

Cloning and characterization of mouse extracellular-signal-regulated protein kinase 3 as a unique gene product of 100 kDa

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MAP (mitogen-activated protein) kinases are a family of serine/threonine kinases that have a pivotal role in signal transduction. Here we report the cloning and characterization of a mouse homologue of extracellular-signal-regulated protein kinase (ERK)3. The mouse *Erk3* cDNA encodes a predicted protein of 720 residues, which displays 94% identity with human ERK3. Transcription and translation of this cDNA *in vitro* generates a 100 kDa protein similar to the human gene product ERK3. Immunoblot analysis with an antibody raised against a unique sequence of ERK3 also recognizes a 100 kDa protein in mouse tissues. A single transcript of *Erk3* was detected in every adult mouse tissue examined, with the highest expression being found

in the brain. Interestingly, expression of *Erk3* mRNA is acutely regulated during mouse development, with a peak of expression observed at embryonic day 11. The mouse *Erk3* gene was mapped to a single locus on central mouse chromosome 9, adjacent to the *dilute* mutation locus and in a region syntenic to human chromosome 15q21. Finally, we provide several lines of evidence to support the existence of a unique *Erk3* gene product of 100 kDa in mammalian cells.

Key words: chromosomal localization, gene cloning, mitogen-activated protein kinase, protein kinase.

INTRODUCTION

Eukaryotic cells have the ability to respond to a wide variety of extracellular signals through the activation of specific transduction pathways. Among them are the extensively studied MAP (mitogen-activated protein) kinase pathways, which are involved in a vast repertoire of responses, including cell proliferation and differentiation, adaptation to environmental stress, apoptosis and embryonic morphogenesis (reviewed in [1–3]). MAP kinase pathways are organized into a three-kinase module architecture, which transmits signals by sequential phosphorylation and activation of the specific components of the module. Many components of these cascades have been conserved from yeast to metazoan cells.

In mammalian cells, six MAP kinase modules have been identified so far. The best described are the ERK (extracellular-signal-regulated protein kinase) pathway, which regulates cell growth and differentiation, and the JNK (c-Jun N-terminal kinase) and p38 pathways, which are associated mainly with the response to stress and inflammation [1–5]. Other protein kinases with high similarity to ERK1/2 have been identified, which define three distinct subfamilies of MAP kinase. These include ERK3 [6–8] and p63^{MAPK} [9,10], ERK5 [11,12] and ERK7 [13]. Much less is known about the regulation and functions of these protein kinases. ERK3 was first cloned from a rat cDNA library as a 543-residue protein with a predicted molecular mass of 62 kDa [6]. The ERK3 protein is approx. 50% identical with ERK1/2 within the kinase catalytic domain and has similar lengths of inserts between conserved subdomains. However, one important structural feature that distinguishes ERK3 (and p63^{MAPK}) from the other MAP kinases is the presence of an SEG motif (one-letter amino acid codes) instead of the highly conserved TXY motif within the activation loop. It has been shown that ERK3 autophosphorylates *in vitro* and is phosphorylated *in*

in vivo on Ser-189 [14,15], the residue equivalent to the activating phosphorylation site Thr-183 in ERK2. Subsequently, two groups isolated a cDNA encoding a human homologue of ERK3 [7,8]. Sequence analysis revealed that human and rat ERK3 are 92% identical over their shared length but the human protein contains a unique C-terminal extension of 178 amino acids. Translation of the human *ERK3* cDNA *in vitro* generates a protein of approx. 100 kDa [7]. Intriguingly, the human and rat ERK3 sequences display high nucleotide similarity (approx. 82%) at their C-terminus, despite the fact that the two open reading frames are not parallel. The relationship between the two ERK3 orthologues has remained obscure and it has been hypothesized that multiple ERK3-related genes might exist or that the *Erk3* gene is alternatively spliced to generate two protein products [7,14].

Here we report the cloning and initial characterization of a mouse ERK3 homologue. The mouse ERK3 is a 720-amino acid protein that exhibits high identity to the human enzyme. The expression of ERK3 varies significantly in adult mouse tissues and is acutely regulated during mouse embryonic development. We now provide compelling evidence for the existence of a single gene product ERK3 of approx. 100 kDa.

EXPERIMENTAL

Isolation and sequencing of murine *Erk3* cDNA clones

A mouse pituitary ATt-20 cDNA library constructed in λ gt11 vector [kindly provided by Dr Jacques Drouin (IRCM, Montréal, Canada)] was screened with a 198 bp *AccI* fragment derived from the first exon of the murine *Erk3* gene (B. Turgeon and S. Meloche, unpublished work) as probe. Hybridization and washing of the nylon filters were performed as described previously

Abbreviations used: ERK, extracellular-signal-regulated protein kinase; MAP, mitogen-activated protein; RT, reverse transcription.

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The nucleotide sequence data reported will appear in GenBank Nucleotide Sequence Database under the accession number AF132850.

A

	GAAACACAGCGACAAGGAAGCGAGG	25
AAGCAGAGGGAAGTGTCTCCAGCGCCTTAGGATGCTGCCTCAGGACCCAGTCCAACACTGAATGTATCTGCACCTGTGAGAGGATGCCCGGAGGCGCTGCCGGCGCATATTTATTCAC		145
AGTTTTGTCCCATGTTAAGTCGGTTAGCATAGTAAATCTGAGTGCATACTATGTCATTTTCATCCGTTTGGAGTTTCTTTGAGTGTTCCTTTAAATGTCGAGAGTTGCTGCCCTTCCCTT		265
GAACATATGAGCACTGCAATCTTCTTAATCTCAGTATGAATAGAGATTTTGGAGCTTAAAGTCTGAGGGGAACTCAGCGGCGCTGGTTGGCGTCTGCAATGAACATCAAGAAACCATCGT		385
GCCGTGGGAACTGATGCTTTCTCCCGTTTGGAGAGATCTTCTCTTGGTCCAGTTTCTCCCTTGTACACAAGTCAACAGTTGAAAGGAAAAGGCAATTGTAAGGGTTTCAAAA		505
ATGGCAGAGAAATTCGAAAGTCTCATGAACATTCATGGCTTTGATCTGGGCTCTAGGTACATGGACTTAAACCATTGGGCTGCCGAGGCAATGGCTTGGTCTTTCTGTGTAGACAAT		625
M A E K F E S L M N I H G F D L G S R Y M D L K P L G C G G N G L V F S A V D N	I	40
GACTGTGACAAAAGAGTAGCCATCAAGAAAATTGCTCACCAGTCCCGAGAGTGCACAACTGCTCTCCGTGAAATCAAAATTTATTCGAAGACTTGACCACGATAACATTTGTGAAAGTG		745
D C D K R V A I K K I V L T D P Q S V K H A L R E I K I I R R L D H D N I V K V	II III IV	80
TTTGAATTTCTGGTCCCAGTGAAGCCAGTTAACAGACGATGTGGGCTCTCTCAGGAGCTGAATAGCGTCTACATTGTTTCAGGAGTATATGGAGACAGACTTGCGGAATGTCTGGAG		865
F E I L G P S G S Q L T D D V G S L T E L N S V Y I V Q E Y M E T D L A N V L E	V	120
CAGGGCTCTTTACTGGAAGAGCATGCCAGGCTTTTCATGTATCAGTCTGCTACGTTGGGCTCAAATATATCCATTCTGCAAAACGTACTGCACAGAGATCTCAAGCCAGCTAATCTTTTCATT		985
Q G S L L E E H A R L F M Y Q L L R G L K Y I H S A N V L H R D L K P A N L F I	VIa VIb	160
AACACTGAAGACTTGTGTCTGAAGATAGGTGACTTTGGCCTGGCAGGATCATGGATCTCTATTTCCATAAGGGTCACTTTCTGAAGGATTGGTTACCAAAATGGTACAGATCTCCA		1105
N T E D L V L K I G D F G L A R I M D P H Y S H K G H L S E G L V T K W Y R S P	VII VIII	200
CGGCTTTTACTTTCTCTTAATAATACACTAAAGCCATTGACATGTGGCTGCAGGCTGCATTTTGTCTGAGATGCTGACTGGTAAACCCTCTTTGCAAGTGCACATGAACATGAACAG		1225
R L L L S P N N Y T K A I D M W A A G C I F A E M L T G K T L F A G A H E L E Q	IX	240
ATGCAGCTGATCTCGACTCCATCCCTGTTGTCACGAGGAGGATCGGCAGGAGCTTCTCAGCGTATCCAGTTTACATTTAGAAAACGACATGACTGAGCCACACAGCCGCTCACTCAG		1345
M Q L I L D S I P V V H E E D R Q E L L S V I P V Y I R N D M T E P H R P L T Q	X	280
CTGCTGCCGGGATCAGCCGAGAAGCACTGGATTTCCTGGAGCAGATTCTGACTTTTCAGCCCATGGACCGGCTGACAGCTGAGGAAGCCCTTTCCCATCTTACATGAGCATCTACTCC		1465
L L P G I S R E A L D F L E Q I L T F S P M D R L T A E E A L S H P Y M S I Y S	XI	320
TTCCGACGGATGAGCCATCTCAAGCCACCCTTTCCACATAGAGGACGAAGTGGACGACATTTTGTCTATGGATGAAACACACAGTCAACATTTATAACTGGGAAAGGTACCAGATTGT		1585
F P T D E P I S S H P F H I E D E V D D I L L M D E T H S H I Y N W E R Y H D C		360
CAGTTCTCGGAGCATGACTGGCTATTTCATAAACACTTTGATATCGATGAGGTGCAGCTTTCAGCCAAAGAGCTCTGTCTGACGTCACCGATGAAGAAGAAGTCCAAGTTGATCTCGAAAA		1705
Q F S E H D W P I H N N F D I D E V Q L D P R A L S D V T D E E E V Q V D P R K		400
TACTTGGATGGAGACCGGAGAAGTATCTAGAGGATCCCGCTTTCGACACAGCTACTCTGCTGAACCTGTGCGAGTACCAGATCACCACGAGAACAAGTACTGCGATCTGGAGTGT		1825
Y L D G D R E K Y L E D P A F D T S Y S A E P C W Q Y P D H H E N K Y C D L E C		440
AGCCACACCTGTAACATAAAAACAAGATCATCACCACTATTAGATAAAGTGGTGGAGGGGAGCGAGGTTAACCATTTACTACGAGCCCAAGCTTATTATAGATCTTTCCAAGTGGAAA		1945
S H T C N Y K T R S S P Y L D N L V W R E S E V N H Y Y E P K L I I D L S N W K		480
GAGCAAAGTAAAGAAAATCCGATAAGAGAGGCAAGTCCAAGTGTGAGAGGAACCGGTTGGTCAAGCGCGAGATCGCGCTAGAGGAAGCATCCAGCAGCTGGCTGAGAGGGAGAGGGGC		2065
E Q S K E K S D K R G K S K C E R N G L V K A Q I A L E E A S Q Q L A E R E R G		520
CAAGGCTTCGACTTTGACTCTTCATCGCGGGGACCATTCAGCTCAGTCCCGCAGCATCAGTCTGCTGACGTAGTTGACAAGTTAAACGACTTGAATAGCTCAGTGTCCAGCTAGAATTG		2185
Q G F D F D S F I A G T I Q L S A Q H Q S A D V V D K L N D L N S S V S Q L E L		560
AAAAGCCTGATATCCAAGTCACTCAGCCGAGAAAAGCAAGAAAAGGGAAGGGCTAACCTGGCCAGCTGGGAGCCTGTACCAGTCTCTGGGACAGCCAGTTTGTGAGTGGCGGGGAG		2305
K S L I S K S V S R E K Q E K G R A N L A Q L G A L Y Q S S W D S Q F V S G G E		600
GAGTCTTCTCATCAGTCACTTTTGTGTGAGGTCAGGAAGGACGAGCATGCGGAGAAGGAGAACACCTTACACCAGCTATTTGGACAAGTTTGTAGCAGGAAGGAGGATTCGGAATG		2425
E C F L I S Q F C C E V R K D E H A E K E N T Y T S Y L D K F F S R K E D S E M		640
CTAGAACTGAGCCAGTGGAGGAAGGGAAGCGTGGGGAGAGGCGGTGAGGACGGGCTTCTGAGCGCGGTGGGGAGTTCCCTCCGAGCAAGCAGCTGGAGTCCATAGGCACCCCGCAG		2545
L S T E P V E E G K R G E R G R E A G L L S G G G E F L L S K Q L E S I G T P Q		680
TTCCACAGCCAGTGGGTTCCCACTCAAGTCCATCCAGGCCACCTTTCAGCTTCCGCTATGAAATCTTCCCTCAAATCCCTCACAGACATACAGCAGCATTTGAAACATCTGAAC		2665
F H S P V G S P L K S I Q A T L T P S A M K S S P Q I P H K T Y S S I L K H L N		720
TAAACCTCAGCAGACAGTCG		2685

Figure 1 For legend see facing page.

[16]. Positive clones were plaque-purified and the insert cDNA species were subcloned into pBK-CMV vector (Stratagene). The nucleotide sequences of the clones were determined with a

Thermo Sequenase Cycle sequencing kit (Amersham Pharmacia). Sequence data were compiled and analysed with GeneWorks software (IntelliGenetics).

B

Human p63 ^{MAPK}	MAEKEDCIASVYSGDLGREFVDFQPLGFGVNGLVLSAVDNRACRKRVAKKIKVLTDPQSVKHALREIKIIRRLDHDNIVKVFEILGPSGSQLTDDVGSGLTE	98
Human ERK3	MAEFESLMNIHGFDLGSRYMDLKPLGCGGNGLVFSAVDNDCKRVAIKKIVLTDPOQSVKHALREIKIIRRLDHDNIVKVFEILGPSGSQLTDDVGSGLTE	100
Mouse ERK3	MAEFESLMNIHGFDLGSRYMDLKPLGCGGNGLVFSAVDNDCKRVAIKKIVLTDPOQSVKHALREIKIIRRLDHDNIVKVFEILGPSGSQLTDDVGSGLTE	100
Rat ERK3	MAEFESLMNIHGFDLGSRYMDLKPLGCGGNGLVFSAVDNDCKRVAIKKIVLTDPOQSVKHALREIKIIRRLDHDNIVKVFEILGPSGSQLTDDVGSGLTE	100
Human p63 ^{MAPK}	--SVAIVIQEYMETDLARILEQGITLPEEHAKLFMYQLLRGLKYIHSANVLRDLKPANLFINTEDELVLKIGDFGLARIMDPHYSHKGLSEGLVTKWYRSP	197
Human ERK3	LNSVYIVQEYMETDLANVLEQGPLLEEHLRFMYQLLRGLKYIHSANVLRDLKPANLFINTEDELVLKIGDFGLARIMDPHYSHKGLSEGLVTKWYRSP	200
Mouse ERK3	LNSVYIVQEYMETDLANVLEQGPLLEEHLRFMYQLLRGLKYIHSANVLRDLKPANLFINTEDELVLKIGDFGLARIMDPHYSHKGLSEGLVTKWYRSP	200
Rat ERK3	LNSVYIVQEYMETDLANVLEQGPLLEEHLRFMYQLLRGLKYIHSANVLRDLKPANLFINTEDELVLKIGDFGLARIMDPHYSHKGLSEGLVTKWYRSP	200
Human p63 ^{MAPK}	RLLLSPPNNTKAIMDWAAGCIFAEMLTGKTLFAGAHELEQMLILESIIPVVEEDRQELLSVIVPYIRNDMTEPHKPLTQLLPGISREALDFLEQILTF	296
Human ERK3	RLLLSPPNNTKAIMDWAAGCIFAEMLTGKTLFAGAHELEQMLILESIIPVVEEDRQELLSVIVPYIRNDMTEPHKPLTQLLPGISREALDFLEQILTF	300
Mouse ERK3	RLLLSPPNNTKAIMDWAAGCIFAEMLTGKTLFAGAHELEQMLILESIIPVVEEDRQELLSVIVPYIRNDMTEPHKPLTQLLPGISREALDFLEQILTF	300
Rat ERK3	RLLLSPPNNTKAIMDWAAGCIFAEMLTGKTLFAGAHELEQMLILESIIPVVEEDRQELLSVIVPYIRNDMTEPHKPLTQLLPGISREALDFLEQILTF	300
Human p63 ^{MAPK}	PMDRLTAEALSHFYMSIYSFPTDEPISSHPFHIEDEVDILLMDETHSHIYNWE---RYHDCQFSEHDWPIHNNFDIDEVQLDPRALSDVTDEEVQV	394
Human ERK3	PMDRLTAEALSHFYMSIYSFPTDEPISSHPFHIEDEVDILLMDETHSHIYNWE---RYHDCQFSEHDWPIHNNFDIDEVQLDPRALSDVTDEEVQV	396
Mouse ERK3	PMDRLTAEALSHFYMSIYSFPTDEPISSHPFHIEDEVDILLMDETHSHIYNWE---RYHDCQFSEHDWPIHNNFDIDEVQLDPRALSDVTDEEVQV	396
Rat ERK3	PMDRLTAEALSHFYMSIYSFPTDEPISSHPFHIEDEVDILLMDETHSHIYNWE---RYHDCQFSEHDWPIHNNFDIDEVQLDPRALSDVTDEEVQV	396
Human p63 ^{MAPK}	DPKRDSSHSSSRFLEQ---SHSSME--RAF---EADY---GRSGLMKVGSSEVLDKILWRDNKPHYSSEPKLILDLSSHWKQAAGAPPTATGLADTG	479
Human ERK3	DPRKYLGDREKYLEDPADFTNYSFPCWQYSDHHEKCYDLECSHTCNKTRSSVLDNLVWRESEVNHYYEPKLILDLSSHWKQAAGAPPTATGLADTG	496
Mouse ERK3	DPRKYLGDREKYLEDPADFTNYSFPCWQYSDHHEKCYDLECSHTCNKTRSSVLDNLVWRESEVNHYYEPKLILDLSSHWKQAAGAPPTATGLADTG	496
Rat ERK3	DPRKYLGDREKYLEDPADFTNYSFPCWQYSDHHEKCYDLECSHTCNKTRSSVLDNLVWRESEVNHYYEPKLILDLSSHWKQAAGAPPTATGLADTG	496
Human p63 ^{MAPK}	AREDEEASLFLHIAQWVKSTOGAOSTEARPPPTTESAACLERPPPPGPGGRRORPPVRPGRVHLRPEALHOARGPAGO	557
Human ERK3	RNGLVKAQIALEEASQQLAGKEREKNQGFDFDSFIAGTIQLSSQHEPTDVKLNDLNSVSVLELKSLSIKSVSDEKQEKGMANLAQLGALYQSSWDSQ	596
Mouse ERK3	RNGLVKAQIALEEASQQLAERER--GQGFDFDSFIAGTIQLSQAQOSADVVKLNDLNSVSVLELKSLSIKSVSDEKQEKGMANLAQLGALYQSSWDSQ	594
Rat ERK3	RNGLVKAQIALRRLRKRPSWLRGRGAKALTLMPSSQAPFSSVPSVSLLT	543
Human ERK3	FVSGGEDDFEFLIQFC--EVRKDEQVEKENTYTSYLDKFFSRKELTEMLETEPVEVDEGKLGERRGEEGFLNNSGEFLFNKQLESIGHPQFHSVPVGSPLKSIQA	695
Mouse ERK3	FVSGGEECFEFLIQFC--EVRKDEHAEKENTYTSYLDKFFSRKELTEMLETEPVEVDEGKLGERRGEEGFLNNSGEFLFNKQLESIGHPQFHSVPVGSPLKSIQA	694
Human ERK3	TLTPSAMKSSFPQIPHRTYSSILKHLN	721
Mouse ERK3	TLTPSAMKSSFPQIPHRTYSSILKHLN	720

Figure 1 Mouse *Erk3* cDNA encodes a predicted protein of 720 residues highly similar to human ERK3

(A) Nucleotide and deduced amino acid sequence of the mouse *Erk3* cDNA. Stop codons are underlined. Locations of conserved protein kinase subdomains are indicated by roman numerals. The SEG motif is doubly underlined. (B) Comparison of mouse ERK3 with other ERK3 homologues and p63^{MAPK}. The protein sequences were aligned with GeneWorks software. Amino acid residues that are identical between proteins are boxed. SEG and SPR motifs are shaded.

Reverse transcription (RT)-mediated PCR and genomic PCR

Total RNA from rat tissues was isolated by the guanidinium thiocyanate acid extraction procedure [17]. For synthesis of first-strand cDNA, 5 µg of RNA was heat-denatured at 70 °C for 10 min, chilled on ice and added to a reaction mixture containing 50 µM random hexamers, 1 mM dNTPs, 5 mM MgCl₂, 20 units of RNAGUARD (Pharmacia) and 200 units of Moloney-murine-leukaemia virus (MMLV) reverse transcriptase (Gibco BRL) in first-strand buffer (Gibco BRL) in a total volume of 20 µl. The RT reaction was performed at 37 °C for 90 min, followed by heat-inactivation of the enzyme at 94 °C for 10 min. Rat genomic DNA was isolated from cultured rat aortic smooth-muscle cells by standard methods [18].

Erk3 sequences were amplified by PCR with the oligonucleotide primers 5'-GGGACACTGAGCTATTCAAG-3' (forward) and 5'-GTAGCCATACTTGTAAC-3' (reverse) based on the published rat cDNA sequence [6]. RT reaction (5 µl) or genomic DNA (5 µg) was amplified in 100 µl

reaction volume with the GenAmp PCR kit (Perkin Elmer Cetus) in accordance with the manufacturer's instructions. PCR conditions used were: 94 °C for 5 min followed by 30 cycles at 94 °C for 45 s, 52 °C for 30 s and 72 °C for 2 min. Extension of the PCR products was performed at 72 °C for an additional 10 min. The PCR products were purified by agarose-gel electrophoresis and sequenced directly as described above.

Northern and Southern blot analysis

Total RNA from adult mouse tissues and embryos at different developmental stages was prepared as described above. Each RNA (20 µg) was resolved by electrophoresis in a 1% (w/v) agarose gel containing 1.8% (v/v) formaldehyde, then transferred to Hybond-N membrane (Amersham) and hybridized to a ³²P-labelled 0.9 kb *Bam*HI fragment from the mouse *Erk3* cDNA. Hybridization was performed in hybridization buffer [5 × SSC (SSC is 0.15 M NaCl/0.015 M sodium citrate)/0.1% SDS/5 × Denhardt's solution (Denhardt's is 0.02% Ficoll 400/0.02%

polyvinylpyrrolidone/0.002% BSA)/50% formamide/1 µg/ml single-stranded DNA] containing the labelled probe (2×10^6 c.p.m./ml) for 16 h at 42 °C. The membrane was washed at a final stringency of $0.1 \times \text{SSC}/0.1\%$ SDS at 60 °C and analysed by autoradiography.

Mouse genomic DNA (20 µg) was digested with various restriction endonucleases, fractionated on 0.8% agarose gel and transferred to Hybond-N membrane. The membrane was hybridized with a ^{32}P -labelled 200 bp genomic probe corresponding to the 3' exon-intron boundary of the mouse *Erk3* gene exon encoding residues 235–288 of the protein (B. Turgeon and S. Meloche, unpublished work). Hybridization was performed as described above.

Transcription and translation *in vitro*

The full-length mouse and human [8] cDNA species for ERK3 were subcloned in pBK-CMV vector (Stratagene). Transcription *in vitro* was performed with T7 RNA polymerase followed by translation with a rabbit reticulocyte lysate (Promega) in the presence of [^{35}S]methionine. The labelled proteins were resolved by SDS/PAGE, transferred to a nitrocellulose membrane and detected with a PhosphorImager (Molecular Dynamics).

Western blot analysis

Protein extracts of mouse embryos were prepared by homogenization of the whole embryo in Triton X-100 lysis buffer [50 mM Tris/HCl (pH 7.4)/100 mM NaCl/50 mM NaF/5 mM EDTA/0.1 mM PMSF/1 µM leupeptin/1 µM pepstatin A/1% (v/v) Triton X-100] for 1 min at 4 °C. Homogenates were clarified by centrifugation at 13000 g for 10 min. Equal quantities of lysate proteins (100 µg) were resolved by SDS/PAGE [7.5% (w/v) gel] and transferred to Hybond-C nitrocellulose membrane (Amersham). The membrane was blocked for 1 h at 37 °C in Tris-buffered saline [TBS; 10 mM Tris/HCl (pH 7.5)/150 mM NaCl] containing 0.1% (v/v) Tween 20 and 1% (w/v) BSA before incubation at 25 °C for 1 h with anti-ERK3 antibody (sc-156; Santa Cruz Biotechnology) diluted 1:1000 in blocking solution. After extensive washing in TBS/0.1% Tween 20, the membrane was incubated for 1 h at 25 °C with horseradish-peroxidase-conjugated anti-IgG (1:3000 dilution) in blocking solution. Immunoreactive bands were detected by enhanced chemiluminescence (Amersham).

Chromosomal mapping

The mouse *Erk3* gene was mapped by using the Jackson Laboratory interspecific backcross panel (C57BL/6J \times SPRET/Ei) \times SPRET/Ei (Jackson BSS) [19]. A total of 94 backcross progeny were used to map *Erk3* by using an *MspI* restriction-fragment-length polymorphism. The *MspI* digestion identified a 6 kb allele fragment for C57BL/6J DNA and a fragment of 3.6 kb for *Mus spretus* DNA. Mouse genomic DNA digested with *MspI* was analysed by Southern blotting with the genomic probe described above. The presence or absence of the 6 kb C57BL/6J-specific band was scored in backcross mice. The mapping data were analysed by Mary Barter at the Jackson Laboratory.

RESULTS AND DISCUSSION

Molecular cloning of a murine ERK3 homologue

To isolate a mouse homologue of ERK3, we screened a mouse pituitary cDNA library with a 198 bp *AccI* fragment derived

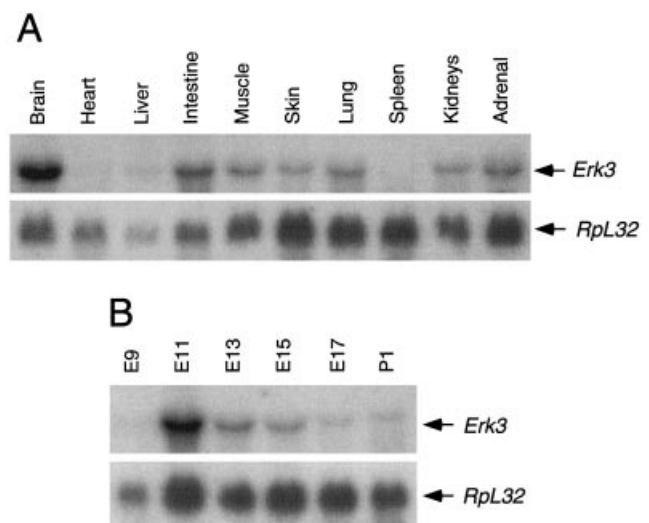


Figure 2 Northern blot analysis of *Erk3* mRNA expression in mouse

Total RNA (20 µg) isolated from various adult mouse tissues (A) and from embryos (B) at different developmental stages [embryonic days (E) 9–17 and postnatal day (P) 1] was analysed by Northern hybridization with a ^{32}P -labelled *Erk3* cDNA probe. RNA levels were normalized by rehybridization of the blot with a ribosomal protein L32 probe. The results are representative of two independent experiments.

from the first exon of the murine *Erk3* gene (B. Turgeon and S. Meloche, unpublished work). Among 10^6 phage plaques, four positive clones were isolated with inserts ranging from 0.8 to 2.6 kb. Partial characterization of these clones confirmed that they were highly similar to previously cloned *Erk3* cDNA species. The largest clone (λMmK3) was characterized further. Nucleotide sequence analysis showed that this clone extends over 2683 nt and contains both initiation and termination codons for translation (Figure 1A). A continuous open reading frame of 2160 nt encodes a deduced protein of 720 residues with a calculated molecular mass of 82.1 kDa (Figure 1A). The coding sequence is preceded by a 505-base 5' untranslated region containing an in-frame stop codon and is followed by a very short 3' untranslated region. Protein sequence alignments revealed that mouse ERK3 shares 94% amino acid identity with human ERK3 and also possesses a C-terminal extension of 177 residues compared with the published rat ERK3 sequence (Figure 1B). Like the human and rat proteins, mouse ERK3 contains the SEG motif in the activation loop and the sequence SPR rather than APE in subdomain VIII, features that distinguish ERK3 from other MAP kinase subfamilies.

Expression of ERK3 in embryonic and adult mouse tissues

The expression pattern of *Erk3* mRNA in various adult tissues and at distinct developmental stages of the mouse was determined by Northern blot analysis. Total RNA was extracted from several mouse tissues and hybridized with a radiolabelled 0.9 kb probe from the 3' end of the mouse *Erk3* cDNA. A single transcript of 4.5 kb was detected in every adult tissue examined but the relative level of expression varied considerably between tissues (Figure 2A). The highest expression was found in the brain.

A developmental study revealed that the expression of ERK3 is acutely regulated during mouse ontogeny. The expression of *Erk3* mRNA increases markedly from embryonic days 9 to 11, followed by a gradual down-regulation up to birth (Figure 2B).

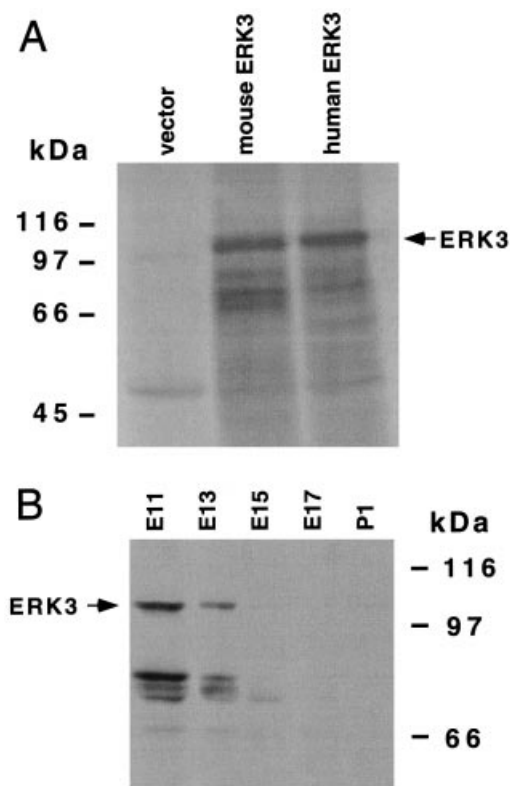


Figure 3 Mouse ERK3 is a unique protein of 100 kDa

(A) Translation of mouse *Erk3* cDNA *in vitro*. The full-length mouse and human [8] cDNA species for ERK3 subcloned in pBK-CMV were translated *in vitro* with a coupled transcription–translation rabbit reticulocyte system in the presence of [³⁵S]methionine. Labelled proteins were resolved by SDS/PAGE, transferred to a nitrocellulose membrane and detected with a PhosphorImager. (B) Immunoblot analysis. Protein extracts (100 µg) of whole mouse embryos at different developmental stages were resolved by SDS/PAGE and electrotransferred to nitrocellulose membrane. The membrane was probed with an ERK3-specific antibody and the proteins were detected by chemiluminescence. The position of ERK3 is indicated. The results are representative of three independent experiments.

These findings are in agreement with previous observations that *Erk3* mRNA is expressed at highest levels in the developing spinal cord and hippocampus of the rat [6]. It has also been shown that *Erk3* transcripts strongly increase on differentiation of P19 embryonal carcinoma cells towards the neuronal or muscle lineage [6]. All these observations support the idea that ERK3 might have unique signalling roles in cell differentiation and development. It is interesting to note that the induction of ERK3 expression, which is detected by days 9–11 after fertilization, is coincident with the period of early organogenesis in the mouse embryo.

ERK3 is a unique gene product of 100 kDa

As mentioned above, two forms of ERK3 have been described so far, a short form of 62 kDa cloned from the rat [6] and a human homologue that encodes a longer protein of approx. 100 kDa [7,8]. We performed a series of experiments to characterize the mouse ERK3 protein and to help resolve the ambiguity about the existence of two putative forms of the enzyme. Transcription and translation of the mouse and human cDNA species for ERK3 *in vitro* generated a specific protein product with an apparent molecular mass of approx. 100 kDa (Figure 3A). To identify ERK3 in intact mouse tissues, we used a commercially

available antibody raised against an internal sequence of ERK3 conserved in mouse, rat and human ERK3, but not in the related protein p63^{MAPK}. Immunoblot analysis of whole embryo extracts detected a protein of 100 kDa, as well as a few other bands that might represent degradation products (Figure 3B). No band was detected at 62 kDa. Similarly, an analysis of cellular extracts from more than 40 human cell lines of diverse histogenesis with the same antibody identified an immunoreactive band at 100 kDa but no 62 kDa protein (P. Coulombe and S. Meloche, unpublished work). The results shown in Figure 3(B) also confirmed that ERK3 protein abundance is maximal at embryonic day 11 and subsequently decreases to very low levels in late embryos and newborns.

Nucleotide sequence alignment of rat, mouse and human cDNA species for ERK3 revealed that the published rat sequence [6] has a missing nucleotide between codons 502 and 503. The addition of a single nucleotide at this position shifts the reading frame of rat ERK3, which now becomes parallel to the mouse cDNA (and human) protein and translates into a protein of 720 residues with 98% overall amino acid identity. To clarify this issue, a pair of primers encompassing the region encoding residues 440–555 of rat ERK3 was used to amplify reverse-transcribed cDNA prepared from rat brain, heart and kidney RNA, as well as rat genomic DNA. A single PCR product of the expected size was obtained with all four templates. Sequence analysis of several PCR products showed that they all contain an additional guanosine residue at codon 503 (Figure 4). The rat *Erk3* gene is therefore unlikely to encode a 62 kDa protein.

In addition to the results presented above, a number of observations are also inconsistent with the hypothesis of two distinct forms of ERK3. Northern blot analysis of RNA from various tissues and cell lines detects only one transcript ([6–8], and this study). The possibility that the human genome contains two ERK3-related genes (other than p63^{MAPK}) is also unlikely because fluorescence *in situ* hybridization analysis with the full-length human ERK3 cDNA revealed a single chromosomal locus [8]. In addition, a recent BLAST search of the dbEST database of the NCBI failed to identify any ERK3-related sequences other than p63^{MAPK}. We conclude from these observations that ERK3 is a unique gene product of 100 kDa.

Chromosomal localization of the murine *Erk3* gene

We have used the interspecific backcross panel BSS from the Jackson Laboratory to determine the chromosomal localization of the gene encoding mouse ERK3. A 200 bp DNA product corresponding to the mouse *Erk3* exon encoding residues 235–288 was used as probe for Southern blot analysis of C57BL/6J and *M. spretus* parental strain DNA. A single hybridization band was detected in each digestion, indicating that the *Erk3* gene is present at a single locus in the mouse genome (Figure 5A). An informative restriction-fragment-length polymorphism was found with this probe in an *MspI* digest (Figure 5A) and was used to type 94 progeny from the Jackson BSS. The position of the *Erk3* gene (approved name *Prkm6*) was unambiguously assigned to central chromosome 9 on the basis of the results of haplotype analysis (Figure 5B). The position and intergenic distances for *Prkm6* relative to other DNA markers on mouse chromosome 9 are shown in Figure 5(C). *Prkm6* co-segregates with previously mapped loci that include *Adam10* and *Myo1e*. *Myo5a*, a marker of the mouse mutation *dilute*, also maps to this region of the Jackson BSS panel. However, this locus presents one double crossover typing that is believed to be a typing error. If the double crossover typing is an error, *Myo5a* co-segregates with *Adam10*, *Myo1e* and *Prkm6*. Comparing the location of

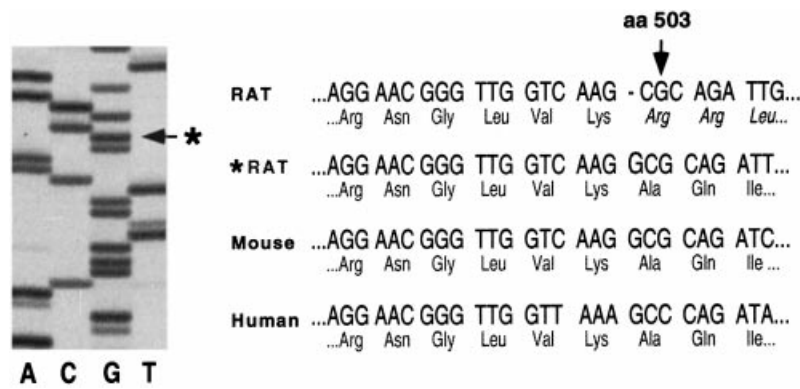


Figure 4 Partial nucleotide sequence of rat *Erk3* cDNA and gene

Total RNA from rat brain, kidney and heart were used as a template for the synthesis of first-strand cDNA with the MMLV reverse transcriptase. Genomic DNA was isolated from rat vascular smooth-muscle cells. This material was amplified by PCR with a combination of primers spanning nt 1710–2056 of rat *Erk3* cDNA sequence [6]. A unique DNA fragment of 345 bp was amplified from each template (results not shown). The PCR products were sequenced directly in the presence of dITP instead of dGTP. Sequences of both *Erk3* cDNA species and gene show the presence of an additional guanosine residue at codon 503 (shown by an asterisk in the left panel) in comparison with the published rat ERK3 nucleotide sequence. The frameshift predicted for this insertion in the rat ERK3 amino acid sequence is shown in the right panel (aa, amino acid). The homologous sequences of mouse and human cDNA species are also shown below.

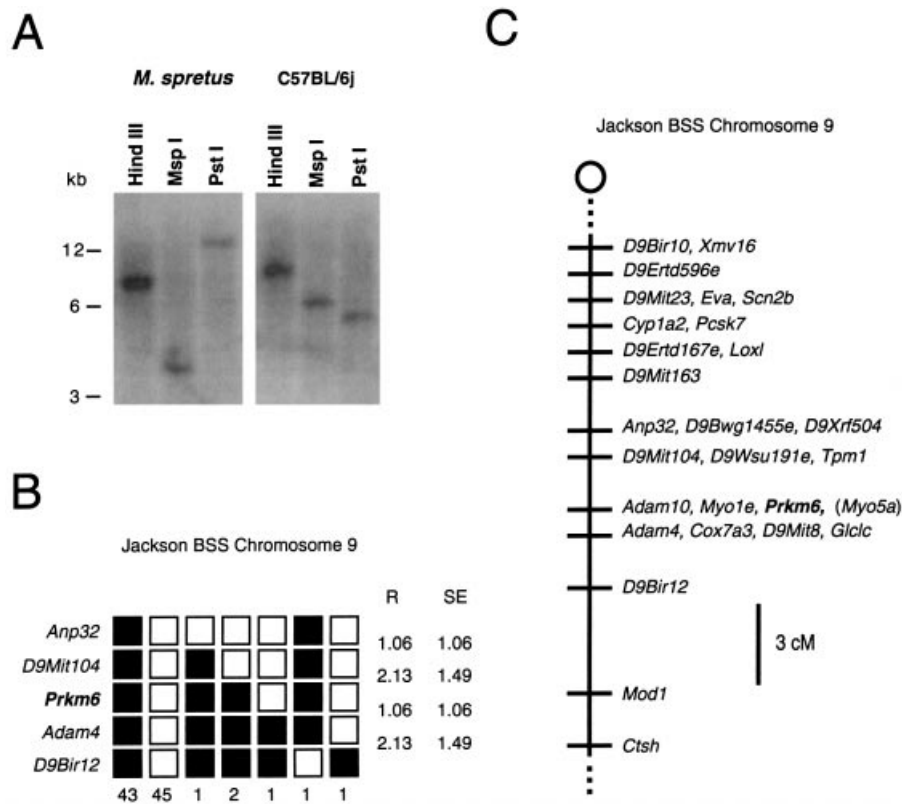


Figure 5 *Prkm6* maps to the central region of mouse chromosome 9

(A) Southern blot analysis of *M. spretus* and C57BL/6J genomic DNA digested with various enzymes. The blot was probed with a 200 bp genomic probe corresponding to the exon encoding residues 235–288 of mouse ERK3. (B) Haplotype figure from the Jackson BSS backcross, showing part of chromosome 9 with loci linked to *Prkm6*. Loci are listed in order, with the most proximal at the top. The black boxes represent the C57BL/6J allele and the white boxes the SPRET/Ei allele. The number of animals with each haplotype is given at the bottom of each column of boxes. The percentage recombination (*R*) between adjacent loci is given to the right of the figure, with the S.E.M. (SE) for each value of *R*. Missing typings were inferred from surrounding data where assignment was unambiguous. (C) Map figure from the Jackson BSS backcross showing part of chromosome 9. The map is depicted with the centromere towards the top. A 3 cM scale bar is shown to the right of the figure. Loci mapping to the same position are listed in alphabetical order. Raw data from the Jackson Laboratory were obtained from its World Wide Web address (<http://www.jax.org/resources/documents/cmdata>). *Myo5a*, listed in parentheses, has one double crossover typing that is believed to be a typing error. The mouse locus symbol *Prkm6* has been approved by the Mouse Nomenclature Committee.

Prkm6 with chromosome 9 data in the Mouse Genome Database (<http://www.informatics.jax.org>) therefore suggests that *Prkm6* is located near the mouse mutation *dilute*. The *dilute* locus is associated with abnormal melanocyte morphology, neurological defects and, in some mutant forms, early death [20]. The human *ERK3* gene has been mapped by fluorescence *in situ* hybridization to chromosome 15q21 [8]; *MYO5A* also maps within this region. Thus mutations at *Prkm6* could contribute to some of the phenotypes associated with the mouse mutation *dilute*.

Note added in proof (received 14 December 1999)

The nomenclature for the MAP kinase family of genes has recently been modified (see <http://www.gene.ucl.ac.uk/users/hester/prkm.html>). The approved name for the mouse gene encoding ERK3 has been changed from *Prkm6* to *Mapk6*.

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