Differential Effects of Drosophila Mastermind on Asymmetric Cell Fate Specification and Neuroblast Formation

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ABSTRACT

During neurogenesis in the ventral nerve cord of the Drosophila embryo, Notch signaling participates in the pathway that mediates asymmetric fate specification to daughters of secondary neuronal precursor cells. In the NB4-2 → GMC-1 → RP2/sib lineage, a well-studied neuronal lineage in the ventral nerve cord, Notch signaling specifies sib fate to one of the daughter cells of GMC-1. Notch mediates this process via Mastermind (Mam). Loss of function for *mam*, similar to loss of function for *Notch*, results in GMC-1 symmetrically dividing to generate two RP2 neurons. Loss of function for *mam* also results in a severe neurogenic phenotype. In this study, we have undertaken a functional analysis of the Mam protein. We show that while ectopic expression of a truncated Mam protein induces a dominant-negative neurogenic phenotype, it has no effect on asymmetric fate specification. This truncated Mam protein rescues the loss of asymmetric specification phenotype in *mam* in an allele-specific manner. We also show an interallelic complementation of loss-of-asymmetry defect. Our results suggest that Mam proteins might associate during the asymmetric specification of cell fates and that the N-terminal region of the protein plays a role in this process.

THE central nervous system (CNS) of the Drosophila embryo provides an important paradigm for investigating the problem of asymmetric division of neural precursor cells during development. In the ventral nerve cord of the Drosophila embryo, ~30 neuroblast (NB) cells in each hemi-segment delaminate in about five successive waves along the mediolateral and anterior-posterior axes in rows and columns in a stereotyped and spatio-temporal pattern (Hartenstein and Campos-Ortega 1984; Doe 1992). Each of these NBs has acquired a unique fate by the time it is formed, and the NB that forms in a given position at a given time always acquires the same fate (reviewed in Bhat 1999). A neuroblast then functions as a stem cell and divides by asymmetric mitosis, renewing itself with each division and producing a chain of ganglion mother cells (GMCs). A GMC does not self-renew; instead it divides to generate two distinct neurons. These postmitotic neurons then undergo cyto-differentiation. At the end of neurogenesis, each of the hemi-neuromeres has ~320 neurons and ~30 glia, the other principal cell type in the CNS (Bossing *et al.* 1996; Schmidt *et al.* 1997). Thus, a complex array of different cell types is formed from relatively few precursor cells.

Genetic and molecular evidence indicate that GMCs generally undergo an asymmetric cell division (Hartenstein and Posakony 1990; Bhat and Schedl 1994; Bhat *et al.* 1995; Hirata *et al.* 1995; Knoblich *et al.* 1995; Spana and Doe 1995, 1996; Buescher *et al.* 1998; Dye *et al.* 1998; Skeath and Doe 1998; Lear *et al.* 1999; Wai *et al.* 1999; Mehta and Bhat 2001). One of the earliest evidences comes from a study on the development of the adult sensilla in which the neurogenic gene *Notch* plays a role in generating asymmetric division of secondary precursor cells (Hartenstein and Posakony 1990). Using the temperature-sensitive allele of *Notch*, it was shown that eliminating Notch activity in sensillum precursors leads to hyperplasia of the sensory neurons at the expense of accessory cells (*i.e.*, shaft, socket cells). Notch, together with Numb (Nb), also regulates asymmetric fate specification to progeny of GMC in the ventral nerve cord (Buescher *et al.* 1998; Skeath and Doe 1998; Lear *et al.* 1999; Wai *et al.* 1999). In the GMC-1 → RP2/sib lineage, loss of *Notch* or *nb* leads to the symmetric division of GMC-1. While both progeny assume RP2 fate in *Notch* mutants, they assume a sib fate in *nb* mutants (Buescher *et al.* 1998; Lear *et al.* 1999; Schuldt and Brand 1999; Wai *et al.* 1999). Nb appears to block the intracellular domain of Notch from being cleaved and translocated to the nucleus, thus allowing that cell to adopt an RP2 fate. These studies have also revealed that the gene product of *mastermind* (*mam*) is downstream of *Notch* and that loss of function for *mam* results in both daughters of GMC-1 adopting an RP2 fate.

Mam is a glutamine-rich nuclear protein essential for

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Notch signaling (Smoller *et al.* 1990; Bettler *et al.* 1996; Helms *et al.* 1999). Mam interacts with the N intracellular domain (N^{intra}) in the Suppressor of Hairless [Su(H)]/CBF1 complex and stabilizes its binding to DNA *in vitro* (Petcherski and Kimble 2000a,b; Wu *et al.* 2000; Kitagawa *et al.* 2001). A recent study to evaluate the activity of the Notch minimal functional enhancer complex in a mammalian chromatin-based, cell-free transcription system indicates that human Mam is an essential component of this complex that associates with histone acetyltransferases (Fryer *et al.* 2002). Mam has one basic domain at the N terminus and two acidic domains, one close to the middle of the protein and another at the C terminus. The basic region of Mam is conserved in fly, mouse, and human, and this region physically interacts with the processed N^{intra} (Petcherski and Kimble 2000a; Wu *et al.* 2000; Kitagawa *et al.* 2001). Truncations in Mam that remove parts of the protein carboxy to the basic region elicit dominant-negative phenotypes when overexpressed in imaginal tissues (Helms *et al.* 1999). A further functional dissection of the Mam protein *in vivo* during neurogenesis has not been done. Given that loss of Mam activity leads to two distinct phenotypes in the Drosophila CNS—neural hyperplasia and loss of asymmetric division of secondary neuronal precursor cells—we attempted to distinguish these functions of the Mam protein *in vivo*. We examined transgenic lines expressing truncated versions of the Mam protein and determined whether they have a neurogenic and or a loss-of-asymmetry phenotype and whether any of the truncated proteins would rescue the asymmetry phenotype of *mam*. We show that while ectopic expression of a Mam truncation induces a dominant-negative neurogenic phenotype, it has no effect on the asymmetric fate specification to daughter cells of secondary neuronal precursor cells. Consistent with this result, this truncated protein rescues the GMC symmetric division phenotype in *mam* in an allele-specific manner. We further show that there is an interallelic complementation of loss-of-asymmetry defect between two alleles of *mam*. Our genetic results suggest that Mam protein might associate with itself during the asymmetric specification of cell fates and that the first two-thirds of the protein is essential in this process.

MATERIALS AND METHODS

Fly stocks and genetics: The following *mam* alleles were used in this study: ethyl methanesulfonate (EMS)-induced allele *cn* mam^{IL42}*bw sp/CyO*, a spontaneous inversion mam^{N2G}*/CyO*, and the hybrid dysgenic allele $mam^{HD\ 10/6}$*/CyO* (Yedvobnick *et al.* 1988; Schmid *et al.* 1996). Heat shock-GAL4 on chromosome 3 was obtained from H. Keshishian. For the *numb* allele, we used nb^{796}. nb^{796}, mam^{IL42} double mutants were generated by recombination.

Heat shock-GAL4/UAS-Mam truncation strains: Construction of Mam truncations in pUAST is described in Helms *et al.* (1999). The Mam protein in the *UAS-MamN* transgene terminates at nucleotide 3884 of cDNA B4 (Smoller *et al.* 1990), encoding through the first acidic charge cluster, as well as an additional 500 residues, and ending at Mam residue 1043. *UAS-MamH* terminates at nucleotide 1489, Mam residue 245, which is 55 residues carboxy to the basic charge cluster. Germline transformants carrying either *UAS-MamN* or *UAS-MamH* on chromosome 3 were mated with a *Hs-GAL4* chromosome 3 strain. Females *trans*-heterozygous for the transgenes were mated to w^{1118} and male recombinants (*hs-MamN* and *hs-MamH*) selected by eye color. Recombinant chromosomes were balanced over *TM3 Sb* and homozygous lines were selected. The strains were tested for phenotypic effects after heat-shock treatment (34° for 5 min) of third instar larvae. Both strains exhibited macrochaete duplications, eye defects, and loss of wing material (see RESULTS) consistent with dominant-negative effects of the Mam truncations described previously (Helms *et al.* 1999).

Heat-shock regimen during embryogenesis: Embryos were collected for 2 hr and then either heat-shocked immediately at 41° for 30 min or aged at various intervals and then heat-shocked (see text for details). Embryos were then aged again for different durations prior to fixation and then stained with anti-Eve or anti-Eve and anti-Zfh-1.

Rescue experiments: *UAS-MamN* and *Hs-GAL4* transgenes were introduced into either the $mam^{HD10/6}$ or the mam^{IL42} background. Embryos from these combinations were collected for 2 hr, aged 6 hr (6–8 hr old) and the *UAS-MamN* was induced by heat shock at 41° for 30 min. These embryos were allowed to grow at room temperature until they reached ~14 hr or older before fixing and staining with anti-Eve.

Sequencing of the mam mutant alleles: mam^{IL42} homozygous embryos were identified by the presence of CNS defects (visualized with Eve staining) and lack of balancer-specific staining. DNA from several individual homozygous embryos were individually prepared and the *mam* coding region between *MamH* and *MamN* (see Figure 2a) was amplified from these individual DNA preparations. The amplified DNA was then sequenced in both directions.

Antibodies and immunostaining: Embryos were stained using standard immunohistochemistry procedures. Embryos were fixed and stained with Eve (1:2000), Eve and Zfh-1 (1:400), or LacZ (1:2000). For light microscopy, alkaline phosphatase or 3,3′-diaminobenzidine-conjugated secondary antibodies were used. For confocal, FITC and Cy5 secondary antibodies were used. Embryos of appropriate genotypes (*i.e.*, mutants or rescue embryos) were identified using blue balancers and or marker phenotypes.

RESULTS

The GMC-1 → RP2/sib lineage: The GMC-1 → RP2/sib lineage, generated by NB4-2, is one of the well-studied neuronal lineages in the ventral nerve cord of the Drosophila embryo (reviewed in Bhat 1999). NB4-2 is delaminated in the second wave of NB delamination during midstage 9 (~4.5 hr old) of embryogenesis (Hartenstein and Campos-Ortega 1984; Doe 1992) and is located in the fourth row along the anterior-posterior axis and in the second column along the medio-lateral axis within a hemi-segment. It generates its first GMC (GMC-1, also known as GMC4-2a) ~1.5 hr after formation. The GMC-1 divides ~1.5 hr later to generate two cells, the RP2 and the sib.

There are several well-established ways to distinguish

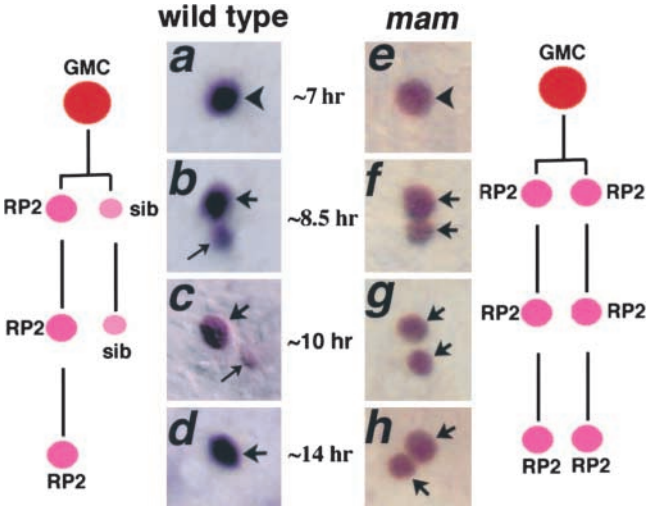

Figure 1.—Symmetric division of GMC-1 in *mam* mutant embryos. Wild-type and *mam* embryos of different ages were stained for Eve. Arrowhead indicates a GMC-1, large arrow indicates an RP2, and small arrow indicates a sib.

a GMC-1, an RP2, and a sib (see Bhat and Schedl 1994; Buescher *et al.* 1998; Wai *et al.* 1999). First, the nuclear division and cytokinesis of GMC-1 that generates the two daughter cells is asymmetric in nearly 97% of the hemi-segments (the number of hemi-segments examined, $N = 400$). Thus, in 7.5- to 10-hr-old embryos, the cell that is destined to become an RP2 is significantly larger compared to the cell that will eventually become a sib (*cf.* Figure 1, b and c). Second is the level of marker gene expression between an RP2 and a sib as well as the temporal dynamics of expression of marker genes. For example, in nearly 99% of the hemi-segments, the future RP2 cell has a stronger expression of markers, such as Even-skipped (Eve), compared to the cell that is destined to become a sib (Figure 1, b and c). We have not encountered a newly formed sib that is the same size as a newly formed RP2 *and* has the same level of expression of marker genes as in an RP2 ($N = >1000$). Third, the cell that eventually assumes a sib identity undergoes a size reduction (Figure 1c) and further downregulation of expression of RP2-specific marker genes. By 13–14 hr of development, the sib loses Eve expression (*cf.* Figure 1d). Finally, RP2 is a motor neuron whereas sib has no axon projection and its eventual fate is unknown.

Loss of function for *mam* causes both loss-of-asymmetry and neurogenic phenotypes: Most *mam* alleles show a neurogenic phenotype (Yedvobnick *et al.* 1988; Schmid *et al.* 1996). We found that one of the EMS-induced alleles, mam^{IL42}, showed a mild neurogenic phenotype whereas it had a strong loss-of-asymmetry phenotype. For example, the GMC-1 of the RP2/sib lineage divides symmetrically into two RP2s in as many as 80% of the hemi-segments, resulting in the duplication of RP2 neurons (Figure 1, f–h, arrows). A slight size asymmetry is present between the two RP2 neurons, which is also the case in *Notch* mutants (Wai *et al.* 1999). The smaller cell is also an RP2 in these mutants as indicated by its RP2-specific axon projection pattern (data not shown; *cf.* Wai *et al.* 1999). The other alleles examined all had a strong neurogenic phenotype (*cf.* Figure 2f); however, these alleles also had the loss-of-asymmetry phenotype, exemplified by the symmetric division of GMC-1 into two RP2 neurons (Figure 2f, arrows).

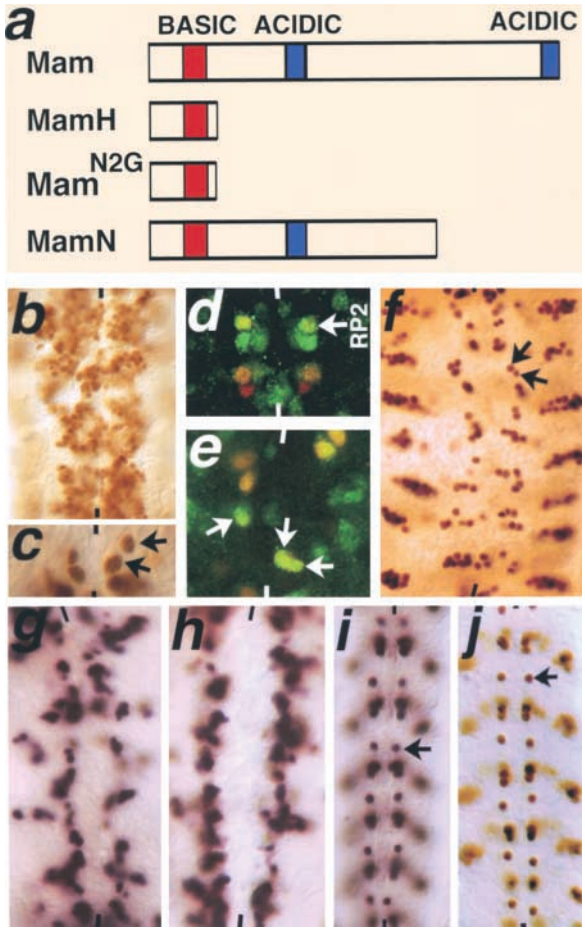

Figure 2.—Effects of expression of truncated *mam* transgenes. (a) Wild-type Mam, the two engineered truncations of Mam (MamH and MamN), and the truncation in mam^{N2G} mutant allele are shown. (b) The neurogenic defect in the embryo where *MamH* was ectopically expressed between 2 and 4 hr of age. (c) The loss-of-asymmetry defect when *MamH* was ectopically expressed between 6 and 8 hr of age. Here, the GMC-1 generates two RP2s (arrows). (d) Wild-type embryo double stained for Eve and Zfh-1 expression. RP2 has both Eve and Zfh-1 (arrow). (e) The GMC-1 divides symmetrically into two RP2s (arrows) in embryos ectopically expressing *MamH* between 6 and 8 hr of age. (f) The neurogenic and loss-of-asymmetry defect (arrows) in embryos mutant for the mam^{N2G} allele. (g and h) The neurogenic defect in an embryo in which *MamN* was ectopically expressed between 2 and 6 hr of age. (i) Ectopic expression of *MamH* between 6 and 8 hr of age has no effect on the asymmetric division of GMC-1. Thus, only one RP2 was seen per hemi-segment (arrow). (j) Wild-type control embryo.

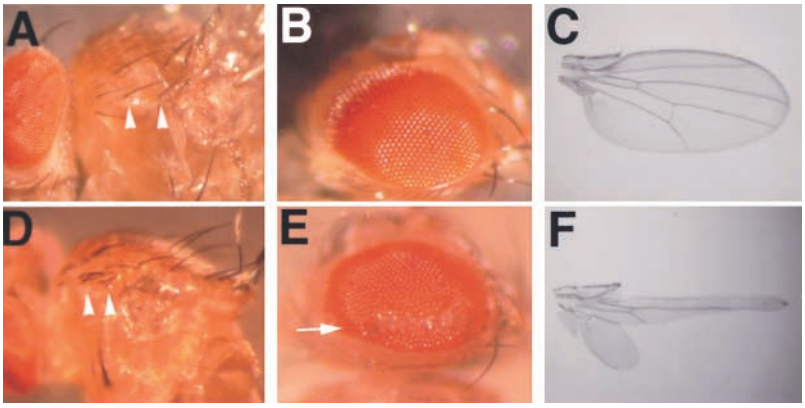

FIGURE 3.—Imaginal effects of MamH. Adult phenotypes produced after the heat-shock induction of *MamH* during third instar larval stages are shown. (A–C) Anterior/posterior notopleural bristles (arrowheads), eye, and wing in wild type. (D–F) Additional anterior/posterior notopleural bristles (arrowheads), eye scar (arrow), and severe wing blade loss in, respectively, *Hs-GAL4; MamH* individuals.

Expression of the MamH truncation interferes with both the neurogenic and the asymmetry functions of wild-type Mam: Mam protein has several distinct domains, such as an N-terminal basic domain, a centrally located acidic domain, and a C-terminal acidic domain (Figure 2a). First, using a transgenic line carrying an N-terminal truncation of Mam (MamH; see Figure 2a) under the control of upstream activator sequence (UAS; HELMS *et al.* 1999), we ectopically expressed this truncated Mam using GAL4 under the control of the heat-inducible *heat shock 70* gene promoter (*Hs-GAL4*) at different developmental time points. Early induction of this transgene (between 2 and 6 hr of development at 22°) resulted in a neurogenic phenotype (Figure 2b). When the transgene was induced during the time in which the GMC-1 of the RP2/sib lineage undergoes asymmetric division (between 6 and 8 hr of development at 22°), it appeared that GMC-1 divided symmetrically into two RP2 neurons (Figure 2c). However, with the *MamH* transgenic line, we encountered a problem: following induction, the embryos failed to retract germ band and therefore we could not ascertain if both the daughters of GMC-1 adopt an RP2 fate by staining for Eve expression alone. Therefore, we double stained these embryos with Eve and Zfh-1. Zfh-1 is a zinc-finger protein and is expressed in a newly formed RP2 but not in a GMC-1 or a sib (WAI *et al.* 1999; MEHTA and BHAT 2001). Double staining of embryos with Eve and Zfh-1 where the *MamH* transgene was induced between 6 and 8 hr of development revealed that both the progeny of GMC-1 have Eve and Zfh-1 (Figure 2e, arrows). The frequency of loss of asymmetry was low (~10% of the hemisegments). Nonetheless, these results indicate that this truncated form of Mam functions as a dominant negative competing with the wild-type Mam and produces both neurogenic and loss-of-asymmetry phenotypes. We also examined an allele of *mam, N2G*, in which an inversion breaks the gene in such a way that it is expected to produce a truncated form of the protein that is 12 amino acids shorter than MamH (Figure 2a). In this allele, we observed both the neurogenic and the loss-of-asymmetry phenotypes (Figure 2f).

Expression of the MamN truncation interferes with the neurogenic function of wild-type Mam but not with its asymmetry function: We next examined a transgenic line carrying a longer form of the Mam protein, MamN (see Figure 2a; HELMS *et al.* 1999). When this transgene was induced using the *Hs-GAL4* driver during early neurogenesis (between 2 and 6 hr of development at 22°), it resulted in a neurogenic phenotype with a large number of neurons forming at the expense of ectoderm (Figure 2, g and h). A similarly truncated version of human Mam produces a neurogenic effect in Xenopus (FRYER *et al.* 2002). However, when the *MamN* transgene was induced between 6 and 8 hr of development at 22°, unlike the *MamH*, it had no effect on GMC-1 division (Figure 2i).

Loss of Mam or Notch activity in imaginal discs has been shown to cause adult phenotypes (see HELMS *et al.* 1999 and references therein). These include macrochaete duplications, eye defects, and wing defects. Induction of Mam truncations in imaginal discs has been shown to cause similar phenotypes (HELMS *et al.* 1999). We repeated these experiments to make sure that the newly constructed *Hs-GAL4, MamH*, and *Hs-GAL4, MamN* recombinant chromosomes behave similarly. As shown in Figure 3, a brief induction of the *MamH* transgene during the third instar larval stage produced macrochaete duplications, eye scarring, and wing defects. Similar results were observed with the induction of the *Hs-GAL4, MamN* transgene (data not shown). These results further indicate that these transgenes behave as loss-of-function *mam* mutations.

MamN rescues the loss-of-asymmetry phenotype in a hypomorphic *mam* allele: The above results indicate that MamN can interfere with wild-type Mam function during neuroblast formation and thus induce a neurogenic phenotype, whereas it cannot interfere with the wild-type protein during asymmetric fate specification. We hypothesized that perhaps MamN has the part of the protein required for specifying sib identity and thus it does not function as a dominant negative during asymmetric fate specification. To test this hypothesis, we sought to rescue the loss-of-asymmetry phenotype in two

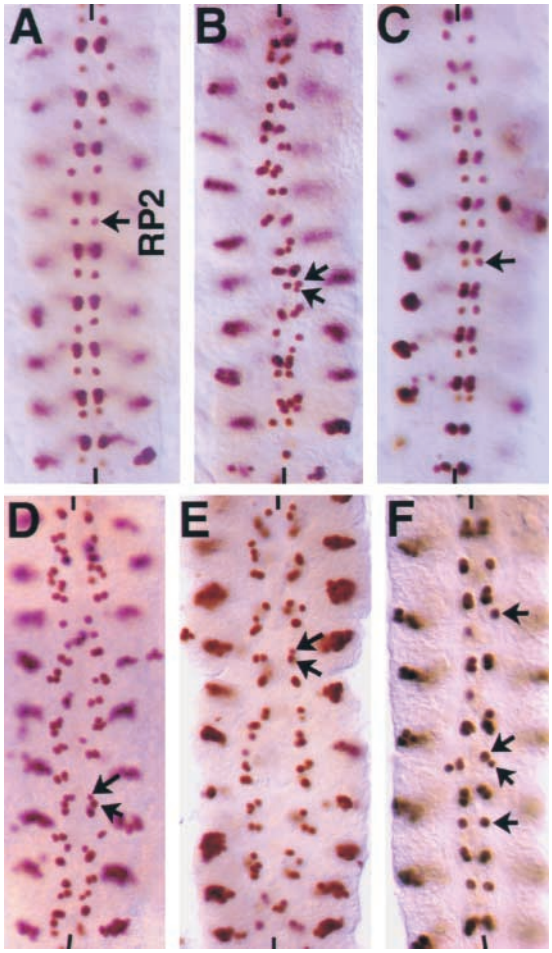

Figure 4.—Expression of MamN rescues the loss-of-asymmetry phenotype in an allele-specific manner. Embryos are stained for Eve. Anterior end is up; midline is marked by vertical lines. Arrows indicate an RP2. All the embryos are ~14 hr old. (A) Wild-type embryo. (B) $mam^{HD10/6}$ embryo. This mutant is a *P*-element insertion allele and shows mostly the loss-of-asymmetry phenotype (arrows). (C) $mam^{HD10/6}$*; MamN* embryo. The loss-of-asymmetry defect is rescued in this allele by the expression of *MamN* between 6 and 8 hr of development. (D) mam^{IL42} embryo. Note the high frequency of duplication of RP2 (arrows). (E) mam^{IL42}*; MamN* embryo. The loss-of-asymmetry defect is not rescued by the expression of *MamN* between 6 and 8 hr of development. (F) $mam^{HD10/6}/mam^{IL42}$ embryo showing the interallelic complementation of an asymmetric division defect. A normal RP2/sib specification occurs in most hemi-segments in this allelic combination (see Table 1).

TABLE 1

The penetrance of the loss of asymmetric division of GMC-1

Genotype	Loss of asymmetry (% of hemi-segments with phenotype)	No. of hemi-segments counted
Wild type	0	288
$mam^{HD10/6}$	75	144
$mam^{HD10/6}$;*MamN*	1.3	144
mam^{IL42}	83	96
mam^{IL42R}	81	96
mam^{IL42};*MamN*	87.5	96
$mam^{HD10/6}$;mam^{IL42}	25	144

The defect was visualized by staining embryos for Eve. Each embryo has 28 hemi-segments; however, we counted 24 hemi-segments per embryo since the defects in the posterior-most and anterior-most hemi-segments of the nerve cord are often difficult to ascertain.

different alleles, $mam^{HD10/6}$ and mam^{IL42}. $mam^{HD10/6}$ is a *P*-element insertion allele in which the *P* element is inserted in the untranslated first exon (Smoller *et al.* 1990). This allele shows the loss-of-asymmetry phenotype (Figure 4B; see also Table 1) but not the neurogenic phenotype (Wai *et al.* 1999); $mam^{HD10/6}$ does show a neurogenic phenotype in combination with stronger alleles (Yedvobnick *et al.* 1988). The Mam protein in this allele is predicted to be wild type but most likely present at reduced levels. mam^{IL42} is an EMS-induced allele and this produced a mild neurogenic phenotype but a strong loss-of-asymmetry phenotype (Figure 4D; see also Figure 1 and Table 1). We introduced *MamN* into these mutant backgrounds and induced the gene using Hs-GAL4 between 6 and 8 hr of development. When these embryos were examined for the RP2/sib lineage division pattern, we found that the asymmetry division defect in the RP2/sib lineage in $mam^{HD10/6}$ was rescued by *MamN* (Figure 4C). However, the asymmetric division defect (as well as the neurogenic phenotype) in mam^{IL42} was not rescued by MamN (Figure 4E). These results suggest that MamN contains the regions necessary for generating asymmetry but in an allele-specific manner. Given this allele-specific difference in the ability of *MamN* to rescue the defects, we sequenced the portion of the *mam* gene implicated in this function in the mam^{IL42} allele. As shown in Figure 5a, this allele had a change from glutamine at position 1038 (within a stretch of glutamines) to a stop codon, predicting a truncated protein seven amino acids shorter than MamN.

mam^{IL42} and $mam^{HD10/6}$ show interallelic complementation: As discussed above, ectopic expression of *MamN* elicits a neurogenic defect but not the loss-of-asymmetry phenotype. Further, *MamN* can rescue the loss of asymmetric division in $mam^{HD10/6}$ but not in mam^{IL42}. These results led us to think that MamN can rescue the asymmetric division defect only in the presence of some wild-type Mam protein. This raised the possibility that MamN might interact with wild-type Mam protein during the specification of sib fate. We tested this idea genetically by looking for interallelic complementation. When two molecules of the same protein interact with one another, this is often revealed by interallelic complementation (*cf.* Tang *et al.* 1998). Therefore, we crossed $mam^{HD10/6}$ to mam^{IL42} and examined the *trans*-heterozygous embryos. As shown in Figure 4F, there was a significant rescue of the asymmetric division defect and thus most hemi-

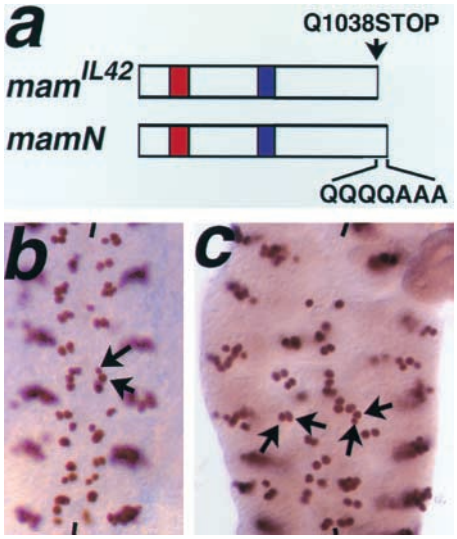

FIGURE 5.—*mam*^{IL42} has a stop codon seven amino acids upstream of MamN truncation. (a) In *mam*^{IL42}, glutamine at position 1038 is changed to a stop codon. This is expected to generate a MamN-like protein. (b) An ~14-hr-old *mam*^{IL42} embryo. Note the high frequency of duplication of RP2 (arrows); the neurogenic phenotype is mild. (c) An ~14-hr-old *mam*^{IL42R} embryo. Note the high frequency of duplication of RP2 (arrows) and also the strong neurogenic defect resulting in a wide nerve cord. The *mam*^{IL42R} allele is derived from *mam*^{IL42} (see text).

segments had normal specification of sib (see Table 1). This interallelic combination is similar to the *mam*$^{HD10/6}$*; MamN* combination and the rescue of asymmetric division defect in these combinations indicates that MamN or Mam^{IL42} associates with Mam to rescue the loss-of-asymmetry defect.

***mam*^{IL42} carries a suppressor of the neurogenic defect but not the asymmetry defect:** The strong neurogenic effect of *MamN* but the absence of a similar strong neurogenic defect in *mam*^{IL42} (which is predicted to produce a truncated MamN-like protein; see Figure 5a) was unexpected. For example, the absence of a strong neurogenic defect in *mam*^{IL42} indicates that the truncated protein has all the necessary function for normal NB formation. However, the dominant-negative neurogenic effect of MamN shows that this truncated protein does not carry the function necessary for normal NB formation. While MamN carries an additional seven amino acids compared to Mam^{IL42}, we considered the possibility that the *mam*^{IL42} mutant carries a suppressor(s) of the neurogenic defect but not a suppressor(s) of loss-of-asymmetry defect. Since a straightforward outcrossing of *mam*^{IL42} does not result in the loss of suppressor(s), the suppressor(s) are likely to be located on the same chromosome as *mam*^{IL42}. Therefore, we subjected *mam*^{IL42} to one round of recombination and examined embryo collections from recombinants. Consistent with the possibility of the presence of a partial suppressor of neurogenic defect, we recovered *mam*^{IL42} chromosomes

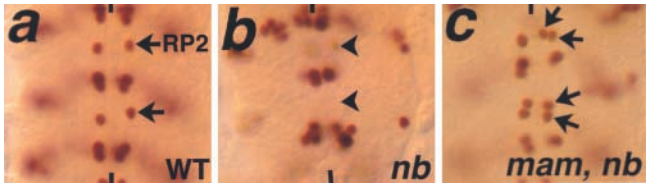

FIGURE 6.—Epistatic relationship between *mam* and *numb*. Embryos that are ~14 hr old are stained with Eve antibody. Anterior end is up, midline is marked by vertical lines. (a) Wild-type embryo. RP2 is marked by an arrow. (b) *nb* mutant embryo. The GMC-1 has symmetrically divided to generate two sibs and as a consequence both daughters have lost Eve expression (the position of cells is marked by an arrowhead). (c) A *mam, nb* double-mutant embryo showing duplication of the RP2 neuron (arrows), similar to *mam* single-mutant embryos. This indicates that *mam* is epistatic to *nb*.

that showed a strong neurogenic defect (Figure 5c). These embryos, however, showed the loss-of-asymmetry defect to the same extent as the embryos from the original *mam*^{IL42} strain (Figure 5c; see Table 1). We have not yet mapped the suppressor(s) nor have we determined that the effect is due to a single locus.

***mam* phenotype is epistatic to the *numb* phenotype:** Recent studies indicate that *inscuteable* (*insc*) and *nb* play a crucial role in the terminal asymmetric division of GMC-1 of the RP2/sib lineage (BUESCHER *et al.* 1998; LEAR *et al.* 1999; WAI *et al.* 1999). The asymmetric divisions mediated by these proteins appear to be tied to their asymmetric localization in GMC-1 and to their asymmetric segregation between two daughter cells during division. For instance, during the division of GMC-1 of the RP2/sib lineage, Insc localizes to the apical end of GMC-1, which in turn segregates Nb to the basal end. The cell that inherits Nb is specified as RP2 due to the ability of Nb to block Notch signaling, whereas the cell that does not inherit Nb (but inherits Insc) is specified as sib by Notch. Thus, in *insc* mutants, both daughters of the GMC-1 adopt an RP2 fate whereas in *nb* mutants they assume a sib fate (BUESCHER *et al.* 1998; WAI *et al.* 1999). Our previous results indicate that the sib cell adopts an RP2 fate in *Notch; nb* double mutants (WAI *et al.* 1999), indicating that Nb is needed to specify RP2 fate only when there is intact Notch. We sought to determine if the same relationship exists between *mam* and *nb*. We generated *mam, nb* double mutants and examined the division pattern of GMC-1. For this purpose, we used the allele *mam*^{IL42}. This *mam* allele, although not a null, shows a very strong loss-of-asymmetry phenotype (83% of the hemi-segments; see Table 1), which can be reliably identified. A null allele gives a severe neurogenic phenotype and makes it far more difficult to determine the double-mutant phenotype. In addition, we had determined the molecular lesion in the *mam* gene in this allele. Since this allele is a loss-of-function allele and the phenotypes of *mam* and *numb* mutants are opposing phenotypes, we reasoned that use of non-null alleles should

not pose any problems for analysis or interpretation of the double-mutant results. Therefore, we used this allele in our double-mutant experiments. As shown in Figure 6c, both the daughter cells of GMC-1 adopted RP2 fate in these embryos.

DISCUSSION

The canonical Notch pathway described for Drosophila (Artavanis-Tsakonas *et al.* 1999) is widely conserved, including *Caenorhabditis elegans* and mammals. The intercellular communication mediated by Notch involves interactions between Delta, the signal, and Notch, the receptor (Fehon *et al.* 1990; Heitzler and Simpson 1993). The signaling involves transendocytosis of the Notch extracellular domain bound to Delta into the signaling cell (Parks *et al.* 2000). A physical perturbation of Notch protein structure during transendocytosis may be needed for proteolytic processing and release of the Notch intracellular domain ($Notch^{intra}$; Mumm and Kopan 2000). Proteolytic cleavage of Notch is mediated by the membrane-bound Presenilin protein (Ye *et al.* 1999). In many but not all contexts (Ramain *et al.* 2001), signaling by Notch occurs in conjunction with the DNA-binding Su(H) protein, the mammalian CBF1/worm Lag-1 homolog (CSL; Henkel *et al.* 1994). In the absence of Notch signaling, Su(H) establishes a default state of gene repression, which appears to be mediated via complexing with Hairless and the corepressors Groucho and dCtBP (Barolo *et al.* 2002). Upon Notch activation, N^{intra} goes into the nucleus, where it dissociates the repression complex and leads to the formation of an activation complex containing Su(H) and Mam.

In the ventral nerve cord of the Drosophila embryo, the Notch pathway mediates terminal asymmetric division of secondary neuronal precursor cells (Buescher *et al.* 1998; Lear *et al.* 1999; Schuldt and Brand 1999; Wai *et al.* 1999). The secondary precursor cells, GMCs, in the nerve cord generally divide by asymmetric mitosis to generate two different daughter cells. For example, in the GMC-1 → RP2/sib lineage during GMC-1 division, the Inscuteable protein asymmetrically localizes to the apical end, which forces Numb to localize to the basal end. Basally localized Numb then segregates to the future RP2. The function of Numb is to prevent the cleaving of the intracellular domain of Notch. In the absence of Numb, the intracellular domain of Notch gets cleaved and then translocated into the nucleus where it specifies a sib fate by complexing with Su(H) and Mam and activating downstream target genes. Previous results also show that for the specification of an RP2 identity Numb is not required, but it is required to prevent that cell from becoming a sib in the presence of an intact Notch pathway.

In this article, we show differential effects of Mam on asymmetric cell fate specification *vs.* neuroblast formation in the ventral nerve cord of the Drosophila embryo. We show that a Mam truncation, which has the basic and the first acidic domain (MamN), rescues the asymmetric cell fate specification defect in an allele-specific manner. These conclusions are based on several lines of evidence. First, a transgene that encodes this truncated Mam protein causes a dominant-negative neurogenic defect, but it does not cause a dominant-negative effect on asymmetric division. Thus, expression of this transgene during the asymmetric division of GMC-1 does not cause a duplication of RP2 as one would expect if this transgene functions as a dominant negative. The same transgene when expressed earlier when NBs are formed causes a neurogenic defect. This indicates that the truncated transgene functions as a dominant negative but only during the earlier neurogenic process. Second, MamN rescues the asymmetry defect in one of the *mam* mutant alleles, $mam^{HD10/6}$. This is a hypomorphic *P*-element insertion allele (Smoller *et al.* 1990), which causes the loss of asymmetric division defect (Wai *et al.* 1999) but does not cause a neurogenic defect except in combination with strong alleles of *mam* (Yedvobnick *et al.* 1988). These results and the fact that the *P* element is inserted in the untranslated first exon suggest that low levels of wild-type Mam are produced by this allele. However, the finding that MamN does not rescue the asymmetry defect in another *mam* mutant allele, mam^{IL42}, which is predicted to produce a truncated Mam protein similar to MamN, indicates that this rescue is allele specific (see below). Thus, some wild-type Mam protein appears to be necessary for the rescue by MamN and it is possible that the two proteins interact to provide the rescue function (see below).

Our sequence analysis of mam^{IL42} suggests that this allele encodes a Mam protein that is similar to MamN (although it is seven amino acids shorter). The inability of MamN to rescue mam^{IL42} argues that this truncated protein in combination with MamN is not sufficient to rescue the asymmetry defect. However, the interallelic complementation between $mam^{HD10/6}$ and mam^{IL42} (a situation very similar to the $mam^{HD10/6}$;*MamN* combination) also suggests that Mam^{IL42} and $Mam^{HD10/6}$ proteins (which are expected to be wild type, but present at reduced levels) interact to rescue the loss of asymmetric division of GMC-1. These results raise the question as to whether or not MamN (which is similar to the Mam protein in the mam^{IL42} allele) has all the necessary function for generating asymmetry. Since it does not rescue the asymmetry defect in mam^{IL42}, clearly it does not have all the necessary information. However, it does have the required function in the presence of some presumably wild-type protein (*i.e.*, in $mam^{HD10/6}$ background). This is consistent with the fact that MamN does not function as a dominant negative during the asymmetric division of GMC-1 but only at earlier stages during the formation of NBs.

There might be some difference between MamN and Mam^{IL42} in their ability to complement loss of asymmet-

ric division in $mam^{HD10/6}$. This is indicated by the findings that while MamN can rescue the asymmetry defect in $mam^{HD10/6}$, the interallelic complementation of the asymmetry phenotype between mam^{IL42} and $mam^{HD10/6}$ is not as complete as rescue of $mam^{HD10/6}$ by MamN (see Table 1). This may, in part, be due to the seven-amino-acid difference between MamN and Mam^{IL42}. Alternatively, there may be a protein-level difference between the two cases; in the former, MamN is expressed at high levels under Hs-GAL4, whereas in the latter mam^{IL42} is under the control of the *mam* promoter. Yet, the seven-amino-acid residues could make some difference, given that these amino acids are mostly glutamine residues, which can be involved in multimerization of proteins (Pascal and Tjian 1991). It is possible that the region of the Mam polypeptide defined by MamN (and Mam^{IL42}) is required to interact efficiently with the full-length Mam during the asymmetric fate specification. The requirement of some wild-type Mam protein for the rescue activity of MamN or Mam^{IL42} also suggests that the remaining portions of Mam are also required for generating asymmetry. The most likely scenario would be that this is a protein-protein interaction, although some other possibilities cannot be excluded. Since the available antibody against Mam recognizes multiple bands on a Western blot of proteins from embryo, we have not performed immunoprecipitation experiments to address protein-protein interaction between Mam molecules.

Our results show that mam^{IL42} carries a partial suppressor of neurogenic defect since a strong neurogenic defect can be restored to this allele upon recombination. This is consistent with the result that expression of MamN elicits a strong dominant-negative neurogenic defect. However, this suppressor in mam^{IL42} has no modifying effect on the loss-of-asymmetry phenotype of mam^{IL42}, as indicated by the fact that there was no change in the penetrance of this defect between the original and the recombinant mam^{IL42}. We have not mapped the location of this suppressor(s) beyond its tentative assignment to chromosome 2.

Previous studies utilizing MamH and MamN have demonstrated that both truncations elicited dominant-negative effects when overexpressed in imaginal tissues (Helms *et al.* 1999). It was later shown that the basic region of Mam is conserved in fly, mouse, and human and that the region physically interacts with the processed intracellular segment of Notch (N^{intra}; Petcherski and Kimble 2000a,b; Wu *et al.* 2000; Kitagawa *et al.* 2001). Mam, N^{intra}, and Su(H)/CSL proteins associate in a ternary complex that binds to HES/E(spl) promoters and activates gene expression (Wu *et al.* 2000; Kitagawa *et al.* 2001). The expression of MamH and MamN presumably leads to transcription complexes containing a defective form of Mam in a complex with Su(H) and N^{intra}. The current results, however, indicate that these interactions may be distinct during the generation of asymmetry. For instance, the MamN polypeptide may lack sequences required for interaction with factors necessary for NB formation but not for asymmetric division.

Finally, our results indicate that the *mam* phenotype in the RP2/sib lineage (symmetrical division of GMC-1 into RP2 and sib) is epistatic to the *numb* phenotype (symmetrical division of GMC-1 into two sibs). During the division of GMC-1, Insc localizes to the apical end of GMC-1, which in turn segregates Nb to the basal end. The cell that inherits Nb is specified as RP2 due to the ability of Nb to block Notch signaling, whereas the cell that does not inherit Nb (but inherits Insc) is specified as sib by Notch. Thus, in *insc* mutants, both daughters of the GMC-1 adopt an RP2 fate whereas in *nb* mutants they assume a sib fate (Buescher *et al.* 1998; Wai *et al.* 1999). The sib cell adopts an RP2 fate in *Notch; nb* double mutants (Wai *et al.* 1999). This indicates that Nb is needed to specify RP2 fate only when there is intact Notch. The *mam, numb* double mutant result is consistent with the above result and extends our previous finding. That is, Numb is needed only when there is intact Mam. This result further indicates that Mam functions downstream of Notch during the asymmetric specification of RP2 and sib, an observation consistent with the prevailing view of the Notch signal transduction pathway.

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