The *Drosophila* neurogenic locus *mastermind* encodes a nuclear protein unusually rich in amino acid homopolymers

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The neurogenic loci of *Drosophila* are required for proper partitioning of ectodermal cells into epidermal versus neural lineages. The loci appear to encode components of a developmental pathway involving cellular communication. In an effort to understand the role of the neurogenic locus *mastermind* in these processes, we have characterized its expression and sequence. The locus produces a number of transcripts that accumulate ubiquitously during early embryogenesis but more specifically in the central nervous system during later stages. Sequence analysis of a major cDNA product predicts an unusual protein containing an abundance of amino acid homopolymers and charge clusters typical of regulatory molecules. Nearly half of the mass of the predicted protein derives from only three amino acids: glutamine, glycine, and asparagine. Immunohistochemical studies of the protein in cell culture and early embryos show that the protein accumulates predominately in the nucleus.

[Key Words: *Drosophila*; neurogenesis; *mastermind*; homopolymers; nuclear]

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The establishment of neural and epidermal lineages within the *Drosophila* ectoderm requires the action of a small group of genes, termed the neurogenic loci (Lehmann et al. 1983; Hartenstein and Campos-Ortega 1984, 1986). Six zygotic neurogenic loci have been identified: *Notch* (*N*), *Delta* (*Dl*), *Enhancer of split* (*E(spl)*), *big brain* (*bib*), *mastermind* (*mam*), and *neuralized* (*neu*), as well as a number of maternal effect neurogenic loci, including *almondex* (*amx*) and *pecanex* (*pcx*) (Perimon et al. 1986; LaBonne et al. 1989). Loss of neurogenic gene function leads to a uniform commitment of cells within the neurogenic region to the neural pathway and the concomitant loss of epidermis, suggesting the hypothesis that these loci encode components of a pathway that represses neural development (Wright 1970). Consistent with this idea, inhibitory interactions among cells of insect neuroectoderm have been demonstrated through laser ablation (Doe and Goodman 1985a,b), and numerous genetic interactions among mutations in the neurogenic loci have been reported (Vassin et al. 1985; de la Concha et al. 1988; Shepard et al. 1989; Brand and Campos-Ortega 1990; Xu et al. 1990). Since the formulation of this hypothesis, the notion that these loci encode molecules involved in the signaling, reception, and transduction of developmental cues has also

received considerable experimental support from molecular studies. The *N* and *Dl* loci code for transmembrane proteins with putative ligand-binding domains related to mammalian epidermal growth factor (Wharton et al. 1985a; Kidd et al. 1986, 1989; Vassin et al. 1987; Koczynski et al. 1988; Johansen et al. 1989). Transfected *Drosophila* cells that express *N* and *Dl* proteins form mixed aggregates, apparently mediated through the physical interaction of these molecules (Fehon et al. 1990). Recent results from mosaic analyses are also consistent with a role for *N* as a receptor or adhesion molecule during these processes (Hoppe and Greenspan 1990). The predicted product of the *bib* locus is similar to transmembrane channel proteins (Rao et al. 1990), and partial sequence of the *pcx* locus is also suggestive of a membrane-spanning product (LaBonne et al. 1989). Finally, DNA sequence analyses have shown that the *E(spl)* region encodes a protein homologous to a mammalian G protein (Hartley et al. 1988), as well as proteins homologous to the vertebrate *myc* family of nuclear proteins (Klammbt et al. 1989).

The object of this study, *mam*, exhibits genetic interactions with several neurogenic loci during central nervous system (CNS) formation (de la Concha et al. 1988). Furthermore, postembryonic interactions have been characterized between alleles of the *N* locus and *mam* (Brand and Campos-Ortega 1990; Xu et al. 1990). Thus, it appears that the functions of the *N* and *mam* loci may be intimately associated during neurogenesis

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and during other developmental processes as well. In an effort to understand the basis for these interactions and the role of *mam* during neurogenesis, a molecular characterization of the product of this locus has been undertaken. Previous molecular studies demonstrated that the *mam* locus is large and unusually rich in repetitive elements, most notably *opa* or (CAX)_n runs (Weigel et al. 1987; Yedvobnick et al. 1988). Here, we report that the unusual genomic organization of *mam* is reflected in its predicted protein product as numerous runs of amino acid homopolymers. Further, *mam* is transcriptionally complex, producing a number of RNAs during and after neuroblast segregation. During early neurogenesis, *mam* products are observed throughout the germ band. In contrast, late *mam* products accumulate in the CNS, implicating the locus in functions beyond the neural-epidermal lineage determination. Immunohistochemical localizations of *mam* protein in cell culture and embryos demonstrate that the product accumulates predominantly in the nucleus.

Results

Definition of the *mam* transcription unit

The molecular cloning of the region containing *mam* was reported previously (Weigel et al. 1987; Yedvobnick et al. 1988). A physical map covering 90 kb of the *mam* region is presented in Figure 1A. The locus is likely to be contained within a 76-kb chromosomal interval (–51 to +25 on the physical map) and, based on transcriptional analyses (summarized below), the locus appears to extend 67 kb.

Initial characterizations of the locus demonstrated a high density of repeated sequences. DNA sequence of several regions revealed two classes of repeat: *opa* (CAX)_n and (CA)_n (Yedvobnick et al. 1988). The repeats complicated the analysis of *mam* transcription, because they are highly transcribed at many genomic sites. Unique sequence regions that were tested for transcription gave negative results, suggesting that particular repeats must be components of exons. This seemed most

Figure 1. Molecular organization of *mam*. (A) The horizontal line depicts 90 kb of DNA from the *mam* region at cytogenetic position 50CD. The *Eco*RI sites are indicated by vertical lines below the coordinate line. The coordinate scale below the line is indicated in kilobases, and the 0 coordinate marks the P-element insertion site cloned previously from the hybrid-dysgenesis (HD)-associated allele HD 11/2 (Yedvobnick et al. 1988). Repetitive regions containing *opa* (CAX)_n elements (open boxes) and (CA)_n elements (cross-hatched boxes) are displayed below the coordinate line. The physical map positions of 10 *mam* alleles are shown above the coordinate line. Seven alleles were described previously: In(2R)N²G, HD 2/3, HD 11/2, HD 13/6, HD 3/1, HD 17/7, and HD 10/6 (Yedvobnick et al. 1988). Arrows associated with breakpoint markers indicate the direction of particular inversions. HD 2/3, HD 17/7, and HD 10/6 are described below and in the text. Three additional *mam* alleles, 1E7, 10E1, and 2B11, were provided by the Jan lab and derived from an enhancer detection screen (Bier et al. 1989). A chromosome breakpoint associated with the *CyO* balancer limits the 3' end of the locus. This breakpoint is spanned by a transcription unit oriented opposite to *mam* transcription that is expressed during early embryogenesis. The proximal (5') region of *mam* is flanked by a maternally expressed gene that is also transcribed opposite to *mam*. (B) Map of the *mam* cDNA B4 and proposed structure of the transcript in three characterized mutants. cDNA B4 is 6333 bp and contains seven exons (312, 127, 1010, 2073, 286, 1090, and 1435 bp), spanning 67 kb of genomic DNA. Sequence analyses of HD 10/6 and HD 17/7 are described in the text. The diagram depicts a P insertion within exon 1 of HD 10/6, a deletion and frameshift within exon 4 of HD 17/7 (open boxes mark regions after deletion that are within frameshift), and an 8-kb deletion that removes exons 5, 6, and 7 in HD 2/3.

likely for *opa* elements, because they are often found within protein-coding regions (Laughon et al. 1985; Wharton et al. 1985b; Pirrotta et al. 1987). Consequently, we sequenced three *opa*-containing regions and searched for open reading frames (ORFs). The map positions of the sequenced regions were -1, -2, and -32 (Fig. 1A). In each case, *opa* comprised a portion of an ORF in which CAX coded for glutamine. The three ORFs had the same orientation, suggesting that they were parts of the same transcription unit. To test this idea, a synthetic oligonucleotide homologous to a nonrepetitive section of each ORF was hybridized to blots of gel-fractionated poly(A)⁺ RNA isolated from 3- to 12-hr embryos, a time period that spans neurogenesis. The probes hybridized to a group of transcripts (see below) and were subsequently utilized to screen cDNA libraries. The largest cDNA isolated, B4 (6.3 kb), was recovered from 8- to 12-hr embryos. The cDNA was mapped against *mam* genomic clones under conditions that eliminate cross-hybridization of repetitive elements. B4 hybridized across 67 kb of DNA (Fig. 1B), and the limits of hybridization coincided well with the estimate of the physical limits of the locus. DNA sequence analysis of B4 and genomic exons indicate that B4 is nearly full length and contains all of the protein-coding information. DNA sequence determinations and physical mapping of three *mam* alleles, HD 10/6, HD 17/7, and HD 2/3, have demonstrated small lesions within exons of B4, strongly implicating the B4 transcript in *mam* function (see below).

Sequence analysis of *mam* P-element mutations and a revertant

To establish a role for B4 in *mam* function, the sequence associated with two P-induced *mam* alleles and a revertant were determined. The *mam* allele HD 10/6 is an insertion of a P element near coordinate -50 on the physical map (Fig. 1A; Yedvobnick et al. 1988). The insertion region from HD 10/6 was cloned and sequenced; this analysis revealed a P element within the first exon of B4 at nucleotide 23 (Figs. 1B and 3). The P element was mobilized through outcrossing to an M strain and complementation testing against a *mam* allele. Approximately 17,000 chromosomes were tested, and two revertants were isolated. Genomic Southern analysis indicated that the P element had excised in both revertants. DNA sequence analysis of one revertant demonstrated a precise excision (data not shown); the second revertant was not analyzed further.

The mutation HD 17/7 was determined to be a small deletion near coordinate -2.0 (Yedvobnick et al. 1988). DNA sequence analysis of the deletion region has demonstrated that this allele is missing 146 bp from exon 4 of B4 (nucleotides 1886–2031; Figs. 1B and 3). The predicted effect of this deletion is to produce a frameshifted and truncated version of the normal B4 protein. In the HD 17/7 product the initial 377 amino acids are normal, followed by 69 amino acids of frameshift, before a premature termination after residue 446. Consistent with

this major perturbation in protein structure, HD 17/7 shows the most severe neurogenic phenotype among the characterized P-induced alleles.

The structure of the B4 product in mutation HD 2/3 is also presented in Figure 1B. The deletion region from this mutant was cloned and mapped to the 3' end of the transcription unit. The deletion extends from an intronic region, between exons 4 and 5, to a site ~2.5 kb distal to the end of B4. The predicted product from deletion HD 2/3 would code for a truncated 923-amino-acid protein.

Transcription of *mam*

Temporal and spatial patterns of *mam* transcripts were determined through hybridization of nonrepetitive sequence segments of cDNA B4 to RNA blots and embryonic tissue sections (Fig. 2). The probes hybridize to a maternal class of 6-kb transcript (0–2 hr) and to four zygotic transcripts (Fig. 2A, A'). Two transcripts of 8.5 and 8.0 kb accumulate to high levels during 2–16 hr of embryogenesis. Two additional transcripts of 7.5 and 6.5 kb are not highly expressed during early stages (0–8 hr); however, both transcripts accumulate over the following 8 hr. The quantity of all four transcripts decreases significantly during later stages, although longer autoradiographic exposures reveal persistent expression late in embryogenesis (data not shown). Higher levels of accumulation are evident again at late larval/early pupal stages.

To determine the spatial distribution of these transcripts during neurogenesis, ³⁵S-labeled antisense RNA probes were hybridized to tissue sections of embryos. Hybridization was not detected with control sense probes of comparable specific activity. High levels of ubiquitous expression were observed during germ-band elongation (~5 hr postfertilization), coincident with neuroblast segregation from the ectoderm (Fig. 2B, B'). For example, RNA accumulates in the developing anterior and posterior midgut, as well as throughout the mesodermal and ectodermal layers of the germ band. The resolution of the hybridization signal did not allow us to distinguish whether neuroblasts were expressing *mam* RNA. However, as embryogenesis proceeds, *mam* transcription is more restricted to neural tissue. During later embryogenesis (~16 hr postfertilization), RNA accumulates almost entirely within the ventral nerve cord and brain of the CNS (Fig. 2C, C'); the signal evident outside the CNS during this stage is significantly higher than that seen with sense control probes. A late function of *mam* in the CNS is unknown; however, it is interesting that the N protein accumulates there concurrently (Johansen et al. 1989; Kidd et al. 1989). A comparison of the RNA blot to the tissue sections suggests that the smaller transcripts (6.5 and 7.5 kb) accumulate specifically in neural tissue.

Analysis of cDNA B4 and genomic DNA

The sequence of cDNA B4 and genomic exons was de-

Figure 2. *mam* transcription patterns. (A, A') Temporal accumulation of *mam* RNAs during embryogenesis. (A) Ten micrograms of poly(A)⁺ RNA from staged embryos (hours are postfertilization at 25°C), or larval stages (L1, first instar; L2, second instar; L3, climbing third instar and early pupae) was electrophoresed, blotted onto nylon membranes, and hybridized with a ³²P-labeled 900-bp antisense RNA probe derived from cDNA B4. (A') Higher resolution RNA blot of embryonic transcripts. (B, B') Spatial accumulation of *mam* RNAs during germ-band elongation (early neurogenesis); neuroblasts segregate from the ectoderm and layer between the ectoderm and mesoderm at this time (~5 hr postfertilization at 25°C). (B) Bright field; (B') dark field. (am) Anterior midgut; (pm) posterior midgut; (e) ectoderm; (m) mesoderm. (C, C') Spatial accumulation of *mam* RNAs during late embryogenesis (~16 hr postfertilization at 25°C). *Top* and *bottom* are horizontal and sagittal sections, respectively, of similar age embryos. (C) Bright field; (C') dark field. (b) Brain; (vc) ventral nerve cord. Sections of embryos were hybridized with a 900-bp ³⁵S-labeled antisense probe derived from the 3' end of cDNA B4.

terminated (Fig. 3). This comparison revealed 16-base substitutions and four changes in the number of glutamines within a homopolymer run. The comparison of cDNA and genomic DNA established intron/exon boundaries; this is presented in Figures 1 and 3. All boundaries matched the consensus for eukaryotes (Mount 1982). The cDNA encodes an ORF of 4788 bp, predicting a

polypeptide of 167 kD (1596 amino acids; see immunoblot, Fig. 5A, below). The ORF is preceded by 753 bp of untranslated sequence and followed by a 791-bp 3'-untranslated region. A poly(A) tail is preceded by several imperfect matches to the consensus polyadenylation signal AATAAA (Proudfoot and Brownlee 1976); that is, TATAAA at position 6290. The ATG at position 754 is

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A second notable feature of the protein is the number and distribution of charged amino acids (Fig. 4B). The protein has fewer charged residues (8.3%) than an average *Drosophila* protein (21.8%), discounting histidine. In *mam* histidine (2.4%) is similar to an average

Drosophila protein (2.8%); however, the frequency of this residue may be influenced by its prevalence within *opa* (CAX) runs: the codons for histidine are CAT and CAC. Most of the histidine residues in *mam* (26/39) occur within polyglutamine regions. It is clear that regions of *mam* are rich in mixed charge, as well as in basic or acidic charges. Two regions of mixed charge concentration occur within residues 127–256 and 466–539. The former region also contains a predominantly basic area (127–190) that is 30% arginine plus lysine, whereas the latter area also contains a predominantly acidic area (466–534) that is 28% aspartic acid plus glutamic acid. A final concentration of acidic charges occurs at the carboxyl end (1559–1592), where 23% are negative. The charged domains are separated by numerous homopolymeric runs of amino acids (Fig. 4B). These runs contain little concentration of charge except, as noted above, for histidine residues interspersed within polyglutamine runs. Two regions, residues 1057–1194 and 1436–1558, contain virtually no charged or hydrophobic residues and may form long, randomly coiled structures.

The *mam* protein accumulates in the nucleus

Data base searches utilizing the *mam* sequence provided

no definitive similarities to other proteins. The most significant matches were to proteins containing amino acid homopolymers. The prevalence of homopolymers in *mam*, in combination with its charge clusters, suggested some structural similarities to a large number of nuclear regulatory proteins (Brendel and Karlin 1989). Consequently, the *mam* protein was localized immunohistochemically, using mouse antibodies directed against *mam* fusion proteins. The sera recognized a predominant protein species on immunoblots in the size range predicted from the sequence analysis (Fig. 5A).

Initially, *mam* protein accumulation was observed in cell culture (Schneider's cell line S2). The coding region from cDNA B4 was cloned into the metallothionein-regulated expression vector, pRmHa-3 (Bunch et al. 1988). This construct was transfected into cells and induced with copper. Cells were assayed for *mam* protein, utilizing *mam* antisera and fluorescein-conjugated secondary antibodies. In a small percentage of cells a strong nuclear signal was evident (Fig. 5B). To ensure that these cells were expressing the *mam* construct, a second clone that expresses *N* (Fehon et al. 1990) was cotransfected. The cells expressing *N* were identified using secondary antibodies conjugated to rhodamine. This demonstrated that cotransfected cells accumulated *N* predominantly in the cytoplasm and membrane (Fig. 5C), as reported

Figure 4. (A) Analysis of ORFs within the sequence of cDNA B4. Solid bars denote ORFs that begin with methionine residues. The vertical positions of pixels above and below bars indicate the degree of similarity or dissimilarity, respectively, to a *Drosophila* codon usage table provided by M. Ashburner, utilizing the GENEPRO program (Riverside Scientific). A single long ORF (4788 bp/1596 amino acid residues), evident in frame 3, shows excellent agreement with *Drosophila* codon usage. (B) Summary of major characteristics of the predicted B4 protein product. The horizontal bar represents the 1596-residue *mam* product with the regions of charge concentration highlighted. Above the bar are vertical hatch marks that indicate (1) the position of five or more identical amino acid residues, for glutamine (Q), glycine (G), asparagine (N), alanine (A), or threonine (T); or (2) the position of alternating Gly–Val (GV) runs; or (3) the position of Gly–Gly (GGX) doublets, which are not parts of longer glycine homopolymers, where X is usually a hydrophobic amino acid.

Table 1. Amino acid composition of predicted *mam* B4 product versus *Drosophila*

	<i>mam</i> (no.)	<i>mam</i> (%)	<i>Drosophila</i> (%)	Ratio ^a
Gln (Q)	347	21.7	5.0	4.3
Gly (G)	269	16.8	7.8	2.2
Asn (N)	155	9.7	4.6	2.1
Pro (P)	123	7.7	5.7	1.4
Ala (A)	108	6.7	7.8	0.9
Met (M)	82	5.1	2.4	2.1
Leu (L)	71	4.4	8.2	0.5
Ser (S)	71	4.4	7.6	0.58
Val (V)	64	4.0	6.0	0.67
Thr (T)	58	3.6	5.5	0.65
Asp (D)	42	2.6	5.1	0.51
Phe (F)	42	2.6	3.4	0.76
His (H)	39	2.4	2.8	0.86
Arg (R)	34	2.1	5.2	0.4
Lys (K)	34	2.1	5.3	0.4
Glu (E)	24	1.5	6.2	0.24
Ile (I)	18	1.1	4.7	0.23
Tyr (Y)	10	0.6	3.2	0.19
Cys (C)	4	0.2	2.3	0.09
Trp (W)	1	0.06	1.1	0.06
Total	1596			

^aRatio = *mam* (%) / *Drosophila* (%). *Drosophila* data were calculated from the codon usage table compiled by M. Ashburner (unpubl.).

earlier (Fehon et al. 1990), and *mam* in the nucleus (Fig 5B). Similar results were obtained in cells cotransfected with plasmids expressing *mam* and β -galactosidase (data not shown).

To confirm that the nuclear accumulation of *mam* in cultured cells was significant, antisera were tested against whole mounts of early embryos. Staining of the cellular blastoderm (~3 hr postfertilization) is presented in Figure 5D. As in the case of the S2 cells, the signal is predominantly nuclear. No regional accumulation of the protein within the embryo is evident at this stage.

Discussion

The neurogenic loci of *Drosophila* function during several developmental stages in numerous tissues and appear to mediate developmental cues that result in the segregation of apparently equipotent cells into different patterns of differentiation (Artavanis-Tsakonas 1988; Campos-Ortega 1988; Simpson 1990). Such processes are fundamental and may be regulated similarly in other systems (Yochem and Greenwald 1989). In an effort to understand the function of the *mam* locus in such processes, we have characterized its expression and determined the sequence of one of its products.

The *mam* locus comprises a 67-kb transcription unit that codes for at least five transcripts accumulating over the first 16 hr of embryogenesis (Fig. 2A, A'). A 6-kb, presumably maternal, transcript has been identified during the initial 2 hr of development, consistent with the predictions of genetic studies (Jimenez and Campos-Ortega 1982). Four zygotic transcripts are evident during the following 14 hr. Transcription from *mam* decreases

significantly during later times until late larval/early pupal stages, when the transcripts reappear, correlated with the imaginal requirement for *mam* (Dietrich and Campos-Ortega 1984; Brand and Campos-Ortega 1990; Xu et al. 1990). Although the sequence differences between the transcripts have not yet been fully characterized, some of the variation appears to derive from alternative 3' and 5' ends (see legend to Fig. 3), and it is clear that the larger transcripts contain most or all of the sequence identified in cDNA B4 (C. Friedel, unpubl.).

Utilizing a probe that recognizes the five transcripts, the spatial pattern of transcript accumulation was observed during neurogenesis (Fig. 2B,C). The transcripts appear ubiquitous in germ-band-extended embryos, a stage that contains segregating neuroblasts. This suggests that *mam* transcription is not limited to a particular cell type during this process. During later embryogenesis, when the ventral cord and brain are condensed, it is clear that *mam* transcription predominates in the CNS, as predicted from the enhancer trap studies of Bier et al. (1989). It appears that the territories of *mam* transcription during embryogenesis overlap significantly with those reported for *N* (Hartley et al. 1987), *Dl* (Vassin et al. 1987; Kopczynski et al. 1989), and particular products of the *E(spl)* region (Knust et al. 1987;

Figure 5. Immunolocalization of *mam*. (A) Antisera raised against *mam* fusion proteins recognize a single protein on immunoblots of embryonic proteins (8- to 24-hr embryos), in the size range predicted from the DNA sequence. Size standards, 205 kD (myosin) and 116 kD (β -galactosidase). (B and C). Immunolocalization of *mam* protein (B) or *N* protein (C) in co-transfected Schneider's S2 cells, expressing products under the control of a metallothionein promoter. *N* and *mam* proteins were visualized by utilizing secondary antibodies conjugated to rhodamine and fluorescein, respectively. (D) Immunolocalization of *mam* in nuclei of blastoderm embryos.

Hartley et al. 1988) and *bib* (Rao et al. 1990). The sites of postembryonic *mam* accumulation, expected to include imaginal disk tissue, are presently under investigation. *N* and *mam* function are required for developmental decisions in the eye and wing, etc. (Shellenbarger and Mohler 1978; Dietrich and Campos-Ortega 1984; Cagan and Ready 1989), and genetic interaction data (Brand and Campos-Ortega 1990; Xu et al. 1990) predict that products of *mam* and *N* will accumulate together in several imaginal tissues. *N* is expressed in these tissues (Johansen et al. 1989; Kidd et al. 1989; Markopoulou and Artavanis-Tsakonas 1989).

In screens for cDNAs, the largest isolated, B4, was derived from 8- to 12-hr embryos and measures 6.3 kb. The cDNA sequence (Fig. 3) demonstrated a single ORF with a codon usage similar to other *Drosophila* genes (Fig. 4A). Nearly half the mass of the predicted B4 product derives from three amino acids, glutamine, asparagine, and glycine, and many of the residues are contained in homopolymer runs. The presence of homopolymers in nuclear proteins of *Drosophila* was noted several years ago (Laughon et al. 1985), and the list is now quite long, including *zeste* (Pirrotta et al. 1987), *cut* (Blochinger et al. 1988), *engrailed* (Poole et al. 1985), and *Deformed* (Regulski et al. 1987), to cite just a few. The list is not limited to *Drosophila*, as yeast regulatory proteins contain similar repeats, that is, ADR6 (O'Hara et al. 1988) and HAP2 (Pinkham et al. 1987), and certain mammalian regulatory proteins as well [i.e., mouse glucocorticoid receptor (Danielsen et al. 1986)].

Another common characteristic of nuclear proteins is regions of alternating amino acids, such as alanine/glutamine runs in *single-minded* (Crews et al. 1988) and *zeste* (Pirrotta et al. 1987). The *mam* sequence has three very uncommon runs of alternating glycine/valine residues. Interestingly, such runs have also been observed in the homeo-domain-containing protein *orthodenticle* (Finkelstein et al. 1990), *Ultrabithorax* of *Drosophila pseudo-obscura* (Wilde and Akam 1987), and herpes simplex virus immediate early protein (Perry et al. 1986).

The function of homopolymeric and alternating runs of amino acid in proteins is still conjecture. Although common in DNA-binding proteins, they are also found outside this class of molecules and may play a generic role, as a spacer or hinge between other protein domains (Beachy et al. 1985; Laughon et al. 1985). The presence of such domains in transcriptional activators might be consistent with the role of a protein in binding both DNA and other regulatory proteins simultaneously. For example, regions devoid of charged or hydrophobic residues may form random coils that provide flexibility to tertiary conformation (Brendel and Karlin 1989). Alternatively, it is conceivable that some proportion of these repeated domains is functionless and derives from processes such as genomic drive. Evolutionary comparisons between genes containing homopolymers, such as polyglutamine, have shown these regions to have high rates of sequence variation (Kassis et al. 1986; Treier et al. 1989), consistent with this interpretation. In *mam*, pre-

liminary comparisons between the sequence in *Drosophila melanogaster* and *Drosophila virilis* also reveal high rates of change within these regions (S. Newfeld and B. Yedvobnick, in prep.). These ideas are not mutually exclusive, and one can imagine that a mechanism promoting variability in the spacing between functional protein domains would be beneficial.

In one case, a function for a polyglutamine-rich region has been reported. The DNA-binding protein Sp1 apparently contains glutamine-rich transcriptional activation domains. Furthermore, it appears that a polyglutamine region from the *Antennapedia* protein, when coupled to the DNA-binding domain of Sp1, can effect transcriptional activation (Courey et al. 1989). In Sp1 the glutamine-rich regions are in close proximity to serine-threonine-rich regions; it is of potential interest that a similar arrangement of residues is found at two locations in the *mam* sequence (residues 890–920 and 1469–1504).

A second feature of the *mam* protein common to many nuclear proteins is the clustered distribution of charged amino acid residues. Although there is very little charge overall in *mam*, the basic and acidic residues reside, for the most part, in three areas (Figs. 3 and 4B). Similar charge concentrations have been described in a number of transcriptional activators (Brendel and Karlin 1989), such as GAL4, where the basic DNA-binding domain is distinct from an acidic activation domain (Fischer et al. 1988), as well as the DNA-binding protein *zeste* (Pirrotta et al. 1987).

Antisera directed against *mam* fusion proteins was utilized to identify the site of protein accumulation. In cultured cells transfected with a plasmid that expresses large quantities of *mam*, it appears that the protein localizes preferentially within the nucleus; in contrast, the same cells accumulate *N* in the cytoplasm and membrane when cotransfected (Fig. 5B,C). Finally, the *mam* antisera stain the nuclei of embryonic blastoderm cells (Fig. 5D), further suggesting that this is the site of action. However, *mam* contains no obvious canonical motifs associated with many nuclear proteins (e.g., homeo domain, zinc finger, leucine zipper, helix-loop-helix, etc.). Consequently, if it functions as a transcriptional regulator, it may contain currently undescribed protein domains involved in such processes. It is also conceivable that an alternative transcript of *mam* encodes a related polypeptide that contains a more common motif of nuclear proteins.

The amino acid sequence and nuclear localization of the *mam* protein suggest a regulatory function. Although the precise role of *mam* in the neurogenic pathway remains to be established, the coincident expression patterns and genetic interactions of *mam* and *N* suggest two principal hypotheses. *mam* may influence the expression of *N* directly or indirectly, or, alternatively, *N* may affect the activity of the *mam* protein. A critical consideration in regard to these hypotheses is whether the interactions observed between these genes are compatible with the notion of nuclear regulation or whether they are more consistent with the idea of phys-

ical interactions between *mam* and *N* proteins, which presumably could occur in the cytoplasm. The available data does not allow a clear resolution between these choices.

Alleles of *N* with mutations in either the extracellular epidermal growth factor (EGF)-like domain, *Ax* and *spl* (Hartley et al. 1987; Kelley et al. 1987), or the intracellular domain, *nd*, *nd*² (Xu et al. 1990), demonstrate interactions with *mam*. The observation that these interactions are not *mam* allele specific suggests that the dosage of *mam* can influence the phenotypic expression of a variety of *N* alleles. In a hypothetical pathway, where *mam* is involved in the regulation of *N* expression, a number of mechanisms are feasible that could explain these interactions. A simple model suggests that particular lesions of *N* lower or elevate the activity of the protein and that reduced concentration of a potential regulator (*mam*) further affects phenotype through changes in the number of *N* molecules.

An alternative hypothesis proposes that particular genetic interactions between *mam* and *N* reflect the physical interactions of their products. In the absence of any knowledge of the molecular nature of the *mam* product, Xu et al. (1990) suggested that *N*, *Dl*, and *mam* may participate in a molecular assembly where proper dosage of each component is critical for function. A similar argument was raised by Brand and Campos-Ortega (1990) following a genetic screen that uncovered an interaction between *mam* and *N*. For the case of *N* and *Dl* proteins, there is very convincing biochemical evidence for physical interactions (Fehon et al. 1990). Our observation that the *mam* protein resides predominantly in the nucleus does not exclude such interactions. For example, it is conceivable that *mam* resides transiently in the cytoplasm after its synthesis. This could provide the opportunity for the *mam* protein to interact with the cytoplasmic domain of *N* prior to relocating to the nucleus, in a manner similar to the proposed roles of *Toll* and *dorsal* (Hashimoto et al. 1988; Roth et al. 1989; Rushlow et al. 1989; Steward 1989). In this regard, it is interesting that the lesions in the *nd* and *nd*² proteins are in the intracellular domain of the *N* molecule (Xu et al. 1990). In this model, interactions between *mam* and the *N* alleles containing perturbations in the extracellular EGF domain (*Ax* and *spl*) would require that changes in this extracellular region influence the function of the intracellular domain. The molecular probes are now available to test the predictions of such hypotheses.

Methods

Isolation of revertants

The *mam* allele HD 10/6 contains a 1.6-kb P element in exon 1 of cDNA B4 (Fig. 1). To revert this allele, HD 10/6 males (balanced over *CyO*) were mass-mated to an M strain containing the second chromosome balancers *Pm* and *Cy*. Groups of HD 10/6 /*Cy* F₁ males were then mated to a *mam* tester allele (balanced over *CyO*), and the progeny were scored for straight-winged survivors. From 17,000 F₂ progeny, two revertants were isolated. The revertant chromosomes were made homozygous and analyzed as described previously.

Nucleic acids

The isolation of DNA and RNA, blotting analyses, and the preparation of genomic libraries from mutant DNAs were performed as described previously (Yedvobnick et al. 1988).

Isolation of cDNA clones

Several embryonic cDNA libraries in a plasmid vector were supplied by Nick Brown (Harvard University); the libraries were screened by using a high-density filter screening protocol (Brown and Kafatos 1988). In some cases, pools of ~30,000 clones were miniprep and screened on Southern blots to identify smaller populations of clones for filter screening. A typical blot contained 20 lanes, totaling ~600,000 individual cDNAs. The initial oligonucleotide probes for *mam* cDNAs were derived from the DNA sequence surrounding three *opa* elements (see Fig. 1), and subsequent probes were derived from unique exonic sections of various cDNAs. cDNAs were mapped across the *mam* chromosomal region under very high stringency conditions to eliminate cross-hybridization due to repetitive elements. Typically, standard hybridization conditions [5 × SSC, (0.75 M NaCl, 0.075 M Na₃C₆H₅O₇, 50% formamide) at 42°C] were followed by high-temperature (80°C) washes in 0.1 × SSC, 0.1% SDS. The largest cDNA isolated, B4, was chosen for DNA sequencing.

DNA sequencing

A shotgun library derived from the sonication of cDNA B4 was constructed in M13, as described by Bankier and Barrell (1983). The sequence of 60 clones produced ~65% of the sequence. The remainder of the sequence of both strands of cDNA B4 was derived from subclone templates and specific priming of templates with synthetic oligonucleotides. Additionally, the sequence of smaller versions of cDNA B4, totaling ~4000 bp, was obtained. Similar strategy was utilized to sequence the corresponding genomic regions, although only a single strand was determined. Standard dideoxy sequencing reactions utilized modified T7 DNA polymerase (Sequenase, U.S. Biochemicals). Sequences were compiled and analyzed by utilizing the Staden Program (Staden 1986) and GENEPRO (Riverside Scientific).

In situ RNA localization

Tissue sections were prepared according to Ingham et al. (1985), with the following modifications. Embryos were collected in mesh baskets with NaCl/Triton rinses (0.03% NaCl, 0.4% Triton X-100), dechorionated in full-strength bleach for 3 min with gentle agitation, rinsed with NaCl/Triton and fixed in heptane/paraformaldehyde fixative, 1 : 1, by rapid shaking for 30 min (Harding et al. 1986). Paraformaldehyde fixative consisted of 4% paraformaldehyde in phosphate-buffered saline [PBS: 0.13 M NaCl, 0.007 M Na₂HPO₄, 0.003 M Na₂HPO₄ (pH 8.0)], 40% DMSO; paraformaldehyde was freshly prepared. After removal of the vitelline membranes (Mitchison and Sedat 1983), the embryos were rehydrated through ethanol (100, 95, 90, 70, 50, and 30%) and postfixed in 4% paraformaldehyde in PBS. Embryos were then dehydrated through the same ethanol series, embedded in paraffin and sectioned to 5 μm. The sections were collected on slides, coated with poly-D-lysine [50 μg/ml] in 10 mM Tris-HCl (pH 8.0)] and stored overnight at 45°C. Slides were dewaxed in xylene (two incubations, 10 min each) and rehydrated through ethanol (100, 95, 80, 60, and 30% PBS, 2 min each). They were then incubated in 0.2 N HCl for 20 min at room temperature, rinsed 30 sec in distilled water, and incubated at 70°C in 2 × SSC for 30 min. The sections were then treated with Pronase [0.5 mg/ml in 50 mM Tris-Cl (pH 7.5), 5 mM EDTA] for 10 min at room temperature, incubated 30 sec in 0.2 mg/ml glycine in PBS, rinsed twice in PBS for 3

min, and fixed in 4% paraformaldehyde in PBS for 20 min at room temperature. The slides were treated with 0.5% (vol/vol) acetic anhydride in 0.1 M triethanolamine (pH 8.0), dehydrated, and air-dried.

Single-strand RNA probes were synthesized according to Hartley et al. (1987), except that the transcription reaction proceeded at 37°C for 30 min.

Hybridizations, rinses, and autoradiography were performed according to Ingham et al. (1985). Coverslips were sealed onto slides with rubber cement, and slides were hybridized upright in a humid chamber. NTB2 autoradiographic emulsion was diluted 1 : 1 with sterile distilled water. After autoradiography, slides were stained with Giemsa [5% (vol/vol) in 0.01 M NaPO₄ buffer (pH 6.8)], rinsed twice in distilled water, air-dried, and viewed under oil.

Immunohistochemistry

Antisera were produced against two *mam* fusion proteins. A 366-bp restriction fragment, inserted into the pUR 291 vector (Ruther and Muller-Hill 1983) produced a fusion of 122 amino acids of *mam* (residues 511–632; see Fig. 3) to β -galactosidase. A 3963-bp restriction fragment inserted into the pGEX3 vector (Smith and Johnson 1988) produced 1321 amino acids of *mam* (residues 245–1565; see Fig. 3) fused to glutathione-S-transferase. Both fusion proteins were purified as insoluble aggregates (Carroll and Laughon 1987) and injected into mice at 3- to 4-week intervals using 50 μ g of protein in Maalox with 3×10^9 killed pertussis organisms per dose (R. Hunter, pers. comm.). Useful antisera were obtained after four to five injections. The 122-amino-acid segment contains no repetitive sequences, and its antisera recognizes a single immunoblot band in 0- to 24-hr embryos. Antisera to the 1321-amino acid segment were preadsorbed to *D. virilis* embryos and produce an identical immunoblot pattern (data not shown). Both sera show qualitatively similar patterns of staining on cultured cells and embryos. All antisera were precipitated with an equal volume of saturated ammonium sulfate solution and resuspended in PBS with 0.02% sodium azide.

For immunoblots, 100 μ g of embryos was collected in Tris-buffered saline (0.3 M NaCl, 0.01 M Tris) with 0.5% Triton X-100 (TBST), dechorionated in 50% bleach, rinsed with TBST twice, and frozen in liquid nitrogen. The embryos were homogenized in 300 μ l of 2 $\times$ Laemmli buffer, boiled for 5 min, and microfuged for 5 min. The supernatant was transferred to a new tube, and 20 μ l was loaded onto a 7.5% SDS-polyacrylamide gel for electrophoresis and transferred to a nitrocellulose filter. The filter was stained with Ponceau S to assure transfer. After staining, the filter was incubated with anti-*mam* antiserum at 1 : 3000 dilution in TBST/5% dry milk, overnight at 4°C. The filter was rinsed three times, followed by two 5-min washes. The filter was then incubated with goat anti-mouse IgG (GAM) antibodies linked to horseradish peroxidase (HRP) for 2 hr at 4°C [Jackson ImmunoResearch]. After washing as above, the filter was stained in 0.1 M diaminobenzidine (DAB), 0.0015% H₂O₂.

For whole mounts, embryos were collected in TBST, dechorionated in 50% bleach, and rinsed twice in TBST and twice in water. Embryos were fixed in 10 ml each of fresh 4% paraformaldehyde and heptane and shaken vigorously for 30 min at room temperature. Paraformaldehyde was removed and replaced with an equal volume of methanol and shaken vigorously for 15–20 sec by hand. Liquid phases and embryos at the interface (which have not lost their vitelline membranes) were removed. Embryos were rinsed three times with methanol, transferred to an Eppendorf tube, and rinsed three times with PBS. Embryos were blocked for 30 min to 1 hr in PBS, 0.1% Triton X-100 (PBST) with 10% normal goat serum (NGS), and

rinsed once with PBS and incubated overnight in primary antiserum diluted 1 : 1000 in PBST with 10% NGS. Primary serum was removed, and embryos were rinsed three times in PBS, followed by a 30-min wash in PBST with 10% NGS. The embryos were then incubated for 2 hr at 4°C in secondary antibodies (GAM/HRP), PBST with 1% NGS. This was followed by three rinses in PBS and a 30-min wash in PBST with 1% NGS. After a final rinse in PBS, 350 μ l of PBS was added, followed by 20 μ l of 10 mg/ml DAB with mixing. One microliter of 3% H₂O₂ was added and mixed thoroughly by inversion. The embryos were allowed to develop for 2–5 min. The reaction was stopped by the removal of the DAB solution and the addition of 70% ethanol. The embryos were then rinsed twice more in 70% ethanol and three times in 100% ethanol. After addition of methylsalicylate, the embryos were mounted (Entellan).

Cell culture

All culture methods are as described (Fehon et al. 1990). S2 cells were grown in M3 media with 11% fetal bovine serum, 100 U/ml penicillin, and 100 μ g/ml streptomycin. Five milliliters of log-phase cells was transfected with 20 μ g metallothionein plasmid DNAs (Bunch et al., 1988) as a calcium phosphate precipitate in 1 ml. The cells were left under the DNA precipitate for 16–18 hr, washed once with complete medium, and allowed to rest for 24 hr before induction with 0.7 mM copper sulfate. After 24 hr, cells were collected by centrifugation, washed in PBS, and fixed in 2% paraformaldehyde for 10–15 min at room temperature. Cells were then centrifuged, rinsed twice in PBS, and incubated for 1 hr in primary antiserum in PBS with 0.1% saponin and 1% NGS (PSN). Mouse anti-*mam* antiserum was used at 1 : 1000, and affinity purified rabbit anti-*Notch* antiserum (provided by the Artavanis lab) was used at 1 : 500. After washing once in PBS, cells were incubated in double-labeling grade secondary antibodies (Jackson) linked to fluorescein or rhodamine, for *mam* or *Notch*, respectively. Secondary antibodies were diluted 1 : 250 in PSN. Cells were then rinsed twice in PBS and mounted in 90% glycerol, 10% 1 M Tris (pH 8.0), and 0.5% *n*-propyl-gallate. Cells were photographed under epifluorescence.

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The *Drosophila* neurogenic locus mastermind encodes a nuclear protein unusually rich in amino acid homopolymers.

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