Molecular Analysis of the Neurogenic Locus *mastermind* of *Drosophila melanogaster*

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ABSTRACT

The neurogenic loci comprise a small group of genes which are required for proper division between the neural and epidermal pathways of differentiation within the neuroectoderm. Loss of neurogenic gene function results in the misrouting of prospective epidermal cells into neuroblasts. A molecular analysis of the neurogenic locus *mastermind* (*mam*) has been initiated through transposon tagging with *P* elements. Employing the Harwich strain as the source of *P* in a hybrid dysgenesis screen, 6000 chromosomes were tested for the production of lethal *mam* alleles and eight mutations were isolated. The *mam* region is the site of residence of a *P* element in Harwich which forms the focus of a chromosome breakage hotspot. Hybrid dysgenic induced *mam* alleles elicit cuticular and neural abnormalities typical of the neurogenic phenotype, and in five of the eight cases the mutants appear to retain a *P* element in the cytogenetic region (50CD) of *mam*. Utilizing *P* element sequence as probe, *mam* region genomic DNA was cloned and used to initiate a chromosome walk extending over 120 kb. The physical breakpoints associated with the hybrid dysgenic alleles fall within a 60-kb genomic segment, predicting this as the minimal size of the *mam* locus barring position effects. The locus contains a high density of repeated elements of two classes; *opa* $(CAX)_n$ and $(dC\text{-}dA)_n \cdot (dG\text{-}dT)_n$. A preliminary study of the transcriptional activity of the *mam* region is presented.

THE ventral and cephalic portions of the central nervous system of Drosophila derive from a restricted section of the ectoderm termed the neurogenic region (POULSON 1950). The ventral region consists of cells situated lateral to the mesoderm at the blastoderm stage, and flanking the ventral midline during gastrulation. Although the neurogenic ectoderm appears homogeneous, it is composed of cell populations destined to form two major tissues. Approximately 25% of the cells will form neuroblasts, the precursors of the central nervous system, whereas the remaining 75% will differentiate into epidermal structures (HARTENSTEIN and CAMPOS-ORTEGA 1984). The choice between these two developmental paths can be disrupted through mutation. Members of the neurogenic class of mutations display an embryonic lethal phenotype associated with a misrouting of epidermal precursors into neuroblasts (POULSON 1950; CAMPOS-ORTEGA 1985). Thus, the neurogenic loci represent a group of genes which effect a specific developmental decision.

Saturation mutagenesis screens for zygotic lethal mutations detected six genes that are mutable to a neurogenic state (LEHMAN *et al.* 1983; JURGENS *et al.* 1984; NUSSLEIN-VOLHARD, WIESCHAUS and KLUDING 1984; WIESCHAUS, NUSSLEIN-VOHARD and JURGENS 1984), including *Notch* (*N*), *Delta* (*Dl*), *Enhancer of split* (*E*[*spl*]), *mastermind* (*mam*), *big brain* (*bib*) and *neuralized* (*neu*). A maternal class of neurogenic mutations, including *almondex* (*amx*) (LEHMAN *et al.* 1983), *pecanex* (*pcx*) and several female-sterile mutations (PERRIMON *et al.* 1986), have also been isolated. An intriguing characteristic of the neurogenic gene class is the interactions observed between certain genes (WELSHONS 1956; CAMPOS-ORTEGA *et al.* 1984; VASSIN, VIELMETTER and CAMPOS-ORTEGA 1985; M. A. T. MUSKAVITCH, personal communication). For instance, *E*(*spl*) enhances the phenotype of a *Notch* locus allele (*spl*), while chromosome breaks associated with reversion of the enhancer phenotype produce a neurogenic lethal allele at the *E*(*spl*) locus (termed $E[spl]^R$). Further, $E(spl)^R$ alleles do not complement *Delta* or *Notch* alleles in double heterozygous combinations, such as $E(spl)^R$ + / + *Dl*. In addition, embryos hyperploid for certain wild type neurogenic loci can reduce the neurogenic phenotype of specific mutant embryos. For instance, homozygous *bib*, *mam*, or *neu* embryos can be partially rescued by the introduction of extra wild-type *Notch* loci. These observations suggest that particular neurogenic loci share a common function and possibly comprise a developmental pathway.

In an effort to determine the function of the neurogenic loci, these genes have become the targets of cloning in several laboratories. At this time, only the *Notch* locus has been well characterized at the

The sequence data presented in this article have been submitted to the EMBL/GenBank Data Libraries under the accession number Y00611.

molecular level. *Notch* spans approximately 38 kb of genomic DNA and encodes a poly(A^+) RNA of 10.5 kb (Artavanis-Tsakonas, Muskavitch and Yedvobnick 1983; Kidd, Lockett and Young 1983; Grimwade *et al.* 1985). cDNAs spanning the entire 10.5 kb transcript have been synthesized and sequenced (Yedvobnick *et al.* 1985; Wharton *et al.* 1985b; Kidd, Kelly and Young 1986). Conceptual translation of the cDNA sequence demonstrated that *Notch* appears to encode a membrane-spanning polypeptide with epidermal growth factor-like repeats in the extracellular domain. The putative structure of the *Notch* protein immediately implicated cell:cell interactions as a component in the process of neurogenic ectoderm differentiation (Wharton *et al.* 1985b; Kidd, Kelly and Young 1986). Subsequent DNA sequence studies of a *Delta* locus cDNA suggested a similar function for the product of this neurogenic locus (Vassin *et al.* 1987). This hypothesis is made more attractive by the observations of Doe and Goodman (1985a,b). Employing laser ablation to eliminate individual neurogenic ectodermal cells in grasshopper embryos, it was demonstrated that neuroblasts appear to inhibit adjacent cells from forming neuroblasts, thereby implicating cell contact as a component in the decision mechanism. Similar conclusions have been reached by Technau and Campos-Ortega (1986, 1987) who reported that transplanted ectodermal cells of early *Drosophila* gastrula exhibit a degree of nonautonomous differentiation, as well as by Hoppe and Greenspan (personal communication) through the analysis of *Notch* mosaic embryos.

The observations cited above have established a provisional model for how ectodermal cells within the neurogenic region enter the neural or epidermal path of differentiation. However, the model does not address the role of maternal vs. zygotic contributions of these genes, nor does it position the neurogenic loci within the larger framework of the regulatory hierarchy that unfolds during early *Drosophila* development. Thus, the establishment of a cell-cell interaction is an event that occurs within a continuum. This interaction must be preceded and followed by other events necessary for proper neural-epidermal dichotomy. It is predicted that certain of these events will involve the transduction of membrane-initiated signals to the cell interior. Consequently, a more definitive description of the process will require the molecular cloning and characterization of DNA sequences containing the other neurogenic loci. In this report, we describe our progress toward cloning one of these loci, *mastermind*, using the method of P-element tagging. During the preparation of this manuscript, Weigel, Knust and Campos-Ortega (1987) reported a similar analysis of the *mastermind* region.

MATERIALS AND METHODS

Strains: Harwich and *seF8*, P and M strains, respectively, were supplied by M. Kidwell. A multiply marked balancer M strain was supplied by V. Finnerty. Males from this stock were outcrossed to P mutants to reduce the background of *P* elements in the stock used for molecular cloning. Mam^{1J113} and bib^{1005} were provided by E. Wieschaus. Mam^{11J14} was provided by C. Nusslein-Volhard. *In(2R)N2G/SM5* was obtained from Cal Tech. Canton S was obtained from Bowling Green. All crosses were performed at 25° on standard corn meal and molasses medium.

***In situ* hybridization and cytology:** Cloned DNA was biotinylated through nick translation and hybridized to polytene chromosome preparations as described by Langer-Safer, Levine and Ward (1982). The hybridized probe was visualized employing a horseradish peroxidase detection system (Enzo Biochemicals) against Giemsa-stained chromosomes. The preparations were photographed using Kodachrome film (ASA 25 or 64) and an NCB 10 filter.

Cuticle preparation: Cuticles from mature embryos were prepared according to the methods of Van der Meer (1977) and photographed using Kodachrome film (ASA 25 or 64) and an NCB 10 filter.

Staining of embryonic central nervous systems: Direct immunofluorescence of embryo whole mounts was performed using a protocol supplied by Marc Muskavitch (Indiana University). It is a variation of a method of T. Karr and B. M. Alberts (UCSF), developed by N. Patel and C. Goodman (Stanford University). Adults were allowed to lay eggs on heavily yeasted bottles for 8 hr at 18°, and the embryos were aged 12–16 hr at 18°. Aged embryos were collected on a nylon screen, washed and transferred to silanized glass culture tubes (12 mm × 75 mm). The embryos were dechorionated in 50% bleach for 5 min, and rinsed with 0.02% Triton X-100. After aspiration of the rinse buffer, the embryos were fixed by suspension in 0.5 ml 4% w/v paraformaldehyde to which were added 1.5 ml heptane. The suspension was vortexed repeatedly for 10–20 min, and the lower phase was removed by aspiration. The vitelline membranes were removed through addition of 0.5 ml 100% methanol for 1–2 min. The solution was aspirated and the embryos were rinsed 2 times in 1.5 ml 100% methanol, and 3 times in 0.5 ml phosphate-buffered saline (PBS: 130 mM sodium chloride, 7 mM dibasic sodium phosphate, 3mM monobasic sodium phosphate). The embryos were rinsed in 200 μl of PNS (PBS + 1% v/v normal goat serum, Worthington Cappel). This rinse was removed and an additional 200 μl PNS was added. One microliter of a preparation of fluoresceinated anti-horseradish peroxidase (rabbit sera, Worthington-Cappel) was added to the suspension, and the tubes were sealed and shaken in the dark at 4° overnight. After removal of the antibody solution, the embryos were washed 3 times (200 μl each) over 30 min, at room temperature. The stained embryos were mounted on glass slides under coverslips in 80% glycerol, 20% PBS, and sealed with nail polish. Preparations were viewed with a Leitz fluorescence microscope and photographed using Kodak Ektachrome (ASA 200) film.

Nucleic acids: The isolation of *Drosophila* RNA and DNA, and Northern and Southern analyses were performed as described previously (Artavanis-Tsakonas, Muskavitch and Yedvobnick 1983; Grimwade *et al.* 1985), except in certain cases in which genomic DNA was isolated from adults using the method of Scott *et al.* (1983). Plasmid preparation and subcloning of phage fragments in plasmid vectors directly out of low melting point agarose gels were

performed according to CROUSE, FRISCHAUF and LEHRACH (1983).

Genomic libraries: The construction and screening of *Sau*IIIA partial genomic libraries in EMBL 3 (Vector Cloning Systems) were performed using the standard methods described in MANIATIS, FRITSCH and SAMBROOK (1982), with the following modification. Partially digested DNA in the 10–22-kb size range was electroeluted from a 0.7% agarose gel and then purified through an Elutip-d column (Schleicher & Schuell).

DNA sequencing: Relevant DNA regions were subcloned into mp 18/19 or Bluescript (Stratagene) vectors and sequenced as described in Wharton et al. (1985a), except standard 8% and 6% gels were utilized rather than gradient gels. Sequencing reactions were electrophoresed on an LKB 2010 macrophor system at 3000 V for 2 or 5 hr. In those cases where deletion subclones were required, they were produced in Bluescript, as described in Stratagene's protocols.

RESULTS

Production of *mam* mutations through hybrid dysgenesis: Our strategy for cloning DNA from the *mam* locus involved transposon tagging with *P* elements. The method and its success rate have been reviewed by ENGELS (1985). The hybrid dysgenesis scheme is outlined in Figure 1. Since the neurogenic loci *mam* and *bib* are both located on chromosome 2 (map positions 70.3 and 34.7 respectively), the initial studies involved a concurrent screen for a mutation at either locus; this was accomplished by constructing a double mutant *bib cn mam sp* chromosomes. The chromosome was then tested for absence of complementation against chromosomes subjected to *P*-element mutagenesis; in single pair matings these lethals can be easily scored as crosses that produce curly winged flies, but not straight winged flies. Approximately 6000 chromosomes were screened and 8 mutations were identified. The 8 lines were tested for complementation against *bib* chromosomes and *mam* chromosomes; these tests demonstrated that all 8 mutations affected the *mam* locus. Consequently, employing the Harwich P strain, it appears that the *mam* locus is a hot spot for hybrid dysgenesis-induced mutation.

Lethal complementation tests to identify hybrid dysgenesis (HD) induced alleles of *mam* did not constitute proof that the *mam* locus was the target of the mutagenic event, because the data do not rule out the possibility of a mutation carried at a different lethal locus, on both the HD and tester *mam* chromosome. Therefore, each of the new HD alleles was tested for embryonic, neurogenic phenotypes. This was accomplished by preparing cuticles from embryos homozygous and heterozygous for the putative HD *mam* alleles (VAN DER MEER 1977). The central nervous system in several of the mutant lines was also examined, using direct immunofluorescence with fluoresceinated anti-horseradish peroxidase (HRP).

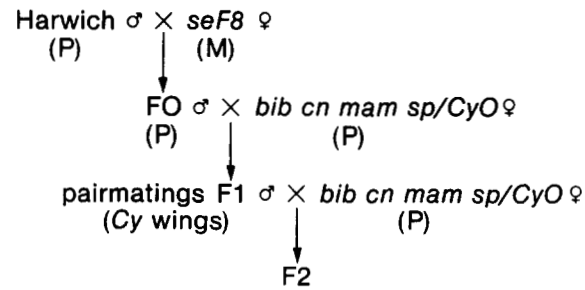

FIGURE 1.—Hybrid dysgenesis scheme for the production of *mam* alleles. The scheme involved the mobilization of *P* elements through a P (Harwich) x M (*seF8*) strain cross. Dysgenic (FO) males were mass mated to a balanced P strain containing the *CyO* chromosome. Individual second chromosomes within F1 males were tested for complementation against a double mutant (*bib, mam*) chromosome. The progeny from such single pair matings were examined for the absence of straight winged flies. Lines showing only straight wings were subsequently tested for complementation against strains containing EMS alleles of *bib* or *mam*.

HD *mam* alleles display typical neurogenic phenotypes: The principal diagnostic characteristic of neurogenic embryos is the absence of cuticle, which in mutants is misrouted toward the central nervous system (LEHMANN *et al.* 1983). The cuticle was analyzed for each of the HD *mam* alleles, as well as the EMS induced allele *11J114*. Six of the HD alleles demonstrated cuticular abnormalities consistent with those described previously (LEHMANN *et al.* 1983). The most prominent defects included the absence of large portions of the cephalic and ventral epidermis; significant variability was observed in the severity of phenotypic expression within single alleles. Figure 2 shows cuticles prepared from a normal embryo (A) and a previously characterized *mam* allele *11J114* (B) which displays a weak neurogenic phenotype (LEHMANN *et al.* 1983). Rows of denticle belts are visible on both embryos; however, the mutant contains large holes in the ventral cuticle and those denticle belts present are disorganized. In addition, major portions of the head epidermis which are visible in the normal embryo are missing in the mutant. Figure 2C–F shows examples of the neurogenic phenotypes exhibited by 4 of the HD alleles (*3/1, 2/3, 6/4, 17/7*), which range from weak to extreme, according to the classification of LEHMANN *et al.* (1983). However, as indicated above, there is significant overlap in the severity of expression among the 5 alleles *HD 2/3, 3/1, 6/4, 11/2* and *13/6* (data not shown); and, the embryos shown in Figure 2 are presented to demonstrate the full range of phenotypes observed. In contrast, *HD 17/7* appeared more consistently extreme in phenotype than any other allele examined. Two of the HD alleles, *10/6* and *15/2*, did not display typical neurogenic cuticular phenotypes at the expected frequencies, but appeared to be embryonic lethals. *HD 10/6*

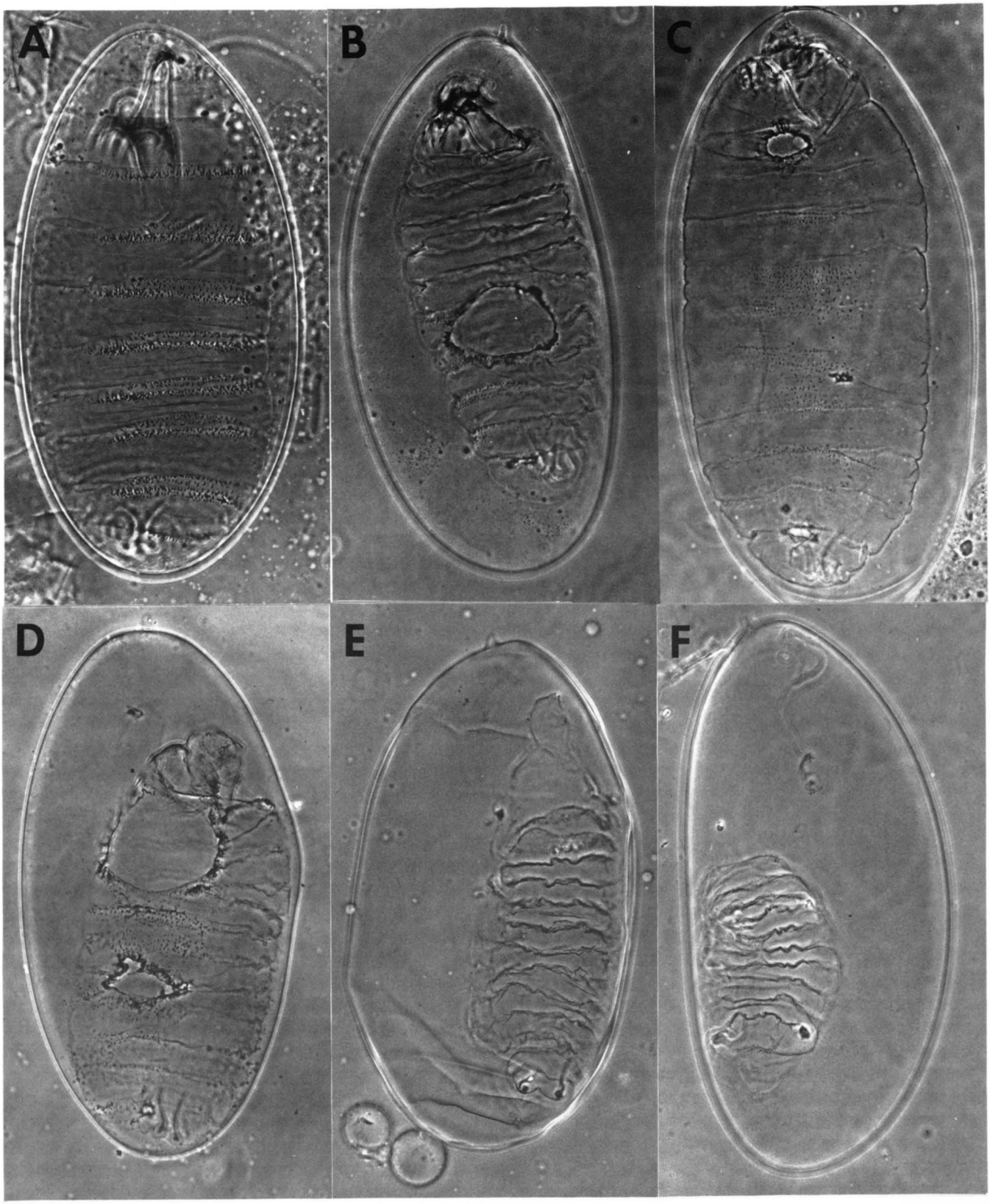

FIGURE 2.—Effects of hybrid dysgenesis-induced *mam* alleles on larval cuticle. Cuticles were prepared from first instar larvae according to the methods of VAN DER MEER (1977). Larvae are oriented anterior end top; the plain of focus is ventral (ABCD) and dorsal (EF). (A) *mam*$^{+}$ (*mam*/*Cy*); (B) EMS *mam* allele *11J114*; (C) *HD 3/1*; (D) *HD 2/3*; (E) *HD 6/4*; (F) *HD 17/7*.

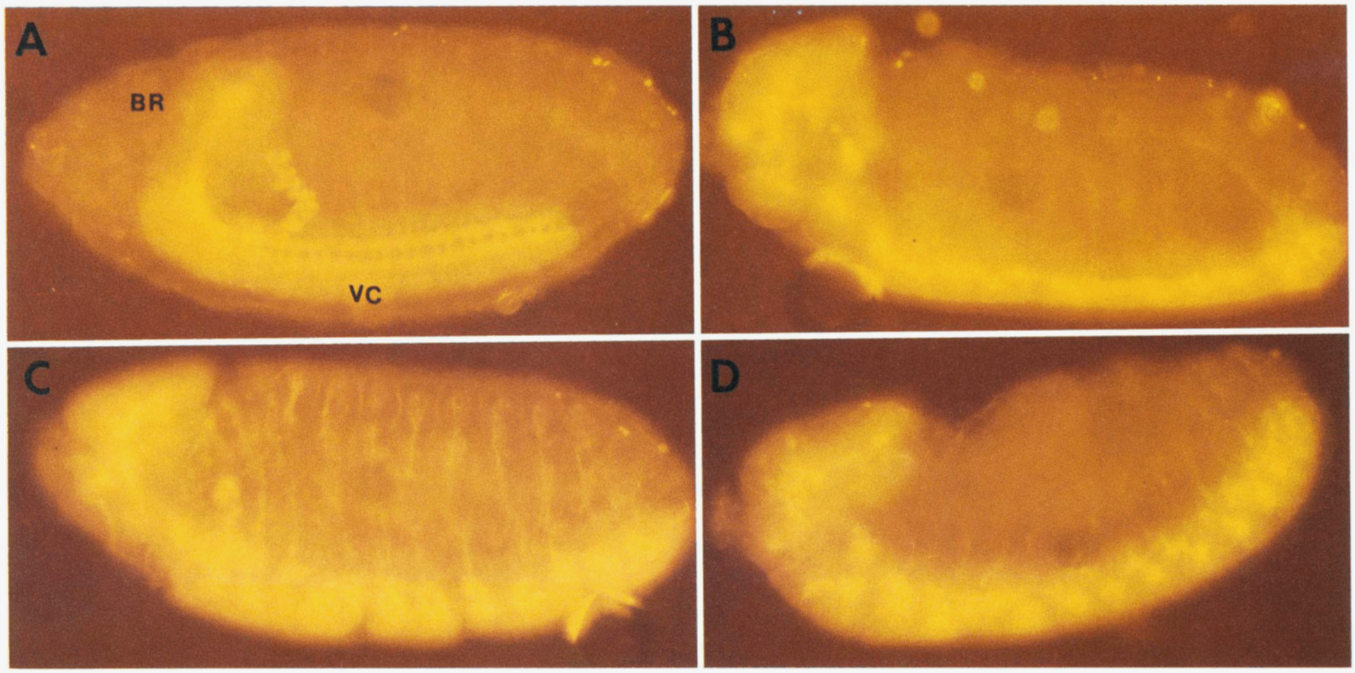

FIGURE 3.—Effects of hybrid dysgenesis-induced *mam* alleles on larval central nervous system. Direct immunofluorescence of embryo whole mounts utilized fluoresceinated anti-horseradish peroxidase as a neural specific marker. Embryos are oriented anterior left, ventral bottom. (A) *mam*$^{+}$ (*mam*/*Cy*), (B) EMS *mam* allele *1J113*, (C) *HD 2/3*, (D) *HD 17/7*. Indicated on the *mam*$^{+}$ embryo are brain (BR) and ventral cord (VC).

was further tested through outcrossing to a known EMS-induced *mam* allele (*1J113*). The progeny of this cross displayed typical neurogenic phenotypes, indicating that *HD 10/6* carries a *mam* allele, and presumably an additional mutation which results in embryonic lethality when homozygous. *HD 15/2*, which showed a high rate of spontaneous reversion, was not further characterized.

The central nervous system was examined in whole mounts of embryos prepared from several of the HD *mam* alleles, as well as the EMS-induced allele *1J113*. Figure 3A demonstrates a normal embryo labeled with neural specific fluorescent antibodies to HRP (JAN and JAN 1982). Evident at the anterior end of the embryo is the brain, which is connected posteriorly to the segmented ventral cord. Distinguishable within the ventral cord are the longitudinal connectives, which run most of the length of the embryo, as well as the lateral commissures contained in each segment. In contrast, the architecture of the central nervous system contained within mutant *mam* embryos appears enlarged and disorganized. Figure 3B–D presents representative examples of the *mam* alleles *1J113*, *HD 2/3* and *HD 17/7*. In each case the anterior end of the embryo is filled with HRP-labeled cells, and the ventral cord appears expanded. Furthermore, the longitudinal connectives and lateral commissures are not properly organized within these embryos. Hypertrophy of the peripheral nervous system, evident in Figure 3C, has been reported previously for *mam* and for other neurogenic mutant alleles (HARTENSTEIN and CAMPOS-ORTEGA, 1986).

Therefore, the analysis of the HD *mam* alleles has correlated the absence of cuticle to hypertrophy of the nervous system, in a manner similar to that of known, EMS-induced *mam* alleles. Consequently, it is concluded that the HD induced mutations are alleles of *mam*. This conclusion is supported by cytological analyses described below.

Identification of *P* element sequences within the *mam* chromosomal region: The production of mutations through hybrid dysgenesis predicts that HD alleles may contain *P* element sequences in or near the mutant locus. However, events such as the mobilization of alternative elements (BINGHAM, KIDWELL and RUBIN 1982) and imprecise excision events (VOELKER et al. 1984; DANIELS *et al.* 1985) can nullify the prediction. A prerequisite for cloning from an *HD* allele is the identification of the associated transposed sequence. Therefore, the 8 *HD* alleles (*2/3*, *3/1*, *6/4*, *10/6*, *11/2*, *13/6*, *15/2* and *17/7*) were examined for the presence of *P* element sequence at *mam*. This was accomplished by *in situ* hybridization of biotinylated *P* element DNA to polytene chromosome squashes of the mutants.

CAMPOS-ORTEGA (1985) reported the cytogenetic position of *mam* as 50C23-50D1. This was based on the proximal breakpoint of *In(2R)N2G*, which apparently causes a *mam* mutation (LEHMANN *et al.* 1983; WEIGEL, KNUST and CAMPOS-ORTEGA 1987; our ob-

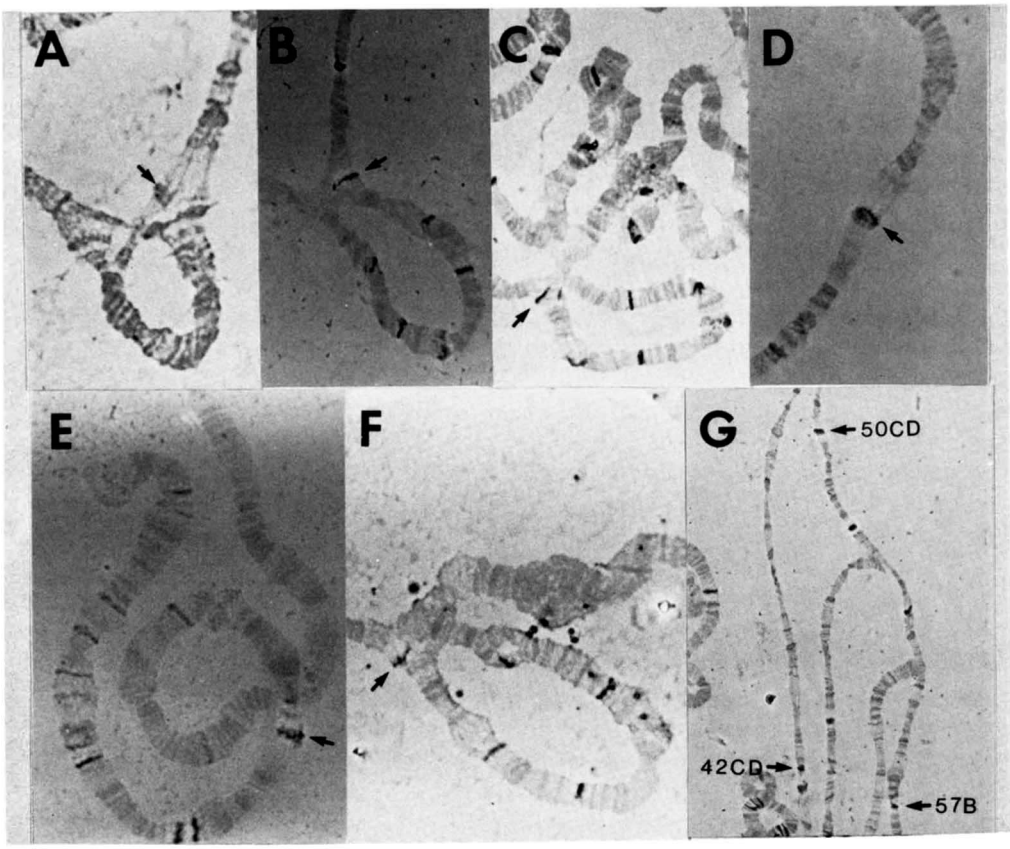

FIGURE 4.—Cytological analysis of *mam* alleles. (A) The cytogenetic position of *mam* is indicated as the proximal breakpoint within the *In(2R)N2G* chromosome (arrow); (B–F) *In situ* hybridization of biotinylated *P* element probe to polytene chromosomes from the hybrid dysgenic induced alleles *3/1*, *6/4*, *10/6*, *11/2* and *13/6*, respectively; (G) *P* element probe hybridized to Harwich. In B–G the *arrows* designate the relevant sites of *in situ* hybridization (see RESULTS).

TABLE 1

Summary of cytological analysis of *mam* alleles and Harwich strain

Strain	Cytological rearrangement	*P* element
HD 11/2	In(2R)50CD-57B	50CD
HD 3/1	In(2R)42CD-50CD	50CD
HD 13/6	In(2R)42CD-50CD	50CD
HD 6/4	Complex	50CD
HD 10/6	None detected	50CD
HD 2/3	None detected	
HD 15/2[a]	None detected	
HD 17/7	None detected	
N2G	In(2R)50CD-54D	
Harwich	None detected	50CD, 57B, 42CD

[a] *HD 15/2* is an unstable *mam* allele. A rebalanced *HD 15/2/Cy* line was produced prior to the cytological analysis. The same line was used for all studies of *HD 15/2* reported here.

servations). The cytogenetic location of the proximal breakpoint of *In(2R)N2G* to the 50CD juncture was confirmed (Figure 4A). Chromosome preparations from five of the mutants, *HD 3/1, 6/4, 10/6, 11/2* and *13/6*, showed a strong *in situ* hybridization signal at the 50CD region (Figure 4B–F), whereas the remaining three (*2/3, 15/2* and *17/7*) showed no signal (data not shown). Four of the five mutants which showed a *P* signal also contained chromosome rearrangements with a breakpoint at the 50CD region (*HD 3/1, 6/4, 11/2, 13/6*); in contrast, *HD 2/3, 10/6, 15/2* and *17/7* appeared cytologically normal. A summary of this data is presented in Table 1.

ENGELS and PRESTON (1981, 1984) provided evidence that hotspots for *P* element-induced mutations can be resident sites of *P* DNA, and that the mutations are associated with chromosomal rearrangements. In 4 of 8 of the HD *mam* alleles examined, mutation was associated with a chromosomal breakpoint at 50CD, and unselected second sites. This suggested that the 50CD region of the Harwich strain may be a site of residence of a *P* element. Consequently, polytene chromosome preparations from the Harwich strain were examined for a *P in situ* signal at this position. A weak hybridization signal was detected at 50CD, suggesting that this strain carries a defective *P* element in or near the *mam* locus (Figure 4G). In addition, signals were observed at 57B and 42CD, the unselected sites of the rearrangements in *HD 11/2, 3/1* and *13/6* (Table 1, Figure 4G).

Finally, the *mam* allele *In(2R)N2G* was tested for the presence of a *P* sequence. This inversion was isolated in the wild (IVES 1947). *In situ* hybridization to this inversion failed to show a *P* element at either breakpoint or at any other genomic site (data not shown).

Molecular cloning of the 50CD-57B juncture from the *HD 11/2 mam* chromosome: The experiments described above established that the *mam* alleles *HD 3/1, 6/4, 10/6, 11/2* and *13/6* each contained a *P* element

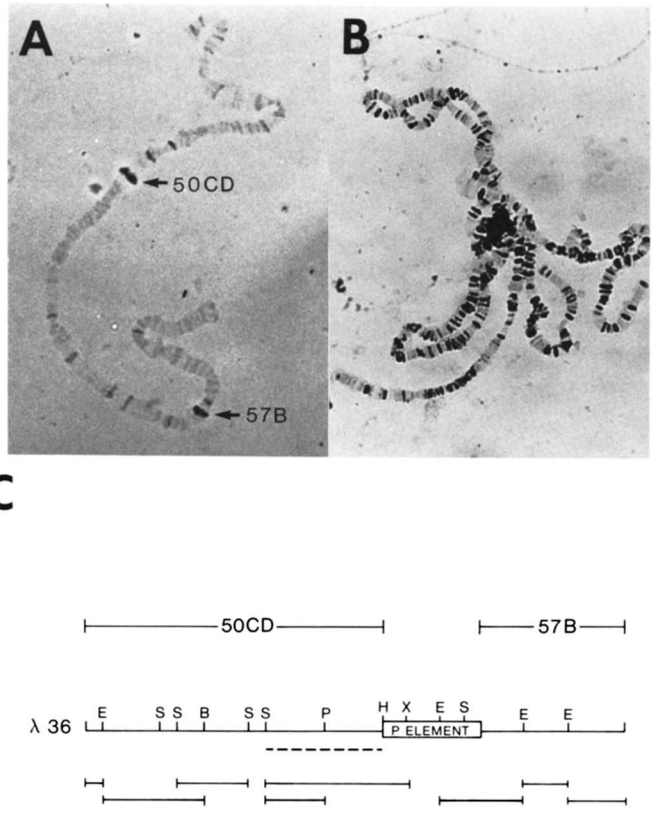

FIGURE 5.—*In situ* hybridization of the *P* element-containing phage λ36 to M Strain (Canton S) chromosomes. The phage was isolated from an *HD 11/2* genomic library and labeled with biotin. (A) Two predominant sites of hybridization (50CD and 57B) are evident; these sites are the inversion breakpoints contained within *HD 11/2*. (B) The majority of nuclei showed multiple hybridization sites, due to the presence of the repetitive element *opa* within this phage. The signal due to *opa* is quite variable in different nuclei from the same gland, presumably due to the short length (<100 bp) and possible divergence of the repeats. (C) Individual regions of λ36 were separately hybridized to M strain chromosomes in order to identify a sequence derived uniquely from 50CD. The horizontal lines below the restriction map represent single *in situ* hybridization probes used in the analysis. The 50CD and 57B regions in λ36 are separated by a full, or nearly full, length *P* element. The *dotted line* indicates the repetitive region.

in or near the cytogenetic position of *mam*. *HD 11/2* was chosen for further molecular cloning work since this line gave consistently strong *in situ* signals.

It was estimated that *HD 11/2* contained 50–60 sites containing *P* sequences. In order to eliminate most of these sites prior to cloning, *HD 11/2* females were repeatedly outcrossed to a multiply marked balancer M strain (see MATERIALS AND METHODS). The reestablished *HD11/2/Cy* stock was analyzed again through *in situ* hybridization. This cleaned up strain still contained a strong *P* signal at 50CD and approximately 25 other sites.

Genomic DNA was purified from *HD 11/2* flies, partially digested with *Sau*3A, and cloned into the vector EMBL3. The recombinants were packaged into phage and the genomic library was plated and screened with a *P* element DNA probe. From 200,000 phage, approximately 150 positive signals were obtained. Sixty phage, representing a wide range of signal intensities, were purified and amplified for DNA isolation. The phage DNAs were then individually biotinylated and singly hybridized to Canton S (M strain) polytene chromosome preparations. The 36th *in situ* (phage 36) showed a strong hybridization signal at 50CD and 57B, as well as several hundred other sites more weakly (Figure 5, A and B). Purified fragments and subclones derived from phage 36 demonstrated that the left and right ends hybridized *in situ* uniquely to 50CD and 57B, respectively, whereas the central portion contained the *P* element and a repeated sequence (data summarized in Figure 5C). A subcloned fragment derived uniquely from 50CD was used as a probe to initiate a chromosome walk in this genomic area. Concurrent with this walk, a second walk was begun in the 50CD region utilizing a cDNA (130H8, gift of M. WOLFNER and D. HOGNESS). The cytogenetic map position of 130H8 (MERRIAM 1984) was corroborated as 50CD, near the *In(2R)N2G* breakpoint. The two walks overlapped and accumulated approximately 120 kb of contiguous DNA from the MANIATIS library, spanning the *N2G* inversion breakpoint (see below).

Characterization of DNA surrounding the *HD 11/2 mam* chromosomal breakpoint: Efforts focused on a series of overlapping genomic segments spanning approximately 90 kb of DNA surrounding the *HD 11/2* breakpoint of the 50CD region. A restriction map of this region is presented in Figure 6C. The 50CD segment of the breakpoint containing phage 36 is aligned above the map (Figure 6A); the juncture of 50CD and *P* element sequence was assigned coordinate 0 on the physical map. The orientation of the physical map relative to the chromosome was determined through separate *in situ* hybridizations of the segments (−14.2 to −12.5 and −38.3 to −47.0) to *In(2R)N2G*. The results of this experiment are presented in Figure 6G. The signal is moved to position 54D in the inversion chromosome, but only employing the first (1700 bp) probe. This established the centromere-telomere orientation of the walk and also confirmed the molecular map position of the *N2G* inversion breakpoint (see below).

This region of DNA was analyzed for the presence of repetitive sequences and the position of DNA lesions associated with *mam* alleles. In addition, this region was scanned for transcriptional activity (see *discussion*).

The *mam* region contains a high density of repeated sequences: Through reverse Southern, genomic Southern, and DNA sequence analyses, 14 regions of repeated DNA were identified within this chromosomal segment; the positions of these sequences are indicated in Figure 6, D and E. One repeat was mapped to 10 positions spanning 60 kb

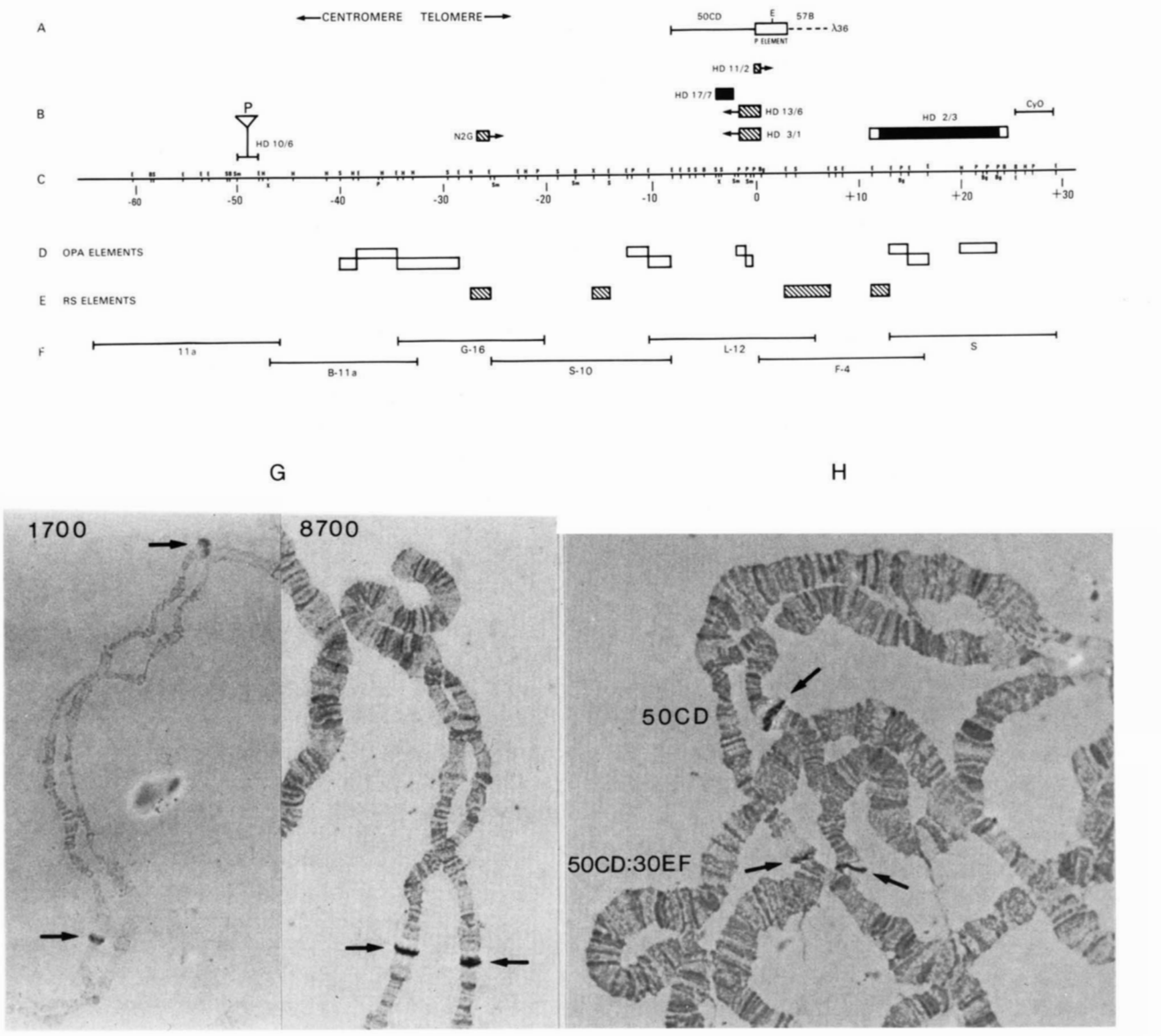

FIGURE 6.—Physical map of the *mam* chromosomal region. Only 90 kb of the 120-kb chromosome walk are displayed. A composite restriction map of this DNA interval is shown in C. The entire region was mapped with the enzymes *Eco*RI (E), *Bam*HI (B), *Sal*I (S) and *Sma*I (Sm). In addition, selected portions were mapped with *Hin*dIII (H), *Xho*I (X), *Pst*I (P), and *Bgl*II (Bg). The O coordinate on the restriction map represents the juncture of Drosophila genomic sequence with *P* element sequence, as determined through DNA sequencing of λ36. (A) Alignment of the *P* element-containing phage λ36 at the P insertion site on the physical map. (B) Physical alteration of DNA correlated with specific *mam* mutations. The hatched boxes represent the positions of chromosome inversion breakpoints; the *arrow* points in the direction of the unselected breakpoints. *Solid boxes* indicate the position of DNA deletions; in the case of *HD 2/3* the limits of the deletion fall within the *open boxes* and the deleted segment is approximately 12 kb. *HD 17/7* appears to be a 100-bp deletion within the indicated restriction fragment. *HD 10/6* is associated with a *P* element insertion (*open triangle*) within the indicated region. The position of *P* insertion in *HD 10/6* was confirmed by cloning the region from *HD 10/6* genomic DNA. (C) Restriction map of *mam* chromosomal region. (D) *Open bars* indicate positions of *opa* elements. (E) *Hatched bars* indicate positions of RS elements. (F) Overlapping λ phage isolated during chromosome walk. (G) Confirmation of the *In(2R)N2G* breakpoint and orientation of physical and cytogenetic maps. DNA segments flanking the putative *N2G* breakpoint (−14.2 to −12.5 and −38.3 to −47.0) were separately hybridized *in situ* to *N2G*/Canton S chromosomes. The signal evident from the former (1700 bp) probe is rearranged, only on the *N2G* chromosome, toward the telomere. In contrast, the 8700-bp probe signal is not rearranged within the *N2G* chromosome. The centromeric-telomeric orientation relative to the physical map was confirmed through *in situ* hybridization of DNA probes, separated by 100 kb, to Canton S chromosomes (data not shown). (H) Identification of the *CyO* breakpoint within the 50CD region. A probe from the distal limits of the chromosome walk (+25 to +29) was hybridized *in situ* to chromosomes from a *CyO* balanced strain. The nonrearranged chromosome contains a single signal at 50CD; the *CyO* chromosome contains two signals, each at a 50CD:30EF juncture, as predicted by the band order in *CyO* (LINDSLEY and GRELL 1968).

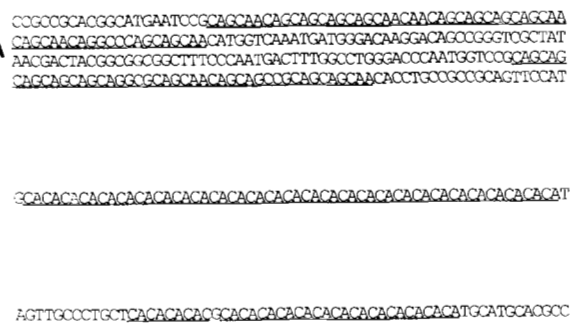

FIGURE 7.—DNA sequence of the *opa* and RS elements. (A) The *opa*-homologous genomic fragment (−0.9 to −1.6) was sequenced. Within the 240-bp region shown here two stretches of *opa* (CAX) repeats were identified (underlined). (B) Identification of a consensus sequence in two RS elements. The two most strongly cross-hybridizing RS regions (−27.3 to −25.5 [G16] and −15.7 to −14.2 [S10]) were further analyzed to delimit the regions of homology. These regions were sequenced and searched for homologies. The most striking homology was a common $(dC\text{-}dA)_n \cdot (dG\text{-}dT)_n$ repeat of 58 bp and 33 bp (one G in stretch of 34), which is underlined.

of DNA (Figure 6D). This sequence was initially identified as the repeated element of clone 36 (Figure 5B), which hybridized to hundreds of sites throughout the genome. This *in situ* pattern was similar to that reported earlier for *opa*, an element found at *Notch* and numerous other *Drosophila* loci (WHARTON *et al.* 1985a). Consequently, *Notch opa* was cross-hybridized to the genomic clones and 9 areas of homology were observed. DNA sequence determination of one cross-hybridizing region (−1.6 to −0.9) demonstrated the presence of CAXn repeats (Figure 7A), as described earlier for *opa* and the M repeat (WHARTON *et al.* 1985a; MCGINNIS *et al.* 1984). A tenth *opa* region (−40) did not cross hybridize to *Notch opa*. DNA sequence analysis of this repetitive segment revealed an *opa* element containing a high proportion (17 of 26) of CAA repeats in homogeneous stretches (data not shown).

In addition to *opa*, 4 other areas contained repetitive sequences (RS, Figure 6E), which also showed significant cross-homology on Southern blots. In order to identify a consensus sequence for RS, two regions were analyzed in more detail. A series of deletion subclones were produced from the RS region −27.3 to −25.5. Through reverse Southern analysis, the repetitious region was delimited to approximately 500 bp; the same 500 bp segment showed cross-hybridization to RS at position −15.7 to −14.2. DNA sequence analysis of cross-hybridizing portions of these clones revealed a $(dC\text{-}dA)_n \cdot (dG\text{-}dT)_n$ stretch of 58 bp and 34 bp (Figure 7B).

Molecular mapping of DNA lesions associated with *mam* mutations: Representative genomic Southern analyses of DNA isolated from the 8 *mam* alleles, and control strains, are presented in Figure 8; the physical positions of DNA breakpoints associated with these alleles are indicated in Figure 6B. The genomic segment initially characterized was that surrounding the *HD 11/2* breakpoint (Figure 8A); the region contains a high frequency of restriction site polymorphisms. The parental chromosomes Harwich and Canton S (identical to *sef8* in this region) contain hybridizing bands of 8.3 kb and 7.5 kb, respectively. The *CyO* chromosome contains the identical *Eco*RI pattern as Harwich, whereas the *Pm/Cy* strain contains a polymorphism which produces a 6.5 kb fragment.

The *HD* alleles *3/1*, *11/2* and *13/6* contain a novel sized restriction fragment (9.5 kb), not associated with any of the parental chromosomes. The 9.5-kb band is consistent with the restriction map of clone 36 derived from the *HD 11/2* strain, and is produced through cleavage at an *Eco*RI site within the *P* element (Figure 6A). Subsequent digests have delimited the breakpoints of these 3 *HD* alleles within a 2-kb *Pst*I-*Bgl*II fragment (data not shown), indicated in Figure 6B. In contrast, *HD 6/4* does not display a novel sized restriction fragment in this region and shows only the *Pm/Cy* control 6.5-kb band, consistent with a deletion within the *mam* genomic region. The limits of this deletion have not been identified on the physical map and possibly fall outside our cloned segment. Several restriction digests have established that *HD 2/3* is also associated with a deletion, approximately 12 kb in length (coordinates +11.5 to +23.5); a representative genomic Southern mapping the proximal end of the deletion is presented in Figure 8B.

HD 17/7 DNA reproducibly demonstrated a slight (100 bp) mobility change within the region −2.0 to −3.6, employing several different restriction enzymes. A typical example of this fragment shift is shown in Figure 8C. Therefore, it has been provisionally concluded that this DNA lesion is associated with the *17/7* mutation, and this segment is presently being cloned from the mutant in order to corroborate this observation.

A physical change in *HD 10/6* was mapped to an *Eco*RI restriction fragment −47.8 to −53 (data not shown). The region was cloned from an *HD 10/6* genomic library and *P* element sequences were identified within this DNA segment (−47.8 to −50.4, Figure 6B), consistent with the *in situ* hybridization analysis of *HD 10/6* which revealed a strong signal at 50CD (see above). Physical changes associated with *HD 15/2* have not been detected; however, the strain is unstable and the *mam* allele appears to be lost at a high frequency. Attempts to identify physical changes in DNA from rebalanced *HD 15/2/Cy* lines have not been successful.

In addition to the 8 HD induced *mam* alleles, genomic DNA derived from the spontaneous *mam* allele *N2G* was analyzed. As discussed earlier, *In(2R)N2G* contains chromosomal breakpoints at

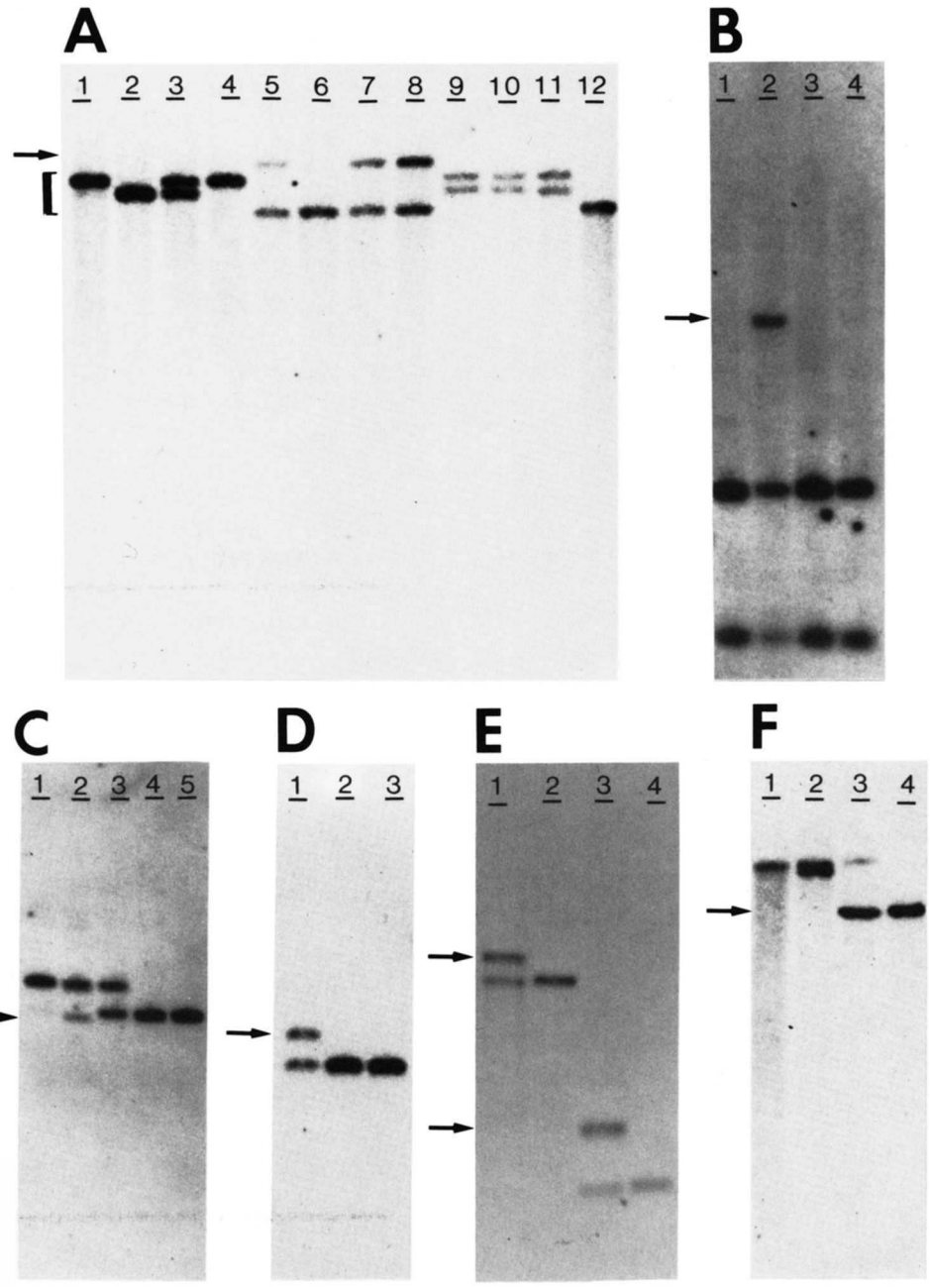

FIGURE 8.—Analysis of genomic DNA from *mam* mutants and parental strains. Genomic DNA (1 μg) was digested with the indicated enzymes, gel fractionated, blotted to nitrocellulose and then probed with the appropriate *mam* region sequence. (A) DNA from the eight hybrid dysgenesis-induced *mam* alleles and control strains were digested with *Eco*RI + *Bgl*II and probed with sequences −5.1 to −7.4. The brackets mark the positions of the wild type control bands (8.3, 7.5 and 6.5 kb). The 7.5-kb Canton S (and *sef8*, not shown) band matches the cloned sequence pattern from the Maniatis library; the 8.3-kb (Harwich, *CyO*) and 6.5-kb (*Pm*/*Cy*) bands are polymorphisms in this region (see RESULTS). *HD 3/1, 11/2* and *13/6* each contain a novel 9.5-kb restriction fragment (*arrow*); analysis of the cloned sequences derived from *HD 11/2* (λ36) demonstrated that the 9.5-kb fragment derives from the *Eco*RI site within the inserted *P* element. The restriction pattern of *HD 6/4* is consistent with a deletion covering at least 25 kb; the limits of this deletion have not been identified (see RESULTS). *HD 3/1, 6/4, 11/2* and *13/6* are balanced over *Cy* (from *Pm*/*Cy*) and contain the expected 6.5-kb control band; 1) Harwich, 2) Canton S, 3) Canton S/*CyO*, 4) *HD 2/3*, 5) *HD 3/1*, 6) *HD 6/4*, 7) *HD 11/2*, 8) *HD 13/6*, 9) *HD 10/6*, 10) *HD 15/2*, 11) *HD 17/7*, 12) *Pm*/*Cy*. (B) DNA from *HD 2/3* and control strains was digested with *Pst*I and probed with sequences +11.2 to +13. A novel fragment of 6.6 kb (*arrow*) is detected with this probe. This region marks the proximal limit of the *HD 2/3* deletion, which extends approximately 12 kb distal (Figure 6B); 1) Harwich, 2) *HD 2/3*, 3) Canton S/*CyO*, 4) Canton S. (C) DNA from *HD 17/7* and control strains was digested with *Eco*RI + *Sma*I and probed with sequences −3.5 to −7.3. In addition to the predicted *CyO* band, a novel fragment (*arrow*) approximately 100 bp shorter than the control bands is evident; 1) Harwich, 2) *HD 17/7*, 3) Canton S/*CyO*, 4) *seF8*, 5) Canton S. (D) DNA from *In*(*2R*)*N2G*/*SM5*; Canton S/*SM5*; and Canton S was digested with *Eco*RI and probed with sequences −26.6 to −25.5. A novel 4-kb fragment (*arrow*) is detected with this probe. The position of the *N2G* breakpoint was confirmed with additional restriction digests (data not shown) as well as *in situ* hybridization analyses (see RESULTS and Figure 6G). 1) *N2G*/*SM5*, 2) Canton S/*SM5*, 3) Canton S. (E) DNA from Harwich and Canton S was digested with *Xho*I plus *Bgl*II (XB) or *Sma*I plus *Bgl*II (SB) and probed with sequences −0.55 to +0.55. Canton S DNA

50CD and 54D. The 50CD breakpoint on the physical map was provisionally positioned within coordinates −25.5 to −27.3 (Figure 6B) using several different restriction enzymes; an example demonstrating a novel restriction fragment specific to the *N2G* chromosome is shown in Figure 8D. However, it was not possible to rule out the possibility that these restriction fragment length alterations were merely polymorphisms, since the parental strain of the *N2G* mutant is unavailable. Therefore, in order to confirm the positioning of the *N2G* inversion breakpoint, it was necessary to perform *in situ* hybridization analyses. DNA probes containing sequences which flank the putative *N2G* breakpoint were individually hybridized to polytene chromosome preparations of *N2G*, as described in Figure 6G. Hybridization of the 8.7kb probe (−38.3 to 47.0) revealed a signal at 50CD in both the *N2G* and Canton S homologs, whereas hybridization of the 1.7-kb probe (−12.5 to −14.2) resulted in the transposition of the signal to 54D on the *N2G* homolog. These results corroborate the genomic Southern analysis which mapped the *N2G* breakpoint between these coordinates on the physical map. The physical analyses of these *mam* alleles define the minimal length of the *mam* functional unit as 60 kb of DNA (coordinates −47.8 to +12), barring position effects (figure 6B and 6C).

Finally, it was observed that the Harwich strain is polymorphic for a small insertion (approximately 400 bp); the position of the insertion was mapped to an 1100bp *Sma*I-*Bgl*II restriction fragment (Figure 8E) within coordinates −0.55 to +0.55. This region encompasses the breakpoint of *HD 11/2* (and probably *HD 3/1* and *13/6*), and was also implicated as a factor in *mam* mutation by WEIGEL, KNUST and CAMPOS-ORTEGA (1987). An insert-bearing phage was subsequently cloned from Harwich and found to contain a *P* element sequence within this segment (data not shown), establishing a basis for the *mam* hotspot within this P strain.

The distal limit of *mam* is marked by a CyO breakpoint: During genomic Southern analyses of the *mam* chromosomal region a restriction fragment alteration was detected that appeared specific to the *CyO* chromosome; the pattern was consistent with an inversion. The probe fragment mapped near the distal limits of the chromosome walk (+25 to +29), no more than 15 kb beyond the distal most *mam* allele, *HD 2/3* (Figure 6B). The *CyO* chromosome is known to contain a breakpoint in 50CD, yet is not carrying a lethal *mam* allele (LINDSLEY and GRELL 1968). If the +25 to +29 region contained the *CyO* breakpoint, this would provide a distal limit for a functional *mam* locus. Therefore, subcloned sequences containing this segment were hybridized *in situ* to chromosomes from a strain balanced over *CyO* (Figure 6H). The results of the *in situ* hybridization demonstrated that sequences from +25 to +29 span the *CyO* breakpoint in 50CD, as two signals are evident on the *CyO* balancer chromosome in the expected cytological postions. This is in agreement with the report of WEIGEL, KNUST and CAMPOS-ORTEGA (1987).

Analysis of a spontaneous *HD mam* allele revertant: During the course of this study several revertants of the *HD 11/2* allele were identified; these appeared as non-curly winged progeny within the *HD 11/2/Cy* stock. A single revertant chromosome, *HD Rev.6*, was used to produce a stock for further analysis. Cytological examination of polytene chromosomes from *HD Rev.6* showed no rearrangements in the 50CD or 57B regions; thus, the original inversion contained within *HD 11/2* was no longer evident. Furthermore, *in situ* hybridization of *P* element DNA to such preparations failed to show a signal at 50CD, despite a large number of *P*-positive sites within the chromosomes (data not shown). It was essential to determine if this cytological change could be correlated with a molecular alteration of DNA sequences within the cloned region, as this would provide further evidence that the genomic segment derives from the *mam* locus. Whole genomic Southern analysis of DNA from *HD 11/2*, *HD Rev.6*, and Harwich are represented in Figure 8F. The mutant *HD 11/2* contains a 15-kb *Bgl*II restriction fragment homologous to a probe (−5.1 to −7.4) derived from the breakpoint region. This restriction fragment is a composite of DNA sequences spanning 50CD, *P* element and 57B sequences. The parental strain, Harwich, contains a 9-kb restriction fragment homologous to this probe, which is indistinguishable from the fragment observed in *HD Rev.6*. Therefore, *HD Rev.6* appears to derive from a reinversion of *HD 11/2* at both the cytological and molecular level; the precision of the reinversion at the DNA sequence level has not yet been determined.

DISCUSSION

The neurogenic loci comprise a group of genes which influence a critical developmental decision during early embryogenesis, the choice between epidermal and neural differentiation (POULSON 1950, CAMPOS-ORTEGA 1985). The molecular analysis of one neurogenic locus, *Notch*, has provided a reason-

contains the predicted 1.1-kb SB band; Harwich contains bands of approximately 1.1 kb and 1.5 kb; *arrows* designate the positions of the insert-containing bands. The slight increase in mobility of the Harwich 1.1-kb band is reproducible; 1) Harwich (XB), 2) Canton S (XB), 3) Harwich (SB), 4) Canton S (SB). (F) DNA from *Pm/Cy*, *HD 11/2*, *HD 11/2 revertant 6*, and Harwich was digested with *Bgl*II and probed with sequences −5.1 to −7.4; the *arrow* indicates the position of the 9-kb restriction fragment observed in the Harwich and revertant chromosomes. 1) *Pm/Cy*, 2) *HD 11/2*, 3) *HD 11/2 revertant 6*, 4) Harwich.

able hypothesis for how certain members of this gene class act; the *Notch* protein product is probably involved in cell:cell communication at the cell surface (WHARTON *et al.* 1985b; KIDD, KELLEY and YOUNG 1986). The recent analysis of a *Delta* cDNA suggests a similar function for the protein product of this locus (VASSIN *et al.* 1987). However, a more complete description of these processes will require the molecular cloning of additional neurogenic loci and a study of their potential function(s). This report summarizes progress toward cloning one of these loci, *mastermind* (*mam*) through *P* element tagging. The data on the molecular organization of *mam* in wild-type, *N2G* and Harwich strains are in agreement with the report of WEIGEL, KNUST and CAMPOS-ORTEGA (1987).

Employing a relatively small F_2 screen (Figure 1) of 6000 chromosomes, it was observed that *mam* is a hotspot for *P* mutagenesis (8 mutations) using the Harwich strain. The hybrid dysgenic induced (HD) *mam* alleles show typical neurogenic cuticular and neural abnormalities (Figures 2 and 3), and in five of the eight cases examined, the mutant chromosome contains a *P* element in or near the cytogenetic position of *mam* (Figure 4). In 4 of 5 of these mutants, a chromosomal rearrangement was observed with one breakpoint at 50 CD (*mam*), and another at an unselected site (Figure 4, Table 1).

ENGELS and PRESTON (1981, 1984) presented convincing evidence that hybrid-dysgenesis induced chromosomal rearrangements occur at resident sites of *P*. *In situ* hybridization analysis of the parental Harwich strain did reveal a *P* element at 50 CD, as well as the unselected breakpoint sites 57B and 42CD (Figure 2G). Genomic Southern analysis (Figure 8E) and subsequent cloning of the relevant *mam* region from the parental Harwich strain confirmed the presence of *P* element sequences at the breakpoint site; the detailed structure of this region will be reported elsewhere. These observations are consistent with the predictions of ENGELS and PRESTON (1981, 1984), and partially explain the basis for a *mam* hot spot within the Harwich strain. However, some of the *P*-induced alleles appear to derive from events at other sites within the *mam* locus, including insertions and possibly imprecise excisions (see below). A full description of the processes leading to the production of these alleles will require more detailed analyses of their structure. However, it seems likely that the large size of the *mam* locus, coupled with the proximity of a defective *P* element are significant factors. The presence of multiple repeated sequences within the locus is an additional factor that may contribute to the production of rearrangements within the region (ROTHSTEIN, HELMS and ROSENBERG 1987). Reversions of *P*-induced chromosome rearrangements have also been described by ENGELS and PRESTON (1984). The reversion of *HD 11/2* occurred spontaneously and resulted in a rectification of the chromosome at the cytological and molecular level (Figure 8F). As the reversion did not derive from a dysgenic cross, the *HD 11/2* line may exhibit low levels of transposition activity; however, alternative explanations, such as homologous recombination between *P* elements cannot be excluded.

The presence of a defective *P* element within the *mam* locus of Harwich does not alone produce a *mam* allele. Only larger disruptions of the region, associated with chromosome breakage, elicit a *mam* phenotype (Table 1). This suggests that the site of *P* residence is intronic. Preliminary DNA sequence analysis of the P insertion region of *HD 11/2* is consistent with this suggestion (P. YOUNG and B. YEDVOBNICK, unpublished data). Similar phenomena have been observed for several loci, in particular *Notch* (KIDD, LOCKETT and YOUNG 1983; GRIMWADE *et al.* 1985; KIDD and YOUNG 1986; K. MARKOPOULOU and S. ARTAVANIS-TSAKONAS, personal communication).

Five of the eight *HD mam* alleles characterized contained a *P* element at the cytogenetic position of *mam*. Therefore, one of these stocks, *HD 11/2*, was used to tag genomic sequences derived from 50CD. Sixty *P* positives were isolated from an *HD 11/2* genomic library and 36 were tested, via *in situ* hybridization, for sequences homologous to 50 CD. Phage 36 showed strong hybridization to 50CD and 57B (the unselected breakpoint of *HD 11/2*) (Figure 5). Unique sequences derived solely from 50CD were identified and used to initiate a chromosome walk in the Maniatis library. In combination with a more proximal walk, started from a cDNA clone, 120 kb of contiguous DNA were isolated. This region was then characterized for repetitive elements and mutant breakpoints.

Utilizing reverse Southern and genomic Southern analyses 14 regions containing repeated DNA were identified (Figure 6, D and E). Ten of these regions are related to the *Notch opa*, an element containing a stretch of CAX repeats which encode polyglutamine within the putative *Notch* polypeptide (WHARTON *et al.* 1985a). DNA sequence determination of one *opa/mam* cross-hybridizing segment revealed 2 CAX stretches within a 750-bp region (Figure 7A). The significance of *opa* repeats within the *mam* region remains to be determined; however, it is noted that the 750-bp region contains a 648-bp open reading frame when glutamine is used to establish the frame. The open reading frame is preceded by a consensus splice acceptor sequence. In addition to *opa*, 4 additional repetitive regions were identified, interspersed across the walk, which show cross hybridization. DNA sequence analysis of two of these RS regions identified a consensus $(dC\text{-}dA)_n \cdot (dG\text{-}dT)_n$ repeat (Figure 7B). As the DNA sequencing of the four RS regions has

not been completed, it is not certain that this is the sole consensus sequence within these regions. However, the $(dC\text{-}dA)_n \cdot (dG\text{-}dT)_n$ repeat is distributed in *Drosophila* in a nonrandom, conserved fashion, suggesting it plays some role in chromosome structure or function (PARDUE *et al.* 1987). An additional possibility is that the repeat encodes a homopolymeric stretch of amino acids; however, the present sequence information is too incomplete to support this contention. In either case, the genomic region that constitutes *mam* contains an unusual sequence organization.

Genomic Southern analysis was used to map the DNA lesions associated with 7 hybrid dysgenic-induced *mam* alleles and one spontaneous allele (Figures 6B and 8). In three of the mutants, the lesion fell within the same 2.2-kb fragment (*HD 3/1, 11/2* and *13/6*), presumably at the site of residence of the defective *P* element; in the case of *HD 2/3* the DNA change was mapped approximately 11 kb distal to the breakpoint of *HD 11/2*. The *HD 2/3* genomic blot hybridization signals were consistent with a small DNA deletion in this area. Since *HD 2/3* does not contain a detectable *P* element sequence at 50CD, hybrid dysgenesis cannot be fully implicated in the production of this mutation. However, it is conceivable that *HD 2/3* originated from the insertion and imprecise excision of a *P* element (VOELKER *et al.* 1984; DANIELS *et al.* 1985). Likewise, *HD 17/7* appears to be a small (100 bp) deletion that may have derived from such events.

A *P* element insertion associated with *HD 10/6* was identified and cloned, consistent with the predictions of the *in situ* hybridization analysis. The *HD 10/6* chromosome is not rearranged, and showed a strong *P* signal at 50CD. In contrast, no cytological or molecular alterations were correlated with *HD 15/2*. However, this allele is unstable and a *P* associated change may be lost at a high frequency. No changes were observed in DNA from a rebalanced *HD 15/2 / Cy* line, which suggests that the original allele may contain a very small lesion. As the *HD 15/2* chromosome is unstable, yet does not contain a *P* element at 50CD detectable through *in situ* hybridization, it may represent the insertion of a defective *P* element not resolved in the analysis. An alternative explanation, that the mutant maps outside the analyzed region, is unlikely but cannot be ruled out.

The final *HD* allele, *6/4*, could not be precisely localized and appears to be a large deletion covering a substantial portion of this region. Cytologically, *HD 6/4* is complex with possibly more than one breakpoint in 50CD (Figure 2C), and it is conceivable that the region between the 50CD breaks was lost during the rearrangement. The analysis of the spontaneous mam allele, *N2G*, revealed that its breakpoint fell within the limits of the locus established by the HD alleles. Barring position effects, these breakpoints define the minimal length of the *mam* functional unit as 60 kb. In combination with the molecular data of WEIGEL, KNUST and CAMPOS-ORTEGA (1987), whose maps are concordant with those presented here, 17 *mam* breakpoint alleles fall within the 60-kb interval established by the *HD* alleles.

The absolute distal limit of the *mam* functional unit is no more than 15 kb distal to this 60-kb interval. The *CyO* balancer breakpoint in 50CD does not inactivate the *mam* locus, yet maps within 15kb of the *HD 2/3* deletion (Figure 6, B and H). Although no definitive limits exist for the proximal end of the *mam* locus, the position of sequences homologous to cDNA 130H8 (−75 to −76) represent a reasonable first approximation. This cDNA is homologous to transcripts expressed in ecdysone-treated salivary glands of third instar larvae (M. WOLFNER, personal communication). Therefore, the *mam* locus presumably falls within a 100-kb chromosomal region (−75 to +25). A summary of the organization of the *mam* region is presented in Figure 9; the figure also shows the results of a preliminary transcriptional analysis of the region, discussed below.

The transcriptional activity of unique sequences within a 105-kb segment (−76 to +29) of the *mam* chromosomal region have been characterized. Subclones and fragments spanning this segment were hybridized to blots of gel fractionated poly(A^+) RNA isolated from staged embryos and postembryonic periods. A significant portion of this DNA is, however, refractory to this analysis because of the presence of repeated and transcribed *opa*/RS elements. As *opa* has been found within exonic segments in several other genes (MCGINNIS *et al.* 1984, WHARTON *et al.* 1985a, LAUGHON *et al.* 1985, PIRROTTA *et al.* 1987), the analysis excludes potential exonic regions that lie in close proximity to the repeats; consequently, 15% of the 105-kb segment was not assayed for transcription. Unique sequence probes spanning the 60-kb *mam* region (−38 to +23) failed to show significant hybridization to staged samples of poly(A^+) RNA covering the entire 24-hr period of embryogenesis. In contrast, probes derived from near the termini of the region hybridized to several transcripts (D. SMOLLER and B. YEDVOBNICK, unpublished data).

The left (proximal) end of the cloned region contains two segments of DNA (−55.5 to −74.5 and −50.5 to −60) that hybridize to 5-kb and 3-kb poly(A^+) transcripts, respectively. Both RNAs accumulate maternally, whereas the 5-kb transcript also shows peak expression from 2 to 8 hr of embryogenesis and early pupation. The distal region contains a sequence (+23.2 to +25) which hybridizes to two poly(A^+) RNAs, a maternal species (8.4 kb) and a maternal plus zygotic species (9.2 kb). The 9.2-kb transcript accumulates during the first half of em-

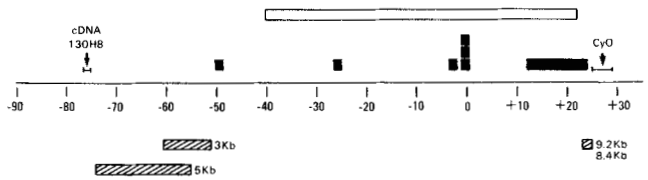

FIGURE 9.—Transcriptional analysis of the *mam* chromosomal region. Probes spanning a 105-kb region (−76 to +29, excluding repeated regions) were hybridized to blots of poly(A$^+$) RNA isolated from embryonic and postembryonic stages. The positions of the repeat-containing region (*open bar*) and mutant breakpoints (*closed bars*) are indicated above the coordinate line. Also indicated are the distal limit (*CyO*) and putative proximal limit (130H8 cDNA) of the *mam* locus. Regions showing hybridization to the indicated size class of RNA are represented by the *hatched bars* below the coordinate line.

bryogenesis and during early pupation. The periods of expression of these transcripts (maternal, early embryonic, and early pupal) overlap with those predicted for a *mam* product as they are concurrent with periods of *mam* locus function; *mam* is required maternally (JIMENEZ and CAMPOS-ORTEGA 1982), zygotically (NUSSLEIN-VOLHARD, WIESCHAUS and KLUDING 1984; CAMPOS-ORTEGA 1985), and within imaginal epidermal cells (DIETRICH and CAMPOS-ORTEGA 1984). However, as exonic segments of these transcripts have not been identified within the 60-kb region, it is possible that some or all of these products derive from flanking transcription units.

It is expected that the 60-kb unit defined by the *mam* breakpoint mutations is largely intronic and that a *mam* transcript will contain sequences that span these breakpoints. Since only 85% of this genomic region was isolated from *opa* and RS elements for use as unique sequence probes, it is possible that the remaining, repetitious portions contain exon elements of the *mam* transcription unit. This is consistent with putative open reading frames observed surrounding and including six *opa* elements within the locus (example: Figure 7A); however, these data are by no means definitive evidence of a *mam* exon. *Opa* elements within *mam* may be situated intronically, or within 5′/3′ noncoding exonic sequences [see WHARTON *et al.* (1985a) for a discussion of *opa* distribution]. These possibilities cannot be resolved with the information available at this time, but will require extensive DNA sequence analyses of the repetitive elements and their flanking regions, which is in progress. Ultimately, a proper definition of the *mam* transcription unit and its products will require the sequence characterization of cDNAs homologous to this region, as well as the determination of the spatial and temporal accumulation patterns of their cognate transcripts.

Finally, the position of sequences homologous to the proximal transcripts (5 kb and 3 kb) fall outside the present limits of *mam* as defined by the molecular analysis of breakpoint alleles. If these transcripts derive from the *mam* locus, then the region has not yet been sufficiently tested for the potential physical limits of *mam* alleles. Therefore, we are presently characterizing the physical map positions of a new collection of x-ray induced *mam* alleles to determine if any breakpoints fall outside the 60-kb interval defined by this study.

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