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Cloning and characterization of myr 6, an unconventional myosin of the dilute/myosin-V family

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ABSTRACT **We have isolated cDNAs encoding a second member of the dilute (myosin-V) unconventional myosin family in vertebrates, myr 6 (myosin from rat 6). Expression of myr 6 transcripts in the brain is much more limited than is the expression of dilute, with highest levels observed in choroid plexus and components of the limbic system. We have mapped the *myr 6* locus to mouse chromosome 18 using an interspecific backcross. The 3′ portion of the myr 6 cDNA sequence from rat is nearly identical to that of a previously published putative glutamic acid decarboxylase from mouse [Huang, W. M., Reed-Fourquet, L., Wu, E. & Wu, J. Y. (1990) *Proc. Natl. Acad. Sci. USA* 87, 8491–8495].**

Myosins are oligomeric proteins consisting of one or two heavy chains and varying numbers of light chains. The families of myosins have a similar amino terminal (head) motor domain, but diverge greatly in their carboxyl terminal (tail) domains. Over 40 myosin cDNAs in many organisms have been identified to date. Similarities in head region amino acid sequences have allowed phylogenetic analyses to be performed on sequences of members of the myosin superfamily (1, 2). These analyses indicate that some, if not all, of the myosin families (at least 11) diverged before the divergence of yeast and vertebrates.

The first myosin shown to have a role in vertebrate nervous system function is encoded by the dilute gene. Mutations at the dilute locus cause a diluted coat color phenotype (3, 4). The alteration in coat color appears to result from a lack of dendritic processes in melanocytes that transport pigment to the hair bulb (5). However, our recent results (D. W. Provance, M. Wei, V. Ipe, and J.A.M., unpublished results) indicate that dilute melanocytes are defective in melanosome localization, not in the extension of processes. The dilute myosin is a member of the myosin-V class, with chicken p190 (6), yeast *MYO2* (7), and yeast *MYO4* (8).

Mice homozygous for null dilute alleles are normal, except for their diluted coat color, until about 8 days of age, when they exhibit apparent tonic–clonic seizures with opisthotonus (9). The mutant mice die at approximately 3 weeks of age from unknown causes. No abnormality has been found in the brains of the dilute mutant mice at the time the phenotype first appears, although there are later changes, including demyelination (10). The lack of morphological abnormalities is even more surprising when contrasted with the early and ubiquitous expression of dilute mRNA (4).

A potential cellular role for the dilute myosin in neurons has come from the work of Kuznetsov *et al.* (11), who have demonstrated actin-dependent organelle movement in squid axoplasm. A member of the dilute/myosin-V family has been associated with these organelles by cofractionation studies (12). More recently, myosin-V (p190, dilute) has been localized to small vesicles in the growth cone (13) and its disruption by laser inactivation has been shown to affect retraction of filopodia in growth cones (14). Myosin-V also has been shown to copurify with synaptosome preparations from rat cerebral cortex (15).

The presence of an α-helical coiled-coil heptad motif in the dilute sequence (4) suggested that the dilute protein exists as a dimer. This prediction was confirmed by Cheney *et al.* (16) by rotary-shadowed electron microscopy of purified chicken p190 protein. The dilute sequence has multiple tandem repeats following the motor domain that are very similar to those of the calmodulin (light chain) binding region of brush border myosin I (4), which were later named "IQ motifs" (6). The p190 protein was shown to be complexed with at least four calmodulin light chains (16), with evidence suggesting the presence of one or two additional non-calmodulin light chains.

Two members of the dilute/myosin-V family have been described in *Saccharomyces cerevisiae*: *MYO2*, mutations which were found to disrupt progression through the cell cycle (7), and *MYO4*, which plays an important role in the asymmetric distribution of mating-type regulatory factors (17, 18). Given the presence of two members of this family in yeast, it is reasonable to hypothesize that multiple members of this family will be found in vertebrates. In this study, we characterize the second member of the dilute/myosin-V family, myr 6 from rat, and present evidence indicating that the putative glutamic acid decarboxylase cDNA described by Huang *et al.* (19) represents the 3′ (tail) domain of the mouse ortholog of myr 6.

MATERIALS AND METHODS

cDNA Cloning. A brainstem/spinal cord library in λ ZAPII (Stratagene) was screened with an myr 2 cDNA head domain probe (nucleotides 324-510) (20) as described (21) to obtain cDNA clones 10/1 and 22/1, among clones representing many other myosins. The myr 2 cDNA probe was derived by PCR using oligo MB 11 and oligo MB 20 (20). The 415-5113 myr 6 clone was obtained by screening a rat brain cDNA library in λ ZAPII, a gift from Jessie English (Department of Pharmacology, University of Texas Southwestern Medical Center).

RNA Expression Analyses. *In situ* hybridization was performed on adult rat brains and mouse embryo sections by the

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Abbreviations: MGD, Mouse Genome Database; GAD, glutamic acid decarboxylase.

Data deposition: The sequence reported in this paper has been deposited in the GenBank data base (accession no. U60416).

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method described by Sperry and Zhao (22). A Northern blot with poly(A) RNA isolated from mouse organs was purchased from CLONTECH and hybridized with a ^{32}P-labeled myr 6 cDNA probe (nucleotides 4456-5204 of the myr 6 sequence) by standard methods.

Chromosomal Mapping and Mutant DNA Analysis. DNA from The Jackson Laboratory Backcross DNA Panel Mapping Service (23) was used for mapping the *myr 6* locus. DNA from *bouncy* homozygous mice and wild-type littermate controls was purchased from The Jackson Laboratory Mutant DNA Resource. Restriction digests and Southern blots were performed by standard techniques. The gel-purified insert from myr 6 clone 22-1 was labeled with ^{32}P-dCTP with the Decaprobe kit (Ambion, Austin, TX) and used as probe.

RESULTS

cDNA Cloning and Sequencing. The assembled cDNA sequence has been submitted to GenBank (accession number U60416). The cDNA clones 22-1 (nucleotides 573-4519) and 10-1 (nucleotides 1-3782) were sequenced. The overlapping sequence had 77% nucleotide identity with the head, or 5′ region, of the dilute cDNA (4), consistent with the initial characterization of myr 6 as a member of the dilute/myosin-V family from a small portion of the sequence. The myr 6 tail region sequence has extremely high (96%) nucleotide sequence identity with the putative murine L-glutamate decarboxylase (GAD) cDNA sequence published by Huang and colleagues (19, 24).

We therefore used a 5′ PCR primer from the 3′ end of 22-1 and a 3′ primer based on the GAD sequence to extend the myr 6 sequence in the 3′ direction by PCR, using rat brain cDNA as template. This strategy produced a PCR fragment of the expected size (748 bp, nucleotides 4456-5204) that was cloned and sequenced. It was 96% identical to the murine GAD cDNA sequence in the 3′ overlapping region. When the same primers were used to amplify mouse brain cDNA, the overlapping portion was identical to the GAD cDNA nucleotide sequence, with the exception of the 52 bp at the 5′ end of the GAD sequence.

To complete the cDNA sequence, the insert from 22-1 was used to screen a rat brain cDNA library. This screen produced a clone (415-5113) whose overlapping sequence was identical to that of the other myr 6 cDNAs. The final remaining 3′ sequence (4456-6078) was cloned by the rapid amplification of cDNA ends technique (25).

The deduced myr 6 amino acid sequence is clearly very closely related to dilute, its homolog in chicken, p190, and the other members of the myosin-V family, *S. cerevisiae MYO2* and *MYO4* (2). The deduced amino acid sequences of the amino terminal head, or motor, domain sequences are more similar within the family than those of the tail domains. Table 1 shows the percentage of amino acid sequence identities among the five family members and Fig. 1 illustrates the relationship between them graphically.

The calmodulin light chain-binding neck and the putative cargo-binding tail regions of myr 6, dilute, p190, and the

Table 1. Amino acid sequence similarities among members of the dilute/myosin-V family

	dilute	p190	myr 6	*MYO2*
dilute				
p190	95			
myr 6	78	78		
MYO2	63	64	62	
MYO4	61	63	61	69

Full-length (both head and tail) amino acid sequences were aligned with the GAP program of the Genetics Computer Group suite 26 with a gap creation penalty of 2.0 and a gap extension penalty of 0.1.

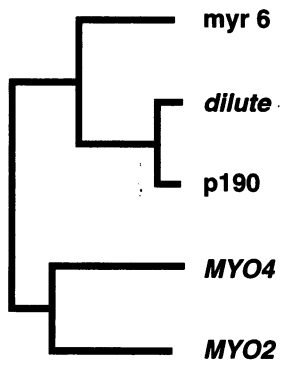

FIG. 1. Phylogenetic tree produced by CLUSTAL W 1.60 (27) multiple-sequence alignment of myr 6, mouse dilute (X57377), chicken p190 (Z11718), *S. cerevisiae MYO4* (M90057), and *MYO2* (M35532) entire deduced amino acid sequences.

putative GAD are shown aligned in Fig. 2. Six IQ motifs are found in the neck region, followed by an α-helical coiled-coil sequence, a PEST calpain cleavage site, and a second α-helical coiled-coil domain. The myr 6 carboxyl-terminal domain shares the homology between dilute and both human AF-6 and *Drosophila* canoe described by Ponting (28) and noted in Fig. 2.

Similarity with Murine GAD cDNA. The C-terminal 755 amino acid residues of myr 6 are nearly identical to the putative GAD sequence published by Huang and colleagues (19, 24) and shown in Fig. 2. The myr 6 sequence also has regions of 26 and 12 residues (shown in boldface type in Fig. 2) that appear to represent alternatively spliced exons, which are not found in the putative GAD sequence.

The 26-amino acid exon is absent in the brain cDNAs of all the other members of the family. However, the alternative splicing pattern found in dilute transcripts from melanocytes (29) contains an exon (D) that is very similar in sequence to the 26-amino acid exon, shown above the alignment in boldface type. This exon is not present in dilute transcripts from brain. The 12-amino acid exon, found within the carboxyl-terminal region that is homologous to AF-6 and canoe, appears to be present, although of shorter length, in all the other family members.

When the N-terminal 6 residues of the deduced GAD sequence and the inserted sequences of 26 and 12 amino acids from myr 6 are eliminated, there are only 25 amino acid differences (96.5% identity, 98.5% similarity) between myr 6 and the putative mouse GAD. These data strongly suggest that the original GAD clone (19, 24) was truncated and that it represents the mouse ortholog of rat myr 6.

RNA Expression Analyses. Northern blot hybridization of myr 6 cDNA to poly(A)$^+$ RNA from mouse organs is shown in Fig. 3. Strong expression of a 7-kb myr 6 transcript was observed in testis, kidney, liver, lung, and heart. Smaller amounts of myr 6 transcripts were detected in muscle and brain. Hybridization of a mouse GAD probe (corresponding to nucleotides 4459-5201 of the rat myr 6 sequence) derived by PCR amplification of mouse brain cDNA yielded a band of indistinguishable size and tissue distribution (data not shown).

In situ hybridizations with myr 6 antisense and control sense RNA probes were performed on adult rat brain sections. A representative dark-field photomicrograph of the antisense hybridization is shown in Fig. 4. The most striking concentrations of myr 6 RNA in the adult brain are in the most ventral portion of the dentate gyrus and in the choroid plexus (Fig. 4). The dorsal choroid plexus, medial mammillary nuclei, and amygdala also hybridized strongly. The sense probe showed no evidence of specific hybridization (data not shown).

Genetic Mapping of the *myr 6* Locus in Mice. The *myr 6* locus was mapped using The Jackson Laboratory BSS interspecific

FIG. 2. CLUSTAL W 1.60 (27) multiple-sequence alignment of myr 6, mouse GAD (GenBank accession number M55253), mouse dilute, and chicken p190 deduced tail-region amino acid sequences. Features, including IQ motifs, α-helical coiled-coil domains, PEST calpain cleavage domains, and the AF-6/canoe homology (DIL) domain are identified above the aligned sequences.

backcross (23). Hybridization of a cDNA probe (nucleotides 415-5113) to C57BL/6J and *Mus spretus* *Bam*HI digests of genomic DNA yielded a restriction fragment length polymorphism. The cDNA probe was hybridized to Southern blots of *Bam*HI digests of genomic DNA from 94 N_2 progeny from a (C57BL/6J × *M. spretus*)F_1 × *M. spretus* backcross. Each digest was scored for the presence or absence of the C57BL/6J band. The mapping data are summarized in Figs. 5 and 6. The mouse *myr 6* locus, which has been assigned the Mouse Genome Database (MGD) name *Myo5b*, was mapped to chromosome 18 at approximately position 48 on the MGD composite map (http://www.informatics.jax.org/mgd.html). The bouncy (*bc*), shaker-with-syndactylism (*sy*), plucked (*pk*), and chronic multifocal osteomyelitis (*cmo*) loci also are found on chromosome 18 and map to MGD positions 25, 36, 22, and 45 respectively. There is very little integration between the maps of classical phenotypic loci and molecular markers on this chromosome; therefore, it is not prudent to eliminate any of these loci by its MGD position alone.

Of these candidate mutants, *bc* appears to have the phenotype most consistent with a loss of myr 6 function. Homozygous mutant mice are small, with tremors and a bouncing gait (30). Comparison of genomic DNA from *bc/bc* mice carrying three independent *bc* mutant alleles (*bc*, bc^{2J}, bc^{3J}) with that of wild-type control littermates using 18 different restriction endonucleases yielded no polymorphisms. We currently are testing DNA from *cmo* and *sy* mutants for polymorphisms.

DISCUSSION

Our search for more members of the dilute/myosin-V family was prompted by the discovery of two members of this family

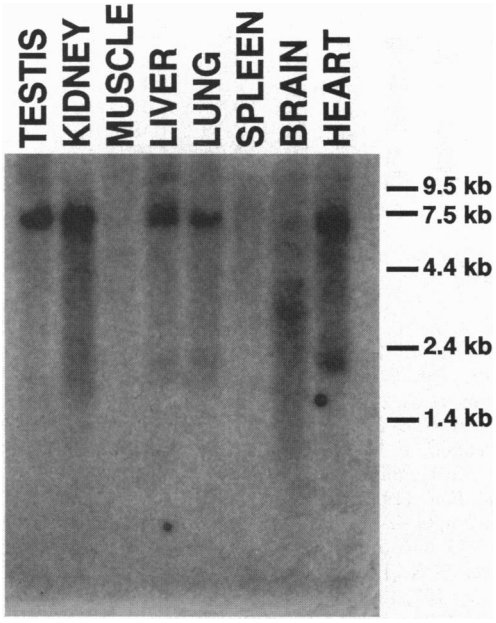

FIG. 3. Autoradiogram of a Northern blot of poly(A)$^+$ RNA from mouse tissues hybridized with a ^{32}P-labeled myr 6 cDNA probe. Marker sizes are shown at right.

in yeast and the mildness of the dilute mutant phenotype when contrasted with the widespread temporal and spatial expression of dilute. These data suggested that dilute is a member of a family of myosins with overlapping functions.

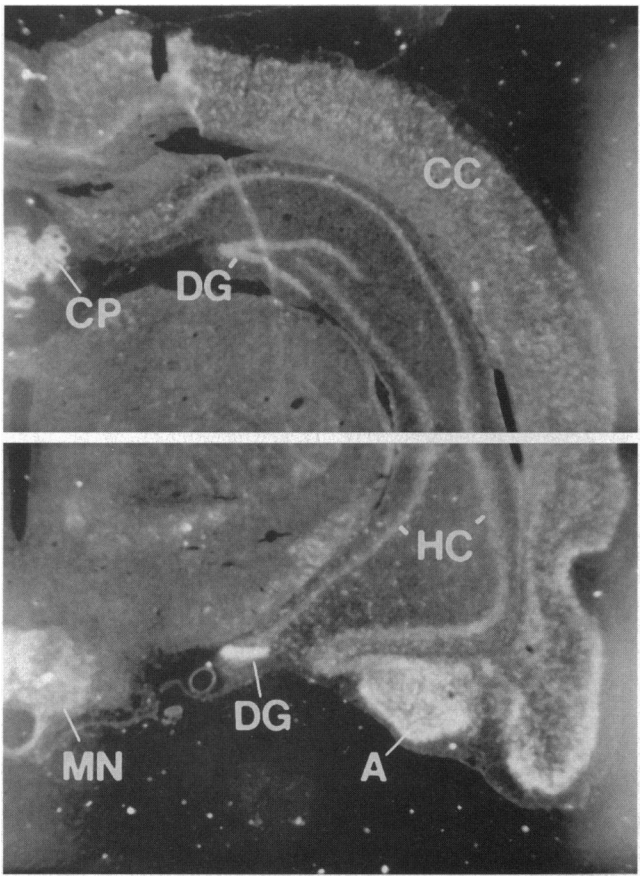

FIG. 4. Dark-field photomicrograph of an adult rat brain section hybridized with a ^{35}S-labeled antisense RNA synthesized from the 10-1 myr 6 cDNA, dipped in Kodak NTB-2 emulsion, and exposed for 5 days at 4°C. Cerebral cortex (CC), choroid plexus (CP), dentate gyrus (DG), hippocampus (HC), amygdala (A), and medial mammillary nuclei (MN) are labeled.

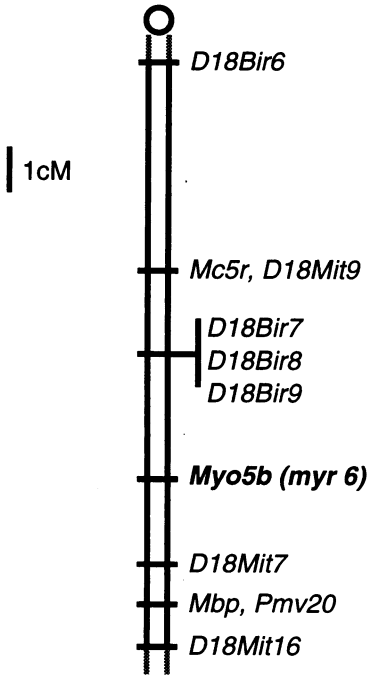

FIG. 5. Linkage map of mouse chromosome 18 showing the position of the *myr 6* (*Myo5b*) locus.

The deduced amino acid sequence of myr 6 shows that it is a close relative of dilute and p190, and less closely related to the yeast members of this family, *MYO2* and *MYO4*. Among the myosins, the greatest divergence is seen in carboxyl terminal, or tail, sequences both within and between families (2). However, the tail sequence of myr 6 is very closely related to those of dilute and p190 (Table 1, Figs. 1 and 2). Bement *et al.* (31) isolated multiple-myosin head region cDNAs from human Caco-2$_{BBe}$ epithelial cells by degenerate PCR, including two members of the dilute/myosin-V family, HuncM-VA and HuncM-VB. These cDNAs encode 33 amino acids of the head domain. Of the two sequences, HuncM-VA is more closely related to dilute (29 of 33 identical amino acid residues) than is HuncM-VB (27 of 33). In isolation, the data of Bement *et al.* (31) suggest that HuncM-VA is the ortholog of dilute; however, the myr 6 sequence is more closely related to the HuncM-VA sequence (31 of 33) than the HuncM-VB sequence (24 of 33). These data, despite the short length of the human sequences, suggest that myr 6 is the ortholog of HuncM-VA and that HuncM-VB represents a third member of the dilute/myosin-V family.

The pattern of expression of myr 6 differs dramatically from that of dilute. In adult brain, myr 6 expression is highly localized to dentate gyrus, with higher expression ventrally than dorsally. The level of myr 6 expression in dentate gyrus is much higher than that of the hippocampus; most gene expression studies show similar levels of expression in these two structures. High levels of expression also were observed in the amygdala, medial mammillary nuclei, and choroid plexus. With the exception of the choroid plexus, the interconnection of the structures expressing myr 6 transcripts in the limbic system is intriguing considering the apparent seizure phenotype observed in dilute mutants. Although the dilute neurological phenotype appears to represent a form of epilepsy, homozygous mutant mice have normal electroencephalograms (Marks, Shaffery, and J.A.M., unpublished results), suggesting either that the seizures are of subcortical origin or that the dilute phenotype may be more analogous to human movement disorders than it is to epilepsy. We have observed no increase

FIG. 6. Haplotype analysis for the *myr 6* (*Myo5b*) locus. Black and white boxes represent inheritance of C57BL/6J and *M. spretus* alleles, respectively. Recombination percentages and standard errors are given for each interval.

in myr 6 expression in the brains of dilute–lethal mutants (data not shown).

The publication of the putative mouse GAD cDNA cloning and sequencing in 1990 and 1991 (19, 24) occurred at approximately the same time as our publication of the dilute sequence (4), so that neither publication acknowledged the obvious homology between the two sequences. The GAD cDNA clone was selected from a λgt11 expression library by reactivity with an anti-GAD polyclonal antibody and GAD activity in the phage lysate. This sequence also has no similarity to any of the other GAD sequences that have been published to date (32–35). To our knowledge, no further characterization of this putative GAD isoform has been published since the original report. The hybridization of a mouse cDNA PCR probe based on the GAD sequence of Huang *et al.* (19) to a 7-kb transcript indistinguishable from the myr 6 transcript also is consistent with our hypothesis that the GAD clone is a truncated myr 6 cDNA. The publication of the p190 sequence from chicken by Mooseker and colleagues (6) addressed the homology with the GAD sequence by performing GAD assays on extracts of bacteria expressing the corresponding region of p190; they found no GAD activity.

Genetic mapping using The Jackson Laboratory interspecific backcross (23) placed the *myr 6* locus on mouse chromosome 18; no polymorphisms were observed in DNA from mice homozygous for mutant alleles at the bouncy locus, which maps near to *myr 6* and whose phenotypes are consistent with our general hypotheses for myr 6 function.

Because the myr 6 tail sequence is very similar in sequence to that of dilute, we believe that it is very likely that myr 6 and dilute will have overlapping functions. The presence in both dilute and myr6 of the AF-6/canoe DIL domain (28) is consistent with this hypothesis. AF-6 (36) and canoe (37) are DHR/PDZ (Discs-large homology region/PSD-95, Discs-large, ZO-1) domain proteins that are hypothesized to bind to protein 4.1 and its relatives and function in signal transduction pathways (38, 39). In particular, canoe mutations show genetic interactions with mutations in genes in the Notch pathway (37) and both AF-6 and canoe have recently been shown to bind to Ras *in vitro* (40). The homology suggests the possibility that AF-6 can interact with the cargoes of myr 6 and dilute, perhaps as a submembrane dock.

We currently are constructing a mouse *myr 6* mutant by gene targeting techniques and will construct a double mutant with dilute to test the overlapping function hypothesis directly. The availability of many different mutant alleles of varying severity at the dilute locus makes this approach an attractive one.

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