mau-2 acts cell-autonomously to guide axonal migrations in Caenorhabditis elegans

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Summary

The gene mau-2 has been found to be required for the guidance of cellular and axonal migrations along both the anteroposterior and the dorsoventral body axes during the development of the nematode C. elegans. We show that mau-2 encodes a novel, previously uncharacterized protein that is highly conserved among animals. Maternal mau-2 gene expression is sufficient for normal development until the fourth larval stage, and a MAU-2::GFP fusion protein localizes to the cytoplasm of neurones. mau-2 is ubiquitously expressed in embryos by late gastrulation and becomes predominantly expressed in the nervous system as morphogenesis progresses. Expression of mau-2 within individual neurones rescues the guidance defects of mau-2 mutants, indicating that mau-2 functions cell-autonomously. Altering the activity of both the dorsal repellent slt-1 and mau-2 leads to the abnormal dorsal projection of the AVM axon, a phenotype that is novel and specific to the interaction of these two genes, indicating that mau-2 participates in the guidance of AVM by a slt-1-independent mechanism. Taken together, mau-2 defines a novel guidance factor that might be involved in the intracellular processing of guidance cues encountered by migrating cells and axons during development.

Key words: mau-2, Migration, Guidance, slt-1, slt, Caenorhabditis elegans, Maternal effect, Cell autonomous

Introduction

Understanding how nervous systems develop is a central issue in biology. Genetic studies using C. elegans have yielded remarkable insights into the processes of neuronal differentiation, including how migrations are guided and how synaptic connectivity is established. Although most cells of the worm are born close to their final positions as a result of cell division and differentiation, a number of neurones and their growth cones, as well as mesodermal cells, undergo stereotyped long-range migrations during development (Hedgecock et al., 1987). Neurones migrate between the hypodermal cell membrane and the basement membrane secreted by the hypoderm, and mesodermal cells migrate along the basement membrane on the side facing the pseudocoelomic cavity.

A number of genes that are known to be required for proper migration in C. elegans were identified through screens for behavioural mutants (Brenner, 1974), the subsequent characterization of which revealed specific neuroanatomical defects (e.g. Hedgecock et al., 1990; Hedgecock et al., 1985; McIntire et al., 1992). Other screens have used surrogate markers such as transparent larvae or withered tails (Forrester et al., 1998; Wightman et al., 1997). Yet more genes were identified through screens for mutants that affect the position of particular neurones and axons, regardless of their behavioural consequences, such as sax-3, which affects the development of the nerve ring (Zallen et al., 1998), and nid-1, which affects the position of nerve bundles (Kim and Wadsworth, 2000).

The molecular characterization of some of these genes has revealed that a wide range of proteins contribute to the proper development of the nervous system of the worm (reviewed by Antebi et al., 1997; Blelloch et al., 1999). Some proteins act as guidance cues or as receptors for these guidance cues, other are components of the extracellular matrix or regulate cell adhesion, and yet other proteins participate in the cellular response to guidance cues. A number of proteins required for the guidance of migrations are novel, such as MIG-13 (Sym et al., 1999), CED-12 (Gumienny et al., 2001), UNC-53 (Stringham et al., 2002), VAB-8 (Wolf et al., 1998), and UNC-76 (Bloom and Horvitz, 1997), and their biochemical functions remain unknown.

Most of the genes required for the development of the nervous system identified so far are zygotic. In an attempt to identify genes involved in the earliest stages of the patterning of the nervous system, a genetic screen for maternal-effect behavioural mutants was carried out (Hekimi et al., 1995). Three mutations (qm4, qm5 and qm40) identified in this screen define the gene mau-2 (mau stands for maternal-effect uncoordinated).

mau-2 mutants are severely uncoordinated and egg-laying defective, and these phenotypes are completely penetrant (Hekimi et al., 1995; Takagi et al., 1997). In addition, approximately one-fifth of the larvae fail to complete development and become filled with fluid (the Lid phenotype). At the anatomical level, mau-2 mutants display defects in the...
positions of cell bodies and processes that belong to the locomotory, egg-laying and osmoregulation systems. In fact, numerous cells that undergo long-range migrations during the development of the worm are misplaced in mau-2 mutants, including neurones (e.g. AVM, PVM, SDQR/L and HSNR/L), axons (e.g. of AVM, PLM, and CAN), as well as the distal tip cells (DTCs) (Takagi et al., 1997). mau-2 mutations affect migrations that occur embryonically as well as post-embryonically. For example, the embryonic migration of the cell body of HSN and the extension of the axons of PLM and CAN, are affected in mau-2 mutants. In addition, the extension of the axon of AVM, the projection of motoneurone axons into the dorsal cord, the migration of the Q5 descendants and of the DTCs, all of which occur during larval development, are abnormal in mau-2 mutants. In addition, migrations that occur along both the longitudinal and the circumferential body axes of the worm, oriented anteriorly-, posteriorly-, ventrally- or dorsally-, are all affected in mau-2 mutants. Thus, rather than being involved in a specific subset of migrations, mau-2 is broadly required for the proper migration of cells and axons.

Importantly, migrating cells frequently undergo excessive migrations in mau-2 mutants, such that their final position is beyond the target region where the migration should have halted. For example, the SDQR and HSN neurones, which undergo anteriorly directed migrations during development, are frequently placed too anteriorly in mau-2 mutants (Takagi et al., 1997). In addition, the axon of AVM, which normally projects ventrally, can instead extend posteriorly for long distances in mau-2 mutants. Thus, mutations in mau-2 do not impair the motility of the cells per se, but rather affect the proper guidance of these migrations. Here, we report that mau-2 encodes a novel protein that functions cell autonomously to guide migrations during the development of the nervous system.

Materials and methods

Nematode strains

Worms were grown as described (Brenner, 1974) at 20°C unless otherwise specified. The wild-type strain was N2. mau-2 alleles (qm4, qm5, qm40 and qm160) were outcrossed at least five times. Strains used were:

che-3(e1214), dpy-5(e61), fer-1(hc1), lin-10(e1439), unc-13(e51), unc-29(e1072); unc-55(e402), glp-4(bn2ts), fem-2(b245ts), fem-3(q20ts), unc-6(e78), unc-6(e400), sli-1(eh15), unc-5(e151); zd154 IV (an integrant of mec-4::gfp, a gift from Dr Clark and Dr Bargmann);

kyIs218 X and kyIs209 X [two integrants of myo-3::sli-1 misexpressing sli-1 in all body wall muscles (a gift from Dr Bargmann) (Yu et al., 2002)];

mgls71 (an integrant of tph-1::gfp) (Sze et al., 2000);

unc-31::gfp (Takagi et al., 1997);

and see below.

Hermodrphidates were injected with cosmids, deletion constructs and pRF4 (Mello and Fire, 1995) at concentrations of 5, 50 and 100 ng/µL, respectively. Insert orientation in cosmid C09H6 was determined by restriction mapping.

Neuroanatomical observations

The position of neurones, axons and fascicles was examined using the reporter unc-31::gfp, which is expressed in virtually all neurones and in vulval muscles (Takagi et al., 1997); the axon of HSN, using tph-1::gfp [mgls71; (Sze et al., 2000)]; AVM, using the mec-4::gfp reporter. In tissue-specific rescue assays, plasmids for mec-4::gfp or unc-31::gfp was injected at 100 ng/µL. Examination of maternally rescued animals was carried out using an integrated unc-31::gfp. For the analysis of genetic interactions with unc-40::myr and slt-1, zdIs4 was used. The morphology of the axon of AVM was scored only when the position of the AVM cell body was normal.

Molecular analysis

General molecular manipulations were carried out as described (Sambrook et al., 1989). Pfu polymerase was used for PCR (Stratagene). All cloned inserts were sequenced.

mau-2 transcript

SL1- or SL2-specific primers, combined with primers specific for predicted exons, were used on two independent first-strand mixed-stages cDNA libraries. RT-PCR products and yk462a (gift of Dr Kohara) were sequenced. mau-2 encodes a single message of 2010 bp (not including SL1, nor the poly A tail). The 5′UTR is SL1 transcribed at the acceptor site CAG, 6 bp upstream of the initiating ATG. The polyadenylation signal is probably AAAAAA, located 211 bp downstream of the TAA Stop codon. The polyadenylation cleavage site is 8 bp downstream of the polyadenylation signal, resulting in a 222 bp long 3′UTR. yk462a lacks the 5′UTR and the first 9 coding base pairs, and part of the 3′UTR.

mau-2 mutant alleles

A library of UV-TMP-mutagenized worms was screened by nested PCR with primers specific for the gene mau-2 and qm160 was isolated (Edgley et al., 2002). The primer sequences correspond to the following bases on cosmid C09H6: 27984-28007, 32138-32161, 27507-27530 and 31892-31915. qm160 fails to genetically complement qm5.

The region of mau-2 was sequenced on two independent PCR products amplified from genomic DNA of N2, qm4, qm5, qm40 and qm160.

Northern blot analysis

Worm populations were synchronized at different developmental stages as described (Wood, 1988). Total RNA was isolated using Trizol (Gibco). A mau-2-specific probe made by 32P-radiolabelling (Ready-to-go kit, Pharmacia) using the entire insert of yk462a was used for northern blotting.

Anti-MAU-2 polyclonal antibodies and western analyses

A BSA-conjugated peptide located (ASRRMLSVENLPTL-VANMPASK, residues 552-573 near the C terminus of MAU-2) was synthesized (Sheldon Center, McGill University) and injected into two rabbits (Animal Resources Center, McGill University). Polyclonal antibodies were affinity purified from crude serum on an Affigel column (BioRad) (Harlow and Lane, 1999). The purified antibodies detect the conjugated antigen of an expected size of 70 kDa, free peptide at ~2.5 kDa, a band of the expected size of MAU-2 (~68 kDa) in N2 but not in mau-2(qm160) extracts, and a band at ~68 kDa in mau-2(qm160) mutants carrying wild-type copies of mau-2 on an extrachromosomal array. Few other non-specific bands were detected.

Total protein extracts were prepared by grinding. Affinity purified anti-MAU-2 antibody (see below) was used at a 1:5 dilution, and donkey anti-rabbit IgG antibody (Jackson Laboratories) at 1:1000, followed by detection with ECL (Amersham). Analysis MAU-2 in maternally rescued animals: 400 Dpy worms for each genotype and each stage were picked, and denatured in sample buffer.

Reporter constructs for mau-2

For translational fusions between mau-2 and gfp, synthetic Prl and BamHI sites on primers allowed directional cloning of bases 27054-32154, 24119-32154, or 22063-32154 of C09H6 into pPD95.77.
Constructs for tissue-specific expression of mau-2

Extrachromosomal arrays carrying mau-2(+) do not give maternal rescue (non-transgenic arrays derived from a transgenic mau-2 hermaphrodite are invariably Mau). In addition, a promoterless transgene containing the entire mau-2 cDNA (pCB42) cannot rescue the mau-2 mutants.

pCB42 contains the entire mau-2 CDNA from the ATG to the last codon, excluding the Stop, fused in frame to gfp. yk462a was the template, and the missing 5′UTR, along with a primer. The 3′ primer contained a synthetic BamHI site for cloning into pPD95.77.

Plasmids were obtained by cloning promoters in front of mau-2 in pCB42. Pdpy-7::mau-2::gfp contains a 0.9 kb fragment corresponding to the promoter of the gene dpy-7 (Gilleard et al., 1997). Pmyo-3::mau-2::gfp, a 2.5 kb fragment for the myo-3 promoter (Okkema et al., 1993); Punc-119::mau-2::gfp, a 1.2 kb region for the unc-119 promoter (Maduro and Pilgrim, 1995); Pmec-7::mau-2::gfp, a contains a 0.8 kb fragment for the mec-7 promoter (Hamelin et al., 1992).

These constructs were injected into N2 and qm160 at 10 ng/µl, along with PRF4 (100 ng/µl) and mec-4::gfp or unc-31::gfp at 100 ng/µl.

Results

Larval and locomotory defects but not egg-laying defects of mau-2 mutants can be maternally rescued

mau-2 mutants are severely uncoordinated and egg-laying defective, and these phenotypes are completely penetrant (Hekimi et al., 1995; Takagi et al., 1997). In addition, approximately one-fifth of the larvae fail to complete development and become filled with fluid (the Lid phenotype). mau-2 mutants were recovered in a screen for mutations that displayed a maternal rescue effect (Hekimi et al., 1995), i.e. homozygous mutant animals issued from a heterozygous mother appear phenotypically wild type (maternally rescued animals are denoted as m+z− throughout the paper). The maternal effect is not strict: heterozygous animals derived by mating from a homozygous mother are fully wild type. Several aspects of the phenotype of mau-2 mutants can be completely rescued maternally, while others aspects are not. For example, maternally rescued mau-2 animals complete larval development normally and have a wild-type locomotion, but they remain severely egg-laying defective (Takagi et al., 1997). This failure of maternal rescue of the egg-laying defect points to a role of mau-2 in the development of the relevant structures. As the egg-laying system develops relatively late (L3 and L4 larval stages), it is conceivable that the maternally supplied product has run out by that time and that these structures develop abnormally. Alternatively, mau-2 could be required for the function of the neurones and muscles required for egg laying in adults, rather than for the development of these structures per se.

To gain insight into the function of mau-2, we determined to what extent the development of m+z− animals proceeds normally. We examined mau-2(qm5) maternally rescued animals at the anatomical level using the reporter unc-31::gfp, which allows the visualization of the nervous system and the vulval muscles. In contrast to mau-2(qm5) mutants, the nervous system of m+z− is virtually indistinguishable from that of the wild type: the ventral nerve cord, the commissures and laterals are normally organized, the dorsal cord is always fasciculated, and the cell body of migratory neurones such as AVM, SDQR and HSN, as well as of the DTCs, are normally positioned (Fig. 1). The defect of the axon of AVM is also completely maternally rescued. Thus, the parts of the nervous system that underlie locomotion develop normally in maternally rescued animals. As the excretory and locomotory systems are laid down largely during embryogenesis or shortly after hatching (L1 and early L2 larval stages), it appears that sufficient wild-type mau-2 product can be supplied by the mother for the early development of its progeny to occur normally. However, maternally rescued mau-2 animals display anatomical defects in their egg-laying system, consistent with their abnormal egg laying. In particular, at least one out of the four vm1 vulval muscles is abnormally placed in 24% of the maternally rescued animals (Fig. 1). In addition, the axon of one of the two HSN neurones projects abnormally in 21% of maternally rescued animals, often extending laterally from the cell body first, and projecting ventrally at a position anterior to the vulva, instead of posterior to it. Other aspects of the egg-laying system, such as the placement of the vm2 vulval muscles and uterine muscles, which are more difficult to examine, might also be affected in mau-2 m+z− animals, possibly accounting for the completely penetrant egg-laying defect.

Positional cloning of mau-2

mau-2 was previously mapped between dpy-14 and unc-29 on linkage group I, outside the chromosomal deficiency nDf23, but inside nDf24 (Hekimi et al., 1995). We mapped mau-2 to the right of unc-55 (2.35 cM), tightly linked to lin-10 (2.56 cM) and che-3 (2.52 cM) (Fig. 2A, see Table S1 in the supplementary material). Physical mapping of the left end of the chromosomal deficiency nDf23 (Whitfield et al., 1999), which genetically does not delete mau-2, indicated that mau-2 lies to the left of ceh-8T01G9.2. Cosmids covering the physical region spanning from unc-55 to ceh-8 were assayed for transformation rescue of mau-2 mutants (Fig. 2B). Cosmid C09H6 and the deletion construct C09H6∆NarI fully rescued mau-2 animals for larval development, locomotion and egg laying. A PCR fragment containing the only predicted gene on C09H6∆NarI rescued mau-2 mutants. The gene C09H6.3, currently annotated in WormBase, corresponds to mau-2 and encodes a predicted protein of 593 amino acids.

mau-2 mutations lead to a complete loss of function

We identified the molecular lesions that underlie the mau-2 mutants. Allele qm54 carries a nonsense mutation at codon 20, qm40 has a nonsense mutation at codon 279, and qm4 is a missense allele substituting a Gly to an Arg at codon 53 (Fig. 2C). To unequivocally determine the null phenotype of mau-2, we isolated a knockout allele in the gene mau-2, from a PCR-based screen for the isolation of deletions. Allele qm160 deletes 1922 bp of the gene (bases 28330 to 30252 on cosmid C09H6). In qm160, codon 66 in exon 1 is joined to the last 54 bp of intron 5, introducing two in-frame Stop codons. A translated product, if any, would be at most 68 amino acids long. It is worth pointing out that a construct coding for the first 84 amino acids does not rescue any of the mau-2 mutants (data not shown), making it unlikely that a translated product encoded by mau-2(qm160) would be functional. Therefore, mau-2(qm160) is a molecular null allele.

The mutant phenotype of all four mau-2 alleles is identical
for phenotype penetrance and expressivity, at both the dissecting microscope and neuroanatomical levels. We have previously described the phenotype of qu4, qu5 and qu40 (Takagi et al., 1997), and now describe qu160. Like the other alleles, all mau-2(qm160) animals are uncoordinated and egg-laying defective (41% of the Egl animals actually become bags of worms, n=359), and 17% of the larvae acquire a straight and translucent appearance and die – the Lid phenotype [n=200 larvae (Takagi et al., 1997)]. In addition, the dorsal cord defasciculation defect is similarly affected in qu160 compared with the other mau-2 alleles and the penetrance of the defect of the guidance of the A VM axon is very similar to that of qu5 and qu4 [see below (Takagi et al., 1997)]. As all four mau-2 alleles have identical phenotypes, it appears that they all result in a complete loss of the gene function.

We examined protein levels in mau-2 mutants compared to the wild type (Fig. 3A). We developed affinity-purified polyclonal antibodies directed against MAU-2 and carried out immunoblot analysis on worm extracts. We believe that a band of ~68 kDa, the expected molecular weight for MAU-2, corresponds to the full-length MAU-2 protein as it is detected in wild-type worms, absent in the deletion allele mau-2(qm160), and detected in mau-2 mutants that carry wild-type copies of mau-2. MAU-2 is undetectable in the nonsense alleles mau-2(qm5) and mau-2(qm40), indicating that they are likely molecular nulls, although we cannot exclude that undetected truncated proteins may be produced in these two nonsense mutants. Finally, the level of MAU-2 is strongly reduced in the mis-sense allele mau-2(qm4), indicating that the qm4 mutation severely affects the stability of MAU-2, and probably also the function of the remaining mutant protein, as this allele is as severe as the other mau-2 mutants. Interestingly, the Gly residue that is mutated in qm4 is conserved through evolution in all animals.

mau-2 encodes a novel highly conserved protein

Database searches for proteins homologous to C. elegans MAU-2 revealed that it is similar to predicted polypeptides across metazoans (Fig. 2D). Thorough database searches have uncovered only one protein homologous to MAU-2 in each species for which a complete genome sequence is available, suggesting that the genes encoding proteins of the MAU-2 family are orthologues. C. elegans MAU-2 and the related vertebrate proteins are 25% identical. MAU-2 is extremely well conserved among vertebrate species: the MAU-2 proteins are 95% identical between the fish Fugu rubripes and humans, and are more than 99% identical among mammals. Comparisons between the proteomes of F. rubripes and H. sapiens reveal that only 5% of the vertebrate proteins display more than 95% amino acid similarity (Aparicio et al., 2002), indicating that MAU-2 is among the best conserved proteins through vertebrate evolution.

Extensive searches using prediction programs detect no functional domains, conserved motifs or subcellular localization signals within C. elegans MAU-2, other than a tetratricopeptide-like domain (IPR008941, residues 485-495). A tetratricopeptide (TRP) repeat is detected in vertebrate MAU-2 proteins (IPR001440, residues 443-476 in F. rubripes and 459-492 in H. sapiens). TRP repeats occur in diverse proteins and are believed to mediate protein-protein interactions.
interactions and the assembly of multiprotein complexes. However, common features in the interaction partners remain undefined (D’Andrea and Regan, 2003; Lamb et al., 1995), providing no clue to what the biochemical function of MAU-2 might be. All four mau-2 mutant alleles can be rescued with a transgene that carries the first five exons of mau-2, which code for a truncated protein from residues 1 to 371 of MAU-2. Thus, at least when overexpressed from an extrachromosomal array, this N-terminal region of MAU-2 is sufficient to carry out fully the identified functions of mau-2, suggesting that it is functionally the most important.

### Abundant mau-2 transcripts accumulate in the oocytes

We examined the abundance of the mau-2 transcript throughout development in wild-type worm populations by northern blot analysis. A single band of 2 kb, which is the expected size for the mau-2 mRNA, is detected at all stages (Fig. 3B). The mau-2 transcript is abundant in the embryonic and young adulthood stages, whereas it is present in low amounts throughout the larval stages.

Given the maternal contribution of mau-2 to development and the high transcript level in young adults, we investigated the mau-2 mRNA levels in the germline by comparing wild-type young adults with adults carrying mutations that affect the germline (Fig. 3B). The mau-2 transcript level is extremely reduced in glp-4(bn2ts) mutants, which fail to develop a germline at 25°C (Beanan and Strome, 1992), indicating that most of the mau-2 mRNA in young adults is in the germline. Furthermore, the level of the mau-2 transcript is very high in fem-2(b245ts) mutants that make only oocytes at 25°C, indicating that the oocytes are enriched with mau-2 transcript. However, the mau-2 transcript is very low in fem-3(q20ts) adults that make sperm only at 25°C, indicating that the mau-2 transcript is not enriched in sperm. Taken together, these results indicate that oocytes accumulate high levels of mau-2 mRNA, presumably as a store to be used by the embryo.

Consistent with our observations, in situ hybridization data reported in the Nematode Expression Pattern Database (http://nematode.lab.nig.ac.jp/) for clone yk462a, which corresponds to mau-2, show an intense signal in the germline of adults, particularly in the pachytene region, which is a major site of transcription in the germline (Lerner and Goldstein, 1988). In addition, a strong level of mau-2 mRNA is detected at different embryonic stages, including as early as the two-cell stage.

We further characterized the expression pattern of mau-2 by

![Fig. 2. Positional cloning of mau-2.](image-url)

(A) Genetic markers used to map mau-2. (B) Cosmids and subclones of C09H6 used for rescue assays (rescuing clones in bold). (C) Genomic structure of mau-2 and the lesions of the four mau-2 alleles. (D) An alignment of the predicted amino acid sequence of *C. elegans* MAU-2 with its homologues from *D. melanogaster* (CG4203-PA), *F. rubripes* (SINFRUP00000081396), *M. musculus* (NP_083269) and *H. sapiens* (KIAA0892). Identical residues in all five species are shown in dark grey; identical residues in three or four species are shown in light grey. The mau-2(qm4) mutation affects the conserved Gly residue (indicated by an asterisk). The *C. elegans* protein is about 25% identical to the vertebrate and the fly proteins. The sequence in the fish is 95% identical to human MAU-2, and the mouse sequence is more than 99% identical to human MAU-2.
MAU-2 and tubulin is a migration artefact. Worms loaded. For the late L4 stage worms, the double band both for dpy-5(e61) mau-2(qm5)/++ hermaphrodites. In each lane, 400

mau-2 detected in transgenic worms carrying wild-type copies of the gene. The expression pattern of mau-2 is similar through all developmental stages, including at times of cellular and axonal migrations. Upon repeated immunoblot analysis, there appeared to be a slight enrichment in the embryos and L1 larvae compared with other larval stages, which could reflect a higher need for MAU-2 during the time when numerous cellular migrations occur.

We examined the level of MAU-2 in the germline mutants determining the levels of protein throughout development by immunoblot analysis (Fig. 3C). Overall, the content of MAU-2 protein is similar through all developmental stages, suggesting a requirement for MAU-2 at numerous stages of development, including at times of cellular and axonal migrations. Upon repeated immunoblot analysis, there appeared to be a slight enrichment in the embryos and L1 larvae compared with other larval stages, which could reflect a higher need for MAU-2 during the time when numerous cellular migrations occur.

We examined the level of MAU-2 in the germline mutants glp-4(bn2ts), fem-3(q20ts) and fem-2(b245ts) compared with the wild type (Fig. 3C). The level of MAU-2 in germline mutants is similar to the wild type, indicating that MAU-2 is not particularly enriched in the germline. Thus, the maternal mau-2 product that is provided to the zygote via the oocyte appears to be principally RNA. Maternally contributed mau-2 mRNA is probably responsible for the maternal rescue effect observed with mau-2 mutations.

Abundant MAU-2 protein is detected in maternally rescued animals up to the L3 larval stage

We examined the level of MAU-2 protein in mau-2(qm5) maternally rescued animals at the L3 and L4 stages compared with the wild type (Fig. 3D). Given that no MAU-2 protein is detected in mau-2(qm5), any MAU-2 protein detected in m+z− animals derived from a qm5/+ mother must result from a maternal contribution. MAU-2 protein is detected in maternally rescued animals, and up to the third larval stage, the level of MAU-2 in maternally rescued animals is very similar to that of the wild type. This indicates that maternally contributed mau-2 is sufficient to sustain a wild-type protein level up to the L3 stage. By the L4 larval stage, the level of MAU-2 is reduced in m+z− animals compared with the wild type: a low level is present in early- to mid-L4 larvae, and it is no longer detected in late-L4 larvae, while it is detected in the wild type. Thus, the maternal contribution of mau-2 product runs out at the L4 stage in maternally rescued worms. In addition, these results indicate that new MAU-2 protein is translated at the L4 stage in wild-type worms, as the level of MAU-2 is higher in L4 than L3 in wild-type larvae (400 worms were loaded in each lane). Thus, mau-2 is re-expressed at the time of the development of most of the egg-laying system.

A functional mau-2::gfp transgene is expressed ubiquitously by mid-embryogenesis and subsequently becomes restricted to the nervous system

To gain more insight into the expression pattern of mau-2, we generated transgenes containing the entire genomic region of mau-2, including a region of upstream sequence, as well as all exons and introns, fused to the green fluorescent protein (gfp) gene (Fig. 4A). In three different constructs, mau-2::gfp was driven by 1 kb, 4 kb and 6 kb of the putative mau-2 promoter region. All three translational fusions expressed from extrachromosomal arrays completely rescued the defective locomotion, egg laying and larval development of all four mau-2 mutant alleles (but did not recapitulate the maternal rescue effect) and produced an identical expression pattern. The expression level of mau-2::gfp is ~10 fold stronger when driven by 4 or 6 kb of putative mau-2 promoter, suggesting that regulatory elements for the transcription of mau-2 reside more than 1 kb upstream of the gene.

The mau-2::gfp transgenes display a dynamic expression pattern (Fig. 4B). mau-2::gfp expression is first observed by mid-embryogenesis, at 200-300 minutes of development, where it is ubiquitous. A broad expression is also detected during the comma stage of embryogenesis. By the end of the twofold stage and during the threefold stage of embryogenesis, mau-2::gfp expression is largely restricted to the nervous system. Throughout the larval stages and adulthood, mau-2::gfp is expressed in virtually all neurones, including in the
neurone AVM, although the expression level decreases in adults. Occasionally, *mau-2::gfp* expression is transiently observed in hypodermal cells of the developing vulva of L3 and L4 larvae, and at a very low levels in the excretory canal, body wall and vulval muscles.

In addition, the MAU-2::GFP fusion appears to entirely and uniformly fill the cytoplasm of all neurones, including along their axonal projections, but excluding the nucleus. The human counterpart of MAU-2 (KIAA0892), expressed as a GFP fusion under the control of the Pcmv promoter in transiently transfected COS7 cells, also appeared cytoplasmic, and excluded from the nucleus (data not shown).

**Neuronally expressed mau-2 is necessary and sufficient to rescue the mau-2 mutants**

*mau-2* is required for the guidance of cellular and axonal migrations during the development of the worm, and rescuing *mau-2::gfp* transgenes are expressed, among other cells, in cells that migrate during development. It is possible therefore that *mau-2* could function inside the migratory cells to participate in their guidance. To directly determine where *mau-2* is required during development, we expressed *mau-2(+) in distinct tissues of the worm and assessed where the activity of *mau-2* is required for normal development. We generated constructs that drive the expression of *mau-2(+) in the hypodermis, the body wall muscles, the entire nervous system, or in six mechanosensory neurones, by placing the *mau-2* cDNA fused to the *gfp* gene under the control of the promoters of the genes *dpy-7*, *myo-3*, *unc-119* and *mec-7*, respectively. We verified the expression pattern of these constructs carried on extrachromosomal arrays and found that they were identical in both the wild-type and *mau-2(qm160) backgrounds (Fig. 5A). *P*dpy-7::*mau-2::gfp* was expressed in hypodermal cells from the early comma stage in embryogenesis onwards, *P*myo-3::*mau-2::gfp* was expressed in the body wall muscles from the comma stage onwards, *P*unc-119::*mau-2::gfp* was
A

<table>
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<th>Expression</th>
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<td>No 1/11 lines</td>
</tr>
<tr>
<td>Hypodermis</td>
<td>No 0/5 lines</td>
</tr>
<tr>
<td>Body Wall Muscles</td>
<td>No 0/5 lines</td>
</tr>
<tr>
<td>Pan-neuronal</td>
<td>Yes 14/14 lines</td>
</tr>
<tr>
<td>Mechanosensory neurons</td>
<td>No 0/7 lines</td>
</tr>
</tbody>
</table>

B

Fig. 5. mau-2 functions cell autonomously for the guidance of AVM. (A) Constructs used to express mau-2(+) in different tissues. The expression of each transgene was monitored with gfp. Rescue of the locomotion, egg laying and larval development are indicated. (B) Phenotype of the axon of AVM in mau-2(qm160)-expressing mau-2(+) in different tissues. AVM guidance was examined with mec-4::gfp. Four independent transgenic lines were scored (n>200 for each construct). Light grey indicates that the axon projected anteriorly; dark grey, posteriorly. The AVM guidance defect observed for each construct, data not shown). Light grey indicates that the axon projected anteriorly; dark grey, posteriorly. The AVM guidance defect observed in mau-2 is rescued by expression of mau-2(+) in the nervous system and in the mechanosensory neurons alone. Error bars indicate standard error of the proportion. The effect of expressing mau-2(+) under these different promoters was also examined in the wild-type background. The transgenic animals were completely wild type for mau-2(+).

Expression of mau-2 in AVM can rescue the ventral guidance of the AVM axon

To distinguish between a non-autonomous role of mau-2 between cells within the nervous system and an autonomous role of mau-2 within the migrating cell themselves, we assessed whether expression of mau-2(+) in the neurone AVM could rescue the axonal guidance defect that is displayed by mau-2 mutants. The neurone AVM is born and its axon migrates during the L1 larval stage (Sulston and Horvitz, 1977; Hedgecock et al., 1987). In the wild-type L1 larva, the axon of AVM pioneers its ventral migration along the body wall, from the lateral aspect of the body towards the ventral nerve cord. The axon then turns anteriorly towards the nerve ring (Hedgecock et al., 1987; White et al., 1986) (Fig. 6A). In ~30% of the mau-2 mutants, this axon fails to migrate ventrally and instead runs anteriorly or posteriorly along the body wall (Takagi et al., 1997) (Fig. 6A). We expressed mau-2(+) exclusively in AVM and five other mechanosensory neurones under the control of the promoter of the gene mec-7 (mec-7::mau-2::gfp). This expression of mau-2(+) strongly rescued the projection of the AVM axon, of both posteriorly and anteriorly misguided AVM axons in three independent transgenic lines (Fig. 5B). Thus, mau-2 functions within the AVM neurone to guide its axon ventrally. As expected, the narrow expression of mec-7::mau-2::gfp did not rescue the locomotion, egg laying and larval development of the mau-2 mutants. Expression of mau-2(+) under the mec-7 promoter also rescued the abnormal projection of the PLM axons (data not shown), which instead runs anteriorly, as in the wild type, run ventrally in 40% of the mau-2(qm4) mutants (Takagi et al., 1997). This suggests that mau-2 functions cell autonomously in other cells as well. In addition, consistent with a cell-autonomous role for mau-2, overexpression of mau-2(+) in muscles, hypodermis or the nervous system in a wild-type mau-2(+) background leaves the guidance of AVM entirely unaffected.

mau-2 acts independently of slit/slt-1 to guide AVM ventrally

Previous work has established that two guidance cues, netrin/unc-6 and slit, and their receptors, unc-40 and sax-3, respectively, are required for the ventral guidance of the axon of AVM (Chan et al., 1996; Hao et al., 2001; Hedgecock et al., 1990; Ishii et al., 1992; Zallen et al., 1998). In fact, this axon relies heavily on these two cues for its ventral migration, as evidenced by the fact that it fails to migrate ventrally in 95% of the cases, and instead extends laterally, in the double mutants unc-6 slt-1 and unc-40;slt-1 (Hao et al., 2001). Given that mau-2 functions cell autonomously in AVM and that MAU-2::GFP is cytoplasmic, it is possible that mau-2 may function as a cytoplasmic effector of the netrin and/or slit cues. The dorsal cord was normally fasciculated (n=180, 94% normal) and the soma of the HSN neurones was normally placed (n=126, 100% normal). It is worth noting that the complete rescue exhibited by these transgenic animals does not result from a maternal rescue effect of the transgene, as the non-transgenic animals (non Rol) from these lines were completely Mau-2. Thus, zygotic expression of mau-2(+) in the nervous system is necessary and sufficient for normal development.
To address this issue, we determined whether mau-2 is required for the signalling of netrin and slit in the ventral guidance of AVM.

We first asked whether mau-2 is required for signalling through the netrin receptor UNC-40. Expression of a hyperactive form of the netrin receptor UNC-40::MYR in mechanosensory neurones leads to profound defects in AVM guidance, and also results in highly abnormal cell-body shapes and branching patterns of AVM and PLM (Gitai et al., 2003). We found that complete loss of mau-2 function does not suppress the effects caused by expression of UNC-40::MYR (in a strain carrying the extrachromosomal array kyEx456). In mau-2(qm160), kyEx456, the severity and penetrance of the defects was similar to those observed in a wild-type background [83% abnormal in 108 mau-2(qm160) young adults, and 88% abnormal in 92 mau-2(+ ) young adults]. Thus, the function of mau-2 is not absolutely required for the signalling downstream of the unc-40 guidance gene.

Consistent with this, we found that a mau-2(qm4); unc-6(e78) double mutant, where e78 is a results in a partial loss of function of unc-6 (Lim and Wadsworth, 2002), displays migration defects that are more severe than either of the single mutants (data not shown), indicating that mau-2 probably acts in parallel to unc-6 in the guidance of at least some cells and axons during their migration. We could not, however, determine the phenotypic consequences of the complete loss of function for both genes [ev400 is a null allele of unc-6 (Wadsworth et al., 1996)], as the mau-2(qm160); unc-6(ev400) double mutant is unviable.

Next, we asked whether mau-2 is required for the signalling of slit-1. SLT-1 is secreted from the dorsal muscles and functions to repel the A VM axon, thus contributing to its ventral migration (Hao et al., 2001). Misexpression of slit-1 in all of the body wall muscles of the worm, i.e. in ventral as well as dorsal muscles, results in the lateral projection of the AVM axon in about 30% of the animals (Hao et al., 2001) (Fig. 6).

We asked whether the complete loss of function for mau-2 could suppress the effect of misexpressing slit-1. We found that mau-2(qm160) does not suppress the defects caused by misexpression of slit-1, indicating that mau-2 is not absolutely required for the signalling of slit-1. By contrast, mau-2(qm160) enhances the AVM axon guidance defects generated by slit-1 misexpression (Fig. 6A,B). In mau-2(qm160) that misexpress slit-1, the defect of the AVM axon is profoundly enhanced.

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**Fig. 6.** The action of mau-2 is independent of that of slit-1 to guide AVM ventrally. (A) AVM was visualized with mec-4::gfp. Anterior is towards the right, ventral at the bottom. (a) A wild-type AVM axon in which the axon first projects ventrally and then turns anteriorly. The axon of AVM fails to migrate ventrally (b-d): the axon of AVM projects anteriorly in mau-2 and slit-1 single mutants (b), or posteriorly in mau-2 single mutant (c), and dorsally in mau-2;slt-1 double mutants and in mau-2(qm160) misexpressing SLT-1 under the myo-3 promoter (d). (e) The axon of AVM projects dorsally (past the dorsoventral position of the neurone ALM) and, although not in the plane of focus here, then turns anteriorly, migrating alone for a distance and finally joining the axon of ALM. A short anterior branch is also present. Arrows and arrowheads in a-e indicate AVM and ALM, respectively. (B) Phenotype of the axon of AVM in mau-2;slt-1 double mutants and in mau-2(qm160) misexpressing SLT-1 under the myo-3 promoter (d). (C) The function of mau-2 appears to be acting in parallel to those of slit-1 and unc-6 to guide the axon of AVM. The AVM neurone is shown in the right-hand panel. Its cell body rests at the boundary between the ventral muscle quadrant and the lateral hypodermis. The axon of AVM normally projects ventrally (towards the bottom), alongside the ventral muscle quadrant, and then turns anteriorly (towards the right). The broken arrows indicate that the axon of AVM can project abnormally (anteriorly, posteriorly or dorsally) in some mutant situations. Loss of function of unc-6, slit-1 or mau-2 results in abnormal anteriorly projecting AVM axons; loss of function of unc-6 or mau-2 can also result in posteriorly oriented axons. When the activity of both mau-2 and slit-1 are altered, the axon of AVM can project dorsally. It appears that three mechanisms (unc-6-mediated attraction, slit-1-mediated repulsion and that involving mau-2) are partially redundant to guide AVM ventrally. Scale bars: 5 µm.
Discussion

The role of mau-2 in the guidance of migrations is cell autonomous

The function of mau-2 is broadly required for the proper guidance of cells and axons that undergo long-range migrations during the development of the worm. In fact, mau-2 mutants display defects in migrations that occur both embryonically and post-embryonically, and that occur along both the longitudinal and the circumferential axes of the body of the worm. The protein encoded by mau-2 is novel, yet highly conserved evolutionarily, and has not been previously studied in any organism. Although the biochemical role of the protein MAU-2 remains unknown, MAU-2 appears to function within the migrating cell and to localize to the cytoplasm, where it may participate in the intracellular interpretation of guidance cues encountered by a cell or axon during its migration.

Our results demonstrate that mau-2 functions cell-autonomously to guide the axon of the AVM neurone. First, mau-2 mutations impair the proper axonal guidance of AVM. Second, a functional mau-2::gfp transgene is expressed in the AVM neurone, including at the time of its axonal migration. Third, expression of mau-2(+) in AVM, in otherwise mau-2 null mutant animals, is sufficient to rescue the axonal guidance defect of AVM. Similar observations were made for the axon of the PLM neurone.

Several lines of evidence suggest that mau-2 may function within other migrating cells to participate in their guidance. mau-2 mutants display defects in the guidance of a number of cellular migrations, including numerous neurones and their axons, and mau-2 is expressed in the affected cells at the time of their migration. Neuronal migration and axon outgrowth start from around 370 minutes and the major nerve tracts form from around 450 minutes. Expression from the functional mau-2::gfp transgene is visible in the embryo, including in neuroblasts and neurones, starting about 300 minutes after the first cleavage. Functional mau-2::gfp keeps being expressed in the nervous system, from the threefold embryonic stage (after 500 minutes) onwards, coincident with the occurrence of additional neural migrations, including the integration of new axons in the dorsal cord at the L1 stage. For example, the positions of the embryonically migrating cell body of HSN and the embryonically extending PLM and CAN axons, is abnormal in mau-2 mutants, and mau-2::gfp is expressed in these cells, including at the time when their migration is occurring in the embryo. Importantly, pan-neuronal expression of mau-2(+) under the unc-119 promoter rescues the mau-2 mutant defects (e.g. locomotion, egg-laying, Lid larval lethality, nervous system organization), indicating that mau-2 functions in other neurones as well. Taken together, these observations suggest that mau-2 may function cell autonomously in other migrating cells as well. Consistent with this, nonautonomous activity of mau-2 is unlikely, as the mau-2 mutant defects are not rescued by expression of mau-2(+) in the mechanosensory neurones, in the hypodermis or in the body wall muscles.

Furthermore, mau-2 may function cell autonomously in cells that develop later, such as those constituting the egg-laying system. In mau-2 mutants, the vulva is frequently asymmetrical and the vulval muscles are often misplaced. Interestingly, our analysis of wild-type and maternally rescued animals revealed that new MAU-2 protein is also produced at the L4 larval stage, and de novo mau-2::gfp expression was observed in the muscle cells, and occasionally in hypodermal cells, of the developing vulva in L3 and L4 larvae. Expression of mau-2 at the L4 stage appears to be necessary for the normal development of the vulva, as vulval muscles attach abnormally in maternally rescued animals.

mau-2 acts in parallel to slt-1 to prevent AVM from projecting dorsally

Simultaneous perturbation of the activities of mau-2 and slt-1 profoundly affects the guidance of the axon of AVM. As many as ~80% of the double null mutants display guidance defects, which is twice as many as in each of the single mutants, indicating that mau-2 and slt-1 function in parallel pathways. Importantly, the AVM axon projects dorsally in a significant number of animals in which both mau-2 and slt-1 activities are perturbed. This was observed in the mau-2; slt-1 double mutants, as well as in mau-2 mutants misexpressing slt-1 in all body wall muscles [slt-1 is normally secreted only from dorsal muscles to repel the AVM axon (Hao et al., 2001)]. This indicates that slt-1 and mau-2 act redundantly to prevent the dorsal projection of AVM.

The dorsal projection of AVM is a guidance defect that has not previously been reported for any single or double mutant that affects the guidance of the AVM axon. In fact, mutations in the genes unc-6, unc-40, slt-1, sax-3, unc-34 and mau-2 that affect the guidance of AVM, result in the lateral projection of
A VM in ~25-40% of the cases (Hedgecock et al., 1990; Takagi et al., 1997; Yu et al., 2002). In these mutants, when the axon of A VM fails to project ventrally, it instead extends anteriorly. The A VM axon can project posteriorly as well in the single mutants mau-2 and unc-6 [in ~7-10% of the animals (Takagi et al., 1997; Yu et al., 2002)]. In addition, the axon of A VM can extend posteriorly in animals that misexpress slt-1 in all body wall muscles (Hao et al., 2001). The axon of A VM has been observed to migrate dorsally and reach the dorsal cord, and then turn anteriorly along the dorsal cord in animals that misexpress UNC-5 in A VM under the control of a heterologous promoter (Hamelin et al., 1993). UNC-5 is a netrin receptor that mediates the repulsion of a growth cone of migrating motor axons away from the netrin source, but is not expressed in A VM (Killeen et al., 2002; Su et al., 2000) and does not appear to normally participate in the guidance of the A VM axon (Leung-Hagesteijn et al., 1992). Thus, our analysis of the interactions between mau-2 and slt-1 reveals that the A VM axon is capable of migrating dorsally as a result of the loss of function of genes implicated in its guidance. Our results also indicate that the ventral guidance of A VM does not only rely on unc-6 and slt-1, as previously reported (Yu et al., 2002).

Our results furthermore indicate the existence of a new mechanism, involving mau-2, that functions in parallel to slt-1 to repel A VM from the dorsal area of the body wall (Fig. 6C). The effects of perturbing both mau-2 and slt-1 gene activities are observed even in the presence of unc-6(+) activity, indicating that the unc-6 mediated attraction of A VM is insufficient to prevent dorsal migration by itself. Although we could not fully characterize the interactions between mau-2 and unc-6, it appears that, unc-6-mediated repulsion, slt-1-mediated repulsion and a mechanism involving mau-2, are all partially redundant in the ventral guidance of A VM. It is also possible that the mechanism involving mau-2 may impinge upon both the unc-6 pathway (for example, upstream of unc-40) and the slt-1 pathways, rather than being a fully independent pathway.

It will be interesting to find out how mau-2 participates in the dorsoventral guidance of the A VM axon and, specifically, to determine if it is involved in a guidance mechanism entirely distinct from those of netrin and slit. For example, one question is whether mau-2 might interact with clr-I, a receptor tyrosine phosphatase that has been recently implicated in the regulation of netrin signalling (Chang et al., 2004).

Maternal expression of mau-2

The zygotic activity of mau-2 is sufficient for the normal development of the worm. Indeed, heterozygous animals produced by a homozygous mutant mother are completely wild type. In addition, functional mau-2::gfp transgene expression is first detected as late as by mid-embryogenesis, which is well past the time at which the zygotic genome is turned on [at the onset of gastrulation (Edgar et al., 1994; Powell-Coffman et al., 1996; Kaltenbach et al., 2000)]. However, mau-2 mutants can be profoundly maternally rescued, and high levels of mau-2 transcript are loaded into the oocytes and still detectable in the very early embryo. Why is mau-2 maternally contributed at all?

It is possible that mau-2 might play role in very early development that may be masked by the function of redundant, yet non-homologous, genes. It is also conceivable that the maternal contribution of mau-2 serves to ensure a sufficient level of mau-2 expression at the time of the start of mau-2 expression in the zygote. The effect of such a stabilization might not easily be detected in the experimental setting, but might be of sufficient magnitude to have been evolutionarily favoured.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/131/23/5947/DC1

References


