may produce global changes in many

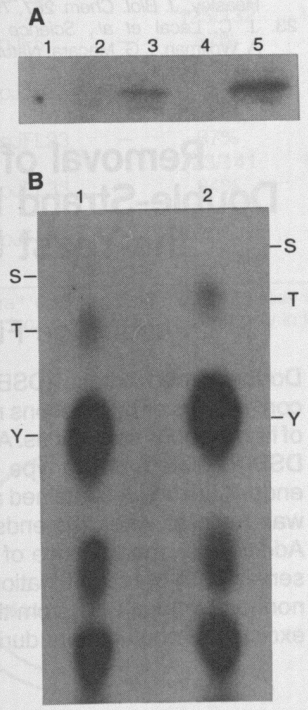

Fig. 2. Enzymatic activity of bacterially produced MEK. The MEK cDNA was subcloned into the Bam HI and Sac I sites of the glutathione-S-transferase (GST) bacterial expression system (pGEX-KG) (*26*). Eight amino acids from Bluescript SK+ and 13 amino acids from the 5′ sequence upstream of the starting methionine in MEK are included in the GST-MEK fusion protein, which was purified as described (*27*). (**A**) Phosphorylation of GST-ERK (a catalytically inactive form of ERK in which Lys^{63} is changed to Met, K63M) by GST-MEK. Lane 1, GST-MEK alone; lane 2, GST-ERK(K63M) alone; lane 3, GST-MEK and GST-ERK(K63M); lane 4, purified murine MEK (pool A) alone (*2*); lane 5, purified murine MEK (pool A) and GST-ERK(K63M). Kinase reactions were performed at 30°C for 20 min in a solution containing 50 mM tris-HCl (pH 8.0), 5 mM dithiothreitol, 3 mM magnesium acetate, 50 μM ATP, 25 μCi of [γ-^{32}P]ATP, ovalbumin (0.1 mg/ml), GST-ERK(K63M) (5 μg), and GST-MEK (0.1 μg) or purified MEK (0.08 ng) (*2*). (**B**) Phosphoamino acid analysis of GST-ERK(K63M) phosphorylated by GST-MEK (lane 1) and mammalian-purified MEK (lane 2). GST-ERK(K63M) was phosphorylated under similar conditions as in (A) but with 200 μCi of [γ-^{32}P]ATP for 60 min. The phosphoamino acids (S, phosphoserine; T, phosphothreonine; and Y, phosphotyrosine) of GST-ERK(K63M) were analyzed as described (*7*).

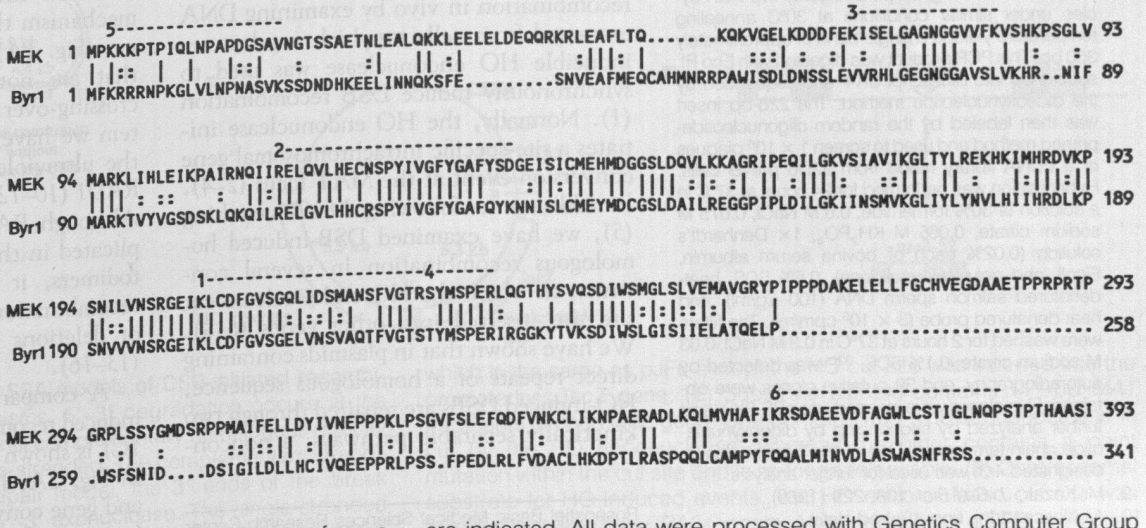

Fig. 3. Comparison of the amino acid sequences MEK and Byr1. The six tryptic peptide sequences obtained from purified MEK (*2*) are marked above the sequence (----). MEK and Byr1 are 60% similar and 45% identical over their entire length. The amino acid sequence of MEK is 60% similar and 41% identical to residues 264 to 710 of Pbs2 and 61% similar and 39% identical to residues 97 to 515 of Ste7 (*10*). A partial cDNA sequence from human brain that encodes a polypeptide nearly identical to amino acids 245 to 323 of MEK has been reported (*28*). A single peptide sequence from a *Xenopus* enzyme corresponding to the adenosine triphosphate binding site of MEK was reported (*29*). The percent similarity and identity were obtained with the GAP program at gap weight 3.0 and gap length weight 0.1. Identical amino acids (solid bars) and conserved amino acids (dots) are indicated. All data were processed with Genetics Computer Group (University of Wisconsin) programs (*30*). Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

pathways not influenced by normal molecules (*24*). Multiple pathways may lead to MEK activation. The protein product expressed by the *MEK* cDNA should facilitate additional studies of this pathway.

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8. Degenerate oligonucleotide primers were synthesized on the basis of amino acid sequence of two peptides recovered from a tryptic digest of purified MEK (*2*). Primer AC1, CGAATTCC(T/G)(T/C)TC(A/C/T/G)GG(A/C/T/G)(C/G)(T/A)CAT (A/G)TA (512 possible permutations), based on peptide 4 (*2*), and primer AC3, CCGAATTCCA(T/C)GA (A/G)TG(T/C)(T/A)(G/C)(A/C/G/T)CC (256 possible permutations), based on peptide 2, were used in a PCR (50 μl) containing 20 mM deoxynucleotide triphosphates, primers AC1 and AC3 (10 mm), 1× Promega Taq buffer, 1 unit of Taq polymerase, and a sample of lambda gt10 murine pre–B cell library 22D6 (provided by D. Schatz, Yale University), representing approximately 6.4×10^4 plaque-forming units. Thirty cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min and extension at 72°C for 1 min were preceded by 3 min at 94°C to disrupt the phage particles and followed by 7 min at 72°C for a final extension. The resulting heterogeneous 300- to 500-bp product was reamplified for 25 cycles under the same conditions as above but at 60°C annealing temperature and without the initial 3-min denaturation step. The resulting heterogeneous 300- to 500-bp product from this amplification was used as a template for a PCR with primers AC1 and AC2—CGAATTCTG(T/C)TTT(A/T)(G/C)(C/A/G)CC(A/C/G/T)TA(T/C)AT (T/C/A)G. Both primers AC2 and AC3 encode segments of peptide 2 (*2*); however, the AC2 sequence is COOH-terminal to AC3. Therefore, AC2 can be used in a PCR with AC3:AC1 PCR products as starting template. Twenty-five PCR cycles under similar conditions at 30°C annealing temperature yielded a single band of approximately 350 bp. This PCR product was digested with Eco RI, subcloned into Bluescript SK+ and sequenced by the dideoxynucleotide method. The 228-bp insert was then labeled by the random oligonucleotide-primed method and used to screen 1×10^6 plaques of a cDNA library made from 22D6 pre–B cells. Hybridization was performed for 24 hours at 37°C in a solution of 30% formamide, 0.6 M NaCl, 0.075 M sodium citrate, 0.065 M KH_2PO_4, 1× Denhardt's solution (0.02% each of bovine serum albumin, Ficoll, and polyvinylpyrrolidone), 0.5% SDS, heat-denatured salmon sperm DNA (100 μg/ml), and heat-denatured probe (3×10^5 cpm/ml). The filters were washed for 2 hours at 37°C in 0.3 M NaCl, 0.03 M sodium citrate, 0.1% SDS. ^{32}P was detected by autoradiography, and 39 putative clones were obtained. Five clones were inserted into pBS-SK+ and further analyzed by sequencing by dideoxynucleotide chain termination. One of the longest clones, designated 4-3, was used for further analysis.
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Removal of Nonhomologous DNA Ends in Double-Strand Break Recombination: The Role of the Yeast Ultraviolet Repair Gene *RAD1*

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Double-strand breaks (DSBs) in *Saccharomyces cerevisiae* can be repaired by gene conversions or by deletions resulting from single-strand annealing between direct repeats of homologous sequences. Although *rad1* mutants are resistant to x-rays and can complete DSB-mediated mating-type switching, they could not complete recombination when the ends of the break contained approximately 60 base pairs of nonhomology. Recombination was restored when the ends of the break were made homologous to donor sequences. Additionally, the absence of *RAD1* led to the frequent appearance of a previously unobserved type of recombination product. These data suggest *RAD1* is required to remove nonhomologous DNA from the 3′ ends of recombining DNA, a process analogous to the excision of photodimers during repair of ultraviolet-damaged DNA.

We have developed an approach to study recombination in vivo by examining DNA extracted from cells in which a galactose-inducible HO endonuclease was used to synchronously induce DSB recombination (*1*). Normally, the HO endonuclease initiates a site-specific intrachromosomal gene conversion event at the *MAT* locus (*2–4*). In addition to following *MAT* switching (*5*), we have examined DSB-induced homologous recombination in several constructs in which the HO endonuclease cut site was inserted into other genes (*6–9*). We have shown that in plasmids containing direct repeats of a homologous sequence, HO-induced DSBs are repaired through two kinetically separable pathways: a nonconservative single-strand annealing (SSA) mechanism that produces a deletion product (Fig. 1A) and gene conversion events that are not accompanied by reciprocal crossing-over (Fig. 1B) (*9*). With this system we have now examined the role that the ultraviolet (UV) excision repair gene *RAD1* (*10–12*) plays in these two pathways. Although *RAD1* has been principally implicated in the excision repair of UV photodimers, it also is involved in mitotic recombination, especially in the formation of deletions between repeated sequences (*13–16*).

A comparison of the kinetics of HO-induced recombination of plasmid pJF6 (Fig. 1C) is shown for wild-type and *rad1* strains in Fig. 2A. In wild-type strains, deletions and gene conversions are formed efficiently. In contrast, the *rad1* derivative showed no detectable gene conversion product and a fourfold reduction in the amount of deletion product (*17*). A genetic analysis (Table 1A) further substantiates the physical data. HO

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