Approaches to Identify Genes Involved in *Drosophila* Embryonic CNS Development

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SUMMARY

Many of the steps involved in formation of the *Drosophila* embryonic central nervous system (CNS) have been identified by both descriptive and experimental studies. In this review we will describe the various approaches that have been used to identify molecules involved in CNS development and the advantages and disadvantages of each of them. Our discussion will by no means be exhaustive; but rather we will discuss our experiences with each approach and provide an overview of what has been learned by using these methodologies. Finally, we will discuss methods that have been recently developed and how they are likely to provide further insight into CNS development. © 1993 John Wiley & Sons, Inc.

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INTRODUCTION

The central nervous system of a *Drosophila* first instar larvae represents the culmination of highly complex and dynamic developmental processes, collectively referred to as *neurogenesis.* The steps involved in this process will be outlined briefly in this introduction. For more comprehensive reviews of neurogenesis in *Drosophila,* readers are referred to Campos-Ortega and Hartenstein (1985), Doe, Kuwada, and Goodman (1985c), Goodman and Doe (1992), and Doe (1992).

In *Drosophila,* the embryonic territories that give rise to the various tissue types are specified during the blastoderm stages [Fig. 1(A)]. Following initial fate specification, neuronal and glial precursors (neuroblasts and glioblasts) segregate from both the neurectodermal and mesectodermal regions (Campos-Ortega and Hartenstein, 1985). The mesectodermal region gives rise to a population of neurons and glia which inhabit the ventral midline of the CNS, whereas the neurectoderm gives rise to the remainder of neurons and glia of the CNS, as well as to the ventral epidermis and its derivatives. Of the approximately 1800 cells in the neurectodermal region, roughly one-quarter become neuroblasts. The ability of a neurectodermal cell to assume a neuroblast fate is controlled both spatially and temporally by a group of genes collectively referred to as the *proneural genes.* Subsequently, determinative events assure that only one cell in each group will assume the neuroblast fate. Laser ablation experiments in the grasshopper have shown that once a cell is determined to become a neuroblast, it laterally inhibits its neighbors from assuming a neural fate [Fig. 1(B); Doe and Goodman, 1985b]. This process of lateral inhibition, which is necessary for proper formation of the ventral epidermis, is controlled by a set of genes referred to as the *neurogenic genes* (Lehmann, Jimenez, Dietrich, et al., 1983).

The spatial and temporal patterns of neuroblast segregation are stereotyped within each of the bilaterally symmetric hemisegments which comprise a

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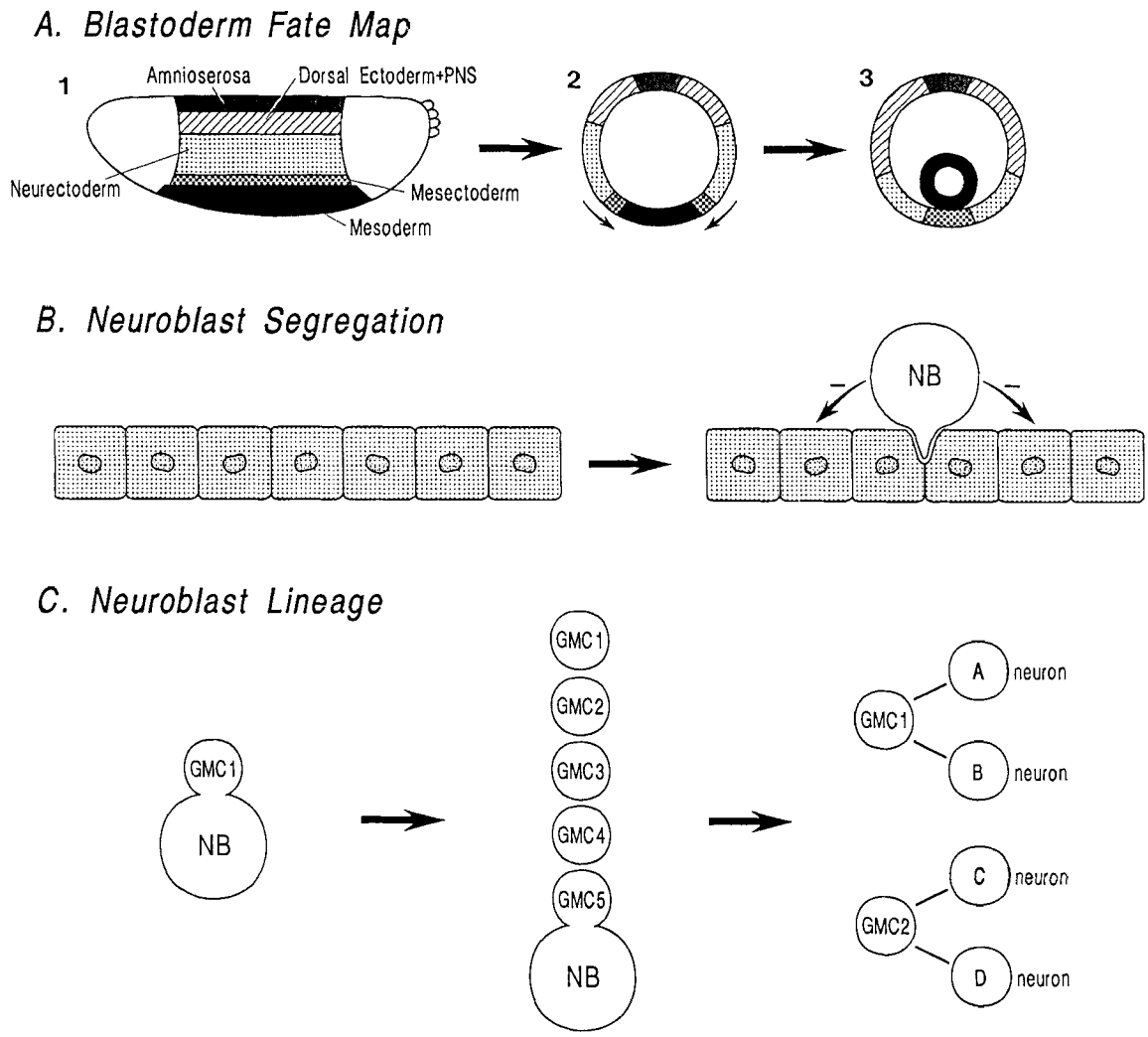

Figure 1 Development of the embryonic CNS. (A) Fate map of the blastoderm. (1) A lateral view of the blastoderm. (2) A transverse section at the cellular blastoderm stage. (3) A transverse section during early gastrulation [adapted from Ingham (1988)]. During blastoderm stages, cells that initially occupy the ventral-most region give rise to all mesodermal derivatives. On either side of the mesoderm lies a narrow region which produces all mesectodermal derivatives. The mesectoderm (Poulson, 1950) is a specialized group of cells that eventually produces the cells occupying the ventral midline. Like the mesectoderm, the neurectoderm is initially comprised of two lateral regions which give rise to neural and glial lineages, as well as ventral epidermis and its derivatives. Dorsal to the neurectoderm is the dorsal ectoderm from which the peripheral nervous system (PNS) is also derived. Finally, in the dorsal-most region of the blastoderm is the amnioserosa. During gastrulation, the mesodermal region invaginates, bringing together the two mesectodermal regions at the ventral midline. The mesectoderm then divides the two regions of neurectoderm. (B) Neuroblast segregation. Initially, cells within the neurectoderm have the potential to give rise to neuroblasts or epidermoblasts. During segregation, the enlarged neuroblast inhibits its neighbors from entering the neuroblast fate through the process of lateral inhibition. (C) Neuroblast lineage. Neuroblasts give rise to ganglion mother cells (GMCs) through asymmetric cell divisions. Each neuroblast generates an average of five GMCs, each having a unique identity. Each GMC divides symmetrically, giving rise to two postmitotic neurons (Goodman and Doe, 1992) [adapted from Doe et al. (1985c)].

segment of the CNS called a *neuromere.* Positional cues are thought to be responsible for the specification of neuroblast identities within the neuromeres. The pair-rule genes, as well as a subset of the segment polarity genes, have been shown to be involved in the assignment and maintainance of neuroblast identity (Doe et al., 1988a,b; Patel, Schafer, Goodman, et al., 1989).

The neuroblasts undergo asymmetric cell divisions, with each generating an average of five daughter cells, referred to as *ganglion mother cells* (GMCs). Each GMC will then divide once, symmetrically, generating two daughter cells which differentiate into neurons [Fig. 1(C); Goodman and Doe, 1992]. The birth order of each GMC appears to be invariant, with each cell giving rise to a unique subset of neurons. In the grasshopper when a neuroblast is ablated after it has produced two or more GMCs, it will be replaced by an adjacent neurectodermal cell. However, this new neuroblast will again generate GMCs beginning with the first, GMC-1 (Doe and Goodman, 1985b).

In contrast to what has been observed in higher vertebrates, cell migration plays a limited role in determining the final position of most cells in the *Drosophila* embryonic CNS. Most neurons acquire their final positions passively, by displacement, due to the birth of other neurons. However, there are some examples of limited cell migration in *Drosophila.* For example, the aCC and pCC neurons (Fig. 2) have been shown to migrate anteriorly across the segment border to reach their final positions in the posterior half of the adjacent neuromere (Thomas, Bastiani, Bate, 1984). Similarly, one type of glioblast migrates from its lateral birthplace to a more medial position in the neuromere in which it was born (Jacobs, Hiromi, Patel, et al., 1989). Thus, while cell migration plays a role in the embryonic CNS formation of *Drosophila,* it is not to the same extent, or over the same relative distances as has been observed in vertebrates.

One aspect of neuronal differentiation is the extension of axons to specific targets. In both the grasshopper and *Drosophila,* the first axons to extend, called *pioneer axons,* may rely on different guidance cues to find their targets than do the axons that extend later. Pioneer axons extend filopodia along the basement membranes of epithelial and mesodermal cells and have been observed to contact and grow over glial cells. These observations have led to the hypothesis that the pioneer axons use a scaffold of glial cells, which prefigures the axon tracts, as guideposts for their growth cones (Jacobs and Goodman, 1989b). Axons that are extended later in development may find their targets by recognizing and following guidance cues provided on the surfaces of specific axons (Bastiani and Goodman, 1986). Alternatively, some combination of guidance cues on specific axons, a preformed glial scaffold, and/or tropic factors, may support axonal pathfinding.

The fully mature embryonic ventral nervous system has a characteristic, ladder-like array of axon tracts [Figs. 2, 3(B)]. Two bilaterally symmetric, longitudinal axonal tracts extend along the antero-posterior axis connecting adjacent neuromeres. Within each neuromere, two commissural tracts (anterior and posterior) extend across the midline joining the longitudinal tracts. Likewise, exiting each neuromere are two major axonal tracts, the segmental and intersegmental nerves, which link the CNS to the peripheral nervous system (PNS).

Descriptive and experimental analyses have provided us with substantial insight into embryonic CNS development in *Drosophila.* Having identified many of the processes involved in constructing the CNS, we are now able to ask what the specific components of these processes are, and how they function. In this review we will outline the various approaches used to address some of the basic questions in CNS development, such as the following:

1. What gene products are involved in neuroblast determination and segregation, and are they distinct from the molecules required for lateral inhibition?
2. Once a cell has been determined to enter a neuroblast rather than epidermal fate, what gene products are required for a specific neuroblast to assume its particular identity and carry out its unique lineage?
3. What guidance cues are required for axonal pathfinding, and what genes direct this process?

Of the different approaches that have been used to address these questions, some are unique to *Drosophila,* whereas others have been adapted from a variety of experimental systems. Here we provide a general overview of these approaches, noting both their advantages and disadvantages, and we review some of the molecules identified by ourselves, and others utilizing these methods. Finally, we discuss some promising new techniques that will undoubtedly provide further insights into the mechanisms controlling embryonic CNS development.

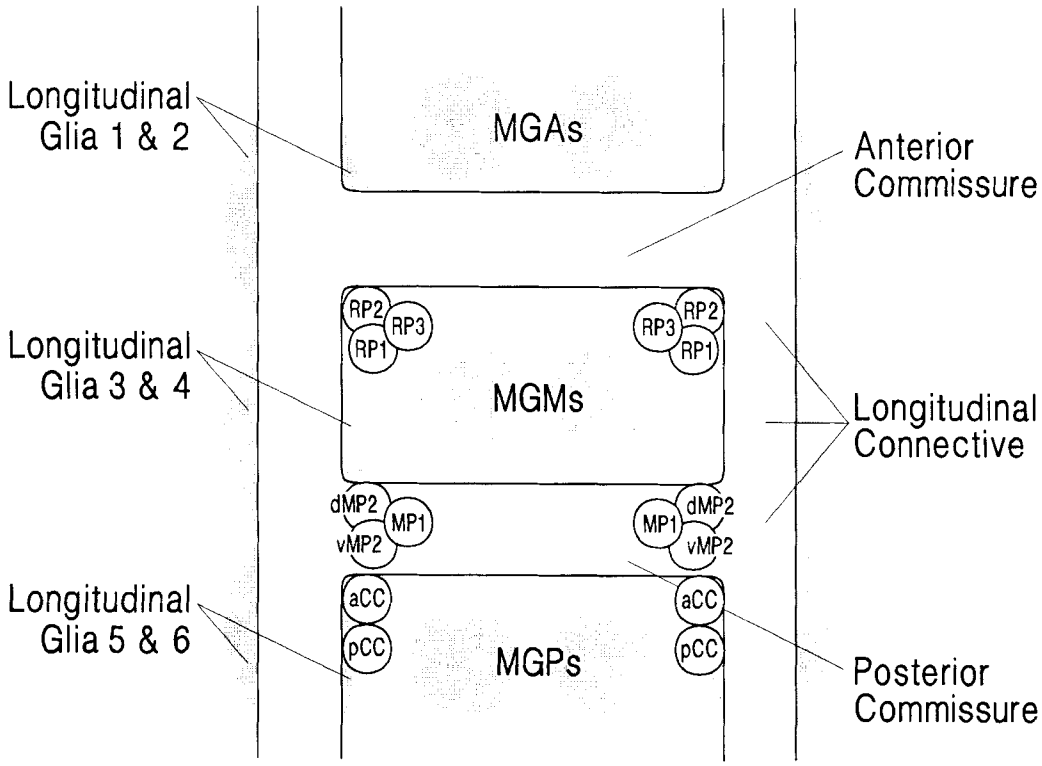

Figure 2 Schematic representation of a single neuromere. The major axon tracts in the *Drosophila* embryonic CNS are the longitudinal connectives and the anterior and posterior commissural axon tracts. The longitudinal connectives run along the anterior–posterior axis, connecting the neuromeres. The commissures cross the midline between the longitudinals. The anterior commissure is actually comprised of two separate axon bundles, whereas the posterior commissure is a single axon bundle. The positions of the three pairs of the mesectodermally derived midline glia (MG) (Jacobs and Goodman, 1989a) associated with the commissures is indicated (MG*A*nterior, MG*M*iddle and MG*P*osterior). The longitudinal glia (three pairs per hemisegment) lie dorsal to the longitudinal connectives (Jacobs and Goodman, 1989a) and develop from a neurectodermally derived lateral glioblast. The relative positions of the RP, MP, aCC and pCC neurons, referred to in the text, are also shown.

THE GENETIC APPROACH

Genetic Screens

One of the advantages of using *Drosophila* as a system to address basic developmental questions is that it is genetically amenable. Investigators interested in various aspects of embryonic development have performed large-scale mutant screens which have generated a large collection of recessive, embryonic lethal mutations (Nusslein-Volhard and Wieschaus, 1980; Wieschaus, Nusslein-Volhard, and Jurgens, 1984; Nusslein-Volhard, Wieschaus, and Kluding, 1984; Jurgens, Wieschaus, Nusslein-Volhard, et al., 1984). Molecular, genetic, and developmental characterizations of many of the genes identified in these mutagenesis experiments have elucidated the underlying mechanisms controlling embryonic axes formation and segmentation (see reviews by Akam, 1987; Ingham, 1988; St. Johnston and Nusslein-Volhard, 1992).

Given the success of this approach in dissecting embryonic segmentation, many investigators interested in CNS development have chosen a similar approach. The existing collections of embryonic lethal mutations provided a valuable resource in which to search for genes that function during development of the embryonic CNS. Initially, antibodies against horseradish peroxidase (HRP), which specifically label neuronal cell bodies and axons of both the CNS and PNS (Jan and Jan, 1982), were used to identify mutations associated with abnormal neural development. This search revealed that many mutations that had been previously identified as playing a role in axes formation or segmentation also function during CNS develop-

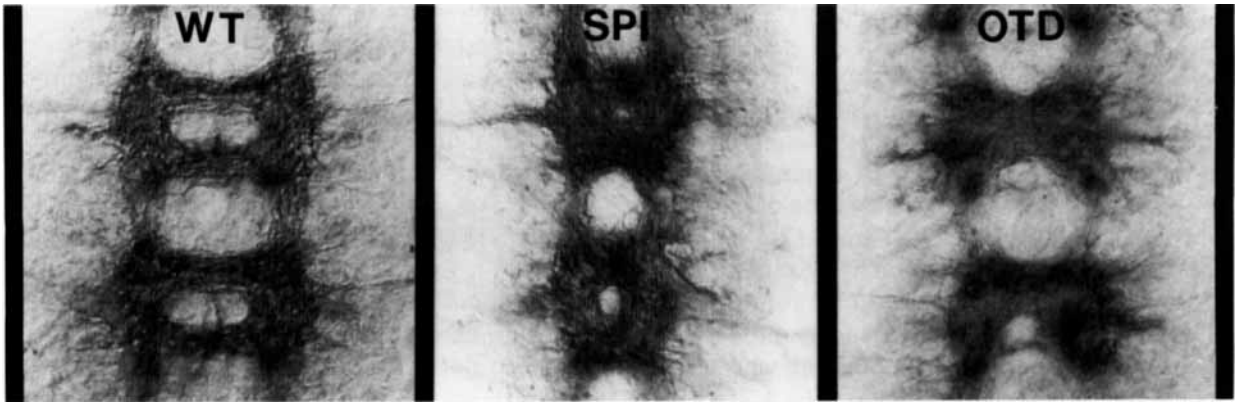

Figure 3 CNS phenotypes in wild-type (WT), *orthodenticle* (*otd*), and *spitz* (*spi*). The array of axonal tracts revealed using MAb BP102 in wild-type, *otd*, and *spi* mutant embryos. See text for detailed descriptions of the *otd* and *spi* phenotypes.

ment (D. T. Smouse, N. Perrimon, A. P. Mahowald, and C. S. Goodman, unpublished results).

Other genetic screens were initiated specifically to identify genes functioning during CNS development. One such screen generated a collection of 331 X-linked, embryonic lethal mutations (E. Noll and N. Perrimon, unpublished results). These ethyl methane sulfonate-induced mutations were also examined using antibodies against HRP and were found to include alleles of genes previously identified as playing a role in CNS formation, as well as some previously unidentified loci. Collectively, the screens outlined above identified a set of mutations that has a range of effects on the longitudinal or commissural axon tracts or both. Several of these genes will be discussed in more detail below.

Genes Affecting the Ventral Midline

The ventral midline of the CNS, comprised of both neurons and glia, is derived from the mesectoderm region of the blastoderm stage embryo (Poulson, 1950) [Fig. 1(A)]. The midline cells play a crucial role in the proper formation of the axon tracts (Klambt, Jacobs, and Goodman, 1991; Fig. 2), perhaps comparable to that of the vertebrate floor plate (Tessier-Lavigne, Placzek, and Lumsden, et al., 1988, Bovolenta and Dodd, 1991). Mutations in a number of loci disrupt various subsets of ventral midline cells, and, in each case, reproducible defects of the major axon tracts are observed. These loci include *orthodenticle* (*otd*) (Finkelstein, Smouse, Capaci, et al., 1990), *spitz* (*spi*) (Rutledge, Zhang, Bier, et al., 1992), *rhomboid* (*rho*) (Bier, Jan, and Jan, 1990), *single-minded* (*sim*) (Crews, Thomas, and Goodman, 1988; Thomas, Crews, and Goodman, 1988), *slit* (*sli*) (Rothberg, Hartley, and Walther, et al., 1988), *Star* (*S*) and *pointed* (*pnt*) (Mayer and Nusslein-Volhard, 1988).

Of the loci effecting the development of the ventral midline, *sim* plays a key role. In *sim* mutant embryos, mesectodermal cells do not delaminate into neuronal and nonneuronal midline precursors. As a result, both the commissural and longitudinal axon tracts fail to form correctly (Thomas et al., 1988; Nambu, Franks, and Hu, et al., 1990). Molecular characterization of *sim* revealed that it encodes a transcription factor, of the basic helix-loop-helix family (Nambu, Lewis, and Wharton, et al., 1991). Consistent with its characterization as a transcription factor, *sim* has been shown to control the expression of several other midline-specific genes (Nambu et al., 1991). Taken together these results suggest that *sim* may be a master regulatory gene controlling mesectodermal identity.

sim mutant embryos fail to express *slit* protein. Hence, *sli* is thought to be one of the genes regulated by *sim* (Nambu et al., 1990). Similar to the phenotype of *sim*, the mesectoderm in *sli* mutant embryos fails to differentiate, which has profound consequences on the formation of the major axon tracts. Based upon the lack of *sli* protein in *sim* mutant embryos and the similarity of phenotypes in both mutations, absence of the *sli* protein is thought to be the primary cause of the phenotype observed in both mutations (Nambu et al., 1990). *sli* encodes a transmembrane protein with multiple EGF repeats, suggesting that cell–cell interaction may play a role in proper mesectoderm differentiation (Rothberg et al., 1988).

Based on the similarity of the larval cuticular mutant phenotypes, the genes *spi, S, pnt,* and *rho* have been placed in the "spitz group" (Mayer and Nusslein-Volhard, 1988). Among this group, *spi* and *rho* have been characterized in the most detail. In the CNS of both *spi* and *rho* mutant embryos, the most noticeable defect is the fusion of the anterior and posterior commissural tracts (Fig. 3). This appears to be due to disruption of the midline glial cells which normally lie between the commissures (Fig. 2) (Klambt et al., 1991). In *rho* mutant embryos the midline glia fail to develop, whereas in *spi* mutant embryos, the midline glia fail to undergo their proper migration.

Molecular and developmental characterization of both *spi* and *rho* suggests that these genes may function within the same developmental pathway. The *spi* gene encodes a protein containing a putative signal sequence, an EGF domain and a potential transmembrane domain (Rutledge et al., 1992). Due to its structural similarity to the EGF-like growth factor, TGFα, the *spi* gene product may function as a ligand initiating a signal transduction pathway. *rho* has been shown to encode a putative transmembrane protein (Bier et al., 1990), suggesting that *rho* may be a receptor for the putative *spi* ligand. The mutant phenotypes of *spi* and *rho,* as well as the wild-type expression patterns, are consistent with the proposed ligand/receptor model. The *spi* protein is ubiquitously expressed throughout embryogenesis, with enrichment within the mesoderm and ventral midline (Rutledge et al., 1992). The *rho* protein, in contrast, is restricted to the muscle cells and cells of both the CNS and PNS which are affected by mutations in either *spi* or *rho* (Bier et al., 1990).

Interestingly, a number of alleles of the *faint little ball* (*flb*) gene display a fusion of the anterior and posterior commissural tracts similar to that observed in *spi* and *rho* (Raz and Shilo, 1992). *flb* encodes a protein believed to be the *Drosophila* homologue of the mammalian EGF receptor (Livneh, Glazer, Segal, et al., 1985). Among the cells that express the *Drosophila* EGF receptor (also called *DER*) are the midline glia, which are disrupted in *flb* mutant embryos (Zak, Wides, Schejter, et al., 1990; Raz and Shilo, 1992). Given the structural similarity between the spitz protein and TGFα, a known ligand for the mammalian EGF receptor (reviewed in Massague, 1990), and the similarity of mutant phenotypes of *flb* and *spi,* it is possible that *spi* may also be a ligand for *DER.*

As in the case of the "spitz group" genes, mutations in the X-linked gene *otd* were identified by the disruption of larval cuticular structures. *otd* was subsequently determined to be required for normal development of a subset of mesectodermal derivatives (Wieschaus et al., 1984; Finkelstein et al., 1990). In *otd* mutant embryos, the posterior commissures do not form and the longitudinal connectives are closer together than in wild-type embryos (Fig. 3; Finkelstein et al., 1990; Klambt et al., 1991). At least two types of mesectodermally derived CNS cells degenerate early in development in *otd* mutant embryos: the ventral unpaired median neurons (VUMs) and the progeny of the median neuroblast (MNB). Further, the normal development of the midline glial cells, which are also mesectodermally derived, is disrupted. Although the specific role of *otd* in the development of these mesectodermal cells is not yet clear, molecular analysis has shown that *otd* encodes a protein with a homeodomain, suggesting that it may function as a transcription factor which specifies cell identities during CNS development (Finkelstein et al., 1990).

Determination of Neural Versus Epidermal Cell Fates

Among the collections of embryonic lethal mutations discussed earlier (Wieschaus et al., 1984; Nusslein-Volhard et al., 1984; Jurgens et al., 1984), six were associated with hyperplasia of the nervous system at the expense of the ventral epidermis (Lehmann et al., 1981, 1983). These "neurogenic loci" [*mastermind (mam), neuralized (neu), big brain (bib), Delta (Dl), Enhancer of split* [*E(spl)*] and *Notch (N)*] restrict the number of cells that enter the neuroblast pathway. As discussed in the introduction, the neurectoderm initially requires the proneural genes to define domains that are competent to produce neuroblasts (Cabrera et al., 1987; Romani, Campuzano, Macagno, et al., 1989). Following the influence of the proneural genes, cells within the neurectoderm acquire the neuroblast fate as their default state. The neurogenic loci then control cell–cell interactions via a mechanism of lateral inhibition, which prevent more than one-quarter of neurectodermal cells from segregating and entering into the neuroblast fate.

Additional neurogenic loci were obtained in screens conducted to detect the maternal effects of mutations that exhibited female sterility (Perrimon et al., 1986; Schupbach and Wieschaus, 1989) or the maternal effects of late zygotic lethal mutations (Perrimon, Engstrom, and Mahowald,

1989). In addition to the six neurogenic loci that are known to function zygotically, maternally derived products from *fs(1)almondex (amx), fs(1)pecanex (pcx), fs(1)M53, l(1)zeste-white 4 (zw4),* and *l(1)brainiac* (Perrimon et al., 1986; 1989; Goode et al., 1992) are required to properly regulate the number of cells that take on the neuroblast fate. If any of these gene products are lacking in the maternal germline, then the progeny will display the neurogenic phenotype.

Transplantation experiments and genetic mosaic analyses have demonstrated that six of the neurogenic genes (*amx, mam, neu, bib, Dl,* and *N*) function either as an inhibitory signal, or in the transmission of that signal, from a cell which has taken on a neuroblast fate, to its neighboring cells (Hoppe and Greenspan, 1986; Technau and Campos-Ortega, 1987; Campos-Ortega and Haenlin, 1992). In contrast, *E(spl),* has been postulated as functioning on the receiving end of the signal inhibiting the neuroblast fate.

Although many of the neurogenic genes have been cloned, the exact role of each in the lateral inhibition process has yet to be clearly defined. However, the nature of their gene products supports the notion that they are involved in signal transduction. For example, one of the transcripts of the *E(spl)* complex is related to β-transducin (Hartley, Preiss, and Artavanis-Tsakonas, 1988), and both *N* and *Dl* encode transmembrane proteins with multiple EGF repeats (Wharton, Johansen, Xu, et al., 1985; Vassin, Bremer, Knust, et al., 1987; Kopczynski, Alton, Fechtel, et al., 1988).

Determination of Neuronal Cell Identity

Following acquisition of a neural fate, each neuroblast needs to assume a specific identity and thereby generate a lineage from which particular neurons arise. There exists at least two mechanisms by which identity can be assigned: lineage or position. Currently, there is little evidence linking cell fate establishment and lineage. However, in the grasshopper, the identity of a neuroblast can be reliably predicted based solely on its location, suggesting that in insects identity is likely determined by location (Doe and Goodman, 1985a,b).

Among the genes required during CNS development, many have been previously identified as playing key roles in assigning cellular fates during axes formation or segmentation. Not surprisingly many of the segmentation genes, which are initially expressed during blastoderm stages, are expressed again in the CNS during neurogenesis. The pair-rule genes *fushi tarazu* (*ftz*) and *even skipped* (*eve*), both of which encode homeodomain-containing proteins (Hiromi, Kuroiwa, and Gehring, 1985; MacDonald, Ingham, and Struhl, 1986), serve to illustrate how genes involved in segmentation are later utilized in the CNS to specify cell identity. Both *ftz* and *eve* are initially expressed at blastoderm stages in the even- and odd-numbered parasegments, respectively. Loss-of-function mutations in either gene results in the deletion of all structures derived from their blastoderm expression domains (Hiromi et al., 1985; MacDonald et al., 1986; Frasch, Hoey, Rushlow, et al., 1987). Following neuroblast segregation, both *ftz* and *eve* are expressed in overlapping subsets of neuroblasts in each segment, and by their GMC progeny. Interestingly, the GMCs produced by a single neuroblast will not necessarily express the same pair-rule genes. For example, the first GMC (GMC-1) produced by neuroblast 4-2 (NB4-2) expresses both *ftz* and *eve,* but the second GMC (GMC-2) expresses only *ftz* and not *eve* (Doe et al., 1988a,b).

Both *ftz* and *eve* have been found to function in determining neuronal identity. This was demonstrated in the case of *ftz* by constructing a transgene which deleted promotor elements necessary for CNS expression and thereby eliminated *ftz* expression in the CNS, while preserving the initial expression in the blastoderm (Doe et al., 1988a). Under appropriate experimental conditions, this transgene was expressed in an *ftz* mutant background, and the identities of cells within the CNS were determined, using both *eve* expression and morphology as criteria for identification. GMC-1, from NB4-2, normally gives rise to the RP2 neuron (Fig. 2) and another neuron whose fate has yet to be determined. GMC-2, also from NB4-2, gives rise to the RP1 and RP3 neurons. When *ftz* expression in the CNS in eliminated, *eve* fails to be expressed in either GMC-1 or RP2. Furthermore, the RP2 axon projects in a manner that is characteristic of axons from the RP1 and RP3 neurons. This suggests that expression of *ftz* is required for expression of *eve* in GMC-1, and that loss of *ftz* transforms GMC-1 towards the GMC-2 fate (Doe et al., 1988a). In addition, a temperature-sensitive allele of *eve,* which produces an inactive *eve* protein at the nonpermissive temperature, was used to demonstrate that when embryos are shifted to the restrictive temperature during neurogenesis, the axons of RP2 neurons projected like RP1/RP3 axons (Doe et al., 1988b).

It is interesting to note that the regulatory hierarchy between *ftz* and *eve* during blastoderm stages is

not the same as the hierarchy observed in the CNS. In the blastoderm, it has been shown that *eve* is required for the correct expression of *ftz*. The experiments described above demonstrate that *ftz* is required for the proper expression of *eve* in the RP2 neuron. However, the dependence of *eve* expression on *ftz* in the RP2 neuron, is not a generalized rule in the CNS. The aCC and pCC neurons (Fig. 2) also express both *ftz* and *eve*, but elimination of *ftz* expression in these cells does not effect the expression of *eve* (Doe, 1992). This would suggest that individual neurons may have distinctive genetic hierarchies.

A striking observation related to the role of *eve* in determining neural identity involves the phenotype associated with mutations in the *polyhomeotic* (*phm*) gene. Null mutations at the *phm* locus produce a phenotype in which the ladder-like array of axon tracts does not form. The axons are instead confined to the hemiganglion of origin (Smouse, Goodman, Mahowald, et al., 1988; Smouse and Perrimon, 1990. Additional defects are observed in *phm* mutant embryos, including death of the ventral epidermis and global misregulation of both homeotic and segmentation gene expression (Smouse et al., 1988; Dura and Ingham, 1988). For example, in *phm* mutant embryos, expression of the homeotic gene *Ultrabithorax* is suppressed, and *eve* is ectopically expressed in all neurons (Frasch et al., 1987; Smouse et al., 1988). The relationships between the observed changes in gene expression and the axonal phenotype is unclear. However, it may be due to the ectopic expression of *eve* in all neurons.

THE ENHANCER TRAP APPROACH

The enhancer trap approach makes use of *Drosophila* transposable P-elements. P-elements are short sequences of DNA, flanked by specific repeats, which are capable of self-excision and reinsertion into a new genomic location. The existence of P-elements has enabled the development of P-element-mediated enhancer detection, or "enhancer trapping." This technique enables the rapid production of numerous strains of flies which exhibit cell- or tissue-specific patterns of β-galactosidase expression. These strains can be utilized to label various cells and/or tissues, and identify and clone genes of interest (O'Kane and Gehring, 1987; Bellen, O'Kane, Wilson, et al., 1989; Wilson, Pearson, Bellen, 1989; Bier, Vassin, Shephard, et al., 1989).

Briefly, P-elements have been modified such that they contain the *E. coli lacZ* gene, under the control of the weak P-element promoter. This promotor is unable to initiate *lacZ* transcription on its own (Wilson et al., 1989). However, when the P-element inserts in the genome near an endogenous enhancer-like element, *lacZ* will often be transcribed in a pattern reminiscent of the flanking gene (O'Kane and Gehring, 1987). Further, this modified P-element has been designed to allow for rapid cloning or "rescue" of the flanking genomic DNA. This "rescued" DNA can be analyzed directly in Southern, Northern, or *in situ* hybridizations, or it can be used to generate probes for screening genomic or cDNA libraries.

When the P-element inserts directly into the coding region of a gene, or into sensitive promoter regions, a mutant phenotype is often observed. Likewise, once inserted near a gene, a P-element can be remobilized often generating small deletions in the region due to imprecise excision. Stable fly stains containing these modified P-elements are easily maintained since the P-elements become mobile only in the presence of another transposon encoding a transposase activity (Cooley, Keeley, and Sprading, 1988; Robertson, Preston, Phillis, et al., 1988).

Numerous enhancer-trap screens have been performed in an effort to isolate stable fly strains that exhibit tissue-specific patterns of β-galactosidase expression (Bellen et al., 1989; Bier et al., 1989; Perrimon, Noll, McCall, et al., 1991; Klambt et al., 1991). Along with the identification of previously unknown loci involved in CNS development, a number of strains recovered from these screens have proved invaluable for cloning previously identified genes such as *rhomboid* [Bier et al., 1990; Fig. 3(A)], *spitz* (Rutledge et al., 1992), and *prospero* (Vassin, Grell, Wolff, et al., 1991; Doe, Chu La-Graff, Wright, et al., 1991).

Depending on which modified P-element is used, anywhere from 65% to 93% of the novel insertion strains express β-galactosidase in a restricted pattern during embryogenesis (Fig. 4) (Perrimon et al., 1991). These strains are useful markers for determining the effects of various mutations during development (Ghysen and O'Kane, 1989; Fig. 4). For example, Finkelstein et al., (1990) used a nervous system-specific P-element insertion strain to study the effects of *otd* mutant alleles on neurogenesis. This study revealed that the positions of specific midline cells are disrupted in *otd* mutant embryos. Similarly, Klambt et al. (1991) used a number of P-element insertion

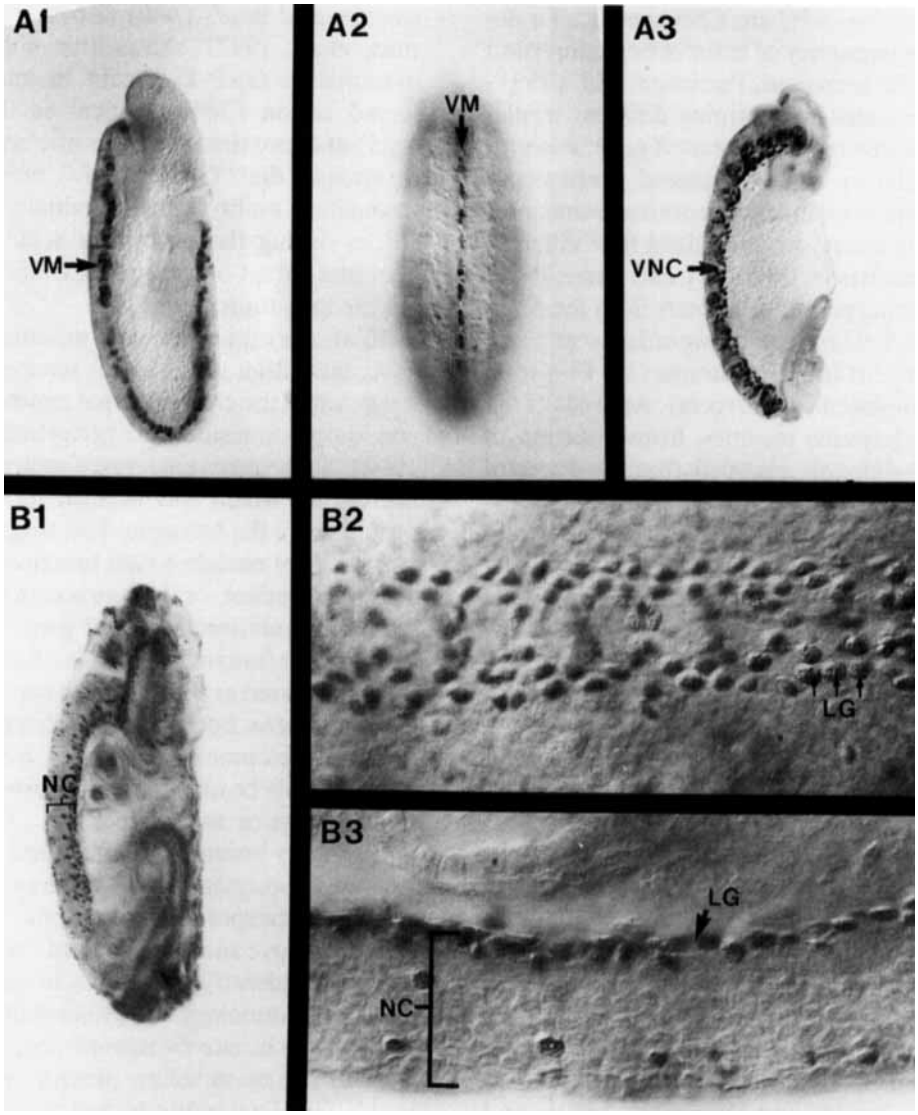

Figure 4 CNS expression of enhancer trap strains. The β-galactosidase expression of three enhancer trap lines recovered in mobilization of the *P[LArB]* transposon (Perrimon et al., 1991 and unpublished) is shown. (A) 2A18 expresses β-galactosidase in a subset of mesectodermal cells. (A1) A lateral view (40×) of a germ band extended embryo. Position of the ventral midline (VM) is indicated. (A2) A ventral view of a germ band retracted embryo. (A3) A lateral view of the embryo shown in A2. Position of the ventral nerve cord (VNC) is indicated. Line 2A18 is an insertion of the *P[LArB]* transposon in the *rhomboid* locus (Bier et al., 1990; E. Noll and N. Perrimon, unpublished observations). (B) 1B6 expression of β-galactosidase. (B1) A lateral view (40×) of a mature embryo showing the restricted expression of 1B6 in the nerve cord (NC). (B2) A dorsal view (100×) of the embryo in B1. Among the cells which express β-galactosidase in this line are the longitudinal glia (LG). (B3) A lateral view (100×) showing the dorsal position of most glial cells labelled by 1B6. Ventral is down in this picture.

strains to conduct a detailed analysis of the development of the ventral midline.

To recover P-element insertion strains, we note that specifically labeling subsets of cells in the CNS requires the generation, and subsequent examination of a large number of insertion strains. Using the P-element construct *P[LArB]*, Perrimon et al. (1991) determined that approximately 5% of insertion stains recovered express β-galactosidase in a single tissue type. Among lines with such tissue-

specific expression, 65% are CNS specific. To determine if the frequency of tissue-specific insertion strains could be increased, Perrimon et al. (1991) mobilized P-elements containing different regulatory elements upstream of the *lacZ* gene. Reasoning that regulatory elements placed upstream of *lacZ* might interact with endogenous genomic promoters or enhancers, we mobilized four different P-element constructs, *P[LArB]* and three P-elements containing partial promoters from the genes *even-skipped, fushi-tarazu,* or *engrailed* to generate 1426 independent insertion strains (see Perrimon et al., 1991 for specific constructs). Analysis of the transcription patterns resulting from insertion of these four P-elements revealed that the types of patterns generated, as well as the frequency of tissue-specific patterns recovered, were significantly affected by the presence of additional promoter elements. Thus, future use of P-elements containing partial promoters would permit the rapid recovery of a larger percentage of strains with CNS-specific β-galactosidase expression and aid in the identification of genes functioning in the CNS.

THE CLONING BY HOMOLOGY APPROACH

Evolutionary conservation of functional domains, especially at the amino acid level, has allowed the identification of many *Drosophila* homologues of vertebrate genes. Among these are tyrosine kinases (Hoffman-Falk, Einat, and Shilo, 1983; Livneh et al., 1985), serine threonine kinases (see review by Siegfried, Ambrosio, and Perrimon, 1990), and protein tyrosine phosphatases (Yang, Seow, Bahri, et al., 1991; Tian, Tsoulfas, and Zinn, 1991).

Using standard techniques, probes from interesting vertebrate genes or proteins can be used to isolate related *Drosophila* sequences. The chromosomal location of the related *Drosophila* sequence can then be determined by *in situ* hybridization to salivary gland polytene chromosomes. Once located, it can be determined whether existing *Drosophila* mutations are available in the region or, if not, a "reverse genetic" approach can be undertaken in which mutations in the region can be induced and subsequently analyzed.

Using this approach, Ng, Perkins, Conboy, et al. (1989) identified a *Drosophila* gene related to the mammalian *GAP-43* gene. The mammalian *GAP-43* is normally expressed at high levels in the growth cones of extending axons and is induced in regenerating nerves. Overall, the *Drosophila* gene, *bangles and beads* (*bnb*) (Eberl, Perkins, Engelstein, et al., 1992), shows little homology to the mammalian GAP-43 except in one highly conserved region (76% identical at the nucleotide level) of approximately 20 amino acids which was designated the "GAP motif." *bnb* is expressed throughout embryogenesis, initially in epidermal stripes during the process of segmentation, and later in a subset of cells in the CNS, among which are the longitudinal glia.

In an attempt to identify mutations in the *bnb* gene, saturation mutagenesis screens for all essential genes in the chromosomal region in which *bnb* was known to reside were performed (Eberl et al., 1992). Subsequent analyses of each complementation group which was isolated failed to identify mutations in the *bnb* gene. This suggests that either *bnb* does not encode a vital function, that its function is redundant, or that the screen simply did not recover a mutation in the *bnb* gene. If *bnb* encodes a redundant function, then it is a formal possibility that a mutation at the *bnb* locus was induced in the genetic screens, but like some other genes involved in CNS development (see below), a mutant phenotype will only be observed in the presence of mutations in one or more other loci. Thus, although screening by homology can be used to identify interesting *Drosophila* genes, it may be difficult to generate corresponding mutations.

Others have successfully used "reverse genetic" screens to identify mutations in genes that were cloned by homology. Hoffman-Falk et al. (1983) were able to isolate *Drosophila* sequences homologous to the mammalian protein tyrosine kinase, *c-abl.* The *Drosophila* homolog, *D-abl,* is highly conserved in the non-catalytic SH2 and SH3 domains, as well as in the catalytic tyrosine kinase domain. Although *D-abl* is expressed in a subset of axons of the CNS, null mutations in *D-abl* result only in late pupal lethality (Gertler, Bennett, Clark, et al., 1989). Given its strong evolutionary conservation and embryonic axonal expression pattern, such late lethality was unexpected. However, second-site mutational analysis subsequently revealed that the embryonic *D-abl* function is partially redundant (Hoffman, 1991).

THE ANTIBODY APPROACH

Production of monoclonal antibodies (MAbs) to *Drosophila* proteins has been successful in identifying molecules involved in CNS development. Once the labor-intensive task of generating hybrid-

oma cell lines, and subsequent supernatant collection is complete, large numbers of MAbs can be screened on whole embryos. Once identified, an interesting MAb can be useful in both the biochemical characterization of the protein it recognizes, as well as to help visualize CNS development in both wild-type and mutant embryos.

Two approaches permit the identification of a gene encoding a protein recognized by a specific MAb. First, the MAb can be used to screen cDNA expression libraries. Alternatively, once immunopurified, the protein can be microsequenced, and the resulting sequence can be used in the design of degenerate oligonucleotides which will serve as probes for screening libraries. As discussed in the cloning by homology section, a "reverse genetic" approach is required to isolate mutations in genes identified in this manner.

MAbs have been successfully used in the identification of a number of putative cell adhesion molecules in insects, among which are *fasciclin I (fas I), fasciclin II (fas II), fasciclin III (fas III),* and *neuroglian (nrg)* (Bastiani, Harrelson, Snow, et al., 1987; Bieber, Snow, Hortsch, et al., 1989; Grenningloh, Rehm, and Goodman, 1991; Patel, Snow, and Goodman, 1987).

fas I has no significant homology to any known protein (Zinn, McCallister, and Goodman, 1988) and although it contains a convincing signal sequence, it has no obvious transmembrane domains. Biochemically, *fas I* behaves as a membrane-associated molecule (Zinn et al., 1988), and ultrastructural analysis indicates that it is tightly localized to the external face of the membranes of axons, growth cones, and filopodia, even when these are not in contact with other cells (Bastiani et al. 1987).

Molecular characterization has revealed that *fas II* (Harrelson and Goodman, 1988; Bastiani et al., 1987), *fas III* (Patel et al., 1987), and *nrg* (Bieber et al., 1989) are members of the immunoglobulin superfamily. In the grasshopper, *fas II* is expressed transiently on the surface of the MP1, dMP2, and vMP2 neurons (Fig. 2) just prior to the appearance of the first growth cones (Harrelson and Goodman, 1988). These cells are responsible for pioneering the first two longitudinal axon fascicles. The *Drosophila fas II* gene encodes at least two highly related proteins, the larger form containing a transmembrane domain. The smaller form, however, has amino acids at its carboxy terminus which are characteristic of proteins that are attached to the cell membrane via a phosphatidylinositol (PI) anchor (Grenningloh et al., 1991).

The *neuroglian* antigen is expressed on many cell types in the developing embryo. Within the midline, the RP1, RP2, and RP3 neurons clearly express *neuroglian,* as do the longitudinal glia that are in contact with the RP neurons (Fig. 2). The three pairs of midline glia also express *neuroglian,* but only on surfaces coming in contact with *neuroglian* expressing axons or cell bodies (Bieber et al., 1989). The exact roles of these four putative cell adhesion molecules during axonal outgrowth remains unclear. However, the dynamic expression patterns exhibited by all four, early in the process of axon outgrowth, is consistent with them acting as selective recognition molecules (Bastiani and Goodman, 1986).

THE *IN VITRO* APPROACH

Remarkably pure populations of neuroblasts can be isolated from *Drosophila* embryos, and it has been demonstrated that these cells undergo many aspects of normal development *in vivo* (Furst and Mahowald, 1985, Huff, Furst, and Mahowald, 1989; Bray, 1989; Fredieu and Mahowald, 1989) (Fig. 5). Once isolated, these relatively pure populations of cells can be used to generate tissue-specific probes for both screening cDNA libraries and Northern analyses.

The validity of using *in vitro* approaches to study *in vivo* processes relies on the assumption that *in vitro* processes resemble those found *in vivo.* Previous studies have shown that neuroblasts *in vitro* undergo all or most of the developmental events that are observed *in vivo.* Gastrula cells in culture have been observed to differentiate into distinct cell types including neurons, myocytes, fused myotubes, fibroblasts, and macrophage-like cells (Shields and Sang, 1970; Seecof, Teplitz, Gerson, et al., 1971; Chan and Gehring, 1971). Neuroblasts from these cultures were observed to divide asymmetrically, extend neurites, and form neuromuscular junctions with adjacent myocytes, just as they do *in vivo* (Seecof et al., 1971, 1972, 1973). Further, the spatial and temporal appearance in culture of the neurotransmitters acetylcholine, serotonin, and dopamine, and their associated enzymes, appear to mimic that observed *in vivo* (Dewhurst and Seecof, 1975; Furst and Mahowald, 1985; White and Valles, 1985; Scholnick, Bray, Morgan, et al., 1986; Huff et al., 1989). Additionally, the electrical properties of cultured neurons (Solc, Zagotta, and Aldrich, 1987), some surface antigens (Furst and Mahowald, 1985, for exam-

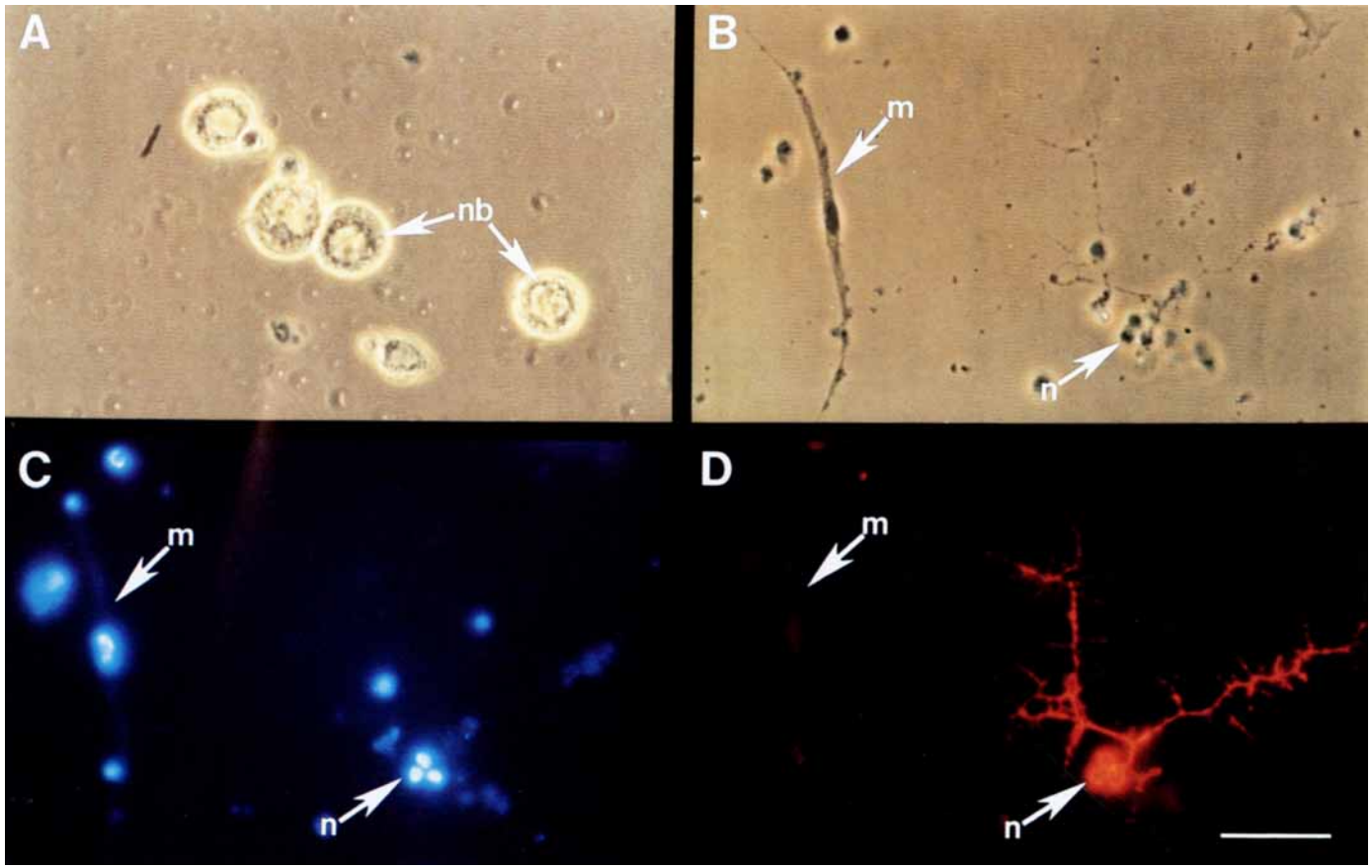

Figure 5 Cultured neuroblasts and pre-elutriated mature cell cultures. For panel (A), gastrula-staged cells were elutriated and the large cell fractions were pooled and photographed prior to the selective adhesion step. Four presumptive neuroblasts (nb) are shown, two of which (upper left) have divided once, asymmetrically to produce a larger neuroblast and a smaller ganglion mother cell. The ganglion mother cell will subsequently divide to produce two neurons. For panels (B), (C), and (D), gastrula-staged cells were prepared, plated prior to elutriation, and allowed to mature overnight in culture. The same cells were photographed in phase (A and B), and double stained with Hoechst DNA stain for cell nuclei (C), and antibodies against HRP (D). Note that the elongated muscle cell (m), the negative control, does not stain with HRP, whereas membrane staining of the three cell neural cluster is apparent on both the cell bodies and extending neurites. Scale bar = 20 μm.

Methods: Gastrula cells cultured overnight were double-labelled with a DNA stain and antibodies against HRP (Jan and Jan, 1982). Nuclear staining was performed by diluting an aliquot of a Hoechst stock solution to a final concentration of 15 μM and added directly to the culture media, followed by a 1 h incubation at 26°C. The cells were then washed in BSS (Chan and Gehring, 1971) for 20 min, fixed with fresh 4% paraformaldyde for 10 min, washed in BSS for 30 min, incubated overnight at 4°C with directly conjugated FITC antibodies against HRP (from Cappel), washed three times in BSS over 4 h, and mounted for visualization in aquamount.

ple), and the expression of some characterized RNAs (L. A. Perkins, unpublished data) correlate well with the expression pattern observed *in vivo.*

Briefly, as outlined in Figure 6, neuroblasts can be isolated from dissociated embryos after fractionation in an elutriation system [Fig. 5(A); Furst and Mahowald, 1985]. This population of cells can be further purified by selecting only those cells that adhere to glass (a known property of neuroblasts). Poly A^+ RNA can then be isolated from the neuroblasts or from neuroblasts that have been allowed to divide and differentiate into neurons (Table 1). We (L. A. Perkins and A. P. Mahowald, unpublished observations) utilized these two classes of RNAs to generate radiolabeled cDNA probes used in differential screening of cDNA libraries for clones expressed in segregating neuroblasts. Two embryonic cDNA libraries prepared from embryos undergoing neuroblast segregation were selected for screening (Poole, Kauvar, Drees, et al., 1985; Yedvobnick, Muskavitch, Wharton, et al., 1985). Replica filters were screened with the labeled cDNA probes, and clones were selected which hybridized with cDNAs derived from undifferentiated neuroblasts but not with cDNAs derived from differentiated neurons. Following screening of approximately 1.2×10^5 plaques per library, a total of 20 clones remained likely candidates for neuroblast-expressing clones.

Clearly, following the screen, only minimal information is obtained concerning the tissue specificity of a selected clone. Therefore, two approaches were subsequently employed to determine the specificity of the isolated clones. First, *in situ* hybridization to embryos was used to reconfirm that the clones were expressed in neuroblasts, and to determine if the clones were expressed in embryonic tissues outside the nervous system. Second, hybridization to developmental and tissue-specific RNA blots was used to determine the transcriptional complexities of the selected clones, their differential regulation throughout development, as well as their regulation within specific tissues as determined by hybridization to RNA from neuroblasts, myoblasts, and RNA from differentiated neurons and muscles (Table 1).

We isolated two clones which were characterized in some detail. The first clone, *c321,* identified the *heat shock cognate 4* gene (*hsc4*), which encodes a predicted protein product that is 85% identical to the human *heat shock cognate 70* protein (Perkins, Doctor, Zhang, et al., 1990). Heat shock cognate proteins are not, or are only minimally, heat inducible. They appear to be important in all cellular compartments for altering protein conformations, which affects protein–protein interactions, and the translocation of polypeptides across specific membranes (reviewed in Lindquist and Craig, 1988). Additionally, *hsc70* is involved in the ATP-dependent uncoating of clathrin from endocytotic vesicles (Rothman and Schmid, 1986; Ungewickell, 1985). Northern analysis revealed that *hsc4* transcripts are very abundant throughout all developmental stages, and *in situ* hybridization to embryos revealed an enrichment of *hsc4* transcripts in newly segregated neuroblasts of both the head and germ band, as well as nonneural cells active in endocytosis or undergoing rapid growth and morphological changes (Perkins et al., 1990). Thus, *hsc4* likely functions during some cellular process which is shared by neuroblasts and other developmentally dynamic cell types.

The second clone characterized from the screen for genes expressed in neuroblasts, *c336,* identified the neurogenic gene *Delta.* Developmental Northern blots reveal that *c336* hybridizes to several developmentally regulated poly A^+ transcripts which are expressed both maternally and zygotically, and one which is strictly maternal in origin (Fig. 7). Tissue-specific Northern analyses reveal that the larger maternal and zygotic transcript is enriched in neuroblasts, as well as myoblasts, and, therefore, may encode an embryonic "blast cell"-specific function, rather than a function restricted to regions with neurogenic capabilities (Fig. 7).

A "blast cell"-specific function of *Delta* gene products is also consistent with the work of Corbin, Michelson, Abmays, et al. (1991). Their observations demonstrate that the loss of *Delta* function in the embryo results in the overproduction of a subset of mesodermal cells which express the muscle-specific gene *nautilus,* and a concomminant loss of the non-*nautilus*-expressing mesoderm. Although the exact nature of this defect is not clear, it is interesting to note the parallels between the *Delta* muscle phenotype and the *Delta* neurogenic phenotype, where there is an overproduction of neuroblasts at the expense of the nonneural ectoderm (discussed in the section on Determination of Neural Versus Epidermal Cell Fates).

In vitro assays have provided clues into the nature of a "blast cell" function served by *Delta,* as well as another neurogenic gene, *Notch* (Fehon, Kooh, Rebay, et al., 1990). *Notch,* like *Delta,* encodes a protein with multiple extracellular, EGF-like repeats (Wharton et al., 1985; Vassin et al., 1987, Kopczynski et al., 1988). *In vitro* transfection experiments placing either *Notch* or *Delta*

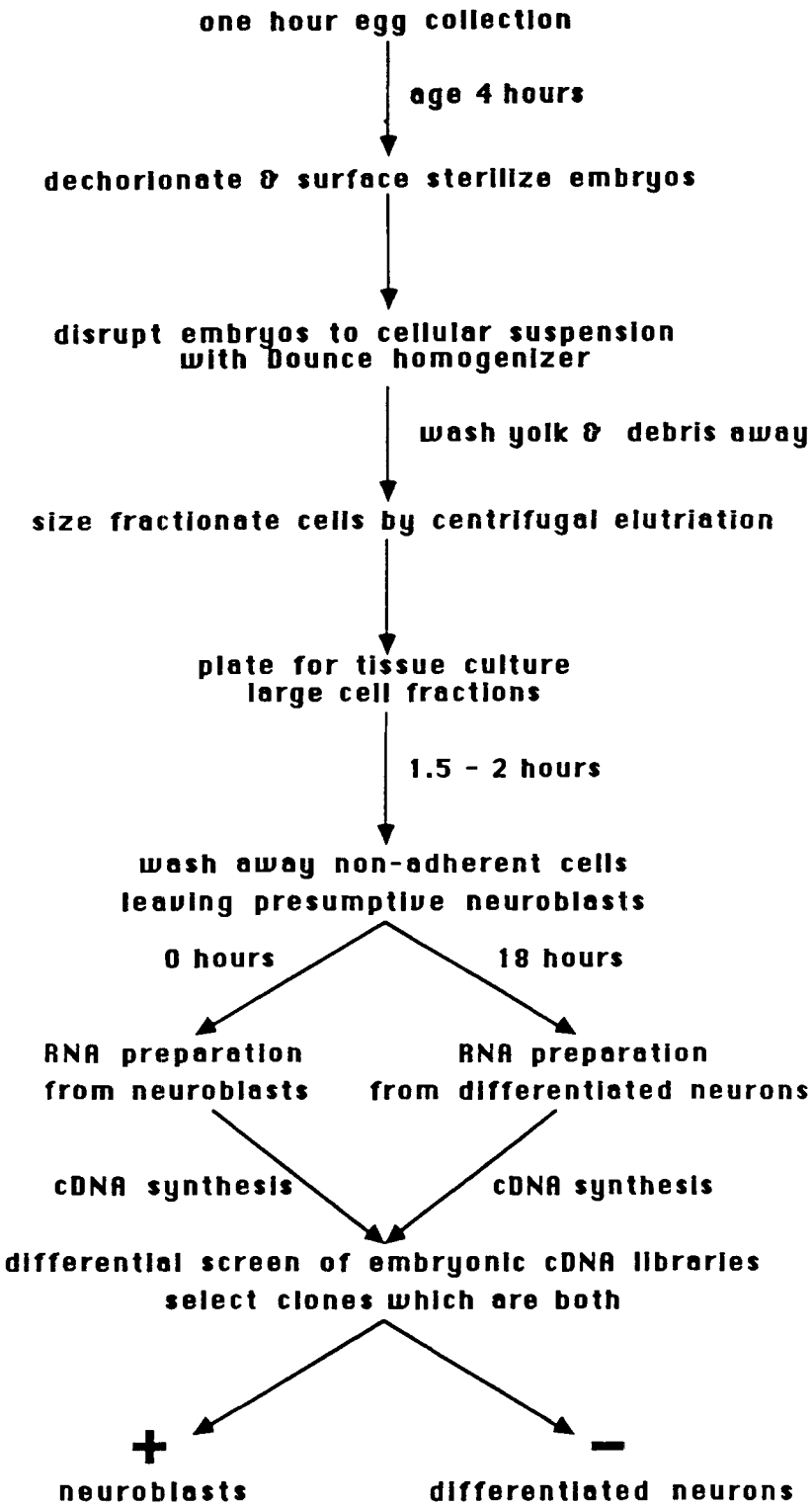

Figure 6 Outline of the preparation of gastrula-staged cells and differential screening procedure. The procedure used to prepare cultured cells from *Drosophila* gastrulae is described in detail in Furst and Mahowald (1985). Briefly, eggs from a wild-type strain (*Oregon R*) are collected for 1 h and aged 4 h to obtain gastrula-stage embryos. Following washing, dechorionation, and surface sterilization, the embryos are disrupted with a Dounce homogenizer. Noncellular debris is washed from the resulting cell suspension by centrifugation in BSS (Chan and Gehring, 1971). Cells are then size fractionated by centrifugal elutriation as described by Furst and Mahowald (1985). Approximately 1×10^9 cells, prepared from embryos 4–5 h after fertilization, are injected into a Beckman JE-6 elutriation rotor at 2800 rpm at 15°C. The counter flow of BSS is then increased by increments ranging from 12.5 ml/min to 25.0 ml/min. The cell fractions are collected, pelleted by centrifugation, and plated for cell culture in a

minigenes into *Drosophila* Schneider's 2 (S2) cells (which do not express detectable levels of *Notch* or *Delta* endogenously), reveal that cells expressing only *Notch,* or only *Delta,* do not form cellular aggregates. However, when *Notch*-expressing cells are mixed with *Delta*-expressing cells, calcium-dependent cell aggregation is observed. This aggregation is probably the result of a direct protein–protein interaction since these two proteins form stable, immunoprecipitable intermolecular complexes, both from transfected S2 cells and from embryonic cells. This protein–protein interaction may be mediated by the EGF-like repeats of both *Notch* and *Delta* (Fehon et al., 1990). EGF-like repeats have been previously shown to be involved in protein–protein interactions in the laminin B1 chain (Graf, Iwamoto, Sasaki, et al., 1987) and in the LDL receptor, where the EGF repeats have been determined to be essential for the binding of one of two ligands (Davis, Goldstein, Sudhof, et al., 1987).

Previously, genetic analyses have provided evidence for a dose-dependent interaction between the *Notch* and *Delta* proteins (de la Concha, Dietrich, Weigel, et al., 1988). For example, in a background that is wild-type for *Notch,* a 50% reduction in the dose of *Delta* results in a widening of the third wing vein, known as the "delta" wing phenotype. Likewise, in a background that is wild-type for *Delta,* a 50% increase in the dose of *Notch* results in the same "delta" phenotype. Thus, the genetic and *in vitro* analyses have provided complementary support of the interactions between the Notch and Delta proteins.

COMPARISON OF THE APPROACHES

The analysis of mutations with specific CNS phenotypes has led to the characterization of many molecules required for such processes as lateral inhibition and the establishment of specific neural identities. The genetic approach relies on the ability to correlate specific mutant phenotypes with restricted gene expression and/or a defined gene product. In some cases such correlations are difficult, or impossible to make. For example, mutations in the gene *crooked neck (crn)* are associated with specific defects in both the commissural and longitudinal axonal tracts (Zhang, Smouse, and Perrimon, 1991). Both sequence analysis of the *crn* protein and the reduced level of cell proliferation in *crn* mutant embryos suggest that *crn* plays a role in cell proliferation. Thus, specific CNS phenotypes can be generated as a result of disrupting rather general functions.

As illustrated in the case of the *crn* gene, phenotypic criteria alone can be misleading. In other instances where a gene expressed in the nervous system is isolated, it may prove impossible to recover the mutant phenotype one expects. For example, a specific phenotype may not be recovered due to genetic redundancy, as demonstrated in the studies on the *D-abl* gene. Given that null mutations for *D-abl* proved uninformative as to its function in the embryonic CNS, a screen was performed to look for dominant enhancers of the pupal lethal phenotype. Four independent mutations, defining three distinct loci were recovered: *disabled (dab), failed axon connections* (*fac*), and *prospero* (*pros*) (reviewed in Hoffman, 1991). *abl,dab* double mutants are embryonic lethal with severely disrupted axonal tracts, lacking commissural and longitudinal axon bundles. This phenotype is not observed in either single mutation alone. Interestingly, a similar interaction is observed between *abl* and *fas I.* Embryos that are mutant for both *D-abl* and *fas I* have grossly abnormal axon tracts, with little or no axonal outgrowth. The phenotype of this double mutant combination is thought to be

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modified Schneider's *Drosophila* medium. Several elutriated fractions are enriched for specific cell types (e.g., neuroblasts and myoblasts) and the fractions with the largest cells are composed of approximately 70% neuroblasts. A further enrichment of these cell types by preferential adhesion is achieved with specific culture substrates (i.e., glass for neuroblasts and tissue culture plastic for myoblasts). Since tight adhesion to glass is a property inherent to neuroblasts, a further enrichment for neuroblasts was achieved by gently washing away cells remaining nonadherent after 1–2 h in culture. If allowed to develop overnight in culture, these neuroblasts continue to divide with most neuroblasts giving rise to a large number of neurons. Confirmation of neuronal identity was demonstrated both by morphology and by immunoreactivity with HRP antibodies [Fig. 5(D)], which are specific for neuronal cell membranes (Jan and Jan, 1982). The two cultured cell populations, enriched neuroblasts, and differentiated neurons were used as the starting material in our selection of cDNA clones which identify genes expressed in neuroblasts.

Table 1 Cell Number and RNA Yields from Neural-Enriched and Muscle-Enriched Elutriated Fractions

Cell Type	Average Cell Number after Elutriation	Average μg RNA	μg RNA Standardized to 1.9×10^8 Cells	Relative % RNA	Relative Blast Cell Volume
N4	4.3×10^7	62.4 ($n = 15$)	275.7	100	2.1
N18	4.3×10^7	131.2 ($n = 4$)	579.7	210	
M4	1.9×10^8	33.1 ($n = 9$)	33.1	12.0	1
M18	1.9×10^8	2.2 ($n = 2$)	2.2	0.8	

Note: On average, 62.4 μg of total RNA can be recovered from 4.3×10^7 cultured neuroblasts. This same number of neuroblasts, when allowed to differentiate overnight, yields approximately 131.2 μg of total RNA. Enriched myoblast cultures, obtainable from the elutriated fractions with smaller cells, and further enriched by preferential adhesion to tissue culture plastic, routinely yielded one-half the amount of total RNA as neuroblast cultures per elutriation. Methods: RNA was prepared by the guanidinium/cesium chloride method (Maniatis, Fritsch, and Sambrook, 1982). Cells were prepared as described in the legend of Figure 5. N4 and M4 are neuroblasts and myoblasts respectively, that were cultured for 2–4 h. N18 and M18 are neuroblasts and myoblasts respectively, that were cultured for approximately 24 h, and, therefore, represent RNA from differentiated neurons and muscle cells.

due to errors in pathfinding (Elkins, Zinn, McCallister, et al., 1990).

Failure to recover an embryonic CNS mutant phenotype may also be attributed to the fact that a gene is maternally provided. Loci whose zygotic functions are masked by maternally stored gene products cannot be identified in screens for embryonic lethal mutations. The neurogenic loci illustrate the extent of this problem. Perrimon et al. (1989) conducted screens to detect the maternal effects of late zygotic lethal mutations on the X chromosome and identified five new neurogenic loci. By extrapolation, one can assume that there are as many as 35 loci in the *Drosophila* genome associated with a neurogenic phenotype, only a dozen of which have been identified.

Identification by virtue of expression avoids the problems inherent to using a genetic approach. However, in some cases it may be difficult to identify mutations in genes isolated by these alternate approaches, thereby making it difficult to determine the gene function. In addition, the functions of many molecules may be associated with some level of redundancy. Therefore, loss of function mutations in these genes may not affect viability. Recently, novel techniques have been developed that can help identify molecular lesions in functionally redundant genes (Ballinger and Benzer, 1989; Gloor, Nassif, Johnson-Schlitz, et al., 1991). Further attempts to analyze the function of redundant genes could involve double mutant analysis or screens designed to identify interacting loci, as described above in the case of *D-abl*.

PERSPECTIVES

The various approaches discussed in this review have resulted in the identification of molecules that function in the embryonic CNS, and in a general understanding of the basic developmental steps required to build a simple nervous system. However, to more fully elucidate the developmental mechanisms that result in a functional nervous system, we must first define specific genetic interactions between the known components, and, in addition, identify new components. Finally, as models for specific gene functions become more sophisticated, it becomes increasingly important to be able to directly test these models *in vivo*.

Design of Novel Genetic Screens

To date, the isolation of mutations that perturb embryonic CNS development have, in large part, relied upon antibodies to HRP to reveal specific CNS mutant phenotypes. While this has been useful in characterizing mutations that affect the axonal ladder in a gross manner, it is not optimal for the detection of mutations associated with more subtle defects. For example, it does not permit the identification of mutations that affect only a specific neuroblast lineage, a subset of neurons, or specific axonal projections. Therefore, genetic screens need to be performed using more discriminating probes. Such probes would label only defined subsets of CNS cells (e.g., the antibody to the *eve* protein which labels only 30 neurons/segment, Frasch et al., 1987; enhancer trap strains, see Fig. 4) or a subset of axonal tracts (e.g., the *fas II* antibody, Grenningloh et al., 1991).

Genetic Dissection of Signal Transduction Pathways

Many studies on signal transduction pathways operating during embryonic development have re-

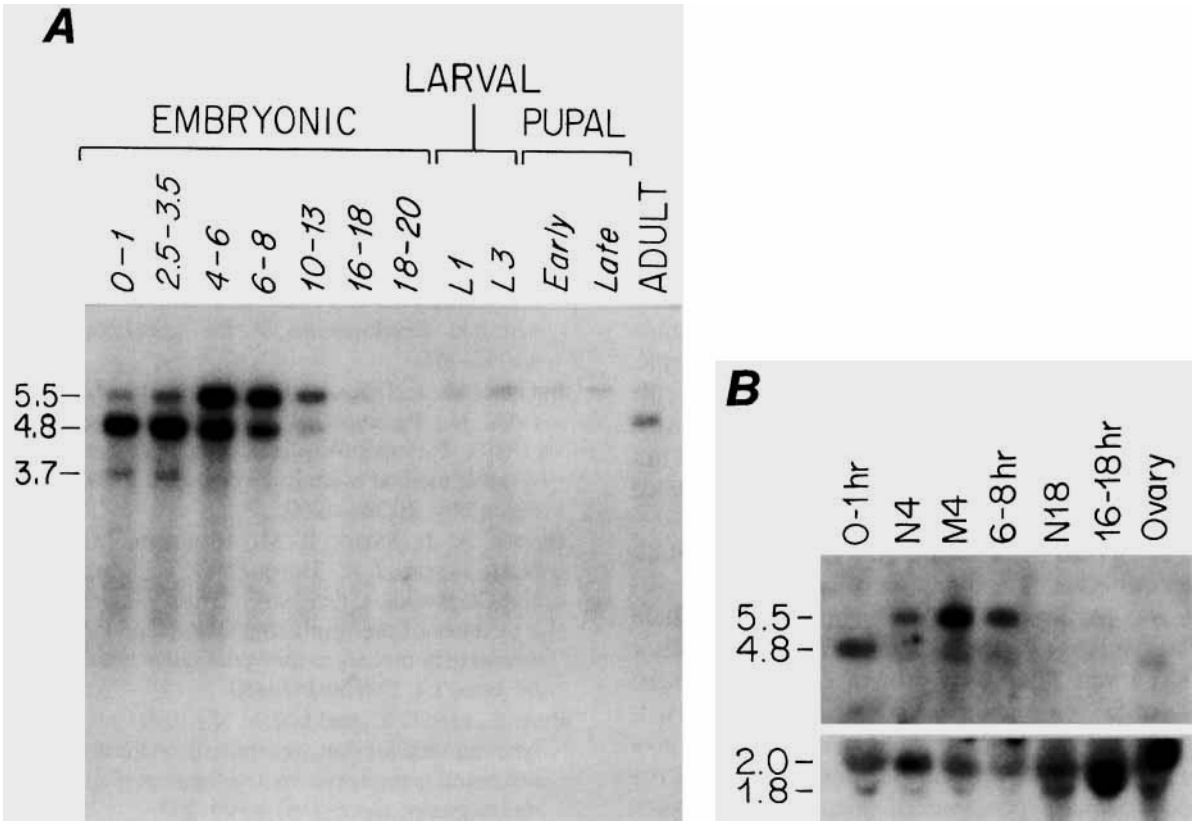

Figure 7 Developmental and tissue-specific RNA analysis of *Delta* expression. (A) Three developmentally regulated transcripts, 5.5, 4.8, and 3.7 kb in size, are detected by cDNA clone 336. The 3.7 kb transcript is most likely maternal in origin. Methods: Ten μg of poly $(A)^+$ RNA, prepared from various developmental stages was loaded in each lane, electrophoresed, transferred to nitrocellulose, and probed with the ^{32}P-labelled cDNA clone 336. The amount of RNA loaded per lane is essentially equal as determined by hybridization with a probe from the *actin 5C* gene (Fyrberg, Mahaffey, Bond, et al., 1983). Lanes are marked according to the specific stage: numbers above lanes refer to hours of development after fertilization; L2 and L3 refer to second and third instar larvae, respectively; early pupae indicates 0–24 h after pupation; late pupae indicates 96–120 h after pupation; and adult RNA is prepared from a mixed population of both males and females. (B) Top of panel shows that *Delta* is expressed early in embryogenesis when it is enriched in neuroblasts (N4) and particularly myoblasts (M4). Later in embryogenesis, expression sharply declines with only faint detection of the larger 5.5 kb transcript in 16- to 18-h embryos and cultured differentiated neurons (N18). Bottom of panel (B) shows as a control for loading the same blot rehybridized with a probe from the *actin-5C* gene (Fyrberg et al., 1983). Ten μg of total RNA, prepared from various elutriated and enriched cell fractions, embryonic developmental stages and ovaries, was loaded in each lane and probed with *Delta* cDNA clone 336. Lanes are marked according to the RNA source: numbers in hours refer to total embryonic RNA from the specified hours of development after fertilization; N4 and M4 refer to cultured and enriched neuroblasts and myoblasts, respectively; N18 refers to cultured differentiated neurons, 24 h in culture; and total ovary RNA was extracted from mature adult females.

lied upon the hypothesis that mutations with similar phenotypes may function in the same developmental pathway (see review by St. Johnston and Nusslein-Volhard, 1992). Extensive genetic and molecular analyses have substantiated this hypothesis. Genetic analyses, such as double mutant combinations and genetic epistasis between mutations with related phenotypes, will help us to understand the signal transduction pathways that operate during CNS development.

A powerful genetic means for identifying new components of specific signal transduction pathways is the ability to isolate second site modifiers (i.e., enhancers and suppressors). Ultimately, this

approach can reveal unsuspected interactions between previously identified genes or even allow the identification of novel molecules involved in signal transduction (see for example, Simon, Bowtell, Dodson, et al., 1991).

In Vivo Assay for Gene Function

As our understanding of the molecules that control specific steps of CNS development increases, it becomes important to test the effect of directed misexpression of these molecules *in vivo*. For example, if, as predicted from mutant analyses, the pair-rule genes, *eve* and *ftz*, control the fates of specific neurons, then ectopic expression of these genes in lineages in which they are not normally expressed may alter their fates.

A method has recently been developed that allows directed misexpression of specific molecules *in vivo* (A. Brand and N. Perrimon, unpublished observations). The yeast transcriptional activator GAL4 was fused downstream of the P-element promoter, producing an enhancer trap construct. This construct expresses GAL-4 under the control of genomic, enhancer-like elements, similar to the site-specific expression of β-galactosidase produced by the *P[LArB]* construct described above. The GAL-4 system also requires a second construct containing GAL-4 binding sites fused upstream of the gene one wishes to misexpress. By simply mating flies that express GAL-4 in an appropriate pattern, to flies which carry the gene of interest downstream of the GAL-4 binding sites, the gene will be misexpressed in all progeny receiving both transposons. In addition, this technique can be used to analyze the developmental consequences of selective ablation of specific neural lineages, by directing the expression of toxic gene products.

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