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9. All of the internal deletions generated and used in this study maintain the correct *fepA* reading frame. Although the Mlu I– and Cla I–cleaved termini are not perfectly complementary, they form two GC pairs and can be efficiently joined. We sequenced the junction regions of *fepAΔMC* and *fepAΔRV* to confirm their structure. The *fepAΔRV*, *fepAΔH261*, and *fepAΔA306* deletions have been described (*7, 10*) The corresponding mutant receptors are designated ΔMC, ΔRV, ΔH261, and ΔA306. The four mutations delete FepA amino acids 202 to 340, 205 to 339, 57 to 142, and 605 to 706, respectively. They remove 112, 108, 58, and 47, respectively, of the 381 predicted surface residues of FepA (*7*). In ΔH261, ΔMC, and ΔRV, only two of the 29 putative transmembrane strands of FepA are completely eliminated; five are eliminated in ΔA306. The peptides created by the ΔH261 and ΔRV junctions have sufficient hydrophobicity and amphilicity for localization in the bilayer (*7*).
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11. FepA and FepA deletion mutants were expressed from pUC18 plasmid vectors (*7, 10*). Although these are high copy number plasmids, the biosynthesis and insertion of FepA into the outer membrane was similarly regulated in all of the strains of interest (Figs. 1 and 2) and did not exceed the expression observed in wild-type *E. coli* with a chromosomal *fepA*$^+$ gene.
12. ΔA306 showed enhanced sensitivity to rifampin as well as some uptake of siderophores at high concentrations, which suggests that the *fepAΔA306* mutation may also expose the FepA pore although to a lesser extent than *fepAΔMC* and *fepAΔRV*.
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15. We cannot exclude the alternative explanation that the deletions alter the thermodynamic equilibrium between the receptor and its membrane environment, inducing NH_2-terminal conformational changes that accommodate this perturbation. The fact that the NH_2-terminal topology of ΔA306 is disrupted by the COOH-terminal mutation (the majority of the epitopes in the receptor's ligand-binding domain are inaccessible to MAb recognition) may support this view. The gross structural changes seen in ΔA306 probably explain why it does not bind ferric enterobactin.
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19. KDF541 was derived from RWB18-60 (*pro, leu, trp, entA, ΔrecA, ΔfepA*) (*10*) by sequential selection for resistance to colicin Ia (*cir*) and bacteriophage T5 (*fhuA*). Hybridization analysis of RWB18-60 shows no evidence of *fepA* sequence (M. A. McIntosh, unpublished data).
20. We created KDF571 by selecting for simultaneous resistance of RWB18-60 to colicins Ia and M. Putative *tonB* mutants were transformed with pRZ540 [*tonB*$^+$; K. Postle and W. S. Reznikoff, *J. Mol. Biol.* **131**, 619 (1979)] and tested for sensitivity to colicins Ia and M and uptake of ferrichrome (*18*). To generate *cir* and *fhuA* markers, which were confirmed by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) of mutant cell envelopes, we sequentially selected the *tonB*/pRZ540 strains for resistance to colicin Ia and bacteriophage T5. The resultant strain, KDF570/pRZ540 (*pro, leu, trp, B1, entA, ΔrecA, ΔfepA, cir, fhuA, tonB/tonB*$^+$) was cured of pRZ540 with acridine orange [J. H. Miller, Ed., *Experiments in Molecular Genetics* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1978), p. 104] and transformed with the desired pUC18 derivatives.
21. MAb 29 binding to bacteria that expressed FepA was not seen in other studies (*7*). However, in this study we used KDF669, a nonreverting *rfa*::transposon *5* (Tn*5*) mutant that produces a deep rough lipopolysaccharide core, as host for the various plasmids. In this background, *fepA*$^+$ cells were weakly fluorescent, which indicates that the epitope recognized by MAb 29 is accessible on the cell surface but lies deep within the core sugars.
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DNA Polymerase β and DNA Synthesis in *Xenopus* Oocytes and in a Nuclear Extract

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The identities of the DNA polymerases required for conversion of single-strand (ss) M13 DNA to double-strand (ds) M13 DNA were examined both in injected *Xenopus laevis* oocytes and in an oocyte nuclear extract. Inhibitors and antibodies specific to DNA polymerases α and β were used. In nuclear extracts, inhibition by the antibody to polymerase β could be reversed by purified polymerase β. The polymerase β inhibitors, dideoxythymidine triphosphate (ddTTP) and dideoxycytidine triphosphate (ddCTP), also blocked DNA synthesis and indicated that polymerase β is involved in the conversion of ssDNA to dsDNA. These results also may have particular significance for emerging evidence of an ssDNA replication mode in eukaryotic cells.

Xenopus laevis oocytes and eggs have been used to study eukaryotic transcription, translation, intracellular transport and localization of molecules, DNA replication (*1*), and DNA repair (*2, 3*). We used *Xenopus* oocytes to study the mechanisms of DNA replication. An extensive component of genomic DNA replication in *Xenopus* embryos appears to be conversion of long segments of ssDNA to semiconservatively replicated dsDNA molecules (*4*). Thus, we used ssM13 DNA molecules as a model for conversion of ssDNA to dsDNA (*5*) in oocytes and in an oocyte nuclear extract. This DNA synthesis appears to be dependent on the activity of DNA polymerase β and the activity of DNA polymerase α; DNA polymerase δ or ε or both may also be required. Polymerase β is a highly conserved DNA polymerase in vertebrates (*6*), generally considered to have a role in gap-filling DNA synthesis in DNA repair (*7*). Recently, polymerase β was shown to substitute for DNA polymerase I in the joining of Okazaki fragments during DNA replication in *Escherichia coli* (*8*).

Model DNA replication systems that use small ds viral DNA molecules (*9*) do not appear to depend on DNA polymerase β because the systems can be reconstituted with purified proteins in the absence of DNA polymerase β. Other replication models (*10*), however, are important to

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consider because genomic DNA replication in *Xenopus*, for example, does not appear to occur exclusively by the bidirectional replication bubble mechanism observed in these viral DNA replication systems (*4*).

Xenopus laevis oocyte nuclei and whole-cell extracts derived from *Xenopus* eggs (*11*) or oocytes (*12*) efficiently synthesize the complementary strand of an ssDNA, whereas only eggs and egg extracts are able to replicate dsDNA (*5*). Our findings demonstrate that in *Xenopus* oocytes and in a nuclear extract polymerases α and β are required for the synthesis of the complementary strand.

Aphidicolin inhibited DNA synthesis on ssM13 DNA in oocytes (Fig. 1A), suggesting that at least polymerase α, δ, or ε (*13*) was required for ssM13 DNA replication. Monoclonal antibodies (MAbs) to human KB cell DNA polymerase α, SJK 132-20 (*14*), and to calf polymerase α (*15*) were then used to determine whether polymerase α was involved (*13*). Both MAbs to polymerase α inhibited DNA synthesis (Fig. 1B). Polyclonal antibody specific to rat polymerase β also abolished DNA synthesis in injected oocytes (Fig. 1B).

We analyzed the early replication products synthesized in oocytes injected along with aphidicolin or antibody to polymerase β by alkaline agarose gel electrophoresis (Fig. 1C). We conclude that polymerases α and β are required for different aspects of DNA synthesis. Polymerase α is required for the earliest events (production of shorter intermediates), and polymerase β is required at a later step.

An oocyte nuclear extract with added deoxynucleotide triphosphates (dNTPs), ribosomal nucleotide triphosphates (rNTPs), and adenosine triphosphate (ATP) (Fig. 2A) also converts ssM13 to dsM13. Alkali or ribonuclease H treatment of ssM13 to remove contaminating short ribonucleotide primers (*11*) before injection (Fig. 1) or before incubation of ssM13 with extract had no effect on subsequent DNA synthesis incubation once dNTPs and rNTPs were added. We eliminated DNA synthesis by the addition of 0.1 mM aphidicolin (Fig. 2A) or by the addition of antibodies to polymerase α or β (Fig. 2B). The antibodies to human DNA polymerase α or to rat polymerase β more effectively inhibited

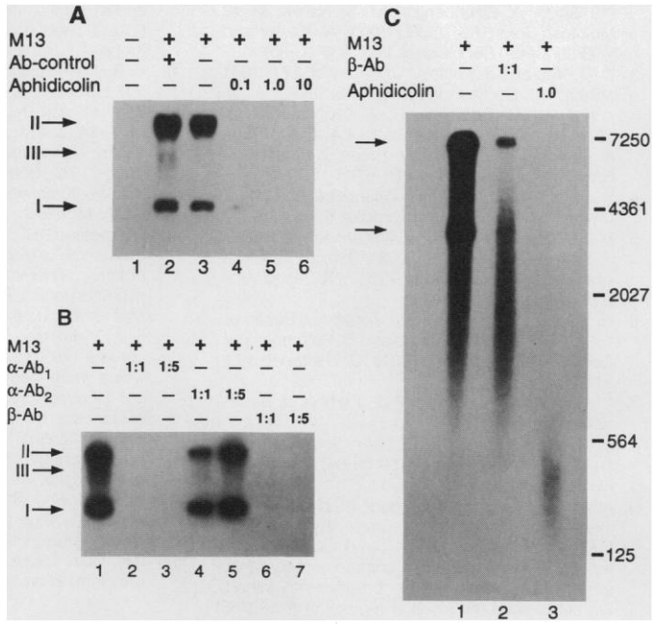

Fig. 1. Antibodies to DNA polymerases α or β inhibit replication of ssDNA in oocytes. Aphidicolin (indicated in milligrams per milliliter) or antibodies (indicated by dilution) were simultaneously injected with 2.5 ng of ssM13 and α-^{32}P–labeled dCTP (*22, 23*); incubation was for 5 hours. We then extracted the DNA (*3*) and analyzed it by electrophoresis on a 1% agarose tris-borate EDTA (TBE) gel. (**A**) Lanes 1 to 6 as indicated; Ab-control in lane 2 was undiluted rat immunoglobulin M (IgM) pre-immune control antibody (*15*). Forms I, II, and III DNA are indicated. (**B**) The α-Ab_1 in lanes 2 and 3 was IgG MAb (SJK 132-20) to KB cell DNA polymerase α (*14*) diluted as indicated; α-Ab_2 in lanes 4 and 5 was IgM MAb to calf thymus DNA polymerase α (*15*) diluted as indicated; β-Ab in lanes 6 and 7 was polyclonal Ab to rat DNA polymerase β diluted as indicated. (**C**) The ssM13 DNA and label were injected into oocytes as described in (A) and were incubated for 3 hours. Recovered DNA was separated by electrophoresis on a 1% alkaline agarose gel, and the resulting autoradiograph was intentionally overexposed to detect the products formed. Lane 1, ssM13; lane 2, ssM13 plus 1 μl of antibody to DNA polymerase β diluted 1:1; and lane 3, ssM13 plus aphidicolin at 1 mg/ml. Molecular weight markers are indicated in base pairs. The top arrow indicates the position of full-length linear ssM13, and the lower arrow indicates the position of circular M13 DNA.

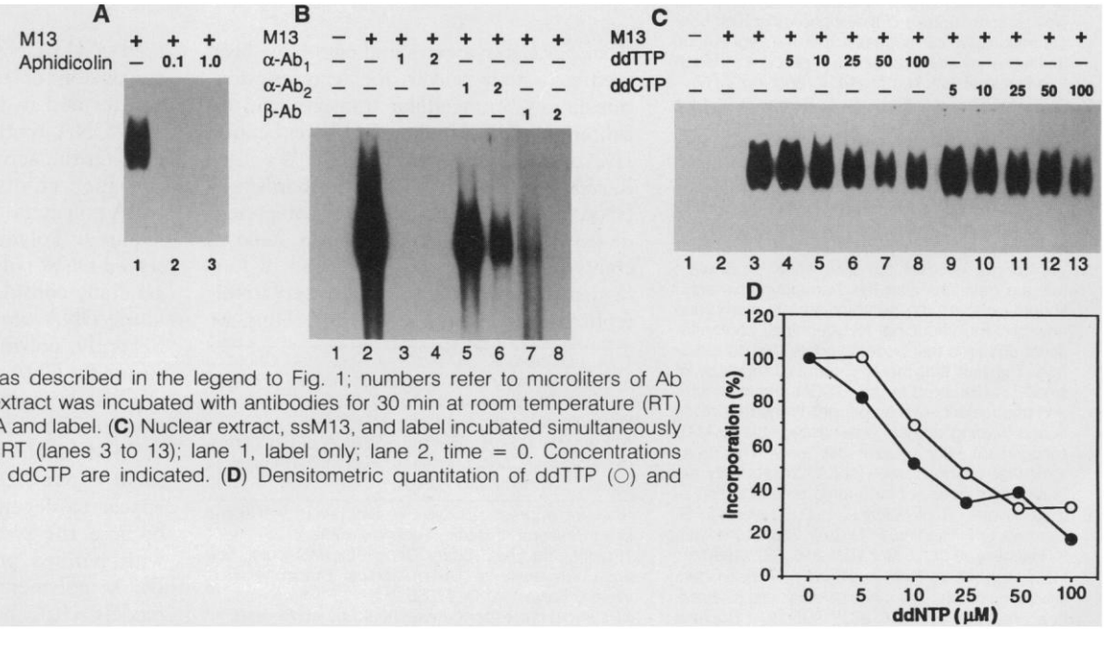

Fig. 2. Antibodies to DNA polymerases α or β inhibit replication of ssDNA in an oocyte nuclear extract (*24–26*). Although a mixture of replication products was observed on native agarose gels, most of the DNA labeled by incorporation of α-[^{32}P]dCTP comigrated with full-length DNA on alkaline agarose gels (*27*). (**A**) Aphidicolin concentration in reaction indicated in milligrams per milliliter. (**B**) Antibodies are as described in the legend to Fig. 1; numbers refer to microliters of Ab added to reaction. Nuclear extract was incubated with antibodies for 30 min at room temperature (RT) before addition of ssM13 DNA and label. (**C**) Nuclear extract, ssM13, and label incubated simultaneously with ddNTPs for 3 hours at RT (lanes 3 to 13); lane 1, label only; lane 2, time = 0. Concentrations (micromolar) for ddTTP and ddCTP are indicated. (**D**) Densitometric quantitation of ddTTP (○) and ddCTP (●) data from (C).

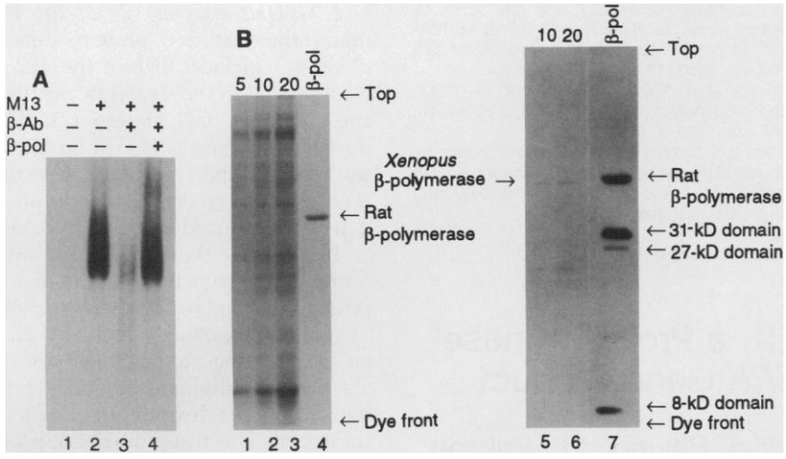

Fig. 3. (**A**) Inhibition of replication by DNA polymerase β antibody (β-Ab) can be reversed by the addition of exogenous polymerase (β-pol). All samples were incubated 3 hours after addition of label and ssM13. Lane 1, no ssM13; lane 2, ssM13; lane 3, incubation of β-Ab and extract for 20 min RT, after which label and ssM13 were added; and lane 4, incubation of β-Ab and extract for 20 min RT, after which 0.3 μg of purified β-pol was incubated 20 min RT, followed by label and ssM13. Addition of polymerase β without extract did not support DNA synthesis (*27*). (**B**) The antibody to rat DNA polymerase β reacted with partially purified *Xenopus* DNA polymerase β but not with purified *Xenopus* DNA polymerase α by protein immunoblot (*27, 28*). We partially purified *Xenopus* DNA polymerase β from liver with procedures established for purification of rat DNA polymerase β—that is, salt extraction and then phosphocellulose and ssDNA agarose chromatography (*29*). The resulting partially purified polymerase fraction was analyzed by SDS–polyacrylamide gel electrophoresis (PAGE) and was stained with Coomassie blue. Lanes 1 to 3 contained 5, 10, and 20 μg of protein, respectively, and lane 4 contained 3 μg of purified rat DNA polymerase β. Protein immunoblot analysis of partially purified *Xenopus* polymerase β (lanes 5 to 7); lanes 5 and 6 correspond to the 10 and 20 μg of protein used in lanes 2 and 3; lane 7 contains a mixture of the well-characterized proteolytic domain fragments of rat polymerase β (*29*) as reference. Nonimmune IgG did not show bands (*27*).

DNA synthesis than did the antibody to calf thymus polymerase α in the nuclear extract (Fig. 2B), which is identical to results obtained in the living cell (Fig. 1B). Therefore, DNA polymerases α and β appear to be required for synthesis of the complementary strand of ssM13 in both oocytes and in the nuclear extract.

We then determined whether this DNA synthesis was sensitive to dideoxynucleotides (ddNTPs), which selectively inhibit polymerase β at appropriate concentrations (*16*). Either 2′,3′-dideoxycytidine 5′-triphosphate (ddCTP) or 2′,3′-dideoxythymidine 5′-triphosphate (ddTTP) was added to the extract along with ssM13 and α-^{32}P–labeled dCTP (Fig. 2C). Both ddCTP and ddTTP inhibited DNA synthesis under conditions where *Xenopus* DNA polymerase α is not inhibited (*17*), implicating DNA polymerase β in the conversion of ssM13 to dsM13. We obtained further evidence that polymerase β activity is required in experiments in which we reversed the inhibitory effect of the antibody to polymerase β (Fig. 3) by the addition of purified recombinant rat DNA polymerase β (*18*). These results demonstrate that the antibody-induced inhibition is due to a specific interaction between the antibody and polymerase β rather than to a nonspecific inhibitory component in the antibody preparation.

DNA replication mediated by non-fork mechanisms is known for some viral systems (*9*), such as adenovirus. It has been suggested (*19*) that a complete description of DNA replication in higher eukaryotic cells will eventually include a role for ssDNA intermediates. There is evidence for strand separation that is not fork-driven in higher eukaryotic DNA replication, and replication in *Xenopus* may involve synthesis from extensive ssDNA regions (*4*). Long regions of ~100-kb ssDNA were isolated from human cells (*20*); these molecules may have been derived from even larger ss or partly ss molecules because ssDNA is sensitive to shearing during isolation. Furthermore, the ssDNA was found primarily in S phase cells and was proposed to represent the displaced strands of parental DNA in asymmetric DNA synthesis (*20*).

Xenopus oocytes efficiently synthesize the complementary strand of injected ssDNA. Our results suggest that polymerase β is involved in ss to ds conversion, and they do not support the possibility that polymerase β is an inactive member of a large complex of DNA replication proteins. Polymerase β may be required in the final steps of genomic DNA replication for gap-filling DNA synthesis after primer removal (*21*). Our in vitro complementation system may be useful for analysis of accessory DNA replication proteins and domain fragments of polymerase β or modified versions of the enzyme obtained by mutagenesis.

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23. Injections and materials as described (*3*). Aphidicolin injected at 0.1 mg/ml corresponds to 5.9 pmol per oocyte (~10^{12} molecules per oocyte), 0.01 mM in the oocyte, or 0.15 mM in the nucleus. Antibodies to human DNA polymerase α cross-react with purified *Xenopus* DNA polymerase α (*27*). We showed that the antibodies were free of nuclease activity by incubating with labeled dsM13 4 hours at 37°C and then by gel electrophoresis analysis (*27*).
24. We prepared oocyte nuclei as described (*25*), except that we substituted J-buffer (*26*) for storage buffer. Each replication reaction (30 μl) used 0.1 μg of ssM13, 2.5 μCi of α-^{32}P–labeled dCTP,

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,

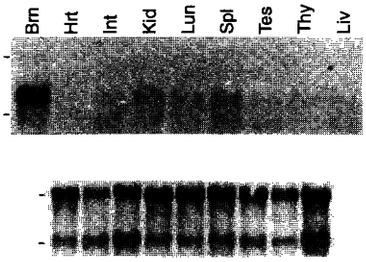

Fig. 1. Tissue distribution of MEK expression. Total RNA (10 μg) from various C57/Bl mouse tissues were Northern blotted and probed with oligonucleotide-labeled full-length MEK cDNA (*25*). Brn, brain; Hrt, heart; Int, intestine; Kid, kidney; Lun, lung; Spl, spleen; Tes, testis; Thy, thymus; and Liv,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

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