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Mammalian Myosin I α , I β , and I γ : New Widely Expressed Genes of the Myosin I Family

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Abstract. A polymerase chain reaction strategy was devised to identify new members of the mammalian myosin I family of actin-based motors. Using cellular RNA from mouse granular neurons and PC12 cells, we have cloned and sequenced three 1.2-kb polymerase chain reaction products that correspond to novel mammalian myosin I genes designated MMI α , MMI β , and MMI γ . The pattern of expression for each of the myosin I's is unique: messages are detected in diverse tissues including the brain, lung, kidney, liver, intestine, and adrenal gland. Overlapping clones representing full-length cDNAs for MMI α were obtained from mouse brain. These encode a 1,079 amino acid pro-

tein containing a myosin head, a domain with five calmodulin binding sites, and a positively charged COOH-terminal tail. In situ hybridization reveals that MMI α is highly expressed in virtually all neurons (but not glia) in the postnatal and adult mouse brain and in neuroblasts of the cerebellar external granular layer. Expression varies in different brain regions and undergoes developmental regulation. Myosin I's are present in diverse organisms from protozoa to vertebrates. This and the expression of three novel members of this family in brain and other mammalian tissues suggests that they may participate in critical and fundamental cellular processes.

THE myosin I family of proteins, like conventional muscle myosins (myosin II), are actin-activated ATPases that generate mechanochemical force within the cell (Korn and Hammer, 1989; Pollard et al., 1991). Unlike the bipolar filaments of myosin II, myosin I's in solution exist as monomeric globular proteins. All myosin I's identified share two important structural properties: a myosin head that contains sites for ATP and actin binding, and a COOH-terminal domain that binds to membranes through interaction with negatively charged phospholipids (Adams and Pollard, 1989). Since the initial report of myosin I in *Acanthamoeba* (Pollard and Korn, 1973), the number and diversity of myosin I family members have grown. Although myosin I's have been well studied in organisms including *Acanthamoeba* (Jung et al., 1987; Lynch et al., 1989), *Dictyostelium* (Jung et al., 1989; Jung and Hammer, 1990; Titus et al., 1989), and *Drosophila* (Montell and Rubin, 1989; Hicks and Williams, 1992), only a single myosin I gene in vertebrates has been cloned and extensively characterized to date (Hoshimaru and Nakanishi, 1987; Garcia et al., 1989). This species appears to be localized exclusively to the brush border of the small intestine (Hoshimaru et al., 1989; Bikle et al., 1991) and has been designated brush border myosin I (BBMI).¹ Recent biochemical findings have pointed to the presence of additional mammalian myosin I family members (Coluccio, 1991; Barylko et al., 1992).

1. *Abbreviations used in this paper:* BBMI, brush border myosin I; EGL, external granular layer.

Biochemical, immunolocalization, and EM studies have suggested that myosin I's play diverse and important roles within cells (for review see Korn and Hammer, 1990; Hammer, 1991; Pollard et al., 1991; Cheney and Mooseker, 1992). For example, in *Dictyostelium*, myosin I was localized to the leading edge of motile cells and to the phagocytic cup upon ingestion of bacteria (Fukui et al., 1989). Similarly, in *Acanthamoeba*, myosin IC was found both at the plasma membrane and the contractile vacuole (Baines and Korn, 1990), and in chick intestinal microvilli, BBMI forms the crossbridge between the actin bundle core and the plasma membrane (Mooseker and Tilney, 1975). In addition, functional loss of the single myosin II gene in *Dictyostelium* did not cause defects in pseudopod extension, cell motility, phagocytosis, or retrograde movement of membrane antigens (DeLozanne and Spudich, 1987; Knecht and Loomis, 1987; Jay and Elson, 1992), all actin-based activities involving interaction with the plasma membrane that could be directed by myosin I's.

We have approached our studies of myosin I's with an interest in their potential roles within the vertebrate nervous system. Cell migration, neurite outgrowth, and vesicular transport are all processes that might involve myosin I's (Mitchison and Kirschner, 1988; Smith, 1988; Pollard et al., 1991) and that are central to neuronal development. Myosin I-like proteins have been localized to growth cones by immunostaining of a neuronal cell line (Miller et al., 1992) and by immunoblotting of fractions enriched in growth cone membranes (Phelan et al., 1991). To begin to address the is-

sue of the role of myosin I's in the brain, we have devised and implemented a PCR-based strategy to clone and characterize novel myosin I genes expressed in the nervous system.

Portions of this work have been presented previously in abstract form (Sherr and Greene. 1991. *J. Cell Biol.* 115:331a; and Joyce et al. 1992. *Soc. Neurosci. Abstr.* 18:221).

Materials and Methods

Generation of PCR Products

The degenerate primers used in this study and their corresponding conserved amino acid motifs were as follows: 5'-ATATCTAGAAGCTTG-GWGCIGGIAARACNGARGC-3' (GAGKTEA); 5'-ATATCTAG-AAGCTTGARGCITTYGGIAAYGCNAAARAC-3' (LEAFGNAKT); 5'-ATAGAATTCATCGATTTKGGYTTNATRCACCTRATRA-3' (YIR-CIKPN); and 5'-ATAGAATTCATCGATACRTTYTCCAGCAGNCC-CAGRTA-3' (YLGLEENV). The two 5' primers contained XbaI and HindIII restriction sites 5' of the degenerate nucleotides, while the 3' primers included the EcoRI and ClaI sites. The primary templates were total RNA from PC12 cells and P6 mouse cerebellar granular neurons. The secondary template was 1–5 ml of unpurified material from the first amplification. The annealing temperature was 48°C in the first round and 54°C in the second. The remaining conditions were those recommended by the manufacturer (Perkin-Elmer Cetus, Norwalk, CT). Bovine BBMI cDNA (Hoshimaru and Nakanishi, 1987), kindly provided by Dr. S. Nakanishi (Kyoto University, Kyoto, Japan), served as the positive control. PCR products of the predicted size were purified from agarose gels (Geneclean, Bio 101, New York University, New York, NY) and directionally cloned into pGEM7Zf+ (Promega Corp., Madison, WI) at Xba I and EcoRI. Clones were analyzed by direct sequencing and by restriction digestion patterns.

cDNA Library Screening

The PCR product pGMMI α was labeled with [³²P]dCTP by random priming according to the manufacturer's instructions (Boehringer Mannheim Corp., Indianapolis, IN). This probe was used to screen an oligo-dT primed λ gt11 library generated from P6 mouse cerebellar granular neurons, kindly provided by Dr. M. Hatten and Dr. S. Vidan. Clones were purified after three rounds of screening (Sambrook et al., 1989) and subcloned at NotI, SfiI sites into pGEM11Zf+ (Promega Corp.). Three unique clones were identified, λ CGN74a, λ CGN82a, and λ CGN15, containing nucleotides 945–3998, 656–3395, and 1212–3403, respectively. By alignment to BBMI, these clones did not encode ~800 nt of the 5' end of MM1 α . To obtain an overlapping clone from this region, a probe containing a novel 5' sequence was generated by PCR. The 3' primer in this PCR amplification was 5'-CCGAATTCGAAATCCCGCTCCAGC-3', contained within the 5' region of λ CGN82a. The 5' primer, 5'-ACAGAATTCAGCTTGNTT-IGTITCNGTNAANCC-3', was a degenerate DNA sequence of the highly conserved amino acid motif, VLVSVP. The specific and degenerate primers were used at concentrations of 2 and 200 nM, respectively. The PCR product of the predicted size was gel purified and subcloned into pBluescript KS. Sequencing demonstrated 100% overlap with λ CGN82a and high homology with BBMI in the novel 5' regions. This fragment was used to screen a λ gt10 random primed adult mouse brain library, kindly provided by Dr. J. Schlessinger (New York University, New York, NY). Three clones were positive after three rounds of purification. These were subcloned into pBluescript KS at the EcoRI site and analyzed by PCR for the extent of the novel 5' sequence. One clone, λ AMB9d, containing nucleotides –197–845, encompassed the remainder of the coding region.

DNA Sequencing and Analysis

The entire coding region was sequenced from both strands using the dideoxy chain-termination method (Sanger et al., 1977). Fragments were either subcloned at convenient restriction sites or digested with S1 nuclease (Erase-base; Promega Corp.). Junctions were sequenced by priming from complementary oligonucleotides. Contigs were assembled and sequences analyzed using the Genetics Computer Group package version 7 for the VAX (Devereux et al., 1984).

RNA Transfer Analysis

Total RNA was purified from tissues and cell lines according to the procedure of Chomczynski and Sacchi (1987). RNA transfer was performed with standard protocols (Sambrook et al., 1989). Probes were labeled by random priming with [³²P]dCTP as described above.

Cell Cultures and Isolation

P19 teratocarcinoma cells (McBurney and Rogers, 1982), N115 neuroblastoma cells (Schrier et al., 1974), U251 and G24–26 astrocytoma cells (Ponten, 1975; Sundarrag et al., 1975), and C17 cells (Ryder et al., 1990) were grown under standard conditions and harvested directly for the isolation of total RNA. PC12 cells (Greene and Tischler, 1976) were treated with NGF (100 ng/ml) for 5 d before harvesting. Astrocytes were purified from rat P6 cerebellum as described (Hatten, 1985).

Animals and Tissue Preparation

P0, P2, P7, P12, and adult mice were deeply anesthetized with an intraperitoneal injection of an overdose of ketacet. The mice were perfused transcardially with 0.1 M PBS, pH 7.2, prepared with diethyl pyrocarbonate-treated water, followed by 4% buffered paraformaldehyde, pH 7.2, also made with diethyl pyrocarbonate-treated water. The brains were carefully removed and placed in fixative overnight, dehydrated, and embedded in paraffin. 8- μ m sections were cut and collected on silanized slides.

In Situ Hybridization

Digoxigenin-UTP-labeled cRNA probes (Genius 4 digoxigenin-UTP RNA Labeling Kit; Boehringer Mannheim Corp.) were generated from a 388-bp KpnI insert (nucleotides 2053–2440) or a XhoI to EcoRI insert (nucleotides 2850–3638), both cloned into pBluescript KS and transcribed with T3 RNA polymerase. All buffers and diluents were prepared using 0.1% diethyl pyrocarbonate-treated distilled water. Deparaffinized sections were treated with proteinase K (20 μ g/ml in 10 mM Tris, 1 mM EDTA [TE]) for 25 min at room temperature. The sections were rinsed in TE, washed in 2 \times SSC for 10 min, and prehybridized in buffer containing 50% formamide, 20 \times SSC, 2 M Tris, pH 7.5, 50 \times Denhardt's solution, and 0.1 mg/ml salmon sperm DNA for 1 h at room temperature. Sections were incubated with the digoxigenin-UTP-labeled probe (4 μ g/ml prehybridization solution) for 16 h at 42°C. The next day, sections were briefly rinsed in 2 \times SSC and incubated in RNase A (200 μ g/ml) in 10 mM Tris, 500 mM NaCl, and 1 mM EDTA for 15 min at 37°C. The sections were then washed with 2 \times SSC for 30 min at room temperature, twice in 0.5 \times SSC for 15 min at 42°C, once in 1 \times SSC for 1 h at room temperature, and briefly in 100 mM Tris and 150 mM NaCl, pH 7.5 (buffer I). The sections were then incubated in 2% normal sheep serum with 0.3% Triton X-100 in Buffer I for 30 min at room temperature. The sections were then incubated with anti-digoxigenin antibody conjugated to alkaline phosphatase (Boehringer Mannheim Corp.) diluted 1:100 in buffer I with 2% normal sheep serum and 1.25% Triton X-100 overnight at 4°C. The next day the tissue was washed in buffer I three times for 10 min each, followed by a 2-min wash in 100 mM Tris, 100 mM NaCl, and 50 mM MgCl₂, pH 9.5 (buffer III). The reaction product was visualized with 0.023% 4-nitro blue tetrazolium chloride, 0.018% 5-bromo-4-chlor-3-indolyl-phosphate (X-phosphate), and 0.024% levamisole. The blue-brown color reaction product was visible within 1–2 h. The sections were then washed in buffer IV for at least 5 min to stop the reaction. Control conditions included omission of the digoxigenin-UTP-labeled probe, labeled sense RNA, and competition with unlabeled antisense RNA. Sections were coverslipped in Aquamount, Lerner Laboratories (Pittsburgh, PA).

S1 Nuclease Protection

Probes including 46 and 51 nucleotides (MM1 β : 5'-CAAGTAGCTCTG-GGGGTTCCGTTCCAAGCCCAGACGACGAAGAGC-3', MM1 γ : 5'-GACTTCAGTTGTGCCCCACACGGATGTAGTTGTAGGATGAGATGATTT-3') corresponding to stretches within pGMM1 β and pGMM1 γ , respectively, that are of low homology among currently described myosin I genes, were synthesized for hybridization to total tissue RNA. S1 nuclease protection was carried out following a standard protocol as described (Greene and Struhl, 1987). Briefly, 2 nmol of the oligonucleotide probes was end labeled with [³²P] α -ATP by polynucleotide kinase (Boehringer Mannheim Corp.). 1.2 \times 10⁵ cpm for each probe was hybridized with 25 μ g of total RNA overnight at 30°C. 300 U of S1 nuclease (Boehringer

Mannheim Corp.) was added for an additional hour and reactions were stopped by boiling in formamide/EDTA. Samples were analyzed on a 10% polyacrylamide/urea denaturing sequencing gel.

Results

Identification of Novel Mammalian Myosin I Genes

Both preliminary biochemical studies (Phelan et al., 1991) and the presence of multiple myosin I's in lower organisms (Pollard et al., 1991) suggested the existence of a family of mammalian myosin I genes in addition to the well-characterized BBMI. To search for such genes, we devised a two-step nested PCR strategy that was biased against amplification of conventional myosin II sequences. Two pairs of degenerate primers from conserved amino acid sequences within the myosin head domain (GAGKTEA and YL-GLEEN; LEAFGN and YIRCIKP) were used in two sequential reactions. Because we sought to identify myosin I genes expressed in the nervous system, total cellular RNA from PC12 cells and postnatal day 6 mouse cerebellar granular neurons served as the templates for the first reaction. The second amplification was conducted with the nested primers using the unpurified products of the first reaction as the templates. 42 subcloned PCR products (30 from PC12 cells and 12 from granular neurons) were analyzed by restriction fragment patterns and partial sequencing. Three unique PCR products were identified and sequenced. Analysis of the nucleotide and predicted amino acid sequences confirmed that these fragments correspond to novel myosin I genes (Fig. 1 A). The clone derived from mouse cerebellar granular neuron RNA, designated pGMMI α , is 66 and 64% identical (based on the predicted amino acid sequence) to bovine and chicken BBMI, respectively. Over the same stretch, pGMMI α is 38% identical to cardiac muscle myosin. Of the conventional myosin sequences contained in Genbank, this myosin II gene is the most similar to pGMMI α . Clones pGMMI β and pGMMI γ , derived from PC12 cells, are 49% identical to bovine BBMI, but only 37% homologous to the cardiac muscle myosin sequence. Additionally, there is a highly conserved stretch within all described myosin I head domains that distinguishes them from myosin II. Sequences similar to this motif, QA-yaRDALAK.iYYSR (Pollard et al., 1991), are present in all three clones. Clone pGMMI γ , which diverges the most from this motif, is still 73% identical in this stretch.

Cloning and Predicted Primary Sequence of Mammalian Myosin I α

As will be detailed below, using clone pGMMI α as a probe we detected a message with a distinctive pattern of regulation in the developing postnatal mouse brain and therefore chose this clone for further study. Screening an oligo-dT primed cDNA library from postnatal day 6 mouse cerebellar granular neurons yielded three overlapping clones ranging from 2.5 to 3.3 kb. Each of the three clones encodes a stretch of the cDNA from within the consensus myosin head through to the 3' untranslated sequence. Two of these clones, λ CGN74a and λ CGN82a (see Materials and Methods), while identical in the coding region, differ in the 3' untranslated stretch, with λ CGN74a containing an additional 503 nucleotides before the polyadenylation site (Fig. 2).

To obtain a clone encoding the 5' end, we screened a random primed mouse brain library with a PCR product that extended 5' from the cDNA clone λ CGN82a (see Materials and Methods). We isolated a 1.2-kb clone (λ AMB9d) that overlapped with λ CGN82a and extended 850 nucleotides to the 5' end. These overlapping clones contain a single long open reading frame that begins with a methionine codon at the designated nucleotide 1. This sequence ACCAATGG is predicted to be a strong translation initiation site (Kozak, 1989). The only in-frame methionine upstream, at position -17, would constitute a weak initiation codon. Translation of this open reading frame predicts a protein of 1,079 amino acids with a molecular mass of 125,160 D (Fig. 2).

Analysis of the predicted MMI α protein reveals three functional domains that are similar to the BBMIs: a myosin head, a calmodulin binding region, and a COOH-terminal tail with a high net positive charge. All myosin heavy chain proteins share a head domain that contains nucleotide and actin binding sites as well as other conserved residues that are probably necessary for the generation of mechanochemical force (Korn and Hammer, 1988). Analysis reveals that MMI α protein contains such a canonical myosin head (Fig. 2). A sequence that participates in ATP binding, GESGAKTE, is conserved throughout all myosins and is also present in MMI α (Fig. 2). Moreover, MMI α mRNA encodes an actin binding site, YIRCIKP(N/K), that is shared by myosin I proteins and is distinct from myosin II. These features provide further evidence that MMI α encodes a member of the myosin I family.

Immediately COOH terminal to the head domain, the predicted MMI α protein appears to encode, by comparison to BBMI, a series of calmodulin binding sites. Calmodulin serves as the light chain for BBMIs and other unconventional vertebrate myosins (Mooseker and Coleman, 1989; Espreafico et al., 1992), and its interaction with Ca²⁺ has been shown to regulate ATPase activity and in vitro motility (Mooseker et al., 1989; Collins et al., 1990). This region was mapped in the chick BBMI by α -chymotryptic mapping and construction of fusion proteins with calmodulin binding capacity (Coluccio and Bretscher, 1988; Halsall and Hammer, 1990). Comparison of this domain in BBMI with the corresponding region in the MMI α protein shows an amino acid identity of 56% and, with conservative substitutions, a sequence similarity of 74%. Prior reports evaluating this domain in BBMI have based estimates of the number of sites on an alignment of this region with the well-defined calmodulin binding domain of neuromodulin (Swanlung-Collins and Collins, 1992), and with a similar motif in neurogranin (Baudier et al., 1991). This comparison was based on the observation that both BBMI and neuromodulin bind calmodulin in the absence of Ca²⁺ (Coluccio and Bretscher, 1987; Alexander et al., 1988). A similar comparison of this region in MMI α reveals five potential calmodulin binding sites (Fig. 2). There are conserved aromatic or aliphatic residues at positions 1, 5, and 8, as well as basic amino acids clustered in the COOH-terminal part of these sites.

The alignment of the calmodulin binding region also reveals a 29 amino acid insert within MMI α that is absent in bovine BBMI. An insert, similar in both length and relative position within the message, was recently reported as a rare splice variant of chicken BBMI (Fig. 2) (Halsall and Hammer, 1990). A synthetic peptide of this fragment was shown

A	MMIα	LEAFGNAKTV	RNDNSSRFGK	YMDIEFDFKG	DPLGGVISNY	LLEKSRVVKQ	PRGERNFHVF	YQLLSGASEE	LLYKCLKLERD	228
	MMIβ	LEAFGNAKTL	RNDNSSRFGK	YMDVQDFDFKG	APVGGHILSY	LLEKSRVVHQ	NHGERNFHVF	YQLLEGGEEE	ALRRLGLERN	80
	MMIγ	LEAFGNAKTN	RNDNSSRFGK	YMDINFDFKG	DPIGGHINNY	LLEKSRVIVQ	QPGERSPHSF	YQLLQGGSEQ	MLHSLHLQKS	80
	BovBBI	LEAFGNAKTI	RNNNSSRFGK	YMDIEFDFKG	FPLGGVITNY	LLEKSRVVKQ	LEGERNPHIF	YQLLAGADAQ	LLKALKLERD	221
	ChkBBI	LEAFGNAKTI	RNDNSSRFGK	YMDVEFDFKG	DPLGGVISNY	LLEKSRIVRH	VKGERNFHIF	YQLLAGGSAQ	LLQQLKLRPD	179
	AcaMIβ	LEAFGNAKTL	LNNNSSRFGK	YFEIHFNRLG	EPCGGRITNY	LLEKSRVTFQ	TRGERSPHIF	YQLLAGASDA	EAQEMQLY.A	218
	CrdMI	LEAFGNAKTV	RNDNSSRFGK	FIRIHFGATG	KLASADIETY	LLEKSRVIFQ	LK A ERNYHIF	YQILSN K KPE	LLDMLLV T N	306
	MMIα	FSRYNYLSLD	S.AKVNGVDD	AANFR T VRNA	MQIVGFLDHE	AEAVLEVVA	VLKLGNI E FK	PESRVNGLDE	SKIKDKIELN	307
	MMIβ	QSYLYLVKG	QFAKVSSIND	KSDWKL L RKA	LSVIDFTEDE	VEDLLSIVAS	VLHLGNI H F	ADEDSNAQVT	TE....NQL	155
	MMIγ	LSSYNIYRVG	A.QLKSSIND	AAEFKVVADA	MKVIGFKPEE	IQT V YKILAA	ILHLGNL K FI	VDGDTPLIEN	GKVVSV....	155
	BovBBI	TGGYAYLNPD	T.SRVDGMD	DANFKVLQSA	MTVIGFSD E E	IRQVLEVAAL	VLKLG N VELI	NEFQANGVPA	SGIRDGRGVQ	300
	ChkBBI	CSHYGYLND	K.SVLP G MD	AANFRAMQDA	MAIVGFAPAE	VTALLEVTA	VLKLG N VKLS	SSFQASGMEA	SSIAEPRELQ	258
	AcaMIβ	PENFNYLNQS	ACYTVDGIDD	IKEFADTRNA	INVMGMTAAE	QRQVPHLVAG	ILHLGNVAFH	DGGKGT....	284
	CrdMI	PYDYAFVSQ	EVS.VASIDD	SEELLATDSA	FDVLGFTAEE	KAGVYKLTGA	IMHYGNM K FK	QKQREEQAE	DGTEDA....	381
	MMIα	EKFASRPASV	KVVLERAFSF	RTVEAKREKV	ST.....TL	NVAQAYYARD	ALAKNLYSRL	FSWLVNRINE	SIKAQ....	376
	MMIβ	KYLTRL L GV	G T LREALTH	RKIIAKGEEL	LS.....PL	NLEQAAYARD	ALAKAVYSAT	FTWLVRKINR	SVPAKDAESP	229
	MMIγ	..IAELLSTK	ADMVEKALLY	RTVATARDII	DK.....QH	TEQEASYGRD	AFAKAIYERL	FGWIVTRIND	IIAVKNYD T T	227
	BovBBI	E.IGELVGLN	SVELERALCS	RTMETAKEKV	VT.....TL	NVIQAQYARD	ALAKNIYSRL	FNWL V NRINE	SIKVG....	368
	ChkBBI	E.ISQLIGLD	PSTLEQALCS	RTVKVRDES	LT.....AL	SVSQGYGRD	ALAKNIYSRL	FDWL V NRINT	SIQVK....	326
	AcaMIβ	...AAVH D RT	PFALKNALLF	RVLNTGGAGA	KKMSTYNVPQ	NVEQAASARD	ALAKTIYSRM	FDWIVSKVNE	ALQKQ..GGS	359
	CrdMI	DKSAYLMGLN	SADLLKGLCH	PRVKV G NEYV	TK.....GQ	SVOQVYYSIG	<u>ALAKSVYEKM</u>	FNMWVTRINA	TLET K	450
	MMIα	TKVRKVMGV	LDIYGFEIFE	DNSFEQFIIN	YCNEKLQQIF	IELTLK E EQE	EYIREDI E WT	HIDY.FNNAI	ICDLIENNT.	454
	MMIβ	SWRSTTVLGL	LDIYGFEV F Q	HNSFEQFCIN	YCNEKL R QLF	IELTLK S EQE	EYEAEGIAWE	PVQY.FNNKI	ICDLVEEK F .	307
	MMIγ	VHGKNTVIGV	LDIYGFEIFD	NNSFEQFCIN	YCNEKLQQLF	IQLVLK Q EQE	EYQREGIPWK	HIDY.FNNQI	IVDLVEQ Q H.	305
	BovBBI	TGEKRVKMGV	LDIYGFEILE	DNSFEQFVIN	YCNEKLQQVF	IEMTLK E EQE	EYKREGIPWV	KVEY.FDNGI	ICNLIE H NQ.	446
	ChkBBI	PGKQRKVMGV	LDIYGFEIFQ	DNGFEQFIIN	YCNEKLQQIF	ILMTL K EEQE	EYVREAIQWT	PVEF.FD N SI	ICDLIEN S K.	404
	AcaMIβ	GDHNNNMIGV	LDIFGFEIFE	QNGFEQFCIN	YVNEKLQQYF	IELTLK A EQE	EYVNEGIQWT	PIKY.FNNKV	VCELI E GKRP	438
	CrdMI	.QPRQYFIGV	LDIAGFEIFD	FNSFEQLCIN	FTNEKLQQFF	NHHMFVLEQE	EYKKEGIEWE	FIDFG M LOA	<u>CIDLIE</u> ..KP	527
	MMIα	NGILAMLDEE	CLR...P G TV	TDETFL E KL.	NQVCATHQHF	ESRMSK S RSF	LNDTTLPHSC	FRIQH Y AGKV	LYQVEG V DK	530
	MMIβ	KGIISILDEE	CLR...P G EA	TDLTFL E KL.	EDTIKHHPHF	LTHKL..ADQ	KTRKSLDRGE	FRL L H Y AGEV	TYSVTG F LDK	381
	MMIγ	KGI I A I LDDA	CMN...P G VV	TDGMFL E AL.	NSKLGK H GHF	SSRKT C ASDK	ILEFDRD..	FRIRH Y AGDV	VYSVIG F IDK	378
	BovBBI	RGILAMLDEE	CLR...P G VV	SDSTFL A KL.	NQLFSK H SHY	ESKVTQ N AQR	QYDHS M GLSC	FRICH Y AGKV	TYNVNS F IDK	522
	ChkBBI	VGILAMLDEE	CLR...P G TV	NEDTFIT K L.	NQIFASHKRY	ESKET L NAKH	FTDVSLPLRC	FRIH H YAGKV	TYNVTG F IEK	480
	AcaMIβ	PGIFSLDDI	CFTMHAQSDG	MDGKFLQKC.	QGGF P SHLHF	RGMN.....NA	FSIKH Y AGEV	TYEAEG F C E K	503
	CrdMI	MGIMSILEEE	CMF...P K A	TDMTFK A KLY	DNHLGK S NNF	QKPRNV K GKQ	EAH.....	PSLVH Y AGTV	DYNILG W LEK	596
	MMIα	NNDLLYRDLS	QAMWKADHSL	IKSLFP....	..EGNPAKVN	L.KRP...P	TAGSQFKASV	ATLMRN L QTK	NPNYIR C IKP	599
	MMIβ	NNDLLFRNLK	ETMCSSTNPI	MAQCFD....	..KSELSD..	.KKRP...E	TVATQFKMGL	LQLVEILRSK	EPAYIR C IKP	448
	MMIγ	NKDTL F QDFK	RLMYNSSNPV	LKNMWP....	..EGKLSITE	VTKRP...L	TAATL F KNS.	427
	BovBBI	NNDLLFRDLS	QAMWKARHPL	LRSLFP....	..EGDPKQAS	L.KRP...P	TAGA Q FKSSV	TTLMK N LYSK	NPNYIR C IKP	591
	ChkBBI	NNDLLFRDLS	QAMWAARHTL	LRSLFP....	..EGDPQ R PS	L.KLP...P	TTGS Q FKASV	ATLMK N LYSK	NPNYIR C IKP	549
	AcaMIβ	NKDTL F DDLI	AVIQESENRL	LVS W FP....	..EDTKQLQ.	.KKRP...T	TAGFK L KTSC	DALMEALSRC	SPHYIR C IKP	571
	CrdMI	NKDPLNETTV	GLYQKSSLKL	MATL F STYAS	ADT G DSGKQ	GGKKK G SS S FQ	TVSALHREN	NKLM T NLRTT	HPHFV R CII P	676
B	MMIα	.SELFKDKKA	LYPSSVGQPF	QGAYLEINKN	PKYK L KDAI	E E KIIIAEV	NKINRANGKS	TSRIFLLTNN	NLLADQKSG	966
	BovBBI	ASELFK G KKA	SY P QSVPIPF	HGDYIGLQRN	PKLQ K LKGE	EGPILMAETV	VKVN R GNAKT	SSRILL L TKG	HVIIT M KNP	929
	ChkBBI	..QLFK D KKA	LYAQSLQQPF	RGEYLGLTQN	RKYQ K LQAVA	KDKLVM A EAV	QKVN R ANGKT	VPRL L LLTTE	HLVLAD P KAA	887
	Consensus	.seLFKdKKA	lYp-Sv.qPF	.G.Yl--.n	pKy-Kk...	e.ki.-AE.V	.K-NRaN g K-	.sRi-LLT..	-l.laD.K..	
	MMIα	QIKSEVPLVD	VTK V SMSSQN	DGFFAVHLKE	GSEAA S GKDF	LFSSDHLIEM	ATKLYRTTLS	QTKQ K LNI E I	SDEFLV Q FRQ	1046
	BovBBI	QAKTVIPLNS	LAGVSVTSFK	DGLFSLHLSE	ISSV G SKGEF	LLVSEHVIEL	LTKICRATLD	ATQ M QLPVTV	TEEF S VK F KE	1009
	ChkBBI	QPKMVL S LCD	IQGASVSRFS	DGLLALHLKE	TSTAGGKGD	LLVSPHLIEL	VTRLHQ T LMD	ATAQAL P LSI	ADQF S TRFPK	967
	Consensus	Q.K.-.pL.d	..-vS-ss-	DG-fa-HLKe	.S.a-sGkdF	L--S.HIIE-	.Tkl.rttl-	-T.q.Lp..i	.deF-v.F..	
	MMIα	DKVCVKFIQ	NQKNGSVPTC	.KRKNR L LE	VAVP*	1080				
	BovBBI	GSLTVKVIQ	PGGGGTGKLS	FKK G SRCLE	VTVQ*	1044				
	ChkBBI	GDVA V TVVES	AKGGDVPVC	.KKR G SHKME	ILVH*	1001				
	Consensus	-.v.Vk-igg	...-G.vp.c	.K-k--r.lE	v.v.*					

Figure 1. Alignment of myosin I head and tail sequences. (A) Predicted protein sequences for MMI α , MMI β , MMI γ , and other myosin protein sequences were aligned within the overlapping head domain using the Pileup program (Devereux et al., 1984) of the GCG package. Where applicable, numbers indicate the amino acid residue according to published sequences. The residue numbers for MMI β and MMI γ start at the 5' ends of the PCR products. The sequences are not listed in the order of their relatedness. MMI γ does not extend to the 3' end because this PCR product was cut at a HindIII site at serine 427 in preparation for subcloning. Examples of residues and domains that are highly conserved in the myosin I but not in the myosin II gene family are underlined. *BovBBI*, bovine BBI (Hoshimaru and

to bind calmodulin, also independent of Ca²⁺ concentration.

The COOH-terminal, or tail, region of BBMI and an equivalent region in invertebrate myosin I's have a high net positive charge and have been shown to bind with high affinity to negatively charged phospholipids (Adams and Pollard, 1989). This interaction has been proposed as a means by which myosin I's associate with membranes in vivo (Miyata et al., 1989; Hayden et al., 1990; Doberstein and Pollard, 1992; Zot et al., 1992). The predicted protein for MMI α also contains a COOH-terminal region with a high net positive charge and a predicted pI of 10.25. In comparison, the pI's of the equivalent regions in chick and bovine BBMI are 10.41 and 10.44, respectively. The similarity within this region extends beyond a simple summation of charge. Alignment of this region with those of chicken and bovine myosin I reveals conserved amino acid stretches that may reflect shared functional domains (Fig. 1 B).

There are also major structural differences between MMI α and most of the myosin I's identified in *Acanthamoeba* and *Dictyostelium*. Unlike these myosins, yet similar to BBMI and the *Dictyostelium* actin-based motor A (Titus et al., 1989), MMI α does not encode an ATP-independent actin binding site (Pollard et al., 1991).

Expression Pattern of MMI α , MMI β , and MMI γ mRNAs

Northern blot analysis of the expression of MMI α mRNAs in various adult murine tissues reveals messages of 4.1, 4.9, and 6.5 kb that are most prominently detected in the brain, lung, and liver (Fig. 3). These messages are also expressed in the heart and testes, but are not detectable or are expressed at very low levels in the kidney, small intestine, skeletal muscle, adrenal gland, or spleen (data not shown). MMI α mRNA also shows variable expression in cultured cells and established cell lines. All three messages are present in the mouse neuroblastoma N115 line, the neural precursor line C17, and the murine teratocarcinoma cell line P19, yet are not detectable in the pheochromocytoma-derived PC12 cell line. MMI α transcripts are not detectable in the astrocytic cell lines U251 and G26-24, nor in isolated astrocytes from P6 rat cerebellum (Hatten, 1985) (data not shown). All three messages are present in normal rat kidney fibroblasts and NIH-3T3 cells, while in the CHO cell line only the 4.1-kb message is detected (data not shown).

The three MMI α messages noted above are detected by probes representing stretches of the coding region of the gene. To evaluate the correspondence of the detected messages with the isolated cDNAs, a subcloned fragment from the 3' untranslated region of clone λ CGN74a that is not contained within clone λ CGN82a (see above) was used to probe total RNA from various murine tissues. As illustrated in Fig. 3 A (lane 7), this probe detects the 4.9- and 6.5-kb messages but not the 4.1-kb message. This indicates that alternate sites

of polyadenylation appear to account for the size difference between the two smaller messages. These findings also demonstrate that the 6.5-kb message contains the longer 3' untranslated segment, but the additional modifications necessary to generate this message are unknown.

MMI β and MMI γ display patterns of mRNA expression that are different both from each other and from MMI α . In PC12 cells, a labeled pGMMI β probe hybridizes to a single 5.0-kb message. This band is detected in all tissues and cell lines that express MMI β mRNA. In tissue an additional 6.9-kb message is also detected with pGMMI β . The pGMMI γ probe also detects a complex pattern of mRNAs, even at high stringency (0.2 \times SSC, 60°C). Hybridization to at least two messages of 5.2 and 7.0 kb is observed, although, as for MMI β , in PC12 cells only the 5.2-kb band is detected. The tissue and cell line distributions of MMI β and MMI γ mRNAs are distinct. MMI β message is widely expressed: it is found in adult lung, kidney, heart, small intestine, adrenal gland, skeletal muscle, and P2 cerebral cortex (Fig. 4), as well as testes, spinal cord, and the adult cerebral cortex (data not shown). The 5.0-kb MMI β message is also present in numerous cell lines of neural, glial, and fibroblastic origin (Fig. 4 B). MMI γ mRNA, though not as widely expressed as MMI β , is found in lung, kidney, small intestine, and adrenal gland, and in the adult cerebral cortex and spinal cord (data not shown). MMI γ is present at relatively low levels in the liver, heart, skeletal muscle, and P2 cerebral cortex. It is also not detected in several cell lines in which MMI β is found: NIH3T3, U251-1, and P19 cells.

S1 nuclease analysis was performed to provide an additional assessment of the tissue distribution of MMI β and MMI γ mRNA, as well as to rule out possible crossreactions in Northern blots. Probes were chosen from regions of pGMMI β and pGMMI γ with very low homology to each other or to MMI α (see Materials and Methods). Bands corresponding to the predicted protected fragment sizes were detected after S1 nuclease digestion of RNA from lung, liver, kidney, skeletal muscle, heart, and small intestine. Furthermore, we did not detect protected fragments of smaller sizes that would suggest crossreaction with a homologous transcript. For both clones, the relative levels of detected signal approximately corresponded to the relative abundance of mRNAs detected by Northern blot analysis with the exception of liver RNA, which had a higher signal by S1 nuclease protection (data not shown).

Developmental Expression of MMI α mRNA in Neurons of the Postnatal Mouse Brain

Examination of MMI α mRNA in the postnatal mouse brain by Northern blot analysis points to a developmental regulation of the steady-state levels for these messages. As shown in Fig. 3 B, all three messages reach a peak at postnatal day 2 (P2) and decline thereafter to relatively low levels in the adult (Fig. 3, A and B). In the cerebellum, a peak of expres-

Nakanishi, 1987); *ChkBBMI*, chicken BBMI (Garcia et al., 1989); *AcaMIb*, *Acanthamoeba* myosin Ib gene (Jung et al., 1987); *CrdMII*, the rat alpha cardiac muscle myosin II (McNally et al., 1989). (B) Alignment of the tail sequences of MMI α protein and both chicken and cow BBMI. In the consensus, a residue is listed as a capital letter if shared by all three sequences, a lowercase letter if shared by MMI α and one other sequence, a dash if shared by both BBMI, and a period if different in all three.

GGACTGGGAAAAAAGACTCTCATAAATACATAAAGAGGGTCTTGGCCAAATGTTGCTACTACTCTCAITTTGAATGCTTCTCTTTGAAGTAAGTTTCTCTTGGGATGACCTCTGGCCT -76
TTGTGGATATGTCAGTGAATCTCGAAGAGAGCTGTGGCTGTCACACATCGCCGATGCTCCCTCGGAAACC 0

ATG GCC AAA ATG GAG GTG AAA TCC TCA CTT CTG GAC AAT ATG ATC GGT GTT GGA GAT ATG GTT CTT TTA GAG CCG CTC AAT GAG GAG ACG 90
Met Ala Lys Met Glu Val Lys Ser Ser Leu Leu Asp Asn Met Ile Gly Val Gly Asp Met Val Leu Leu Glu Pro Leu Asn Glu Glu Thr 30
TTC ATC GAC AAC CTG AAG AAG CCG TCC GAC CAC AAT GAG ATA TAT ACA TAC ATT OGG AGC GTG GTT ATA TCT GTT AAC CCC TAC CGG TCC 180
Phe Ile Asp Asn Leu Lys Lys Arg Phe Asp His Asn Glu Ile Tyr Thr Tyr Ile Gly Ser Val Val Ile Ser Val Asn Pro Tyr Arg Ser 60
TTG CCC ATA TAT TCG CCG GAG AAA GTG GAA GAT TAC AGA AAT AGA AAN TTC TAT GAA CTG AGC CCT CAC ATC TTT GCT TTG TCG GAT GAA 270
Leu Pro Ile Tyr Ser Pro Glu Lys Val Glu Asp Tyr Arg Asn Arg Asn Phe Tyr Glu Leu Ser Pro His Ile Phe Ala Leu Ser Asp Glu 90
GCA TAC AGA TOC CTC CGT GAT CAA GAT AAA GAT CAA TGC ATT CTC ATT ACT GGG GAG AGT GGA GCA GGA AAA ACA GAG GCC AGC AAG CTC 360
Ala Tyr Arg Ser Leu Arg Asp Gln Asp Lys Asp Gln Cys Ile Leu Ile Thr Gly Glu Ser Gly Ala Gly Lys Thr Glu Ala Ser Lys Leu 120
GTC ATG TCC TAT GTC GCT GCC GTG TGC GGA AAA GGA GCA GAA GTC AAT CAG GTC AAG GAA CAG CTT CTG CAG TCC AAC CCT GTC CTG GAA 450
Val Met Ser Tyr Val Ala Ala Val Cys Gly Lys Gly Ala Glu Val Asn Gln Val Lys Glu Gln Leu Leu Gln Ser Asn Pro Val Leu Glu 150
GCT TTT GGA AAT GCC AAT GTC AGG AAT GAC AAC TCA TCT AGA TAT GGC AAA TAC ATG GAT ATT GAA TTT GAC TTT AAA GCG GAT CGC 540
Ala Phe Gly Asn Ala Lys Thr Val Arg Asn Asp Asn Ser Ser Arg Phe Gly Lys Tyr Met Asp Ile Glu Phe Asp Phe Lys Gly Asp Pro 180
CTG GGA GGA GTG ATA AGT AAC TAT CTT TTA GAG AAA TCT CGT GTT GTT AAG CAG CCA AGA GGT GAA AGA AAC TTC CAT GTG TTC TAT CAG 630
Leu Gly Gly Val Ile Ser Asn Tyr Leu Leu Lys Ser Arg Val Val Lys Ile Gln Pro Arg Gly Glu Arg Asn Phe His Val Phe Tyr Glu 210
CTG CTC TCA GGC GCC TCT GAG GAG CTC CTC TAC AAG CTT AAG CTG GAG CCG GAT TTC AGC AGA TAC AAC TAT CTG AGT CTG GAC TCT GCT 720
Leu Leu Ser Gly Ala Ser Glu Glu Leu Leu Tyr Lys Leu Lys Leu Glu Arg Asp Phe Ser Arg Tyr Asn Tyr Leu Ser Leu Asp Ser Ala 240
AAA GTT AAT GGT GTG GAT GAT GCT GCC AAT TTC AGA ACT GTT AGG AAT GCA ATG CAG ATC GTG GGC TTT CTG GAT CAT GAG GCC GAG GCT 810
Lys Val Asn Gly Val Asp Ala Ala Asn Phe Arg Thr Val Arg Val Lys Gln Ile Val Gly Phe Leu Asp His Glu Ala Glu Ala 270
GTC CTG GAG GTA GTG GCA GCC GTT TTG AAA CTG GGG AAC ATC GAG TTC AAG CCT GAA TCA CGA GTG AAT GGT TTG GAT GAA AGC AAA ATC 900
Val Leu Glu Val Val Ala Ala Val Leu Lys Leu Gly Asn Ile Glu Phe Ser Pro Glu Ser Arg Val Asn Gly Leu Asp Glu Ser Lys Ile 300
AAA GAT AAA ATT GAG TTA AAC GAA AAG TTT GCG AGT CGA CCA GCA TCG GTC AAG GGT TTA GAA AGA GCG TTC AGT TTC CGA ACA GTT 990
Lys Asp Lys Ile Glu Leu Asn Glu Lys Phe Ala Ser Arg Pro Ala Ser Val Lys Val Val Leu Glu Arg Ala Phe Ser Phe Arg Thr Val 330
GAA CCG AAG AGG GAG AAA GTT TCA ACC ACC CTG AAT GTG GCT CAG GCT TAT TAT GCC CGT GAT GCC CTG GCT AAA AAC CTC TAC AGC CGC 1080
Glu Ala Lys Arg Glu Lys Val Ser Thr Thr Leu Asn Val Ala Glu Lys Tyr Ala Arg Asp Ala Lys Asn Leu Tyr Ser Arg 360
TTG TTT TCA TGG TTG GTA AAT CGT ATC AAC GAA AGC ATT AAG GCA CAA ACA AAA GTG AGA AAG AAG GTC ATG GGT GTT CTG GAC ATT TAT 1170
Leu Phe Ser Trp Leu Val Asn Arg Ile Asn Glu Ser Ile Lys Ala Gln Thr Lys Val Arg Lys Lys Val Met Gly Val Leu Asp Ile Tyr 390
GGC TTT GAA ATA TTT GAG GAT AAC AGC TTC GAG CAG TTT ATT ATT AAC TAT TGT AAT GAA AAG CTT CAA CAA ATC TTC ATC GAA CTT ACC 1260
Gly Phe Glu Ile Phe Glu Ile Phe Glu Gln Phe Ile Ile Asn Tyr Cys Asn Glu Lys Leu Gln Ile Phe Ile Glu Ala Glu Ala 420
CTC AAA GAA GAG CAA GAG GAA TAT ATT CCG GAG GAC ATA GAA TGG ACT CAC ATT GAC TAC TTC AAC AAT GCT ATC ATT TOT GAC CTA ATA 1350
Leu Lys Glu Glu Gln Glu Gly Tyr Ile Arg Glu Asp Ile Gly Trp Thr His Ile Asp Tyr Phe Asn Asn Ala Ile Ile Cys Asp Leu Ile 450
GAA AAT AAC ACA AAT GGA ATT TTG GCG ATT CTG GAT GAA GAG TCG CTG AGA CCC GGC ACT GTT ACA GAT GAG ACC TTC CTG GAA AAG CTG 1440
Glu Asn Asn Thr Asn Gly Ile Leu Ala Met Leu Asp Glu Glu Cys Leu Arg Pro Gly Thr Val Thr Asp Glu Thr Phe Leu Glu Lys Leu 480
AAC CAA GTC TGT GCC ACC CAC GAG CAC TTT GAG AGC AGG ATG AGC AAG Cys Ser Arg Phe Leu Asn Asp Thr Thr Leu Pro His Ser Cys 1530
Asn Gln Val Cys Ala Thr His Gln His Phe Glu Ser Arg Met Ser Lys GTC Ser Arg Phe Leu Asn Asp Thr Leu Pro His Ser Cys 510
TTC AGA ATC CAG CAT TAT GCT GGC AAG GTG CTC TAC CAG GTG GAA GGA TTT GTT GAC AAG AAC AAT GAC CTT CTG TAC CCG GAC CTG TCT 1620
Phe Arg Ile Gln His Tyr Ala Gly Lys Val Leu Tyr Gln Val Glu Gly Phe Val Asp Lys Asn Asn Asp Leu Leu Tyr Arg Asp Leu Ser 540
CAA GCC ATG TGG AAG GCC GAC CAC TCC CTC ATC AAG TCT CTG TTT CCT GAA GGA AAT CCT GCA AAG GTC AAC CTG AAG AGA CCT CCC ACC 1710
Gln Ala Met Trp Lys Ala Asp His Ser Leu Ile Lys Ser Lys Leu Glu Lys Pro Ala Lys Val Asn Leu Lys Arg Pro Thr Pro 570
GCA GCC TCC CAG TTC AAG CCG TCT GTG GCC ACG CTG ATG AGA AAC CTG CAG ACC AAG AAC CCG AAC TAC ATC AGG TGC ATC AAG CCA AAT 1800
Ala Gly Ser Gln Phe Lys Ala Ser Val Ala Thr Leu Met Arg Asn Leu Gln Thr Lys Asn Pro Asn Tyr Ile Arg Cys Ile Lys Pro Asn 600
GAT AAA AAG GCA GCT CAC ATT TTC AAT GAG AGT CTC GTA TGC CAT CAG ATC AGG TAC CTG GGT CTT TTG GAG AAC GTT CGA GTG AGA CCG 1890
Asp Lys Lys Ala Ala His Ile Phe Asn Glu Ser Leu Val Cys His Ser Glu Ile Arg Tyr Leu Gly Leu Leu Glu Asn Val Arg Val Arg 630
GCA GGC TAT GCT TTC AGG CAG GCC TAT GAA CCT TGC CTG GAA AGA TAC AAG ATG CTT TOC AAA CAA ACG TGG CCT CAC TGG AAG GGA CCA 1980
Ala Gly Tyr Ala Phe Arg Gln Ala Tyr Glu Pro Cys Leu Glu Arg Tyr Lys Lys Met Leu Cys Lys Gln Thr Trp Pro His Trp Lys Gly Pro 660
GCA AGG TCT GCG GTG GAG GTT CTG TTT AAT GAG CTA GAT ATT CCT GTG GAA GAG CAC TCC TTC GGG AGA TCC AAG ATA TTC CTC AGA AAC 2070
Ala Arg Ser Gly Val Glu Val Leu Phe Asn Glu Leu Glu Ile Pro Val Glu Glu His Ser Phe Gly Arg Ser Lys Ile Phe Ile Arg Asn 690
CCA CGG ACA TTA TTC CAA CTA GAA CAG CTA AGG AAG CAG CCG CTG GAG CAG CTG GCC ACT CTC ATT CAG AAG ATT TAT CGA GGA TGG AAA 2160
Pro Arg Thr Leu Phe Thr Leu Glu Asp Leu Arg Lys Gln Arg Leu Glu Asp Leu Ala Thr Leu Ile Gln Lys Ile Tyr Arg Gly Trp Lys 720
TGC COT ACA CAT TTT CTG CTC ATG AAA AGA AGC CAA GTT GTG ATT GCT GCG TGG TAC AGA CGA TAT CCG CAA CAA AAG CCG TAC CAG CAG 2250
Cys Arg Thr His Phe Leu Leu Met Lys Arg Ser Gln Val Val Ile Ala Ala Trp Tyr Arg Arg Tyr Ala Gln Gln Lys Arg Tyr Gln Gln 750
ATA AAG AGT TCA GCC CTG GTG ATT CAG TCG TAC ATC CCG GCG TGG AAG GCT CGA AAA ATC CTG CCG GAG CTG AAG CAT CAG AAG CCG TGT 2340
Ile Lys Ser Ser Ala Leu Val Ile Gln Ser Tyr Ile Arg Gly Trp Lys Arg Ala Arg Lys Ile Leu Arg Glu Leu Lys His Gln Lys Arg Cys 780
AAG GAG GCA GCC ACC ACC ATC CCG GCG TAC TGG CTT GGA CTG AAG GTA CCG AGG GAA TAC AGG AAA TTC TTT AGA GCA AAT GCT GGA AAG 2430
Lys Glu Ala Ala Thr Thr Ile Ala Ala Tyr Trp Leu Gly Leu Lys Val Arg Arg Glu Tyr Arg Lys Phe Phe Arg Ala Asn Ala Gly Lys 810
AAA ATC TAT GAG TTT ACA CTT CAA AGA ATT GTG CAA AAA TAC TTG TTG GAA ATG AAA AAT AAA ATG CCT TCC TTG TCA CCG ATA GAT AAA 2520
Lys Ile Tyr Glu Phe Thr Leu Gln Phe Thr Leu Lys Ile Val Gln Lys Tyr Leu Leu Glu Met Lys Asn Lys Met Pro Ser Leu Ser Pro Ile Asp Lys 840
AAT TGG CCA TCA AGA CCT TAC CTG TTC TTG GAT TCG ACT CAC AAG GAG CTA AAA AGG ATC TTC CAC TTG TGG AGG TGT AAA AAA TAC AAG 2610
Asn Trp Pro Ser Arg Pro Tyr Leu Phe Leu Asp Ser Thr His Lys Glu Leu Lys Arg Ile Phe His Leu Trp Arg Cys Lys Lys Tyr Arg 870
GAT CAA TTC ACA GAC CAG CAG AAA CTT ATT TAT GAA GAG AAG CTC GAG GOC AGC GAA CTC TTC AAA GAC AAG AAG GCT TTA TAT CCT TCT 2700
Asp Gln Phe Thr Asp Gln Gln Lys Leu Ile Tyr Glu Glu Lys Lys Asp Lys Lys Glu Leu Phe Lys Ala Leu Tyr Pro Ser 900
AGT GTT GGG CAA CCA TTC CAA GGA GCT TAC CTG GAA ATC AAC AAG AAC CCA AAA TAT AAG AAA CTC AAA GAT GCC ATT GAA GAG AAG ATC 2790
Ser Val Gly Gln Pro Phe Gln Gly Ala Tyr Leu Glu Ile Asn Lys Asn Pro Lys Tyr Lys Lys Leu Lys Asp Ala Ile Glu Glu Lys Ile 930
ATT ATT GCT GAG GTT GTG AAC AAA ATT AAT CCG GCT AAT GGG AAG AGT ACA TCT CCG ATT TTC CTC TTA ACA AAC AAT AAC CTT CTC CTT 2880
Ile Ile Ala Glu Val Val Asn Lys Ile Asn Arg Ala Asn Gly His Ser Thr Ser Arg Ile Phe Leu Leu Thr Asn Asn Asn Leu Leu Leu 960
GCT GAC CAA AAG TCT GGG CAG ATC AAG TCC GAG GTC CCC CTG GTA GAT GTG ACC AAG GTT TCC ATG AGT TCC CAG AAC GAT GGT TTC TTT 2970
Ala Asp Gln Lys Ser Gly Gln Ile Lys Ser Glu Val Pro Leu Val Asp Val Thr Lys Val Ser Met Ser Ser Gln Asn Asp Gly Phe Phe 990
GCA GTG CAC CTC AAG GAG GGT TCA GAA CCG GCT AAA GGG GAG TTT CTC TTC AGC AGT GAC CAC CTG ATT GAA ATT GCA ACC AAG CTG 3060
Ala Val His Leu Lys Glu Gly Ser Glu Ala Ala Ser Lys Gly Asp Phe Leu Phe Ser Ser Asp His Leu Ile Glu Met Ala Thr Lys Leu 1020
TAT CCG ACG ACT CTC AGC CAA ACT AAA CAG AAG CTC AAC ATC GAG ATT TCC GAT GAG TTC CTG GTA CAG TTC AGA CAG GAC AAA GTA TOT 3150
Tyr Arg Thr Thr Leu Ser Gln Thr Lys Leu Asn Ile Glu Ile Ser Asp Glu Phe Leu Val Gln Phe Leu Val Gln Phe Lys Asp Lys Val Cys 1050
GTG AAG TTT ATT CAA GGC AAC CAG AAA AAT GGG AGC GTG CCA ACC TGC AAA CGA AAG AAC AAC AGA CTC CTT GAA GTC GCT GTC CCT TAA 3240
Val Lys Phe Ile Gln Gly Asn Gln Lys Asp Val Pro Thr Cys Lys Arg Lys Asn Asn Arg Leu Leu Glu Val Ala Val Pro End 1080
GTGAGGCTTCCTCTCTCAGGACTTGTTCCTCGTAAACAGTGCATTTTACTTTGGTTTTATTGGGGTTCACGTATGTTTGGGAATGCCAAAGGCTAACTGTTAAGCCTCT 3360
TGGCACAAATTAAGATTTTGGACTAATTAAGTTTAAATATCAGAATAGTTCATTCCTAAGTCCACATTTCTGCTCTGGCCAGAAATTCCTGTGTGTTGTTGTTTGTGTTTCAAAA 3480
TCTATTTGAAAAGAGATAACCAACATTTAAACATACACATTTATGGTTCAGATCCGGTTTTCACCTTTAGAAAAAGTCACTGAAGCAGGGCTGAGAAATGCTTTGTTCTGGTCCAGAGA 3600
AGCATGAGATAGGACCTGGATGTGCACAGTGTATCCACCAGGCTCATTAGCTTTTGAACCTGCATGATAAAGAGGCTGAGAGCCCTCAGACATCCGACATCTTCTTTGATGTC 3720
AATAAACAATCCAAGATCACTTTTGAAGTGTATTTATTAATATGCACTTTTGTGTATGAGAGGCTGATTTGATCAAGGCTGTATTTTATGTCAGATCAAATTAAGTTAGAAATCGCT 3840
TTTCAGGGCTTTTGTATTTTTTCTTCAAAATTAACAATTAAGGATTCATTTTGAAGAACCATTTTAACTCTGGAATTAATTTGTTTCTTATTTGGGAGGATAATGTAATACATTTGG 3960
GATTATGTTAATAAATAATTTGTTCAATTTGGTCCCA 3998

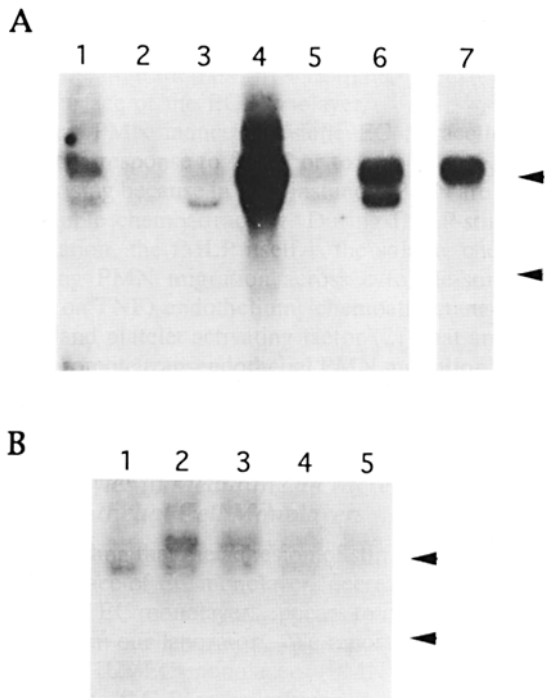


Figure 3. Tissue distribution and development regulation of *MMI α* mRNAs. 15 μ g of total RNA from murine tissues or cell lines was analyzed by Northern blot hybridization with a 789-bp *Xho*I-*Eco*RI probe including amino acids 950–1079, except for the sample in lane 7. Unless indicated, tissue is from adult animals. 28S and 18S rRNAs are indicated by arrowheads. (A) Lane 1, P2 cerebral cortex; lane 2, adult cerebral cortex; lane 3, N115 neuroblastoma cell line; lane 4, lung; lane 5, testes; lane 6, liver; lane 7, lung. The RNA in lane 7 was hybridized with a probe containing only 3' untranslated nucleotides 3638–3998 that are contained within clone λ CGN74a and not clone λ CGN82a. (B) Total RNA from developing mouse cerebral cortex. Lane 1, P0; lane 2, P2; lane 3, P6; lane 4, P10; lane 5, adult.

sion occurs at P6, whereas the amount of message present in the adult is below detection by this approach (data not shown).

To provide further information regarding the cellular, regional, and developmental distribution of *MMI α* mRNA in the brain, we carried out *in situ* hybridization. Paraffin sections of brain tissue from mice ages P0 through adult were hybridized with a digoxigenin-labeled 400-nt cRNA probe containing both head and calmodulin domain sequence. Identical staining patterns are seen with an 800-nt probe against tail sequence. No staining is observed in the presence of excess unlabeled probe or with sense probes (data not shown).

In adult mice, *MMI α* mRNA is widely distributed in neurons throughout the brain. Regions examined included the cerebral cortex, hippocampus, and dentate gyrus, subcortical areas, brainstem, and cerebellum. All stained cells con-

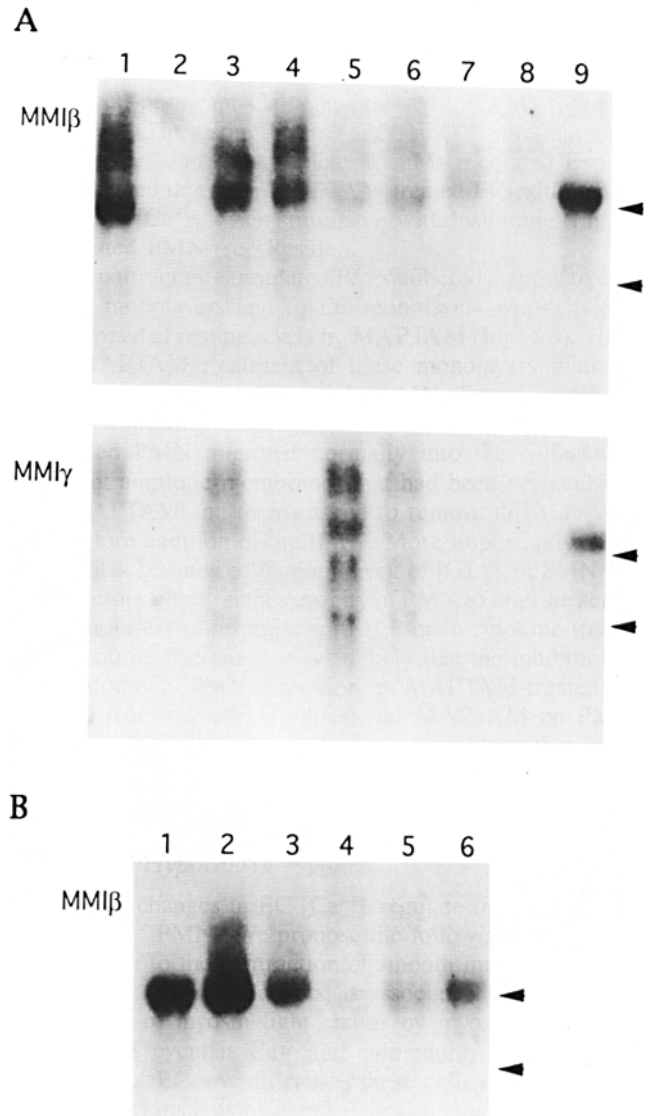


Figure 4. Tissue and cell line distribution of *MMI β* and *MMI γ* . 10 μ g of total RNA from rat tissue or cell lines was analyzed by Northern blot hybridization with probes from the indicated 1.2-kb PCR product. The positions of 28S and 18S rRNA are denoted by arrowheads. (A) Lane 1, lung; lane 2, liver; lane 3, kidney; lane 4, heart; lane 5, small intestine; lane 6, adrenal gland; lane 7, hind leg skeletal muscle; lane 8, P2 cerebral cortex; lane 9, PC12 cells. (B) Lane 1, PC12 cells; lane 2, NIH 3T3 cells; lane 3, U252-1 astrocytoma; lane 4, C17 cells; lane 5, P19 cells; lane 6, P19 cells + 5 μ M retinoic acid.

tain large nuclei and a thin ring of cytoplasm (Fig. 5 *b*), characteristic of neurons; an extensive search did not reveal reaction product in any cells of glial or astrocytic morphology. However, reaction product is found in cells of the choroid plexus. Comparison with adjacent sections stained

Figure 2. Nucleotide and predicted amino acid sequence of *MMI α* cDNA. Full-length *MMI α* cDNA was assembled from overlapping clones λ AM9d, λ CGN82a, λ CGN15, and λ CGN74a. Conserved stretches within the ATP (amino acids 108–115) and actin binding (amino acids 593–600) sites are indicated by double underlining. The five potential calmodulin binding domains spanning amino acids 712–875 are underlined. The 29 amino acid insert within this region includes residues 767–795. The two double underlined adenines at nucleotide positions 3395 and 3998 are potential polyadenylation sites as deduced from analysis of clones λ CNG82a and λ CGN74a.

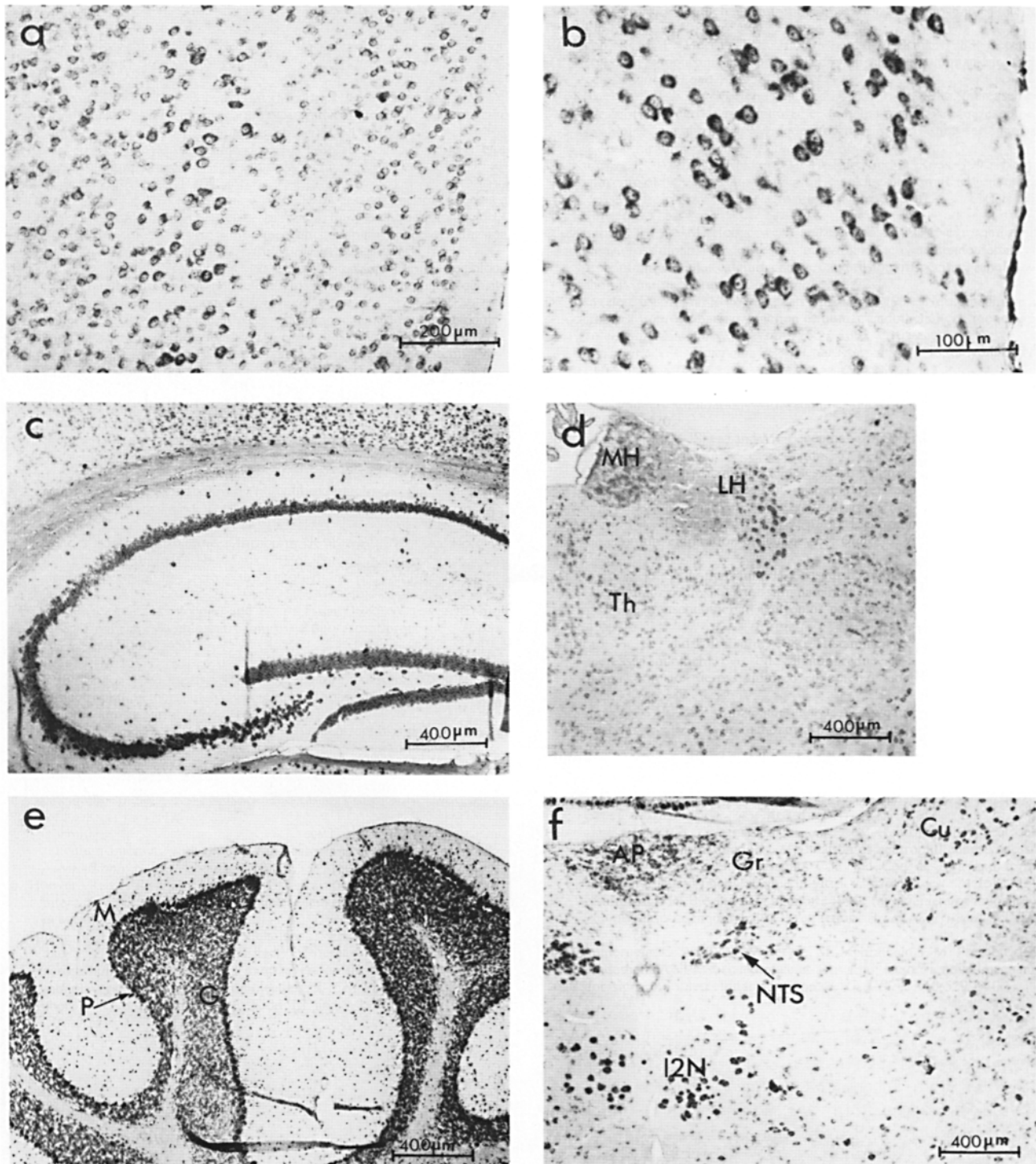


Figure 5. Photomicrographs of in situ localization of $MMI\alpha$ mRNA in adult mouse brain. These coronal sections from an adult mouse depict the in situ localization of $MMI\alpha$ mRNA in the cerebral cortex (a), high magnification micrograph of the cerebral cortex (b), hippocampus (c), subcortical diencephalon (d), cerebellum (e), and brainstem (f). The color reaction in the section of the cerebellum was allowed to develop longer in order to enhance the labeling intensity of the granular layer. *MH*, medial habenula; *LH*, lateral habenula; *Th*, thalamus; *M*, molecular layer; *p*, Purkinje cell layer; *G*, granular layer; *AP*, area postrema; *NTS*, nucleus of the solitary tract; *12N*, hypoglossal nucleus; *Gr*, nucleus gracilis; *Cu*, nucleus cuneatus.

with hematoxylin and eosin (data not shown) reveals that nearly all neurons appeared labeled, although the intensity of staining showed regional differences. For instance, in the cerebral cortex, the intensity of reaction product is greater in neurons of layer three than in neurons of the other cortical

layers (Fig. 5 a). Also, in the cerebellum Purkinje cells and neurons of the deep nuclei stain much more intensely for $MMI\alpha$ mRNA than do granule neurons. In contrast, labeling in the hippocampus and dentate gyrus appears to be uniformly strong in all neuron layers.

Examination of P0, P2, P7, and P12 brains reveals that the proportion of positively stained neurons does not vary significantly during central nervous system maturation. However, there are both regional and temporal changes in expression of $MMI\alpha$ mRNA during development. In the hippocampus a uniformly intense level of staining is detected from P0 through adult. In areas including the cerebral cortex and cerebellum, however, the overall level of staining declines with development. For example, in consonance with Northern blot data, neurons within the cerebral cortex generally show peak levels of expression between days P2 and P7. In cerebellum at day P2, granular neurons exhibit intense and uniform labeling, whereas neurons in the Purkinje layer do not stain uniformly. This situation progressively changes with development so that, by P12 and onward, staining in Purkinje cells is uniformly intense and a relatively light staining pattern is observed in all cerebellar granular neurons (Fig. 6).

Hybridization analysis also indicates expression of $MMI\alpha$ mRNA in at least certain neuronal precursors. In the developing mouse cerebellum, neuroblasts migrate to form the external granular (or germinal) layer (EGL) where they continue to divide postnatally and give rise to granule, stellate, and basket cells (Jacobson, 1991). Throughout the developmental period examined (P0 to P12) all cells in the EGL label with probes to $MMI\alpha$. However, staining was more intense in cells of the superficial layers of the EGL (Fig. 6). It is in this region of the EGL where granule cells continue to undergo mitosis and are just beginning to extend their bipolar processes.

Discussion

Evidence for a Family of Mammalian Myosin I Genes

The characterization of $MMI\alpha$, $MMI\beta$, and $MMI\gamma$ provides strong evidence for the existence of three novel mammalian myosin I genes. These are the first vertebrate myosin I genes described that are expressed outside the intestinal brush border. The classification of these predicted proteins as myosin I's is based on both a high degree of overall homology and the presence of highly conserved domains: some of these are shared by all myosins and others are unique to myosin I's. The latter point is exemplified by the conserved residues, QA.yaRDALAK.iYSR, first described as adjacent to the *Acanthamoeba* myosin I phosphorylation site (Pollard et al., 1991). Given the presence of multiple myosin I genes in at least two invertebrates, the existence of a family of mammalian myosin I genes is not unexpected.

We screened 42 subcloned PCR products to identify these three novel myosin I genes. While this is not an exhaustive search for myosin I family members, this result may suggest that these are the only products that can be obtained from these cell sources given the constraints of the PCR strategy we used. These constraints also would prevent the amplification of unconventional myosins like *NinaC* (Montell and Rubin, 1988) and the high molecular weight myosin I from *Acanthamoeba* (Horowitz and Hammer, 1990).

$MMI\beta$ may be the rat homologue of a newly identified myosin I purified from bovine adrenal glands (Barylko et al., 1992). 12 residues, LSVIDFTEDEVE, have at least 75% identity with a microsequenced fragment from the bovine

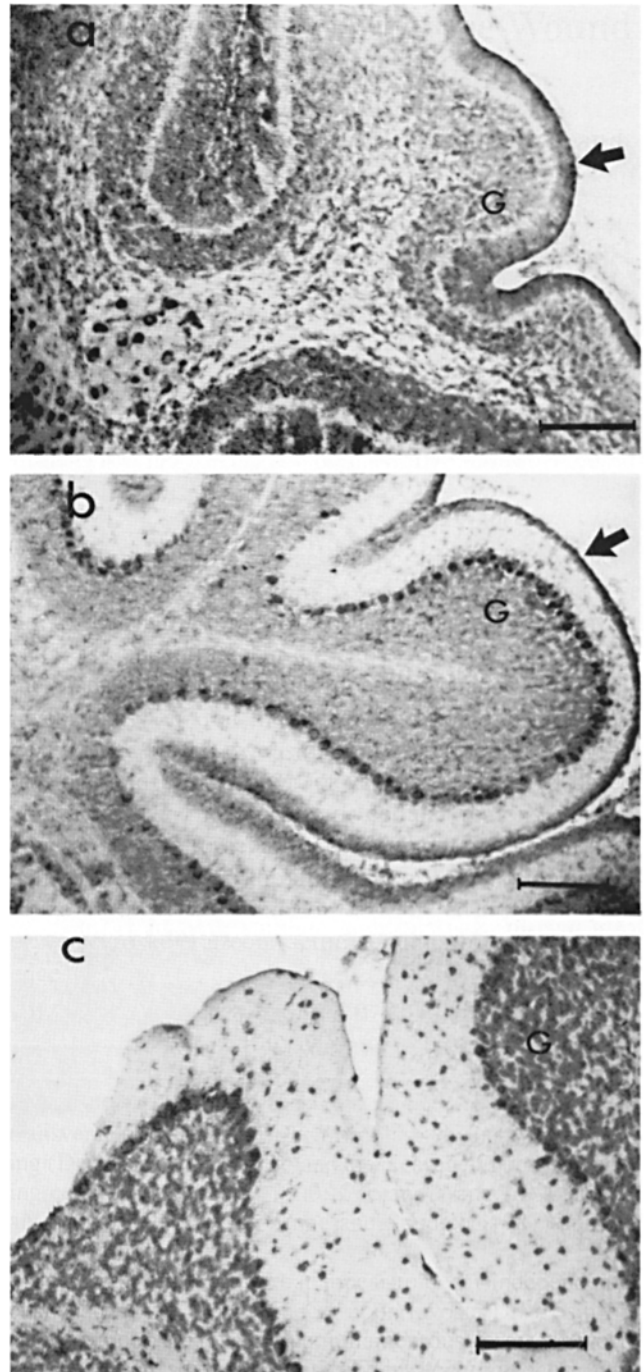


Figure 6. Developmental expression of $MMI\alpha$ in the mouse cerebellum. In situ localization of $MMI\alpha$ transcript in the cerebellum from P7 (a), P12 (b), and adult mice (c). The superficial layer of cells in the P7 and P12 EGL of the P7 and P12 mice (arrows) is more intensely labeled than the deeper layers of this structure. G, granular layer. Bar, 200 μ m.

myosin. A more recent paper from this group (Wagner et al., 1992) provides localization data for this protein in many tissues and cell types that overlap with expression of the $MMI\beta$ transcript.

Expression Pattern of $MMI\alpha$, $MMI\beta$, and $MMI\gamma$

$MMI\alpha$ is highly expressed in the brain, lung, and liver, while

MMI β and MMI γ are detected in diverse tissues including the lung, kidney, small intestine, adrenal gland, and brain. These patterns of expression are distinct from one another and also substantially different from that of BBMI, which appears by both Northern blot and immunochemical analysis to be expressed only in the brush border of the small intestine (Hoshimaru et al., 1989; Bikle et al., 1991). The very different cellular distribution of these messages is consistent with the possibility that each of these products performs separate and unique functions.

The various messages detected by probes derived from MMI α , pGMMI β , and pGMMI γ point to a further complexity within the mammalian myosin I family. One potential concern in interpreting these results is the crossreactivity that may occur by using myosin head sequence as the cDNA probe. However, hybridization was carried out at high stringency, and thus we expect that the bands correspond to alternate transcripts of the same gene or to ones that are highly homologous. This is supported by the different tissue distribution detected with probes to the three different genes. For MMI β and MMI γ , this is further supported by the S1 nuclease protection results. For MMI α , the chance of cross-reactivity is most unlikely because similar results were obtained using multiple probes, including those containing tail and 3' untranslated sequences.

Three messages are detected with MMI α probes. Two of the messages appear to differ by alternate polyadenylation sites. The modifications responsible for generating the largest 6.5-kb message may include an additional coding sequence representing a separate functional domain, or this band may represent unprocessed nuclear RNA. An example of a myosin gene encoding multiple proteins is *ninaC*, in which the two transcripts differ by the presence of the COOH-terminal membrane binding domain (Porter et al., 1992). Using probes derived from pGMMI β and pGMMI γ , we have detected a complex pattern of bands primarily in tissue; the cell lines examined typically displayed only a subset of these messages, suggesting that certain transcripts may be differentially expressed within multiple cell types in a single tissue.

MMI α Encodes a Protein Structurally Similar to BBMI

The cDNAs we isolated for MMI α encode a myosin I containing three functional domains: a canonical myosin head that contains sites for ATP hydrolysis and actin binding, a neck that possesses sites for calmodulin binding, and a positively charged tail that is likely to participate in membrane binding.

Calmodulin is the light chain for BBMI, for the unconventional myosin p190 (Espreafico et al., 1992), and probably for two other vertebrate myosin I proteins that have recently been purified (Coluccio, 1991; Barylko et al., 1992). Association of calmodulin with the heavy chain has been shown to regulate the activity of BBMI (Coluccio and Bretscher, 1987; Collins et al., 1990; Mooseker et al., 1989; Swanlung-Collins and Collins, 1991). The unconventional myosins p190 (Espreafico et al., 1992), dilute (Mercer et al., 1991), and yeast *myo2* (Johnston et al., 1991), as well as BBMI, all encode a region in the neck of the protein that contains four to six related sequences of ~ 23 amino acids. These elements

are homologous to the well-characterized calmodulin binding domain of neuromodulin (Chapman et al., 1991a,b). Like neuromodulin, BBMI and p190 bind calmodulin in the absence of Ca²⁺. Within the analogous neck region, the predicted MMI α protein contains five calmodulin binding sites, and thus also probably binds calmodulin in a Ca²⁺-independent manner. While these five sites possess the common motifs that appear necessary for calmodulin binding, each is more closely related to the homologous site in BBMI than to any of the other four sites within MMI α (and vice versa). This suggests that each site has been uniquely conserved. Moreover, these differences between sites may be reflected by biochemical data for BBMI that demonstrate a complex interaction between Ca²⁺ concentration, the number of calmodulins bound, and motor activity.

The neck region of MMI α also encompasses a 29 amino acid stretch that has high homology, based on sequence and location within the molecule, to a 29 amino acid calmodulin binding insert encoded by a rare transcript of chick BBMI (Halsall and Hammer, 1990). Interestingly, this stretch is encoded by all three MMI α cDNAs we have isolated and sequenced. However, it is possible that there may be additional splice products that vary in this region.

The COOH-terminal region of the predicted MMI α protein contains a high net positive charge. In both BBMI and *Acanthamoeba* myosin I's this region is also highly basic and has been implicated in the binding of these proteins to the plasma membrane. This interaction is mediated at least in part by a high affinity for negatively charged phospholipids found on the inner leaflet of the membrane (Adams and Pollard, 1989; Zot et al., 1992). Thus, MMI α protein would be expected to link the actin cytoskeleton to the cell membrane. Comparison of MMI α to both bovine and chicken BBMI reveals conserved domains within the tail (Fig. 1 B). Interestingly, 65% of these residues are not charged. In contrast, overall charge, but not specifically these domains, is conserved between MMI α and the equivalent regions in *Acanthamoeba* or *Dictyostelium* myosin I's.

We have considered here as myosin I all myosins that possess the positively charged membrane binding domain and do not contain any heptad repeats for dimerization, including all the *Acanthamoeba* and *Dictyostelium* myosin I's and BBMIs. This excludes such molecules as dilute, *ninaC*, and *myo2*, which have been classified within a broader category as "unconventional" myosins (Cheney and Mooseker, 1992). Even this narrow classification for myosin I may permit potential subdivisions. The 65% overall homology of MMI α to BBMI, as compared with 71% between the chick and bovine BBMIs, suggests a high degree of relatedness. This contrasts with the lower degree of homology (47–49%) that pGMMI β and pGMMI γ show to the head domain of BBMI and to each other. Thus, MMI α and the BBMIs can be viewed as a subfamily within the myosin I family as outlined (Espreafico et al., 1992), while MMI γ and MMI β would presently each be single members of separate groupings. Whether these similarities and differences extend to function remains to be determined.

Expression of MMI α in the Nervous System

In situ hybridization and Northern blot analysis reveals that MMI α mRNA is expressed in the mouse brain, from the neonate to the adult. Whereas virtually all brain neurons ex-

press $MMI\alpha$ mRNA, no message is observed in glial cells or in glial cell lines. We cannot, however, rule out the presence of $MMI\alpha$ mRNA in microglia or other relatively sparse populations of nonneuronal cells within the brain.

Although the $MMI\alpha$ message is present at all postnatal stages, expression varies both developmentally and regionally. For example, in situ hybridization in cerebellar granular neurons indicates that $MMI\alpha$ mRNA declines substantially from a peak between P7 and P12 to lower levels in the adult, whereas staining in hippocampal neurons remains equally strong throughout development. Northern blots reveal a general postnatal developmental decline in the expression of $MMI\alpha$ mRNA in the brain. Because most gliogenesis (in contrast to neurogenesis) occurs in the neonate, we suggest that the Northern blot data reflect summation of the actual decrease in average message levels per neuron and the dilution concomitant with the increasing relative percentage of glial RNA.

In addition to postmitotic neurons, neuroblasts in the external granular layer of the cerebellar cortex also stain for $MMI\alpha$ mRNA. These cells continue to divide postnatally; those fated to become granular neurons (the great majority) migrate past the Purkinje cells to form the internal granular layer (Altman, 1972). Interestingly, $MMI\alpha$ mRNA expression is highest in the outermost layer of the EGL (in contrast to the inner layers), which contains those cells that are still dividing and just beginning to extend bipolar processes. We do not yet know whether $MMI\alpha$ is expressed in other neuroblasts and how early this expression commences. It will be of considerable interest to explore these questions through examination of the embryonic brain.

We began our search for novel myosin I genes with the hypothesis that one or more of these motor proteins may participate in processes critical to neuronal development and function such as cell migration, neurite outgrowth, and vesicular transport. The presence of $MMI\alpha$ in all neurons examined and their absence from glial cell are consistent with these roles. The high sequence homology of $MMI\alpha$ with $BBMI$, as well as the high level of $MMI\alpha$ mRNA in neuroblasts just beginning to extend processes, may suggest a role for $MMI\alpha$ at the leading edge of these processes creating motive force between the plasma membrane and the actin cytoskeleton. Whether $MMI\alpha$ can function as a motor for growth cone-like motility is the subject of ongoing investigation.

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