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Molecular Genetic Analysis of Larval Visual System Development and Programmed Cell Death in *Drosophila*

by

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A. B., Biology
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Submitted to the Department of Biology in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Biology

at the

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ABSTRACT

These studies are focused on the development of a simple neuronal circuit, the larval visual system of *Drosophila melanogaster*. In a systematic screen for mutations which affect the development of the larval visual system, the 75C1,2 region of the *Drosophila* genome was identified to be required for proper projection of the larval optic nerve. In embryos homozygous for deletions of 75C1,2, the larval optic nerve projects to an ectopic position in the embryonic brain.

Genetic analysis of the 75C1,2 genomic region has demonstrated that the *head involution defective (hid)* gene, which maps to this interval, is required for the normal projection of the larval optic nerve. The projection defect observed in *hid* embryos is due to the inappropriate localization of the target regions of the larval optic nerves, the optic lobe primordia. In *hid* embryos, the optic lobe primordia migrate abnormally and fail to incorporate into the embryonic brain. The DNA in the 75C1,2 region was cloned by chromosomal walking, and molecular characterization of the *hid* gene reveals that *hid* encodes a novel protein of 410 amino acids. Expression of *hid* mRNA is associated with all head structures that invaginate, including the optic lobe primordia, suggesting that *hid* is required in these tissues for their morphogenetic movements.

The 75C1,2 region of the genome is also required for programmed cell death in *Drosophila*. 75C1,2 deletions block virtually all programmed cell deaths which occur during embryogenesis. Cell deaths which appear to be ultrastructurally normal can be induced in these deletion mutants; thus, the function deleted by the 75C1,2 deficiencies is required for the initiation, but not the execution of a cell death program. We have identified a gene, *reaper (rpr)*, mapping to 75C1,2, which may fulfill this function. *rpr* mRNA is expressed in dying cells, and *in situ* hybridization experiments indicate that *rpr* expression precedes the first morphological signs of cell death by 1-2 hours. Transgene constructs containing the *rpr* gene can restore cell death to deletion mutants. Furthermore, ectopic expression of *rpr* under the control of the hsp70 heat shock promoter can induce ectopic cell death in both wild type and cell death defective mutants, indicating that *rpr* expression is sufficient for the initiation of cell death. We propose that *rpr* is globally required for the initiation of cell death in *Drosophila*.

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Chapter I
Introduction
A remarkable feature of the nervous system is the precision with which neuronal connections are formed. How does this complex neuronal network develop? There are a number of processes involved in building a nervous system. For example, cells acquire specific identities; neurons extend axons which navigate to and recognize correct synaptic partners; functional connections between neurons are established and maintained. The regulated control of cell number and cell survival is also crucial to the development of the nervous system. In addition to events that occur at a cellular level, nervous system development involves the coordination of events at the level of tissues. Specialized structures in the vertebrate nervous system, such as the hindbrain rhombomeres, arise as a consequence of the concerted movements of groups of cells, highlighting the importance of morphogenetic movements to nervous system development.

While some of the steps mentioned above, such as axonal outgrowth and pathfinding, are specific to nervous system development, others, such as the establishment of cellular identity and programmed cell death are re-iterated again and again throughout the development of the organism. Thus, by studying the nervous system, one may gain insight into the general mechanisms of metazoan development.

In this introductory chapter, I review some of the studies which have contributed to our understanding of nervous system development. I begin with the determination of cellular identity, and issues of axonal pathfinding. I then discuss programmed cell death and its general role in development. Finally, I introduce the larval visual system of Drosophila, which is used as a model system for much of the work presented in this thesis.

**Determination of neuronal identity**

One of the earliest steps in the patterning of a nervous system is the determination of neuronal identity. Cells must first acquire the ability to become neuronal, and then undergo further specification into neuronal subtypes. Questions of neuronal cell fate have been addressed in a number of systems, both invertebrate and vertebrate, and have revealed a number of different mechanisms by which neuronal identity is established.

The nematode Caenorhabditis elegans, is particularly well suited to a genetic analysis of cell fate. The ability to recognize individual cells in living animals has allowed the reconstruction of the entire lineage history of the
somatic cells in the adult hermaphrodite (Sulston and Horvitz, 1977; Kimble and Hirsh, 1981; Sulston et al., 1983). Such studies have revealed that each neuronal precursor undergoes a stereotyped sequence of cell divisions, and this pattern is invariant from animal to animal. This suggests that cell fate determination in the worm can be driven by the lineage history of a particular cell.

Genetic analysis has allowed the identification of *C. elegans* genes which are crucial for determining cell fate and have established the link between cell lineage and cell identity in this organism. For example, the gene *unc-86* is required for the specification of certain neurons, and in *unc-86* mutants, one finds transformations of certain cell fates (Finney et al., 1988). Finney and Ruvkun (1990) have shown that UNC-86 protein is found in cells transformed by *unc-86* mutations, suggesting that expression of UNC-86 protein is lineage dependent. *unc-86* encodes a pou domain protein which presumably regulates the transcription of factors important for the control of cellular differentiation (Finney et al., 1988).

It is clear, however, that intercellular interactions also play an important role in the development of *C. elegans*. For example, local cellular interactions are crucial for determining which cells, among a number of equivalent cells, will be induced to form the vulva (reviewed in Greenwald and Rubin, 1992). Another example of the role of cellular interactions in cell fate specification is the establishment of the cellular identity of two gonadal precursors, the anchor cell (AC) and a ventral uterine precursor cell (VU). These cells are derived from the cells Z1.ppp and Z4.aaa, and through a stochastic process, one becomes VU and the other AC (Kimble and Hirsh, 1981). When either Z1.ppp or Z4.aaa is ablated, the other always becomes AC (Kimble, 1981), demonstrating the importance for intercellular signalling in determining cell fate. Mutations in the gene *lin-12* affect this choice; gain of function *lin-12* mutations result in two VU cells, loss of function, two AC's (Greenwald et al., 1983). Mosaic analysis has shown that *lin-12* is autonomously required for VU determination (Seydoux and Greenwald, 1989). *lin-12* encodes a transmembrane protein, hypothesized to be the receptor for a signal determining VU cell fate (Greenwald, 1985). *lin-12* shows structural similarity to the *Drosophila* Notch protein, (Yochem et al., 1988), which is known to be required in the cellular interactions leading to neuronal fate determination in the fly (see below).
Studies of neuronal determination in *Drosophila* have demonstrated the importance of intercellular interactions for fate determination both at the level of neuronal versus non-neuronal decisions, and for the further specialization of neuronal identity.

The neurogenic genes of *Drosophila* are involved in the decision to become either neuronal or epidermal (Lehmann et al., 1983). In embryos homozygous for loss of function mutations in the neurogenic genes, there is hypertrophy of the nervous system and the reciprocal loss of the epidermis (reviewed in Campos-Ortega and Jan, 1991; Jan and Jan, 1992). Some of these proteins have been demonstrated to physically interact, at least in vitro. For example, Notch interacts with Delta in tissue culture aggregation experiments (Fehon et al., 1990) and mosaic analysis suggests that the relationship between Notch and Delta may be that of receptor and ligand (Heitzler and Simpson, 1991). In this study, Notch was shown to be cell autonomously required for epidermal fate. A cell must be Notch$^+$ in order to become epidermal. Delta, on the other hand, functions nonautonomously. Cells mutant for Delta can form epidermis. Thus, Delta may be a ligand for a receptor, presumably Notch (Heitzler and Simpson, 1991). Both Notch (Wharton et al., 1985) and Delta (Vaessin et al., 1987) encode transmembrane proteins. Although it may seem surprising that a nonautonomous "ligand" is a transmembrane protein, this is not without precedent. Another *Drosophila* transmembrane protein, encoded by the bride of sevenless gene, has been proposed to be the ligand for the Sevenless receptor (see below).

Neuronal specification has also been extensively studied in the compound eye of *Drosophila*. The adult compound eye is composed of a limited number of well described and easily identified cell types which are organized into repeating units called ommatidia. Ommatidial assembly has been studied in detail (Tomlinson and Ready, 1987). It has been demonstrated that there are no obligate lineage relationships among cells of a particular type or within an ommatidium (Ready et al., 1976). This suggests a role for intercellular interactions in the determination of neuronal cell fate.

The differentiation of a class of photoreceptor neuron, the R7 cell, has been particularly well characterized (reviewed in Greenwald and Rubin, 1992; Hafen and Basler, 1991). Mutations in the sevenless (sev) gene result in transformation of the cell normally fated to become R7 into a non-neuronal cone cell (Tomlinson and Ready, 1986). Mutations in another gene, bride of
sevenless (boss) also result in the loss of R7 (Reinke and Zipursky, 1988). Mosaic analysis has demonstrated that sev is required cell autonomously in R7 (Tomlinson and Ready, 1987), while boss acts non-autonomously (Reinke and Zipursky, 1988). Analogous to the actions of Notch and Delta, Boss is believed to be the ligand for the Sevenless receptor (Krämer et al., 1991). At the molecular level, sev encodes a receptor tyrosine kinase (Hafen et al., 1987) and boss a protein with seven transmembrane spanning domains (Hart et al., 1990). Boss protein has been demonstrated to be internalized in the R7 cell in a sev dependent manner (Krämer, et al., 1991), further supporting its role as the Sevenless ligand. A number of molecular players in the signal transduction pathway acting downstream of boss and sev have also been identified (see for example, Simon et al., 1991; Gaul et al., 1992).

Cellular interactions are also crucial for the development of vertebrate nervous systems. It has long been known that formation of neural tissue is dependent on inductive interactions and that regional interactions may be responsible for further neuronal patterning (reviewed in McMahon, 1993). Much effort has been put into identifying genes responsible for neuronal patterning. The gene, noggin, was identified by expression cloning for its ability to induce dorsal development in ventralized Xenopus embryos (Smith and Harland, 1992), and recent experiments suggest that noggin may be the long sought endogenous neural inducer (Lamb et al., 1993).

Patterning in the neural tube has been shown to require both cellular contacts and secreted signals. The floorplate is a ventro-medial group of cells in the neural tube and motor neurons are derived more laterally. The induction of floorplate from neural tube has been shown to require direct contact between notochord and neural plate (Placzek et al., 1993); however, motor neuron differentiation can be induced by a diffusible factor present in media conditioned by notochord or floorplate cells (Yamada et al., 1993). This demonstrates that multiple mechanisms are likely to be important for determining cellular identity in the vertebrate nervous system.

**Axonal pathfinding and target recognition**

In addition to neuronal specification, neurons need to be able to find and recognize their correct synaptic partners. This may involve extensive cell migration as well as axonal elongation and pathfinding. How is this
accomplished? A number of models have been put forth to account for the remarkable ability of axons to precisely navigate to their targets.

In the 1940's, Weiss put forth the resonance hypothesis. In this view, neuronal specificity was dependent on activity patterns of axons. Weiss held that targets would selectively respond to those axons that displayed correct activity patterns. Although some aspects of neuronal development clearly are activity dependent, a high degree of neuronal specificity can be achieved even in the presence of activity blockers (reviewed in Goodman and Shatz, 1993).

Other models for axonal pathfinding rely on biochemical cues to act as guidance molecules. In the 1960's, based on his work in the retinotectal systems of amphibians and fish, Sperry put forth the chemoaffinity hypothesis (Sperry, 1963). He proposed that there are intrinsic, position dependent differences of a biochemical nature between neurons. Specificity would arise as the result of selective interactions between pre- and post-synaptic cells with the same chemical address. An extreme interpretation of Sperry's model is that individual cells in the retina are uniquely specified and precisely targeted to particular cells in the tectum. This, however, would necessitate a prohibitive number of biochemical markers. Furthermore, individual retinal cells are not rigidly matched with individual tectal cells, as is evidenced, for example, by the continual rearrangement of retinotectal connections throughout the life of the goldfish (reviewed in Easter, 1983). There are, however, clear preferences of axons for their targets, supporting the notion that position specific differences, perhaps in the form of gradients, are likely to be important in nervous system development.

A large number of mechanisms, some of which can be viewed as variations on the chemoaffinity hypothesis, have been proposed to guide axons (reviewed in Goodman and Schatz, 1993). For example, gradients of diffusible chemical signals may chemotropically guide axons to their targets. Interactions with specific "guide post cells," as well as selective fasciculation with axon bundles may serve to orient elongating axons. In addition it is also possible that axons are guided mechanically by the topology of the tissue through which they migrate. Some of these examples are discussed below.

As mentioned above, pre-existing axonal scaffolds may serve as guidance cues for later growing axons (discussed below). However, a fundamental question of neuronal development is, how do the initial axons
reach their targets? A nerve which grows to its targets without following a previously existing axon has been termed a "pioneer" (Harrison, 1910) and is believed to define the routes along which later developing axons migrate (reviewed in Bentley and Keshishian, 1982b). Pioneer neurons find their targets early in development when distances are very small, circumventing problems of long range navigation (see for example, Bate, 1976). Pioneers have been characterized in a number of invertebrate (Bate, 1976; Jacobs and Goodman, 1989; Tix et al., 1989) and vertebrate (McConnell et al., 1989; Stainier and Gilbert, 1990) species. Often these pioneers are transient in nature (Kuwada, 1986; Klose and Bentley, 1989; McConnell, et al., 1989), and are eliminated after fulfilling their proposed guidance functions.

Despite the fact that pioneers generally don't navigate over long distances, they must still be able to correctly choose their routes. In the grasshopper limb, pioneer neurons rely on specific cellular interactions for pathfinding (as proposed by Bate, 1976). Here, the pioneering peripheral nerve fibers specifically interact with three immature neurons (Bentley and Keshishian, 1982a; Ho and Goodman, 1982). Dye coupling between these cells and the pioneer nerve supports the notion that intercellular communication takes place (Bentley and Keshishian, 1982a). Ablation of one pair of these so-called "guidepost cells" results in the failure of the pioneer neuron to find its targets (Bentley and Caudy, 1983). In the *Drosophila* central nervous system, a scaffold of glial cells is believe to be the template for pioneer axon migration (Jacobs and Goodman, 1989). Candidates for guide post cells have been identified in other systems, yet their requirement for axon guidance has yet to be established (reviewed in Palka et al., 1992).

Although clearly in a position to act as guidance cues, are the pioneer neurons really required for axon guidance? In the grasshopper, elimination of pioneer neurons in the periphery does result in the failure of sensory neurons to reach the central nervous system (Klose and Bentley, 1989) demonstrating the importance of pioneers for accurate pathfinding in this system. In other cases, however, pioneers do not seem to be essential for subsequent axonal targeting (Bentley and Keshishian, 1982a; Kuwada, 1986) For example, ablation of pioneer motorneurons in the zebrafish embryo does not prevent later axons from reaching their targets (Eisen, 1989).

For pioneers to act as templates for subsequent axon ingrowth there must exist mechanisms by which they are recognized. The labeled pathways
hypothesis (Ghysen, 1978; Raper et al., 1983), suggests that axons are
differentially labeled, and that growth cones can recognize and follow these
cues. This proposal is based on observations of neuronal development in
grasshoppers. Here, ingrowing axons selectively fasciculate with certain
groups of axons while ignoring others in the vicinity (Raper et al., 1983).

In support of the labeled pathways hypothesis is the identification of a
number of molecules which are expressed on certain portions of axons or
subsets of groups of axons (reviewed by Harrelson and Goodman, 1988).
Many investigators have used monoclonal antibodies to identify molecules
with restricted spatial or temporal expression patterns in the nervous system.
In vertebrate systems, a number of such molecules have been identified,
including TOP (Trisler et al., 1981), the Jones antigen (Constantine-Paton et
al., 1986), and ROCA (Suzue et al., 1990). Because of their differential patterns
of expression, it seems possible that these molecules are acting to guide axons,
however, it has been difficult to demonstrate the functional relevance of any
of the above for neuronal development.

Some of the best evidence for the labeled pathways hypothesis comes
from studies in invertebrates. Working initially in grasshoppers, Goodman
and his colleagues generated a number of monoclonal antibodies which
recognize specific axon fascicles in the embryonic central nervous system
(CNS). The antibodies were then used to purify the antigens, fasciclins I and
II (Bastiani et al., 1987). Since then, additional proteins with intriguing
patterns of distribution have been identified both in grasshopper and
Drosophila. fasciclins I, II, III, and neuroglian have been characterized and
cloned in grasshopper and Drosophila (Bieber et al., 1989; Grenningloh et al.,
1991; Harrelson and Goodman, 1988; Patel et al., 1987; Snow et al., 1989; Snow
et al., 1988; Zinn et al., 1988) and recently, fasciclin IV has been cloned from
grasshopper (Kolodkin et al., 1992).

Because many of these genes have been identified in Drosophila, it has
been possible to isolate mutations and assess what role they play during
neuronal development. Interestingly, mutations in these genes do not
grossly perturb nervous system development. Mutations in fasciclin I have
no detectable CNS phenotype (Elkins et al., 1990). Null mutations in
neuroglian, while lethal, have no striking neuronal defects (Bieber et al.,
1989); however, occasionally, the chordotonal organs in the peripheral
nervous system (PNS) of neuroglian mutants are abnormally arranged
(Goodman et al., 1992). Fasciclin II mutants also have no striking phenotype, but a subtle CNS phenotype has been reported (Grenningloh et al., 1991).

The lack of gross defects in these mutants despite striking patterns of gene expression suggests that if these molecules are involved in axon guidance, there must be a large degree of functional redundancy in this system. In support of this notion, mutations in fasciclin I interact with mutations in the Drosophila Abelson proto-oncogene (abl). ABL is a tyrosine kinase which is expressed in the axons of the central nervous system during embryonic development (Gertler et al., 1989). Like fasciclin I, mutations in ABL do not result in striking embryonic central nervous system defects (Gertler et al., 1989). Fasciclin I ABL double mutants, however, have severely disrupted nervous systems (Elkins et al., 1990), suggesting that these genes are required for nervous system development and act in pathways which are at least partially redundant.

An interesting feature of a number of these molecules is that they share structural motifs with molecules implicated in vertebrate neuronal development. For example, fasciclin IV (Kolodkin et al., 1992) is similar to collapsin (Luo et al., 1993; discussed below). Fasciclin II (Grenningloh et al., 1991), fasciclin III (Snow et al., 1989), and neuroglian (Bieber et al., 1989) are all members of the immunoglobulin (Ig) super family. Molecules with Ig like domains have been implicated in cell adhesion in both vertebrate and invertebrate development. Because differential cell adhesion has been proposed to be a mechanism for axon guidance (see, for example Letourneau, 1975a; Letourneau, 1975b), much attention has been focused on the identification of molecules which can mediate cell adhesion.

Neural cell adhesion molecule, or N-CAM was the first adhesion molecule with Ig like domains to be described (Thiery et al., 1977; Cunningham et al., 1987). First identified in the chick, it is an integral membrane glycoprotein which is widely expressed in the neural ectoderm. Because of its widespread expression, it is unlikely to be a true guidance molecule. However, N-CAM mRNA is alternately spliced (Cunningham et al., 1987) and there is heterogeneity in its sugar moiety (Rothbard et al., 1982), which may confer some degree of regional specificity to N-CAM throughout the nervous system.

In addition to N-CAM and the Drosophila proteins, a fair number of neuronal adhesion molecules with Ig like domains have been identified (e.g.,
MAG (Johnson et al., 1989), L1/NILE (Lagenauer and Lemmon, 1987), contactin (Ranscht, 1988), and TAG-1 (Furley et al., 1990)). In general, Ig like domains are believed to mediate homophilic adhesion. This has been demonstrated for some of these molecules (e.g., Fasciclin III (Snow et al., 1989), Fasciclin II, Neuroglian (Grenningloh et al., 1990) and N-CAM). In other cases, molecules with Ig like domains, such as some present during endothelial development, are involved in heterophilic adhesive interactions (reviewed in Hynes and Lander, 1992).

The cadherins typify another class of adhesion molecules (see Takeichi, 1990) They can mediate homophilic adhesion and are widely expressed throughout development. The adhesive properties of these molecules are entirely calcium dependent. N-cadherin is the predominant cadherin expressed in the nervous system and has been shown to support neuronal survival (reviewed in Takeichi et al., 1990). Again because of its widespread distribution, N-cadherin is unlikely to be a guidance molecule.

Studies of nervous system development have also focused on extracellular matrix proteins, such as fibronectins and laminins. Genetic analysis in C. elegans has revealed the importance of the extracellular matrix for axonal migration. Mutations in the genes unc-5, unc-6, and unc-40 disrupt circumferential migrations of pioneer axons and mesodermal cells. unc-5 affects dorsally directed growth, unc-40 affects ventrally directed growth, and unc-6 affects both dorsally and ventrally directed growth (Hedgecock et al., 1990). The gene unc-6 has been cloned and encodes a laminin related protein (Ishii et al., 1992). unc-5 encodes a transmembrane protein with immunoglobulin and thrombospondin type 1 domains and is required cell-autonomously in neurons and migrating cells (Leung-Hagesteijn et al., 1992). unc-5 protein may serve as a transmembrane receptor for unc-6 protein to direct dorsal migration.

In vertebrate systems, extracellular matrix proteins have been shown to support neuronal survival and mediate cell adhesion (reviewed in Lander, 1987). While it is tempting to speculate that differential adhesive properties could be a driving force in axon guidance, in some situations, extracellular matrix molecules such as thrombospondin and laminin exhibit anti-adhesive properties (reviewed in Chiquet-Ehrismann, 1991). Furthermore, the propensity of these substrates to promote neuronal migration is not always correlated with their adhesive properties. Some anti-adhesive substrates can
be excellent substrates for neuronal migration (Calof and Lander, 1991). Clearly other mechanisms must be involved in guiding axons towards their targets.

Repulsive interactions may be important for guiding axons. Axons in a number of different systems have been shown to avoid certain substrates or other populations of axons (e.g., Kapfhammer and Raper, 1987; Davies et al., 1990; Moorman and Hume, 1990). Recently one such repulsive guidance cue has been molecularly identified (Luo et al., 1993). Named collapsin for its ability to induce growth cone collapse in vitro, it has a number of features common to adhesion molecules. Its C-terminal region contains an Ig like domain and its N-terminus is similar to fasciclin IV. The collapsing activity of this protein is specific to certain populations of neurons. Sensory growth cones from the dorsal root ganglion collapse upon exposure, whereas retinal ganglion cells are relatively insensitive to this protein.

What is clear from all of these studies is that the nervous system employs several mechanisms to insure specificity of neuronal connections and accurate patterning. Furthermore, it is also clear than any single approach is likely to be unsuccessful in completely elucidating these mechanisms. Purely biochemical studies, such as the identification of molecules with restricted localization, are limited when it comes to determining the function of such molecules. Likewise, purely genetic approaches can be hindered when there is functional redundancy in a system, or if molecules fulfill several different functions throughout development. For example, several genes involved in early embryonic patterning in Drosophila also have roles in nervous system development which were initially masked by the patterning defects observed in embryos mutant at these loci (Patel et al., 1989; Chu-LaGraff and Doe, 1993). Clearly, a variety of strategies is needed to approach questions of axon guidance.

Cell death and neuronal development

One event crucial to the development of a precisely patterned nervous system is to match the number of pre- and post-synaptic elements which participate in functional networks. While it has been demonstrated that neurons, in some cases, actually induce the proliferation of their target cells (Baptista et al., 1990; Selleck and Steller, 1991), it is not clear how general a mechanism this is for the regulation of cell number in the nervous system.
During vertebrate development, a large excess of neurons is generated, and cell death is believed to be critical for the establishment of specific neuronal connections (reviewed in Oppenheim, 1991).

Much of the current thinking about neuronal cell death has been interpreted in the context of the neurotrophic theory of cell survival. In this model, neurons depend on factors provided by their targets for survival. Neurons for a particular target compete with each other for these target derived survival factors. Those that fail to successfully compete die. Evidence for neuronal competition for target factors is fairly broad. Augmenting (Hollyday and Hamburger, 1976) or reducing (O'Leary and Cowan, 1984) a target field either increases or reduces the number of input neurons which survive, respectively. Likewise, decreasing the number of ingrowing neurons for a particular target increases the relative survival of remaining neurons (Pilar et al., 1980).

The neurotrophic theory provides an explanation for how the number of neurons is matched to the number of target cells. Furthermore, this model does not require that the number of cells which participate in functional circuits be precisely predetermined. A number of "size matching" experiments have been carried out in attempts to prove that this is so. By increasing or reducing the size of the target field (and thereby the availability of a trophic factor), one expects to find a proportional increase or reduction in the amount of cell death among ingrowing neurons. While in many instances such experiments do demonstrate that the relationship between axon number and target size appears to be roughly proportional, this is not uniformly true (reviewed in Oppenheim, 1991). Such discrepancies do not discredit the belief that target interactions are important for neuronal survival, but do suggest that other mechanisms of regulating cell number are likely to be important as well (e.g., see Baptista et al., 1990; Selleck and Steller, 1991). Neurotrophism also provides a way for insuring that inappropriate connections are eliminated. Assuming that different targets provide distinct survival factors, and that ingrowing neurons are selectively responsive only to particular factors, neurons which grow to the wrong target will be not be sustained.

Much of the support for the neurotrophic theory has come from studies of nerve growth factor (NGF). NGF has been shown to be secreted from certain target cell populations (Davies et al., 1987) and can be taken up by
ingrowing neurons. NGF has neurotrophic effects both in vitro and in vivo. If NGF blocking antibodies are injected into an animal, most NGF responsive neurons die (Gorin and Johnson, 1979). Likewise, artificially elevating NGF concentrations prevents much neuronal cell death (Hamburger et al., 1981). Only certain populations of neurons, primarily sympathetic and sensory neurons are NGF responsive, providing some degree of specificity for this neurotrophic factor. The search for other target derived factors has revealed that NGF is a member of a family of neurotrophic molecules which includes brain derived neurotrophic factor (BDNF; Leibrock et al., 1989), and neurotrophin-3 (NT-3; Maisonpierre et al., 1990). Both of these factors can promote neuronal survival in vitro (Barde, 1989; Thoenen, 1991; Glass and Yancopoulos, 1993), and the distribution of all three factors in vivo suggests they promote the survival of different, yet sometimes overlapping, populations of neurons. The combinatorial action of these factors may be important for survival in some cases.

How is the presence of a trophic factor detected? In the case of NGF, there are at least two cellular receptors for this molecule, TrkA, the high affinity NGF receptor, and p75NGFR, the low affinity receptor (Hempstead et al., 1991). TrkA is believed to be functionally important for transducing NGF mediated survival and differentiation signals. Recent reports have suggested that p75NGFR may have a role in apoptosis (Rabizadeh et al., 1993). Cells in culture were transfected with p75NGFR. Expression of this construct had no effect on cells grown in the presence of serum. Withdrawal of serum led to enhancement of cell death of the p75NGFR expressing cells as compared to controls, and this death was blocked by adding NGF back to the media. This has led to the suggestion that those cells which express p75NGFR will die if the requisite growth factor is not present; however, it remains to be demonstrated that this is the role of p75NGFR in vivo. The death of cells following reductions in growth factor levels is a theme which is re-iterated in a number instances (see below).

Competition for survival signals is not restricted just to neurons. It has recently been reported that approximately 50% of oligodendrocytes in the rat optic nerve die during development (Barres et al., 1992). Artificially elevating the concentration of certain growth factors increased oligodendrocyte survival, suggesting that these cells normally compete for limited quantities of survival signals.
It is clear that neurotrophic factors are not the only factors which are required for neuronal survival. Electrical activity, afferent inputs, interactions with glial cells and extracellular matrix molecules may act to prevent cell death (reviewed by Oppenheim, 1991).

**Cell death outside the nervous system**

The regulation of cell number is generally required for the development of an organism and is not a problem specific to the nervous system. Cell death has been found to be involved in a number of other processes. For example, cell death is required during morphogenesis to eliminate interdigital webbing (Saunders and Fallon, 1967), and may be important for the elimination of ancestral structures (e.g., the human tail, discussed in Oppenheim, 1991). Cell death is also utilized to create sexual dimorphism in a wide range of organisms, from worms to man. In worms, HSN neurons which are necessary for egg laying in hermaphrodites degenerate in males (Sulston et al., 1983). Likewise, elimination of Müllarian ducts in male vertebrates occurs via cell death (Jost, 1971). The metamorphosis of insects and amphibians also involves extensive cell death and is often hormonally controlled (Tata, 1993; Truman et al., 1993).

Cell death is also required for the development and functioning of the immune system (reviewed Cohen and Duke, 1992). The primary immune response cells, B cells and T cells must be equipped to recognize a tremendous number of foreign antigens. Rearrangement of Ig genes in B cells and T cell receptor (TCR) genes in T cells creates the enormous diversity of molecules necessary to mount an effective immune response. However, it is not enough to be able to recognize foreign antigens; the immune system must also be able to distinguish these antigens from self antigens. Some rearrangements of Ig genes and TCR genes are likely to recognize host antigens and could potentially cause autoimmune disease, and selective cell elimination may have a role in its prevention.

Cell death has been proposed to be involved in the elimination of self-reactive lymphocytes. Studies using transgenic mice have been particularly useful for the investigation of immune tolerance. For example, mice engineered to express Ig's recognizing either hen egg lysozyme (HEL) or a histocompatibility molecule, H-2K\(^k\) only maintain these B-cells if the corresponding antigens are not systemically expressed (Hartley et al., 1991).
Recent work suggests that elimination of these cells involves two steps, arrested development, followed by cell death (Hartley et al., 1993). The authors propose that B cells which recognize "self" antigens undergo a reversible developmental arrest. Cell death then insures that these cells do not escape this arrest and produce auto-antibodies. A prediction of this model is that artificially prolonging the life of self-reactive B cells will increase the chances of escape from developmental arrest and subsequent autoimmune disease. In fact, constitutive expression of the gene bcl-2 (see below) in B cells can significantly extend their life span, and old bcl-2 transgenic mice ultimately succumb from a lupus-like syndrome (Strasser et al., 1991).

Cell death is also important for thymocyte development. T cell receptors recognize antigen in association with major histocompatibility proteins (MHC) on the surfaces of other cells. T cell receptors must first be positively selected for the ability to recognize self MHC proteins and then negatively selected against recognition of other self-antigens (reviewed by Blackman et al., 1990). Clonal deletion is believed to play a significant role in the negative selection of T cells (reviewed by Ramsdell and Fowlkes, 1990), and evidence, again from transgenic studies, suggests that clonal deletion of T cells is apoptotic. In mice engineered to express a self-reactive TCR, there is massive thymocytic cell death upon presentation of the relevant antigen (Murphy et al., 1990).

While cell death is clearly important during the maturation of B and T cells to prevent autoimmunity, cell death is also important in eliminating cells following an immune response. During an immune response, the levels of T cells which recognize a particular antigen increase. These cells are induced to express high levels of interleukin-2 (IL-2) receptor and are dependent on IL-2 for their survival (Gillis et al., 1978). Following an immune incident, IL-2 levels drop. Analogous to the death of p75NGFR expressing neurons in the absence of NGF, T cells expressing high levels of IL-2 receptor undergo cell death in vitro in the absence of IL-2 (Bishop et al., 1985; Cohen et al., 1985; Duke and Cohen, 1986).

Are these just specialized examples or is cell death more generally required by the organism throughout development? It is clear that cell death is more widespread than previously thought. Significant amounts of cell death have now been identified in tissues such as the kidney (Coles et al., 1993) where it was previously thought not to occur. Why has this cell death
only recently been discovered? Despite the fact that a substantial amount of cell death occurs throughout development, at any one point in time, only a few dying cells are visible histologically. This is because apoptotic cells are rapidly phagocytosed and no longer recognizable by light microscopy. Thus, cell death may be occurring in many tissues where it was previously undetected, and its extent is probably grossly underestimated (Raff, 1992).

The widespread occurrence of cell death throughout development has led to the proposal that programmed cell death might occur by default unless cells receive specific survival signals (Raff, 1992). The "social control of cell death" theory proposes that all cells are poised on the brink of suicide and are capable of executing a cell death program. Raff suggests that all mammalian cells, with the possible exception of blastomeres, require exogenous signals to survive (Raff et al., 1993).

"Natural" Cell Death is a Gene Directed Process

It is clear that cell death is a nearly ubiquitous feature of the development and homeostasis of a metazoan organism (reviewed by Ellis et al., 1991; ffrench-Constant, 1992; Raff et al., 1993). Furthermore, cell deaths which occur as part of a developmental program generally occur with an ultrastructural morphology which is distinct from the cell deaths that result from cellular injury (Wyllie et al., 1980). The latter generally occur via a process known as necrosis. Cells undergoing necrotic death swell and burst, and the extracellular release of cytoplasm often causes a marked inflammatory response. "Natural" cell death, as opposed to pathological cell death, often occurs with a characteristic ultrastructural morphology known as apoptosis. Apoptotic cell deaths are characterized by compaction of the cytoplasm, nuclear condensation and fragmentation, and often, degradation of chromatin into nucleosomal ladders. Apoptotic cell corpses are rapidly engulfed by phagocytic cells and such cell deaths do not invoke an inflammatory response (Wyllie et al., 1980). "Natural" cell death is often referred to as "programmed cell death," reflecting the belief that this is a gene directed process.

The best evidence that programmed cell death is under genetic control comes from the genetic studies in the nematode, *Caenorhabditis elegans*. In *C. elegans*, 131 somatic cells undergo programmed cell death. These deaths occur in a stereotyped fashion in each animal with features that generally
resemble that of apoptosis (reviewed in Ellis et al., 1991). Because one can identify individual dying cells in C. elegans, it has been possible to screen for mutations in which these cell deaths do not occur. Such screens have led to the identification of a number of "cell death genes" and have allowed the elucidation of a genetic cell death pathway. Two genes, ced-3 and ced-4 have been identified which are required for the initiation of programmed cell death. The function of both of these genes is necessary for the execution of the cell death pathway. In animals bearing loss of function mutations in either of these genes, these cell deaths do not occur (Ellis and Horvitz, 1986). Mosaic analysis indicates that these genes are autonomously required in cells which will die (Yuan and Horvitz, 1990).

Another gene crucial to the cell death process which has been identified in worms is ced-9 (Hengartner et al., 1992). Where ced-3 and ced-4 are critical for carrying out cell death, ced-9 appears to be crucial for preventing it. Gain of function ced-9 mutations, like loss of function ced-3 and ced-4 mutations block cell deaths in the animal. Loss of function ced-9 mutations have the opposite effect. They are lethal to the organism apparently because cells in the organism are undergoing ectopic cell death. This cell death is dependent on the actions of ced-3 and ced-4.

While there is clearly a genetic cell death program in worms, is this true in other organisms? Oncogenes have been generally implicated in uncontrolled cellular proliferation which can lead to cancer. Because the control of cell number is often achieved via programmed cell death, a number of investigators have recently considered the role that proto-oncogenes might have in the cell death process.

Expression of the c-fos proto-oncogene, as assayed by beta-galactosidase expression from a fos-lacZ fusion in transgenic mice, is found in a number of tissues which undergo cell death during development (Smeyne et al., 1993). Furthermore, expression of the transgene could also be found in cells in which cell death was induced, either pharmacologically or genetically. A functional role for c-fos in cell death, however, has yet to be demonstrated.

Another proto-oncogene, c-myc is also involved in cell death. Although generally implicated in the control of cell proliferation, c-myc induces apoptosis in Rat-1 fibroblasts grown in low, but not high levels of serum (Evan et al., 1992). This has led to the proposal of a "two signal" model whereby c-myc will induce either apoptosis or cell proliferation depending
upon what other cellular signals (e.g., growth factors) are present (Bissonnette et al., 1992). \textit{c-myc} is not able to induce apoptosis in all cell types, suggesting that such signals may be cell type specific.

Much attention has also been focused on the role of the tumor suppressor, p53, in apoptosis. Increased levels of p53 protein have been linked to cell cycle arrest following irradiation, and p53 has been proposed to be required for genome maintenance (Kastan et al., 1992). Under some circumstances, however, increased p53 levels are associated with apoptosis. For example, forced over-expression of p53 in a myeloid cell line can induce apoptosis (Yonish-Rouach et al., 1991) and apoptosis associated with expression of adenovirus 5 E1A protein is accompanied by stabilization of p53 protein (Lowe and Ruley, 1993). p53 has been demonstrated to be required for certain forms of apoptosis (Clark et al., 1993; Lowe et al., 1993a), and the responsiveness of cancer cells to various chemotherapeutic agents is likely to be p53 dependent (Lowe et al., 1993b).

While genes such as p53 and \textit{c-myc} appear to be necessary for the induction of a cell death program under some circumstances, several genes have been identified which are required to suppress that program. One example is proto-oncogene, \textit{bcl-2}. Expression of \textit{bcl-2} has been shown to block programmed cell death under a number of circumstances. For example, \textit{bcl-2} can suppress cell death which results from growth factor withdrawal both in lymphocytes (Nunez et al., 1990) and neurons (Allsopp et al., 1993). \textit{bcl-2} can also suppress cell death induced by the adenovirus E1A protein (Rao et al., 1992) and over-expression of \textit{c-myc} (Bissonnette et al., 1992).

Both genetic and biochemical studies have led to rapid advances in our understanding of how Bcl-2 functions. Gene targeting has been used to induce \textit{bcl-2} mutations (Nakayama et al., 1993; Veis et al., 1993). Studies of the immune system in chimeric mutant mice show that \textit{bcl-2} is required not for lymphocyte maturation, but for lymphocyte maintenance (Nakayama et al., 1993). This result was confirmed in mice homozygous for a similar mutation (Veis et al., 1993). Massive apoptosis in both the thymus and the spleen was found in the \textit{bcl 2-/-} mice, and apoptosis was accelerated in \textit{bcl-2 -/-} thymocytes following γ-irradiation.

Biochemical studies have shown that Bcl-2 may suppress apoptosis by functioning in an anti-oxidant pathway (Hockenbery et al., 1993). Supporting this conclusion, at least two of the pathologies identified in the \textit{bcl-2} mutant
mice, hair hypopigmentation and polycystic kidney disease could be due to defects in redox metabolism (Veis et al., 1993).

Recent studies have also led to the identification of two Bcl-2 related proteins which also have a role in cell death. Bax (Oltvai et al., 1993) was identified as a 21 kd protein which heterodimerizes with Bcl-2 in vivo. Alternative splicing results in a number of different forms of this protein. Remarkably, Bax is similar to Bcl-2, but unlike Bcl-2, Bax does not function to suppress cell death. Instead, overexpression of Bax accelerates cell death, and it appears that the relative ratios of Bax to Bcl-2 affect the rate of programmed cell death in vitro. Another bcl-2 related gene, bcl-x (Boise et al., 1993) was isolated via low stringency hybridization with a bcl-2 probe. bcl-x, like bax, is alternatively spliced. Two forms of bcl-x have been identified, bcl-xL, which can inhibit cell death, and bcl-xS which prevents bcl-2 enhanced suppression of cell death. While neither of the bcl-x proteins have been demonstrated to interact directly with Bcl-2 in vivo, it seems likely that the interplay of bcl-2 like molecules may in part determine whether cells undergo apoptosis.

Because of its role in suppressing cell death, bcl-2 appears to be functionally homologous to ced-9. bcl-2 and ced-9 are also structurally similar (M. O. Hengartner and H. R. Horvitz, Cell, in press)). bcl-2 has been demonstrated to rescue some cell deaths in worms (Vaux et al., 1992). The functional and structural similarities between ced-9 and bcl-2 suggest that a cell death pathway has been conserved evolutionarily. In support of this, it has recently been reported that ced-3 is the structural and functional homologue of IL-1β-converting enzyme (ICE) (Yuan et al., 1993). Both ced-3 and murine ICE can induce apoptosis in Rat-1 fibroblasts in vitro. This apoptosis can be blocked by overexpression of bcl-2 or of crmA (Miura et al., 1993), a cowpox virus gene which acts as an ICE inhibitor (Ray et al., 1992).

Cell death has also been documented in another genetic system, Drosophila melanogaster. Cell death in Drosophila has been shown to depend on a variety of cues, such as hormones (Kimura and Truman, 1990) and inter-cellular interactions (e.g., Fischbach and Technau, 1984; Steller et al., 1987; Magrassi and Lawrence, 1988; Cagan and Ready, 1989; Campos et al., 1992). These observations indicate that cell death in Drosophila, like in vertebrates, is under epigenetic control.

Cell death is also observed during the development of the Drosophila visual system. Cell death occurs in the eye imaginal disc (Cagan and Ready,
1989), as well as in the optic ganglia of wild type flies and mutants in which photoreceptor innervation is reduced (Fischbach and Technau, 1984). Interactions between photoreceptor axons and their targets are required not only for the proliferation of the optic ganglia (Selleck and Steller, 1991), but for their survival as well (Fischbach, 1983; Fischbach and Technau, 1984; Steller et al., 1987). Furthermore, photoreceptor cell survival is also dependent upon interactions with the optic ganglia, suggesting the existence of trophic interactions in the *Drosophila* visual system (Campos et al., 1992).

Recently, genetic analysis has identified three genes which are involved in cell death in the eye imaginal disc. *roughest* and *echinus* were isolated in a screen for abnormal patterns of cell death in rough eye mutants (Wolff and Ready, 1991). Flies mutant at either locus have decreased cell death in the eye which results in the rough eye phenotype. This cell death is likely to be dependent on cell-cell interactions. The *eyes absent* (*eya*) gene has also been proposed to be a cell death gene (Bonini et al., 1993). Loss of function mutations in *eya* lead to massive apoptosis in the eye imaginal disc and adult flies with no eyes (Bonini et al., 1993). Mutations in many genes, however, lead to ectopic cell deaths (e.g., *crumbs* (Tepass et al., 1990) and some segmentation genes (see for example Magrassi and Lawrence (1988)). Thus, induction of ectopic cell death may not be the best criteria for defining a gene as a cell death gene.

A large amount of cell death also occurs during *Drosophila* embryogenesis (Campos-Ortega and Hartenstein, 1985; Abrams et al., 1993). This death is apoptotic and at any given stage occurs in a roughly reproducible pattern (Abrams et al., 1993). Close examination suggests, however, that there is some plasticity as to which cells die when, re-enforcing the notion that intercellular interactions may act to regulate programmed cell death in *Drosophila*.

Genetic analysis of cell death in *Drosophila* embryo is particularly powerful. It allows the identification of essential genes and does not require that the organism live to adulthood, as is necessary for the study of cell death in the adult visual system. It should therefore be possible to identify a number of genes involved in programmed cell death in *Drosophila* through analysis of programmed cell death in the *Drosophila* embryo. The identification of one such gene, *reaper*, is described in Chapters V and VI of this thesis.
The larval visual system as a model for nervous system development

The studies presented in this thesis encompass two major areas, establishment of neuronal patterning, and the role of cell death during the development of the *Drosophila* embryo. Questions of neuronal development have been addressed through the study of a simple neuronal circuit, the larval visual system of *Drosophila*. Clearly, issues of cell fate specification, short range axon pathfinding, and target recognition can be addressed in this system. Furthermore, because the development of the larval visual system involves the movements of tissues, it is important to consider the role morphogenetic movements may have in establishing the projection pattern of the larval optic nerve. Finally, because there is extensive cell death which occurs during head development in the *Drosophila* embryo, it seems likely that programmed cell death may also have a role in shaping the larval visual system. Because so much of the work presented in this thesis focuses on larval visual system development, it is useful to have an understanding of how the larval visual system develops.

The larval optic nerve was first discovered in the house fly, *Musca domestica* by N. Bolwig in 1946 (Bolwig, 1946). It was known for some time that larvae possess a visual response and most commonly exhibit negative phototaxis. Because *Dipteran* larval are acephalic, they do not develop external eyes; thus, exactly what constituted the larval visual organs was a matter of some controversy (discussed in Bolwig, 1946). In order to identify the larval visual organ, Bolwig performed a number of experiments, operating on larvae by various methods and monitoring their visual response. By keeping track of which surgical manipulations eliminated larval vision, he was able to identify the larval optic nerves. Analogous structures have been found in other species including *Phaenicia sericata* (Melamed and Trujillo-Cenoz, 1975) and *Drosophila melanogaster* (Melamed and Trujillo-Cenoz, 1975; Steller et al., 1987). This nerve is now generally referred to as Bolwig's nerve or the larval optic nerve.

In *Drosophila*, each Bolwig's organ consists of twelve photoreceptor cells. In the larva, the cell bodies of the photoreceptors sit near the cephalopharyngeal skeleton. Each photoreceptor sends out an axon. These axons fasciculate together, forming Bolwig's nerve, and project to the region of the optic lobe primordium. Bolwig's nerve and its presumptive targets in
the developing optic lobe constitute the larval visual system. A number of investigators have described development of the larval visual system (Steller, et al., 1987; Schmucker et al., 1992; Green et al., 1993; Campos et al., in prep). Bolwig's organ and the optic lobe primordium differentiate from a group of cells in the dorsal lateral ectoderm (Green et al., 1993). These cells correspond to the optic placode identified by Poulson (1950). During stages 12 - 13, these cells invaginate from the dorsal ectoderm and the larval photoreceptors begin to differentiate, as evidenced by the expression of Krüppel (Schmucker et al., 1992) and various neuronal markers (Steller et al., 1987).

When axon outgrowth from the photoreceptors begins, these cells lie closely opposed to the optic lobe region. Contact between Bolwig's nerve and the optic lobe primordium is established at this time and is maintained throughout development (Green et al., 1993). Throughout the rest of embryonic development, the nerve elongates and the cell bodies move away from the target region. The optic lobe primordia themselves migrate ventromedially and ultimately fuse with the supraoesophageal ganglion (Green et al., 1993).

Because of its simplicity, the larval visual system is ideally suited to genetic analysis. Development of the larval visual system requires most if not all of the steps generally required in nervous system development. Cell fate specification, axon outgrowth, and target recognition are all important for the development of the larval visual system. Furthermore, cell migration as it relates to morphogenesis of the head is also important in this system. Because of the availability of markers for both Bolwig's nerve (e.g., mAb24B10 (Fujita et al., 1982)) and the optic lobe primordia (e.g., anti-fasciclin II antibodies (Grennigloh et al., 1991) and anti-disconnected (Lee et al., 1991)), it is possible to screen for mutations which affect any or all of these processes.

The first mutation to be recognized for its effect on larval visual system development is the disconnected (disco) mutation (Steller et al., 1987). disco was isolated in a screen for adult flies with structural brain mutants (Heisenberg and Böhl, 1979). In flies mutant for disco, the photoreceptors of the compound eye do not connect to the brain. This defect has been proposed to result from the failure of the larval optic nerve to maintain proper connections with its targets (Steller et al., 1987). In wild type larvae, Bolwig's nerve traverses the eye disc and runs through the optic stalk on its way to the brain. This has led to the suggestion that Bolwig's nerve might pioneer this
pathway and serve as a guide for the first ingrowing retinula axons (Melamed and Trujillo-Cenoz, 1975; Steller et al., 1987). Bolwig's nerve does not, however, appear to be strictly required for this process as is evidenced by studies where Bolwig's nerve is either absent (as in glass mutants (Moses et al., 1989; Kunes et al., 1993)) or ablated (Kunes and Steller, 1991). In both cases, photoreceptor cells can travel from the eye disc to the brain. What exactly is the relationship between the embryonic and adult phenotypes in disco remains to be established. The disco gene has been cloned and contains two zinc finger domains (Heilig et al., 1991). The protein does exhibit some degree of specific DNA binding activity (Lee and Steller, in preparation) and is thought to be a transcription factor. It is expressed in the optic lobe region (Lee et al., 1991) and may regulate genes required for the maintenance of interactions between Bolwig's nerve and its targets.

Two other genes, glass and Krüppel have also been shown to be required for larval visual system development. The glass gene encodes a zinc finger protein which is required for differentiation of all photoreceptor cells in Drosophila (Moses et al., 1989). While glass is not required for the expression of general neuronal antigens such as those recognized by mAb 22C10 (Zipursky et al., 1984) and anti-HRP antibodies (Jan and Jan, 1982), it is required for the expression of photoreceptor cell specific markers such as chaoptin and Rhl (Moses et al., 1989). Thus, glass appears to be required for late stages of photoreceptor cell determination. In glass null mutants it is not possible to identify a Bolwig's organ either in embryos or in larvae. Occasionally what are thought to be rudiments of Bolwig's nerve can be found, but these do not differentiate or project normally (Schmucker et al., 1992).

Krüppel, also a zinc finger containing protein, is required for normal fasciculation of the larval photoreceptors (Schmucker et al., 1992). In Krüppel mutants, the cells of Bolwig's nerve differentiate into photoreceptors as is evidenced by expression of chaoptin, but these cells are not arranged as in wild type. Rather than being tightly grouped, the photoreceptors are loosely clustered and do not project normally. Marked defasciculation of the nerve is also observed (Schmucker et al., 1992).
Morphogenesis and the role of head involution in shaping the larval visual system

Development of the larval visual system occurs simultaneously with head formation; thus morphogenesis of the embryonic head may influence the projection pattern of the larval optic nerve. The role that morphogenesis plays in shaping the nervous system is not unique to this system. Development of vertebrate nervous systems involves a large number of tissue movements. Neurulation results in the formation of the neural tube from the previously undifferentiated ectoderm. Formation of the neural tube in vertebrates is comparable to the invagination of the *Drosophila* optic lobe. In both cases, cell shape changes precede and may actually drive invagination of the presumptive neural tissue from the ectoderm (Schoenwolf and Smith, 1990; Green et al., 1993). Another example of the role morphogenesis plays in shaping the vertebrate nervous system is the regionalization of the neural tube. The neural tube changes shape throughout the course of embryonic development. A number of swellings and constrictions form which correspond to different portions of the brain and spinal cord. For example, shape changes at the anterior end of the neural tube allow the identification of forebrain, midbrain, and hindbrain. These structures undergo further subdivisions which also manifest as shape changes. A striking example of this is the development of hindbrain segments, also known as rhombomeres.

In order to fully appreciate the role morphogenesis may have in shaping the larval visual system, it becomes necessary to have an understanding of the process of head involution (Turner and Mahowald, 1979; Campos-Ortega and Hartenstein, 1985). With the formation of the cephalic furrow in stage 6, the head primordium can be identified. Cells anterior to the cephalic furrow undergo particularly complex patterns of differentiation and migration, all of which are required for the formation of the head. During stages 10 - 11, the ventral portion of the cephalic furrow disappears and the gnathal protuberances appear. These are the anlagen for the three gnathal segments, the labial, mandibular, and maxillary segments. Meanwhile in the procephalon (which consists of the non-gnathal region of the head) the clypeolabrum differentiates. The clypeolabrum, first seen at stage 10, is visible in the dorsal half of the procephalon. Its ventral most boundary is formed by the roof of the pharynx which itself is formed by the
stomodeal invagination. Also, at this time the optic placode begins to
differentiate in the procephalon.

Movements in both the ventral and dorsal regions of the presumptive
head constitute the process known as head involution. Ventrally, the gnathal
segments are orally displaced, migrating towards and along the stomodeal
invagination. Some of these cells will ultimately form portions of the atrium
and pharynx. Dorsally, the so-called dorsal ridge begins to differentiate. Each
dorsal ridge (one on either side of the embryo) is visible as a bulge
immediately posterior to the optic placode. The dorsal ridge grows and fuses
across the dorsal midline, forming the dorsal fold. Once formed, the dorsal
fold moves anteriorly over the procephalon. It continues migrating and
ultimately envelops the clypeolabrum, resulting in the formation of the
frontal sac. By the end of this process, the entire head region of the embryo is
internalized, the net result being an acephalic larva. Optic placode
invagination occurs prior to the movement of the dorsal ridge and continues
throughout the course of head involution. Also during head involution, the
photoreceptor cell bodies move medially from the head epidermis and
ultimately end up attached to the portion of the frontal sac which covers the
pharynx (Green et al., 1993).

Much of our understanding of Drosophila development comes from
studies of the obviously segmented thoracic and abdominal regions of the
embryo (reviewed in Akam, 1987). By contrast, the embryonic head of
Drosophila is not organized into easily defined metameric units, making its
development harder to elucidate. The expression of markers such as
engrailed and wingless reveal the segmental nature of head structure
(DiNardo et al., 1985; Diederich et al., 1989; Cohen and Jürgens, 1990; Schmidt-
Ott and Technau, 1992). A number of genes have been identified which are
required for head development in Drosophila (reviewed in Finkelstein and
Perrimon, 1991), and some of them are required for the specification of certain
head segments (e.g., empty spiracles (Dalton et al., 1989), orthodenticle
(Finkelstein and Perrimon, 1990), and buttonhead (Cohen and Jürgens, 1990)).
The morphogenetic movements of head involution have been shown to
require the function of labial (Merrill et al., 1989), Deformed (Merrill et al.,
1989), and head involution defective (Abbott and Lengyel, 1991). A detailed
analysis of the head involution defective gene and the role that it plays in
patterning the larval visual system are presented in Chapter IV of this thesis.
The aims of this thesis

The goal of the work presented here is the study of some of the mechanisms which are required for the patterning of the *Drosophila* nervous system. I initiated this work by screening for mutations which affect the projection pattern of the larval optic nerve of *Drosophila*. The details of this screen are presented in Chapter II. As a result of this mutational analysis, the 75C1,2 region of the *Drosophila* genome was identified as being required for normal projection of Bolwig's nerve. The 75C1,2 region is also required for programmed cell death in the *Drosophila* embryo, and thus I also became involved in the molecular identification of a gene, *reaper*, which is likely to be a central control function for programmed cell death in *Drosophila*. Molecular genetic analysis of the 75C region is presented in Chapter III. In Chapter IV, I present molecular characterization of the *head involution defective* locus, which is the function in 75C1,2 required for normal projection of Bolwig's nerve. The phenotypic consequences of mutations in *hid* as they relate to larval visual system development are also presented in Chapter IV. Chapter V is a phenotypic analysis of the cell death defect found in 75C1,2 deficiency embryos, and in Chapter VI, I present evidence that we have molecularly identified a cell death gene in 75C1,2 interval.
REFERENCES


Ranscht, B. (1988). Sequence of contactin, a 130 kD glycoprotein concentrated in areas of interneuronal contact, defines a new member of the


Yuan, J., Shaham, S., Ellis, H. M., and Horvitz, H. R. (1993). The *C. elegans* cell death gene *ced-3* encodes a progein similar to mammalian interleukin-1β-converting enzyme. Cell 75,


Chapter II
A Systematic Screen for Mutations Affecting
the Development of the Larval Visual System of Drosophila
ABSTRACT

We screened 40% of the Drosophila genome for mutations which affect the projection of the larval optic nerve. We identified four major classes of projection phenotypes which reveal key points in the development of the larval visual system. More specifically, we found that the genomic interval 75B8-11 to 75C1,2 is required for the projection of the larval optic nerve to the ventral brain.

INTRODUCTION

A hallmark of the adult nervous system is its highly complex, yet remarkably precise array of neuronal connections. The assembly of the nervous system involves a number of steps, including, but not limited to: cell fate determination, cell migration, axon outgrowth, and target recognition. We are interested in the identification and study of genes required for nervous system development. We have chosen to approach this problem through studies of the nervous system of the fruit fly, Drosophila melanogaster. Although Drosophila melanogaster is a relatively simple organism, its central and peripheral nervous systems are highly complex. In order to examine in detail the projection pattern and behavior of a single neuron during embryonic development, we use the larval visual system of Drosophila as our model.

The larval visual system forms a simple neuronal circuit. It consists of twelve photoreceptor cells whose axons form the larval optic nerve, or Bolwig's nerve (Bolwig, 1946), and their synaptic partners in the larval brain (Steller et al., 1987). This circuit is bilaterally symmetric. The larval optic nerve follows a highly stereotyped projection pattern which can be identified in wholemount embryos using neuron specific or photoreceptor cell specific monoclonal antibodies (Figure 1). In the mature wild type embryo, the cell bodies of Bolwig's nerve sit in the anterior of the embryo near the larval head skeleton (the cephalopharyngeal skeleton). The nerve projects posteriorly until it reaches the brain hemisphere where it travels around the brain and terminates ventrally. The nerve terminates in the region of the optic lobe primordium which later becomes the adult optic ganglion. In this region of the brain, Bolwig's nerve can be seen to contact at least two groups of cells, the optic lobe pioneers and the so-called central neuron (Campos et al., in prep.). These cells may serve to guide Bolwig's nerve towards its synaptic partner(s) in the central brain.

Wild type development of the larval visual system has been described in detail (Steller et al., 1987; Schmucker et al., 1992; Campos et al., in prep.; Green et
Bolwig's nerve and the optic lobe primordia are derived from a dorso-lateral placode in the *Drosophila* embryo (Green et al., 1993). The so-called "optic placode" is visible in stage 11 embryos as a dense group of cells (Poulson, 1950; Turner and Mahowald, 1979; Campos-Ortega and Hartenstein, 1985). The presumptive larval visual system can also be identified as a group of fasciclin II positive cells situated in the dorsal ectoderm (A. R. Campos, unpublished observations). The optic placode invaginates from the dorsal ectoderm during stages 12 - 13. It is at this time that a group of cells in the ventral portion of the optic lobe invagination begins to differentiate into the larval photoreceptors (Green et al., 1993). They are first identifiable with neuron specific monoclonal antibodies (mAb) during stage 12 (Steller et al., 1987). The cells of Bolwig's organ differentiate in a sequential fashion and are believed to be recruited from surrounding cells, rather than from a common precursor (Schmucker et al., 1992).

Axon outgrowth from the larval photoreceptors is visible as early as late stage 12. At this stage, the larval optic nerve and the optic lobe are in close proximity. The association of Bolwig's nerve with the optic lobe region is maintained throughout the rest of embryonic and larval development. The final projection pattern of the nerve seen in Figure 1 is the result of a complex series of morphogenetic movements which take place during stages 12 - 15 (Figure 2). Once the optic placode has invaginated from the dorsal ectoderm, it continues to descend ventrally and ultimately fuses with the supraoesophageal ganglion in a postero-ventral position. Meanwhile, the larval photoreceptor cell bodies move away from the optic lobe region (although the nerve ending remains in association with it). The axon of Bolwig's nerve continues to elongate throughout the course of head involution, and the photoreceptor cell bodies end up attached to the outer lining of the dorsal pouch (Green et al., 1993). Although the process of head involution may account for some of the relative displacement of the photoreceptor cell bodies and their targets, relocation of the photoreceptor cells from their position in the ectoderm to a more medial position may involve some active migration on the part of the cells as well. During stage 15, the tip of Bolwig's nerve penetrates the brain and synapses with as yet unidentified cells in the central brain (Schmucker et al., 1992).

The *disconnected* (*disco*) gene is one gene which has been identified as playing a role in larval visual system development (Steller et al., 1987). In *disco* mutant embryos, the larval visual nerve fails to recognize and maintain proper
connections with its targets. In order to identify other genes which are required for formation of the larval visual system, we have examined the projection pattern of the larval optic nerve in embryos homozygous for chromosomal deficiencies. By screening through such a collection of "deficiency lines" (courtesy of the Drosophila Stock Center, Bloomington, Indiana), we have been able to rapidly sample mutant phenotypes throughout much of the Drosophila genome (approximately 40%). By studying the behavior of Bolwig's nerve in a wide variety of mutant backgrounds (both in deletion strains and in known single gene mutants) we have not only been able to identify regions of the genome required for normal projection of Bolwig's nerve, but have gained insight into how the larval visual system normally develops. In this study we present a description of the various phenotypes associated with the mutations examined and discuss their significance.

MATERIALS AND METHODS

Drosophila stocks. The collection of deficiency containing stocks used for this screen was provided by the University of Indiana Drosophila stock center. tllL10 was provided by R. Lehmann. Embryos are staged according to Campos-Ortega and Hartenstein, 1985. Marker mutations are described in (Lindsley and Zimm, 1992).

HPR immunohistochemistry. Staining of embryos was performed as previously described (Steller et al., 1987) with the following modifications: embryos were fixed in a 1:1 mixture of 2% paraformaldehyde in 0.1M phosphate buffer pH7.2 (PB) and octane for 30 minutes at room temperature. For 22C10 staining, vitelline membranes were removed as according to (Mitchison and Sedat, 1983) except that octane was substituted for heptane. For 24B10 staining, vitelline membranes were removed by shaking the embryos for 2 minutes in a 1:1 mixture of fresh octane and 85% acetone in PB. Devitellinized embryos were washed 2X in PB and 2X in BSS (0.038M NaCl, 0.053M KCl, 0.012M MgSO4.7H2O, 0.011M CaCl2.2H2O, 0.01M Tricine, 0.02M glucose, 0.05M sucrose, 0.2%BSA, pH 6.95). Embryos were pre-incubated for 30 minutes in BSN (BSS + 0.1% Triton X-100, 5% normal goat serum). Primary antibody was added (1:1 dilution in BSN) and incubated overnight at 4°C. Embryos were washed in 4 changes of BSS over the course of an hour, pre-incubated again in BSN and goat anti-mouse HRP conjugated secondary antibody (BioRad) was added (1:200 dilution in BSN).
Incubation was either 4 hours at room temperature or overnight at 4°C. After washing in BSS (4 changes, 1 hour total), embryos were rinsed for 10 minutes in PB. They were then placed in a solution of 0.5 mg/ml diaminobenzidine and 0.0003% hydrogen peroxide in PB for 1-5 minutes. After one wash in PB embryos were dehydrated through an ethanol series and mounted in DPX (Fluka).

RESULTS

Normal development of the larval visual system is not easily perturbed

In order to visualize the projection pattern of Bolwig’s nerve, we stained pools of embryos with the photoreceptor cell specific mAb 24B10 (gift of S. Benzer; Zipursky et al., 1984)). 24B10 recognizes the photoreceptor cell specific protein, chaoptin (VanVactor et al., 1988). Chaoptin expression is first detectable in embryos around stage 15 and persists throughout the life of the photoreceptor cells. Embryos derived from flies heterozygous for the various mutations examined in this study were collected for 5 hours and aged until the embryos were 12 - 17 hours old at 25°C (corresponding to stages 15-17). The embryos were then fixed and processed for 24B10 staining. We looked for lines in which roughly one quarter of the embryos displayed a particular phenotype, indicating that the phenotype was segregating with the mutant chromosome. In some cases, the expected fraction of mutant embryos was less than 1/4 due to the presence of a duplication of the deleted region in the deficiency stock. This was accounted for in our analysis. The results of our screen are summarized in Table I.

We found that in the majority of lines stained (63/109), the larval visual nerve appears to follow a normal projection pattern. In cases where overall embryonic morphology is generally wild type, minor deviations from normal would have been detected. For example, the projection phenotype in disco embryos, while often fairly subtle, would likely have been identified in our screen. In many of the deficiency lines screened, genes are deleted that are essential for normal morphogenesis. In these cases, embryonic morphology was often grossly abnormal; however, it was sometimes still possible to identify 24B10 positive cells which could be seen projecting to the embryonic brain (see Figure 3a). In such cases, the projection pattern was scored as being wild type. We recognize that in doing so, we might have missed subtle projection defects present in these mutant backgrounds. In some cases, embryonic morphology
was so abnormal that it was impossible to interpret the projection pattern seen. These cases are noted in Table I.

**Mutations which may affect specification of the larval photoreceptor cells**

In 18/109 lines stained, we were unable to identify any 24B10 positive cells. It is possible that these deficiencies delete genes which are required for specifying the cellular identity of the larval photoreceptors. One of these deficiencies, *Df(3R)P14* deletes the gene *glass*. *glass* is known to be required for normal development of Bolwig's organ (Moses et al., 1989). In most embryos homozygous for strong *glass* alleles, including *Df(3R)P14*, Bolwig's nerve is impossible to identify. (In rare cases, rudiments of Bolwig's organ can be found, see (Schmucker et al., 1992)). In the 17 other 24B10 negative deficiencies, it is possible that the larval optic nerve is not missing, but that mutant embryos arrest before the onset of chaoptin expression (chaoptin is the antigen recognized by 24B10). In principle, one can visualize the larval optic nerve before it is fully differentiated using the monoclonal antibody 22C10 (gift of S. Benzer). The peripheral nervous system specific mAb 22C10 (Fujita et al., 1982) stains the larval optic nerve at the onset of axon outgrowth, at least two hours before chaoptin is expressed. We stained all of the 24B10 negative lines with 22C10 in hopes of identifying Bolwig's nerve in these embryos. Because 22C10 stains all head sensory organs, we rely on morphological landmarks to identify Bolwig's nerve with this antibody. Most of the 24B10 negative lines we identified, however, have grossly abnormal morphology; thus, we were unable to unambiguously identify the larval optic nerve in these mutants. This leaves open the possibility that in some, if not all of these lines, the larval optic nerve may be present, but is immature and ectopically positioned.

In one set of three overlapping deficiencies *Df(1)N-8, Df(1)N-71h, and Df(1)N-69h9*, we observed embryos with an excess of 24B10 positive cells (Figure 3b). These deficiencies uncover the neurogenic gene *Notch*. In *Notch* mutants, there is a hypertrophy of the nervous system, at the expense of the epithelium (reviewed in Campos-Ortega and Knust, 1990). The fact that there are more 24B10 positive cells in *Notch* mutants than in wild type suggests that a large number of cells have the potential to become larval photoreceptors and that *Notch* function is required to ensure that the correct number of cells are recruited to form Bolwig's organ. This finding is in agreement with that of Green et al.
(1993), who report that there are roughly twice as many larval photoreceptor cells in Notch mutants than normal.

**Mutations affecting axon - target interactions**

We identified four lines in which the larval optic nerve endings are abnormal (see for example Figure 3c). In all four cases, the terminal region of the larval optic nerve is larger than normal and spread over a much greater area than is ever seen in wild type embryos. Such phenotypes could be indicative of cases where there are more target cells than normal. Alternatively, they could result from a failure of the nerve to properly recognize and interact with its targets. In two of the four cases (see Table 1), the cell bodies of the photoreceptor cells are sometimes ectopically positioned, suggesting perhaps that the phenotype(s) result from general defects in cell adhesion.

**Mutations affecting later development of the larval visual system**

In thirteen lines examined (including two lines with abnormal nerve terminals), the photoreceptor cell bodies are ectopically positioned. This phenotype is fairly variable with anywhere from one to all of the photoreceptor cell bodies being found in abnormal locations. Furthermore, this phenotype is not always bilaterally symmetrical in a given embryo. In at least one case, little or no axon elongation occurs and the photoreceptor cell bodies are located near the embryonic brain (Figure 3d). This deficiency may delete genes required for axonal outgrowth. In the other cases, cell bodies are found spread along the path that the nerve normally follows. Here, defects in cell migration and/or cell adhesion could account for the phenotype.

**Axonal outgrowth from the larval photoreceptor cells can occur in the absence of the brain**

We have examined the projection pattern of Bolwig's nerve in embryos homozygous for mutations in the gene tailless (tll). tll is a zygotic gene of the terminal class that is required for the formation of anterior and posterior structures of the embryo (Jürgens et al., 1984). The tll gene has been cloned and is a member of the steroid receptor super family (Pignoni et al., 1990). In tll null mutants and homozygous tll1 embryos, the posterior half of the procephalic lobe and optic placode are apparently deleted (Strecker et al., 1988). The
supraoesophageal ganglion (or brain) which is formed from these structures (Campos-Ortega and Hartenstein, 1985; Jürgens et al., 1986) is missing as well.

We have identified the larval optic nerve in embryos homozygous for \textit{tll}^{1} (\textit{tll}^{1} = L10.22; Strecker et al., 1988). In \textit{tll}^{1} embryos, Bolwig's nerve is present and sends a projection in a ventral/posterior direction as is seen in wild type embryos (Figure 4); thus, polarity of axon outgrowth from the larval photoreceptors does not depend on proper fusion of the optic lobe precursors to the brain. Most often, the projection of Bolwig's nerve in \textit{tll}^{1} mutants remains short and thick, but in some cases, the projection is quite long.

Bolwig's nerve and the optic lobe primordia are believed to be derived from the optic placode (Green et al., 1993). The optic placode is reported to be deleted in \textit{tll}^{1} embryos (Strecker et al., 1988). Our identification of the larval optic nerve in \textit{tll}^{1} embryos suggests either that Bolwig's nerve is not derived from the optic placode, or, as seems more likely, that the optic placode is not entirely deleted in \textit{tll}^{1} embryos. Perhaps in \textit{tll} mutants, the optic placode is ectopically positioned, thus hindering its identification.

\textbf{Lines in which the larval optic nerve projects to abnormal locations in the brain}

When we set out to examine the projection of Bolwig's nerve in the deficiency backgrounds, we were particularly interested in identifying lines in which the larval optic nerve projects to ectopic locations. We hoped that such phenotypes might be the result of mutations which disrupt normal pathfinding and/or target recognition. We identified six deficiency lines in which Bolwig's nerve projects inappropriately. In embryos homozygous for \textit{Df(2R)eve}1.27, Bolwig's nerve projects to a more central part of the brain than normal. These embryos are morphologically quite abnormal, presumably due to the deletion of the segmentation gene \textit{evenskipped (eve)}.

In \textit{Df(1)JA27} embryos, the larval optic nerve sends its projection anteriorly, before turning and terminating in a posterior region of the head (Figure 5a-c). This deficiency is quite large, however, and the morphology of the mutant embryos is highly irregular. It is likely, therefore, that the anteriorward projection seen in these embryos is not due to a real path finding defect, but is merely the consequence of abnormal morphogenesis. This could be investigated by examining the projection of the larval optic nerve in smaller deficiencies in this interval, seeing if it is possible to identify embryos which are
morphologically more normal, yet display the anteriorward projection of Bolwig's nerve.

Associated with the stock Df(2R)L+48 is a phenotype in which the larval optic nerves on either side of the mutant embryos meet at the midline of the embryo, fasciculate together, and terminate in a dorsal posterior region of the brain (Figure 5d). Preliminary genetic analysis reveals that this phenotype does not segregate with the putative deficiency chromosome, but instead segregates as a temperature sensitive maternal effect mutation. As many known maternal effect mutations affect patterning of the embryo in a global way (for review see Akam, 1987), it is possible that this phenotype is the consequence of abnormal patterning of the embryo as a whole and is not specific to the larval visual system.

Three deficiencies, Df(3L)WR4, Df(3L)WR10, and Df(3L)CatDH104 which overlap from 75B8-11 to 75C1,2 define a region of the genome which is required for proper projection of the larval optic nerve (referred to as 75C1,2). In embryos homozygous for any of the three deficiencies, or in any trans-heterozygous combinations, the larval optic nerve projects to a dorsal portion of the embryonic brain (Figure 5e,f). This is a highly consistent phenotype, and despite pronounced defects in head involution, the overall morphology of the embryos is quite normal. This phenotype could be due to recognition of inappropriate targets by Bolwig's nerve, or to improper placement of the target region itself. We believe that the phenotype cannot be accounted for by the head involution defect alone. In Figure 6 we show two lines identified in our screen which clearly have head involution defects, yet in each case, the larval optic nerve projects ventrally. Because of the highly consistent nature of the projection phenotype in the 75C mutants, the fact that it can be localized to a specific genomic interval (75B8-11 to 75C1,2) and because it is clearly required for the normal development of the larval visual system, we have elected to study this phenotype further. It is the analysis of this mutant phenotype and the 75B8-11 to 75C1,2 interval which forms the basis of the work in the rest of this thesis.
DISCUSSION

In an effort to identify genes required for larval visual system development, we analyzed the projection of the larval optic nerve in stocks bearing chromosomal deficiencies. We screened through a total of 109 lines, which represents approximately 40% genome coverage. When we started our screen, one mutation, *disco*, had been identified which is required for normal projection of Bolwig's nerve (Steller et al., 1987). We reasoned that it should be possible to identify other loci which affect the development of the larval visual system. By systematically screening through stocks in which different chromosomal deficiencies are maintained, we were able to sample a large fraction of the *Drosophila* genome for mutant phenotypes relatively quickly. Screens through collections of deficiency stocks have been used successfully by other investigators to identify genes important for nervous system development (e.g., *daughterless* (Caudy et al., 1988) and *single minded* (Thomas et al., 1988)).

One major advantage to the use of the larval visual system as a model for nervous system development is that the projection of Bolwig's nerve can be analyzed in embryos; therefore, long term survival is not required before a given phenotype manifests itself. We are able to look at mutations which have pleiotropic effects on development and assess how they affect the projection of the larval optic nerve. This becomes particularly relevant in light of the observation that several of the segmentation genes, which are necessary for establishment of embryonic body architecture, are known to be specifically required for nervous system development as well (see, for example, Chu-LaGraff and Doe, 1993; Patel et al., 1989).

Although all of the deficiency stocks examined delete genes essential for embryonic viability, the large store of maternal components in *Drosophila* embryos allows the deficiency embryos to develop long past the point at which the larval photoreceptor nerve has developed (Garcia-Bellido et al., 1983). In fact, almost all of the lines examined (91/109) develop beyond the point at which 24B10 (chaoptin) expression, which is of relatively late onset (approximately stage 15) is detectable.

Because we are looking at stocks in which many genes are deleted at once, more subtle aspects of projection defects might be obscured by general abnormalities in embryonic morphology. Furthermore, our screen is limited to the portion of the genome (approximately 40%) for which deficiencies were available at the time. This screen should be viewed not as a definitive list of all
mutations which affect the projection pattern of the larval optic nerve, but rather as an attempt to rapidly catalogue the kinds of phenotypes one can expect to isolate.

An alternative strategy towards the identification of genes required for larval visual system development would be to chemically mutagenize flies and establish balanced mutant lines which could be screened for projection defects. Such an approach was used by (Nüsslein-Volhard and Wieschaus, 1980) in their search for mutants defective in pattern formation, and has been more recently employed by Seeger et al. (1993) and VanVactor et al. (1993) to identify genes required for various aspects of nervous system development. Although by using a chemical mutagen, one is not constrained to looking for mutations in particular regions of the genome, saturation mutageneses such as those mentioned above are at least ten fold more labor intensive than deficiency screens.

The class of mutations which we have chosen to concentrate on are those in which the larval optic nerve projects to an abnormal location in the embryonic brain. We hope that defects such as these might reflect abnormalities in target recognition or in target morphogenesis. It should be pointed out that despite the fact that the projection of Bolwig's nerve is relatively long and makes a number of turns, the larval visual system is not suited to the study of long range axonal pathfinding. Although the photoreceptor cell bodies and their targets end up in very different portions of the embryo, initially they are very closely opposed and in fact, are believed to derive from the same group of cells (Green et al., 1993). It is at this point, early in development that contact between Bolwig's nerve and its targets is established. Therefore, very little axon guidance or pathfinding is actually required in this system for normal connections to be made. The nerve must, however, send its initial projection in the proper direction and be able to find and recognize its targets. The nerve and its targets also go through a complex series of morphogenetic movements before arriving at the mature projection pattern; thus, the larval visual system is well suited to studies of target recognition and morphogenesis.

We have chosen to concentrate further efforts on the genomic region, 75C1,2. In embryos deleted for 75C1,2 the larval optic nerve projects to a dorsal, rather than ventral portion of the embryonic brain. In Chapter III I present a molecular and genetic analysis of 75C. In Chapter IV I present phenotypic and molecular characterization of the head involution defective locus, the function in 75C which is required for proper projection of the larval optic nerve.
REFERENCES


Figure 1  Projection of the larval photoreceptor nerve in wild type embryos.  
24B10 staining of stage 17 Canton-S embryos. In this and all subsequent figures, 
anterior is to the left. (A) lateral view - the cell bodies of Bolwig's nerve (arrow) 
are seen as a tight cluster of cells which sit near the atrium of the 
cephalopharyngeal skeleton (triangle). The nerve projects posteriorly, turns 
ventrally around the brain and terminates in the region of the optic lobe 
primordium, which is located ventrally. (B, C) - dorsal views of a wild type 
embryo, illustrating the bilateral symmetry of the larval visual system. (B) 24B10 
positive cells can also be seen in each brain hemisphere (dp). These "dorsal 
photoreceptors" are not part of Bolwig's nerve. (C) Bolwig's nerve can be seen 
terminating in a more ventral plane of focus (arrows). Abbreviations:  
BN Bolwig's nerve; br brain; dp dorsal photoreceptors.
Figure 2 - Development of the larval visual system. Lateral view of wild type embryos. (A) The larval optic nerve and the optic lobe primordia are derived from the optic placode (shaded). (B) The optic placode invaginates from the dorsal ectoderm. Bolwig's nerve differentiates and axon outgrowth begins. (C) The optic lobe primordia are closely opposed to the ventral brain. Bolwig's nerve is now fully differentiated and much axon elongation has occurred. (D) The optic lobe primordia are now incorporated into the ventral brain. The mature projection pattern of Bolwig's nerve is seen by this stage. Bolwig's nerve penetrates the optic lobe region and terminates in the central brain. Figure drawn by Kevin Lee.
stage 11 - 7 hr.

stage 14 - 9.5 hr.

stage 15 - 11.5 hr.

stage 17 - 16 hr.
Figure 3 - Representative projection phenotypes. All embryos are stained with 24B10 (A) Df(2R)vgw - lateral view - Although embryonic morphology is clearly abnormal, Bolwig's nerve (arrow) can be seen projecting around the brain. (B) Df(1)N-69h9 - dorsal view - Although there are more 24B10 positive cells than normal, they are still packed into two clusters and send out axons. (C) Df(2L)dp79b - dorsal view - Note how Bolwig's nerve terminates over a much larger region than normal (arrows, compare to Figure 1C). (D) Df(3R)Scr - dorsal view - Here the cell bodies of Bolwig's nerve are located ectopically, close to each brain hemisphere. Abbreviations: BN Bolwig's nerve; br brain.
Figure 4 - The larval optic nerve is present in $tll^1$ embryos. Lateral view of $tll^1$ embryo stained with 24B10. Note the posterior ventral projection of Bolwig’s nerve (arrows) and the absence of the brain hemisphere. The dark structure anterior to Bolwig’s nerve is the cephalopharyngeal skeleton. Abbreviation: $BN$ Bolwig’s nerve.
Figure 5 - Lines in which Bolwig's nerve projects to ectopic locations. All embryos are stained with 24B10. (A-C) Df(1)JA27 - dorsal views of the same embryo. Note how the initial projection from the photoreceptor cell bodies is in an anterior direction (triangle; arrows indicate the position of the cell bodies). (D) Embryo derived from stock Df(2R)L+48 - dorsal view - Here each photoreceptor nerve crosses to the midline of the embryo. They then fasciculate together and terminate between the two brain hemispheres (large arrow). The dorsal photoreceptors (dp) can also be seen in this embryo. (E, F) lateral views of Df(3L)WR10 and Df(3L)WR4 embryos respectively. In each case, the embryos have a defect in head involution and the larval optic nerve terminates dorsally in the brain. Note the striking similarity of the phenotypes in each case, suggesting that the gene(s) responsible for these defects resides in the overlap between the two deficiencies. Abbreviations: BN Bolwig's nerve; br brain; dp dorsal photoreceptors.
Figure 6 - Bolwig's nerve can project ventrally even when head involution is abnormal. 24B10 staining of Df(2L)b87e25 (A) and Df(3R)ry27, Dfd - lateral views. In each case, the larval optic nerve is seen to project ventrally, despite pronounced defects in head involution. (for Df(3R)ry27, Dfd, the head involution defects are most likely due to the Dfd (Deformed) mutation on the chromosome, not to the deficiency.)
Table 1 - Phenotypes Associated With Deficiency Stocks. Deficiency stocks examined and associated phenotypes are organized cytologically beginning at the tip of the X chromosome (1A). In the "Stock/Map" column, the top line of each group of two is the name of the deficiency examined, the bottom line shows the deficiency breakpoints. All other headings refer to the projection pattern of Bolwig's nerve in a given stock. "Normal" means Bolwig's nerve was observed to project as in wild type embryos. This category includes lines in which, despite grossly abnormal embryonic morphology, Bolwig's nerve projects to the brain without any obvious defects. "No Bolwig's nerve" includes all lines where we failed to identify the larval photoreceptors. In the "Ectopic cell bodies" category, photoreceptor cell bodies are abnormally positioned. "Abnormal nerve terminals" includes all lines in which Bolwig's nerve terminates over a larger region than normal. The "Abnormal projection" category includes all cases where Bolwig's nerve projects to inappropriate locations in the brain. Those lines in which abnormal embryonic morphology precludes classification of a projection type are listed under "No consistent phenotype." Deficiencies which show embryos with an excess number of larval photoreceptors because they delete Notch are listed as "Other."
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<thead>
<tr>
<th>Stock Map Position</th>
<th>Normal</th>
<th>No Bolwig's nerve</th>
<th>Ectopic cell bodies</th>
<th>Abnormal projection</th>
<th>Abnormal nerve terminals</th>
<th>No consistent phenotype</th>
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Note a - phenotype does not segregate with the deficiency chromosome
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80
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Totals 63 18 13 6 5 4 3
Chapter III
Molecular Genetic Analysis of 75C1,2
ABSTRACT

In *Drosophila* embryos homozygous for deletions of the 75C1,2 region of the genome, the larval optic nerve projects abnormally. These same embryos lack essentially all embryonic cell deaths, as assayed by a number of histological criteria (see Chapter V). In order to identify the gene(s) responsible for either or both phenotypes, we: 1) performed a chemical mutagenesis to isolate single gene mutations in 75C1,2; 2) cloned the DNA in the 75C1,2 region by chromosomal walking; 3) generated and mapped new X-ray induced breakpoints in the 75C region; and 4) identified a number of putative transcription units in the 75C1,2 interval.

INTRODUCTION

In embryos homozygous for deletions of 75C1,2, the larval optic nerve projects to an abnormal region of the brain (Chapter II). This phenotype is highly consistent: in wild type embryos, Bolwig's nerve projects to the ventral brain; in 75C deletion mutants, Bolwig's nerve projects dorsally. We decided to characterize this phenotype in more detail and identify the underlying genetic defect.

In our lab, two post-doctoral associates, John Abrams and Kristin White, were interested in identifying genes required for embryonic programmed cell death. Using the dye acridine orange as a marker for dying cells (Abrams et al., 1993), they screened through the collection of deficiency stocks described in Chapter II, searching for lines in which 25% of the embryos had abnormal patterns of acridine orange staining. They found that embryos homozygous for 75C1,2 deletions lacked the majority of programmed cell death, as assayed by acridine orange staining. Ultrastructural analysis of mutant embryos confirms this finding (Chapter V). The projection defect and the cell death defect are uncovered by the same set of overlapping deficiencies, *Df(3L)WR4, Df(3L)WR10* (Segraves and Hogness, 1990) and *Df(3L)CatDH104* (Mackay and Bewley, 1989). We therefore decided to collaborate on further characterization of 75C1,2.
MATERIALS AND METHODS

Isolation of single gene mutations in 75C. Males of the genotype st e were allowed to feed on a solution of 0.4 mg/ml ENU, 1% sucrose for 24 hours. Mutagenized males were crossed *en masse* to virgin Sb H/TM2 females. Individual *st e/SbH* males were then crossed to WR4/TM3Sb females and their progeny were scored for visible mutations uncovered by WR4 or for lack of *st e/WR4* individuals, indicating the presence of a recessive lethal mutation when in combination with the WR4 chromosome. Candidates were then tested for lethality or segregation of the visible phenotype by crossing them to WR10 flies. *st e* and Sb H/TM2 stocks were provided by R. Lehmann. WR4 and WR10 (Segraves and Hogness, 1990) were provided by the Indiana Stock Center. All genetic symbols are described in (Lindsley and Zimm, 1992).

Generation of W revertants. _ru W ca red/TM6_ males were X-irradiated with 3-4000 rads and crossed to _y; TM1/TM2_ virgin females. _F1_ progeny were scored for reversion of the _W_ phenotype and recovered over _TM1_ or _TM2_. Lines were established by crossing each revertant to _y; TM1/TM2_ flies.

HRP immunohistochemistry. Staining of embryos was performed as described in Chapter II.

Acridine orange staining of whole mount embryos. Acridine orange stainings were performed as described in (Abrams et al., 1993)

Isolation of genomic clones in 75C1,2. Phage DNA's were isolated from a library of Canton-S genomic DNA in the _λ_ Dash II vector (Stratagene) provided by Ron Davis. Clones 3501 through 3510 were provided by _W_. Segraves. Clone 3501 from the chromosomal walk of Segraves and Hogness (1990) was used as the distal starting point for our walk.

Cosmid DNA's were isolated from a genomic library of _iso-1_ _Drosophila_ DNA in the NotBamNot-CoSpeR vector, provided by Dr. John Tamkun. Yeast artificial chromosome (YAC) clones were provided by D. Hartl.
PCR to isolate the *terminus* locus. Sequence from the published sequence of the *terminus* cDNA (Baldarelli et al., 1988) was used to design primers for PCR (the polymerase chain reaction). The primer sequences were: 1) 5'GCGGAATTCCAGAACAACACGGT and 2) 5'GCGGAATTCGTAGATCTCCACGAT. This primer combination is predicted to amplify a 740 basepair genomic fragment of *terminus*, assuming no introns fall between the two primers. We set up a standard PCR reaction according to the protocol supplied by Perkin-Elmer Cetus. We used 0.26 pmole of each primer and 100ng of genomic *Drosophila* DNA. We used the following cycling profile to amplify *terminus*: 5 cycles of 94°C - 30 seconds, 48°C - 2 minutes, 72°C - 30 seconds, followed by 30 cycles of 94°C - 30 seconds, 58°C - 30 seconds, 72°C - 30 seconds. Using this regimen we specifically amplified a 740 bp fragment corresponding to the genomic *terminus* locus.

**In situ hybridization to polytene chromosomes.** Whole genomic phage were used as probes and hybridized to polytene chromosomes of Canton-S larvae as described in the 1986 version of the *Rubin Lab Methods Book*.

**Preparation of DNA used to map chromosomal breakpoints in 75C.** Embryos homozygous or trans-heterozygous for all rearrangements generated in the 75C region have a pronounced head involution defect. This allows the identification of embryos homozygous for 75C mutations. Overnight collections of embryos were allowed to age for at least 24 hours at 25°C. Embryos were then dechorionated in 50% chlorine bleach and those that had not hatched and undergone head involution properly were selected for DNA preparation. DNA was prepared according to the small scale isolation method in the 1986 version of the *Rubin Lab Methods Book*. Approximately 100 embryos were used per lane for each genomic Southern.

**Identification of putative transcription units in 75C1,2.** Cloned DNA in 75C1,2 was probed with 32P labeled cDNA prepared from poly-A RNA from embryos of 0-7 and 7-12.5 hours old. Hybridizing fragments were then used for conventional Northern analysis to confirm the existence of a transcript and determine its size.
RESULTS

Isolation and preliminary characterization of single gene mutations in 75C1,2

The chromosomal deficiencies WR4, WR10 and CatDH104 overlap from 75B8-11 to 75C1,2 (See Figure 1). For the purpose of discussion, this region will be referred to as the 75C1,2 region. When this region of the genome is deleted, embryos have larval visual system defects and lack essentially all programmed cell death. Development of the head region is also abnormal in these embryos. Based on the banding pattern of polytene chromosomes, 75C was estimated to contain 195.5 kilobases (kb) of DNA (Sorsa, 1988); thus several genes are likely to be deleted in the region defined by the overlap of the three deficiencies. It was possible that the projection defect, the cell death defect, and the head involution defect are due to deletions of the same gene. Alternatively, these phenotypes are genetically separable. In order to identify the gene(s) responsible for each mutant phenotype, we tried to identify single gene mutations with the phenotypes of interest.

To isolate single gene mutations in 75C1,2 we mutagenized flies with the alkylating agent ethylnitrosourea (ENU) and did a standard F2 mutagenesis as outlined in Figure 2. Approximately 23,000 mutagenized third chromosomes were screened, and we identified 20 chromosomes which were lethal in combination with the WR4 chromosome (See Table 1). Each lethal line was crossed to WR10 to identify those lethals mapping to 75C1,2. Of the 20 lethal chromosomes isolated, five are uncovered by both WR4 and WR10. Complementation analysis revealed that all five "75C1,2 mutants" are alleles of a single complementation group. The other 15 lethal mutations isolated fall into two complementation groups: 10 alleles of what we call the "ML1" complementation group and five alleles of the "ML62" complementation group.

We also screened for any recessive visible mutations that might be uncovered by both deficiencies. Cell death is known to play a role in the development of the adult compound eye (Cagan and Ready, 1989) and wing (Spreij, 1971). Thus, viable cell death mutants might manifest themselves as visible defects in these structures. Furthermore, abnormal projection of the larval optic nerve might result in alterations in compound eye morphology. In fact, adults mutant at the disco locus (which is required for proper
connection between Bolwig's nerve and its target region) have deformed eyes (Steller et al., 1987); thus, it is possible that the 75C projection mutants might be viable and have an eye phenotype. For each line, adult flies of the appropriate genotype were scored for eye and wing defects.

We identified nine lines of flies with rough eyes. These were determined to be alleles of roughoid (ru) which is on the WR4 chromosome. We also identified thirteen lines in which flies had abnormal wing veins. This phenotype was also visible in combination with WR10. These lines were tested and did not have any detectable defects in cell death or the projection of Bolwig's nerve.

In a search for mutations in a blastoderm specific transcript mapping to 75C1,2, terminus, (Baldarelli, et al., 1988), Abbott and Lengyel (1991) performed a mutagenesis for lethal mutations in 75C1,2. They found a single lethal complementation group, which they named head involution defective (hid), that was uncovered by WR4 and W10. As is indicated by its name, embryos mutant for hid fail to complete head involution. After completing our mutagenesis, we received a collection of hid alleles from M. Abbott. All five of the 75C1,2 mutations which we generated failed to complement the hid alleles sent by M. Abbott; thus, the 75C1,2 mutations we isolated are alleles of hid.

We tested the hid alleles for defects in the projection pattern of Bolwig's nerve and for defects in the pattern of cell death by staining collections of hid mutant embryos with either mAb24B10 or acridine orange, respectively. All of the hid alleles tested had abnormally projecting larval optic nerves (Figure 3). The details of this analysis are presented in Chapter IV. Of all the hid alleles tested (n=12), only two, H99 and H109, showed any perceptible reduction in the number of acridine orange staining cells. H99 and H109 lack almost all acridine staining and are comparable in phenotype to WR4 and WR10. We subsequently showed that both of these alleles, while appearing cytologically normal (Abbott and Lengyel, 1991) are deletions of approximately 300 kb (see below). Thus, while mutations in the gene hid appear to cause the abnormal projection of Bolwig's nerve, hid mutations alone do not result in gross defects in programmed cell death.
Chromosomal walk through 75C1,2

We cloned the DNA deleted by both WR4 and WR10 by chromosomal walking (Bender et al., 1983) (Figure 4). Segraves and Hogness (1990) had cloned 350 kb of DNA corresponding to the region encompassed by polytene bands 75A6-7 to 75B11-13. Approximately 130 kb of this DNA, extending towards the centromere from the distal breakpoint of WR4 fall in the overlap between WR4 and WR10. We used clone 3501, which was the most proximal clone isolated by Segraves and Hogness to initiate our walk towards the proximal breakpoint of WR10. The origin (0) on Figures 4 and 5 defines the beginning of our walk. We also obtained a WR10 "jump clone" from W. Segraves (Segraves, 1988). This clone was isolated from a genomic library of WR10 DNA and spanned the deficiency breakpoint of WR10, thus defining the proximal boundary of our walk.

The blastoderm specific transcript, terminus, also maps to 75C1,2 (Baldarelli, et al., 1988; Roark et al., 1985). By PCR, we generated a 740 base pair fragment corresponding to the genomic terminus locus. We determined by hybridization that terminus does not map to any of the DNA cloned by Segraves and Hogness, and therefore was useful as another entry point into 75C1,2. We labeled this fragment and used it to isolate additional genomic clones. These clones were mapped to 75C by hybridization to polytene chromosomes (Figure 1). terminus maps approximately 10 kb away from the proximal breakpoint of WR10 (M. Abbott, unpublished observation). We then walked in the proximal to distal direction starting from terminus. Thus, starting from clone 3501 and from terminus, we walked in two directions until each walk merged. The amount of DNA in the overlap between WR4 and WR10 is approximately 510 kb, 380 kb cloned by us, 130 kb by Segraves and Hogness. This is well above the estimated DNA content of 195 kb for all of 75C (Sorsa, 1988). This discrepancy is likely due to the fact that polytene bands 75C1,2 are highly condensed, and it is hard to obtain accurate estimates of DNA content in such cases.

H99 and H109 are genomic deficiencies

While all of the existing hid alleles displayed the Bolwig's nerve defect, only two, H99 and H109 had defects in cell death as well. H99 and H109 were generated by exposing flies to γ-irradiation (Mackay and Bewley, 1989) and were determined to be alleles of hid by Abbott and Lengyel (1991). Because
these mutations had been induced by γ-irradiation, we suspected that they might be chromosomal rearrangements. We prepared DNA from homozygous \( H99 \) and \( H109 \) mutant embryos (identifiable because of pronounced head involution defects), and used this DNA for genomic Southern analysis. We determined that \( H99 \) and \( H109 \) are in fact identical genomic deficiencies (at the level of genomic Southern) in which approximately 300 kb of DNA, extending from clone 3508 to AA31, are deleted (See Figure 5). This 300 kb interval defines the smallest genomic region which, when deleted, results in a dorsally projecting Bolwig's nerve and the absence of most embryonic programmed cell deaths.

The identification of putative transcription units in 75C1,2
At least a portion of the gene(s) responsible for the projection phenotype and the cell death defect are likely to be contained within the \( H99 \) deficiency. Reverse Northern analysis was used to identify DNA within \( H99 \) that might be transcribed. Because the phenotypes are manifest by at least 12 hours of development (at 25°C), we probed genomic DNA contained within the \( H99 \) deficiency with cDNA prepared from RNA of embryos 0-7 and 7-12.5 hours old. Conventional Northern analysis was used to confirm the presence of transcription units identified by the reverse Northern analysis. The transcribed regions represented in Figure 5 were pursued as likely candidates for the genes responsible for the phenotypes of interest.

Generation and mapping of breakpoints in 75C
In an effort to determine which portion of the 300 kb deleted in \( H99 \) is required for embryonic programmed cell death, we generated a number of new X-ray induced breakpoints in the 75C region. X-irradiated flies of the \( ru\ W \text{ca red}/TM2 \) genotype were scored for reversion of the Wrinkled (\( W \)) phenotype. Similar strategies were used by Segraves and Hogness (1990) to generate WR4 and WR10, and by Abbott and Lengyel (1991). \( W \) is a dominant mutation in the 75C region. Adult \( W \) flies have a pronounced wing defect, reversion of which is easily scored in an F1 mutagenesis. Abbott and Lengyel (1991) demonstrated that all \( W \) revertants, including those induced by ethyl methanesulfonate (EMS) are \( hid \) alleles; thus \( W \) is a dominant allele of \( hid \).

We identified 18 \( W \) revertant chromosomes out of 117,197 scored. Only four of the \( W \) revertants, \( X14, X20, X24 \) and \( X25 \), however, were fertile
and lines were established from these. All four newly isolated \( W \) revertants were \( hid \) mutants, consistent with the proposal that \( W \) is a \( hid \) allele. Each \( W \) revertant was also tested for cell death defects, both when homozygous and when put in trans to \( H99 \). In \( X14 \) and \( X24 \) mutant embryos, levels of acridine staining appear to be wild type. \( X20 \) mutants, however, lack most embryonic cell deaths (Figure 6D). This phenotype is comparable to that of the originally identified cell death mutants (\( WR4, WR10, CatDH104, \) and \( H99 \)). \( X25 \) mutants display what we term a "partial phenotype." In \( X25 \) homozygotes, there is a slight decrease in the number of cell deaths seen with respect to wild type (Figure 6B). This phenotype is greatly enhanced when \( X25 \) is placed in trans to \( H99 \). In \( X25/H99 \) mutant embryos, there is a substantial reduction in the number of acridine orange positive cells (Figure 6C). There are still, however, significantly more cell deaths observed in these embryos than are ever found in embryos homozygous for \( H99 \) (Figure 6D).

In order to molecularly map the breakpoints of the \( W \) revertants, we crossed each \( W \) revertant to \( H99 \) and prepared DNA from embryos of the genotype \( W \) revertant/\( H99 \). All of the \( W \) revertants, with the exception of \( X24 \), are embryonic lethal either homozygous or in trans to \( H99 \), and mutant embryos can be reliably identified on the basis of their head involution defects. We used this DNA for genomic Southern analysis and identified the breakpoints shown in Figure 5.

This analysis facilitated further localization of both \( hid \) and a cell death function. Because all \( W \) revertants are \( hid \) alleles, at least a portion of \( hid \) is likely to reside in the DNA which is deleted in all deficiencies, the 80 kb interval defined by the distal breakpoint of \( X20 \) and the proximal breakpoint of \( X14 \). Furthermore, a cell death function should reside in the 85 kb from phage H1-1 to AA31 (see discussion). We have in fact, molecularly characterized both \( hid \) (Chapter IV), and a cell death gene, \( reaper (rpr) \) (Chapters V and VI) whose molecular localizations are consistent with the findings outlined here.
DISCUSSION

The 75C1,2 region of the genome is required for at least three developmentally important events during embryogenesis: head involution, proper projection of Bolwig's nerve and control of programmed cell death. All of the above phenotypes were initially identified in deletion mutants in which several genes are likely to be affected. To determine which functions in 75C1,2 are required for larval optic nerve projection and/or programmed cell death, we undertook a detailed analysis of this genomic interval.

One major question which follows from the study of deletions mutants is whether the observed phenotypes are genetically separable. To address that issue, single gene mutations in the 75C1,2 interval were generated and analyzed. We isolated five alleles of a single lethal complementation group which were uncovered by WR4 and WR10 and demonstrated that these mutations are allelic to hid. hid mutants are defective in the process of head involution and also have abnormally projecting larval optic nerves; thus, the head defect and the Bolwig's nerve phenotype segregate with the same locus. The details of our analysis of these phenotypes and the molecular characterization of the hid locus are presented in Chapter IV.

Only two of the hid alleles, H99 and H109, showed any obvious defects in programmed cell death. We demonstrated that H99 and H109 are genomic deletions of approximately 300 kb, and therefore are expected to delete several genes in addition to hid. hid mutations alone are not sufficient to eliminate a significant amount of programmed cell death in the Drosophila embryo. We have identified at least two null hid mutants (See Chapter IV), neither of which has any detectable cell death phenotype. Mutations in hid clearly cannot account for the global lack of cell death found in deficiency embryos. We cannot, however, exclude the possibility that hid does have a role in programmed cell death (see below).

While we were able to isolate single gene mutations which affect the projection of Bolwig's nerve, we were unable to identify any that dramatically alter the levels of programmed cell death during embryogenesis. There are several different explanations for this. First, when doing our mutagenesis, we made the assumption that a cell death defective mutation would manifest itself as a visible or lethal phenotype. Mutations in the genes roughest and echinus, which dramatically reduce the amount of cell death in the
Drosophila eye result in visible rough eye phenotypes (Wolff and Ready, 1991); thus, we expect viable cell death defective mutants to have rough eyes. We favor the possibility, however, that globally cell death defective mutants in Drosophila would not be viable. There is a large amount of cell death which occurs during Drosophila embryogenesis. We have demonstrated that 75C deletion mutants possess extra cells, presumably reflecting the lack of programmed cell death in these embryos (see Chapter V) and predict that the presence of these cells would interfere with morphogenesis, resulting in lethality. Given that the mutations in the genes ced-3 and ced-4 which block programmed cell deaths in C. elegans are not lethal (reviewed in Ellis et al., 1991), it was also important to screen for visible phenotypes. We did not, however, isolate any lethal or visible mutations which reflected a cell death defect.

Another explanation is that while a cell death mutation might result in lethality or present a visible phenotype, we simply did not saturate the interval for all complementation groups. In our mutagenesis, we isolated several alleles of each of seven complementation groups (See Table 1). We thus assumed that we had saturated 75C1,2 for lethal and visible mutations. Furthermore, in an independent screen, Abbott and Lengyel (1991) identified hid as being the only complementation group mapping to the interval. Of course, the probability of inducing a mutation in a gene is roughly proportional to its size. If the 75C1,2 cell death gene is particularly small, it is unlikely that we would have mutated it. This is particularly relevant in light of our identification of a small transcript, which we call reaper (rpr) which accounts at least partially, if not entirely for the cell death defect in 75C1,2 deletion mutants (see Chapters V and VI).

Another possibility is that there are multiple functions in 75C1,2 which are required for programmed cell death. If this were true, one might only observe striking cell death defects if several genes are deleted simultaneously. In Drosophila, there are several examples of clusters of genes with redundant functions (Cote et al., 1987; Grossniklaus et al., 1992; Schrons et al., 1992). It is for this reason that we sought to induce mutations in 75C1,2 with ionizing radiation. Ionizing radiation (X-rays and γ-rays) is known to be effective for inducing chromosomal deletions (Ashburner, 1989). We were able to generate several new deletions in 75C by reverting a dominant allele of hid, W with X-
rays (see Figure 5) and from these W revertants breakpoints, we were able to further localize both hid and at least one cell death function.

The breakpoints which were molecularly mapped are shown in Figure 5. X20 and H99 lack most programmed cell deaths, either homozygous or in combination. This maps the full cell death defective phenotype to the 300 kb from clone 3504 to AA31. X14 is deleted from D6A2 towards the telomere, at least as far as the distal breakpoint of X20. X14 mutants are apparently wild type for programmed cell death. Deletion of this DNA is not sufficient to confer a cell death phenotype, suggesting that a cell death function must reside between clones D6A2 and AA31. Homozygous X25 mutants display a partial cell death defect. This phenotype is greatly enhanced when X25 is in combination with H99. Because the distal breakpoint of X14 extends beyond that of X25, this suggests that a cell death function must reside between the proximal breakpoints of X25 and H99, i.e., between H1-1 and AA31. We have identified one transcript (rpr) which maps to that interval and propose that rpr is crucial for the activation of a global cell death program (see Chapters V and VI).

Is rpr the only function in 75C which is required for cell death? At least at the level of genomic Southern, the rpr locus appears to be unaffected in X25 mutants. How then can the cell death defect in X25 mutants be explained? One possibility is that there is a small change in the rpr gene which renders it partially non-functional. A second possibility is that the X25 deletion somehow influences expression or function of the rpr gene. The proximal breakpoint of X25 maps at least 70 kb away from the rpr gene. Furthermore, levels and tissue distribution of the rpr transcript appear to be unaffected in X25 mutant embryos (or X25/H99 mutants) when examined by wholemount in situ hybridization with a rpr probe (K. White, unpublished).

An alternative possibility, as mentioned above, is that there are multiple functions in 75C required for programmed cell death, one of which is probably rpr. The model in Figure 7 illustrates how this might work. If there are two cell death functions in 75C, one of which is deleted in X25 and both of which are deleted in H99, the enhancement of the cell death phenotype seen when X25 is in trans to H99 could be explained by a further reduction in "cell death gene" dosage.

Does hid have a role in programmed cell death? We know that null hid mutations do not appear to affect the levels of programmed cell death in
the *Drosophila* embryo. However, because all cell death deletions were generated by reverting *W*, a dominant allele of *hid*, all cell death mutants are *hid* mutants as well. If there are multiple genes in 75C which are required for programmed cell death, this leaves open the possibility that *hid* has a cell death function. To assess the role that *hid* might play in programmed cell death, one needs to know the cell death phenotype of a 75C deletion that has wild type *hid* function. We are in the process of re-introducing the *hid* gene into the deficiency background by P-element mediated gene transformation (see Chapter IV). If *hid* does have a cell death function, a *hid* transgene might be expected to restore some level of programmed cell death to deficiency H99 embryos.

In summary, we have demonstrated that *hid* is required for normal projection of the larval optic nerve. We have been able to further localize at least one cell death function in 75C, and we have cloned (or obtained) all of the relevant DNA in the interval, making possible molecular characterization not only of *hid*, but of the cell death gene(s) as well (see Chapters IV-VI).
REFERENCES


Figure 1 - Deficiencies mapping to polytene bands 75A-F. Deficiencies WR10, WR4, and CatDH104 and their published breakpoints are diagrammed below a photograph of the 75C region of a polytene chromosome. This chromosome has been hybridized with a clone containing the terminus gene. DNA which is deleted in each deficiency is represented as dotted lines. Solid bars represent chromosomal DNA. Embryos homozygous for, or bearing any trans-heterozygous combination of the deficiencies have abnormal projections of the larval optic nerve and lack most programmed cell deaths.
Figure 2 - Mutagenesis scheme used to isolate single gene mutations in 75C

1) Score for the absence of \( \frac{*s t e}{Df(3L)WR4 \ ru \ h \ e \ ca} \) progeny, indicating a lethal mutation

2) Score for visible phenotypes of \( \frac{*s t e}{Df(3L)WR4 \ ru \ h \ e \ ca} \) genotype

3) Recover lethals and visibles over TM3 Sb
Table 1 - Mutations isolated in 75C mutagenesis

<table>
<thead>
<tr>
<th>Lethal mutations recovered</th>
<th>Number of alleles</th>
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<tr>
<td>hid</td>
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<tr>
<td>ML1</td>
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<tr>
<td>ML62</td>
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<tr>
<th>Visible mutations recovered</th>
<th>Number of alleles</th>
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<tr>
<td>ru</td>
<td>9</td>
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<tr>
<td>pp*</td>
<td>19</td>
</tr>
<tr>
<td>wing</td>
<td>13</td>
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Total chromosomes scored: 23,000

ML1, ML62, and wing are our names for previously uncharacterized complementation groups uncovered by Df(3L)WR4

*segregates with TM3 Sb
Figure 3 - In *hid* mutants, the larval optic nerve projects abnormally. Lateral views of *Drosophila* embryos stained with mAb 24B10, anterior is to the left. Arrows indicate the cell bodies of Bolwig's nerve. (A) Wild type embryo - in wild type embryos, Bolwig's nerve always projects to the ventral brain. (B) *Df(3L)H99* embryo - Embryos homozygous for deletions of 75C1,2 have dorsally projecting Bolwig's nerves. (C, D) *hidA22* and *hid H89* embryos, respectively - Note the similarity in projection phenotypes between the *hid* mutants and the 75C deficiency embryo. Embryos with dorsally projecting larval optic nerves can be found in collections of all tested *hid* alleles (See Chapter IV). Abbreviation: *br* brain.
Figure 4 - Chromosomal walk through 75C1,2. Representative clones isolated in chromosomal walks through 75C1,2 and EcoRI restriction sites are shown. Clones 3510 through 3501 were identified and mapped by Segraves and Hogness (1991). "0" defines the start of the DNA we isolated. All clones, with the exception of cosE3A and cosF12 are phage clones. cosE3A and cosF12 are cosmid clones isolated from iso-1 cosmid library of Dr. J. Tamkun. All other clones were isolated from the Canton-S genomic library of Dr. R. Davis.
Figure 5 - Clones and Deficiencies in the 75 region of Drosophila. Deficiencies are shown above a map of cloned DNA in the 75 region. Dotted lines represent DNA which is deleted in each deficiency. Embryos homozygous for all deficiencies shown display abnormal projection of the larval optic nerve. Embryos homozygous for deficiencies represented by solid bars lack most programmed cell deaths as assayed by acridine orange staining. Wild type levels of programmed cell death are observed in embryos homozygous for X14 (open bar). A partial reduction in cell death is observed in embryos homozygous for X25 (stippled bar). This phenotype is enhanced when the deficiency is in combination with H99 (or any of the solid bar deficiencies). Deficiency breakpoints, when known, are shown above each deficiency. We did not determine the proximal breakpoint of X20 or the distal breakpoint of X14. X24, the other W revertant we isolated behaves as an extreme hypomorph of hid and we were unable to detect any chromosomal abnormalities in heterozygous X24 DNA. Below the deficiencies is a representation of cloned DNA in this region. Clones running from 0 to 400kb were cloned in this study. Phage clones from 0 to -250 kb were isolated by Segraves and Hogness(1991). All cosmid clones were isolated in this study from the library of Dr. J. Tamkun. We mapped Yeast Artificial Chromosome (YAC) clones 187 and 657 to the chromosomal walks in this region. YACs were provided by Dr. D. Hartl.
Figure 6 - Levels of cell death in W revertant embryos. Embryos stained with acridine orange to assay relative levels of cell death. (A) wild type embryo. (B) X25/X25 - These embryos still have substantial amounts of programmed cell death as assayed by acridine orange. Note, however, the conspicuous lack of staining in the thoracic region of this embryo. (C) X25/H99 - When X25 is place in combination with H99, the levels of acridine orange staining are dramatically reduced. (D) X20/X20 - These embryos lack most, if not all embryonic cell deaths.
**Figure 7 - Model - Redundant Functions in 75C are required for programmed cell death.** Assume there are two cell death functions, A and B that map to 75C, as shown above. Wild type diploid cells will have two doses each of A and B. *H99*, which lacks most programmed cell deaths, is missing both A and B. *X25* homozygotes show a modest reduction in the number of programmed cell deaths. They have two doses of B and no doses of A. It is possible that having wild type levels of gene B will partially compensate for lack of gene A, resulting in embryos with significant levels of acridine orange staining.
When *X25* is put in combination with *H99*, the level of cell death gene dosage is further reduced, resulting in the enhancement of the cell death phenotype. This model predicts that *H99/+* embryos should show a phenotype comparable to *X25* homozygotes. However, *H99/+* embryos have levels of acridine staining indistinguishable from wild type. Perhaps there is some cooperativity between A and B such that having one dose each of A and B results in greater gene activity than having two doses of B alone.
hypothesical cell death genes: 

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<td>H99</td>
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<td>X25</td>
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<td>X25/H99</td>
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AUTHOR'S NOTE

This work involved the collaboration of a number of people in the lab. John Abrams, Kristin White, and Lynn Young participated in the mutagenesis for single gene mutations in 75C. The \( W \) revertants were generated by Kristin White, Kim Mejia, Dung Vu, and Melissa Rivera. Lynn Young provided technical assistance for the chromosomal walk through 75C1,2, and the transcription units in the 75C region were identified by John Abrams.
Chapter IV
Molecular Genetic Analysis of head involution defective, A Gene
Required for the Development of the Larval Visual System of Drosophila
ABSTRACT

Embryonic head development in Drosophila melanogaster involves a complex series of morphogenetic movements. The head involution defective (hid) gene of Drosophila is required for head morphogenesis as well as for the proper development of the larval visual system. In hid mutant embryos, head involution arrests and the projection pattern of the larval optic nerve (Bolwig's nerve) is abnormal. We show that the projection defect results from a failure of the target region of Bolwig's nerve, the optic lobe primordium, to migrate to its normal position. We have molecularly characterized the hid gene and sequenced a number of wild type and mutant hid alleles. The predicted translation product of this gene is a novel protein of 410 amino acids. hid mRNA expression is associated with all head structures that invaginate, including the optic lobe primordia, suggesting that hid is required in these tissues for their morphogenetic movements.
INTRODUCTION

The proper functioning of a nervous system depends on the formation of precisely patterned neuronal connections. Understanding how these projection patterns are established is a fundamental goal of developmental neurobiology. A wide variety of approaches have been employed in efforts to elucidate the mechanisms which generate neuronal specificity (reviewed in Goodman and Shatz, 1993). Because of its simplicity, the larval visual system of Drosophila is an ideal model system for studying how the projection pattern of a single nerve is formed.

Drosophila larvae, like all Dipteran larvae, are acephalic and do not develop external eyes. Instead they possess bilaterally symmetric internal visual organs. The larval photoreceptor organ, or Bolwig's organ, is the rudimentary larval eye (Bolwig, 1946). Each organ consists of approximately twelve photoreceptor cells. These cells send out axons which fasciculate together, forming the larval optic nerve, or Bolwig's nerve. In the wild type Drosophila embryo, each larval optic nerve projects in a highly stereotyped manner to the ipsilateral brain hemisphere and contacts the brain in the region of the optic lobe primordium. Upon entering the optic lobe primordium, the nerve turns and makes contact with interneurons in the central brain (Steller et al., 1987).

Development of the larval visual system (see Figure 1) has been well described (Steller et al., 1987; Schmucker et al., 1992; Green et al., 1993; Campos et al., in prep.). Bolwig's nerve and the optic lobe primordium are originally derived from the optic placode which invaginates from the dorsal ectoderm during stages 12 - 13 (Green et al., 1993). The cells destined to become the larval photoreceptors differentiate from a ventral portion of this placode (Green et al., 1993), and axon outgrowth from the photoreceptors is visible as early as stage 12 (Steller et al., 1987). Contact between the larval photoreceptor nerve and the optic lobe primordium is established at this time and maintained throughout the rest of embryonic and larval development. As the optic lobe primordia migrate ventrally and medially and the photoreceptor cell bodies move anteriorly, Bolwig's nerve elongates. Ultimately, the optic lobe primordia fuse with the supraoesophageal ganglia in a postero-ventral position and the photoreceptor cell bodies end up situated near the cephalopharyngeal skeleton (Campos-Ortega and Hartenstein, 1985).
Genetic analyses have revealed several genes which are crucial for certain aspects of larval visual system development. For example, glass is required for cellular specification of the larval photoreceptors (Moses and Rubin, 1991), disconnected for the maintenance of contact between Bolwig's nerve and its targets (Steller et al., 1987), and Krüppel for proper fasciculation of the photoreceptor cell axons (Schmucker et al., 1992).

Much of the development of the larval visual system occurs during the process of head involution (a process described in detail by Turner and Mahowald, 1979; Campos-Ortega and Hartenstein, 1985) and the relationship between larval visual system development and head involution is not well understood. Head involution is a complex series of morphogenetic movements by which the head is enveloped into the larval body (see Figure 2). At the start of head involution (Figure 2A), the most prominent anterior structures are the clypeolabrum (cl) and the gnathal segments (gn). Head involution can be viewed as essentially two processes. Ventrally, the gnathal segments are orally displaced, i.e., they move into and along the stomodeal invagination (st), forming, among other things, portions of the atrium (at) and the pharynx (ph). Dorsally, an epithelium known as the dorsal fold (df) moves anteriorly across the procephalic lobe (Figures 2B-D). The anterior progression of the dorsal fold continues until the entire head region is enveloped. Much of the relative displacement of the larval photoreceptor cell bodies and the optic lobe primordia occurs concomitant with head involution (Steller et al., 1987; Green et al., 1993).

In a screen for mutations affecting the development of the larval visual system, we determined that the genomic region 75C1,2 is required for proper projection of the larval optic nerve (Chapter II). We have demonstrated that the head involution defective (hid) gene, which maps to 75C1,2 (Abbott and Lengyel, 1991), is essential for the normal projection of the larval optic nerve (Chapter III). In this chapter, we report a detailed analysis of the mutant phenotype and the molecular characterization of the hid gene. We show that the aberrant projection of the larval optic nerve is due to incomplete migration of the optic lobe primordia; thus, hid is required not only for the movements of head involution but for the invagination of the optic lobe primordia as well.
MATERIALS AND METHODS

Drosophila stocks. Df(3L)H99, Df(3L)H109, hidA22, hidA206, hidA329, In(3LR) hidH89, In(3L)hidWR+X1, hidWR+E1, hidWR+E2, hidWR+E4, and hidWR+E6 were obtained from M. Abbott and J. Lengyel. hidML23, hidML66, and hidML87 were generated in this study (see Chapter III). l(3)05014 and l(3)00225 were the gift of A. Spradling. hid40C and hid8D were generated by excision of the 05014 P-element. All other stocks used in this study were obtained from the Drosophila stock center, Bloomington, Indiana.

Generation of excisions of the 05014 insertion. The 05014 P-element was mobilized using A2-3 (99B), a genomic source of P-transposase (Robertson et al., 1988). Excision chromosomes were identified by loss of the ry+ marker. Reversion of the hid phenotype was scored by placing the excision chromosome in trans to Df(3L)H99. 45% of excision events resulted in reversion of the hid phenotype.

Immunohistochemistry. Monoclonal antibody (mAb) 24B10 staining was performed as described in Chapter I. All other immunohistochemical stainings of embryos were performed as previously described (Steller, et al., 1987). To detect engrailed expression, we used mAb4D9 (Patel et al., 1989) which recognizes both the engrailed and invected proteins. These proteins are expressed coincidentally (Coleman et al., 1987), and staining with this antibody is referred to as "engrailed" expression. The primary antibodies used in this study were used at the following dilutions: mAb24B10 (gift of S. Benzer) and mAb4D9 (gift of D. Staininer) - 1:1; affinity purified rabbit anti-disconnected, monoclonal anti-fasciclin II (gift of C. Goodman), and mAb22C10 (gift of S. Benzer) - 1:3; and rabbit polyclonal anti-bsh (gift of W. McGinnis) - 1:1000 (after preadsorption at 1:20 against 0-2 hour embryos). Secondary antibodies (HRP-conjugated anti-rabbit immunoglobulin (Ig) from Bio-Rad, Rhodamine-conjugated anti-rabbit Ig from Boehringer Mannheim and FITC-conjugated anti-mouse Ig from Cappel) were used at a dilution of 1:100. Light microscopy was done using a Zeiss Axioptot microscope. Confocal microscopy was done using a BioRad MRC600 laser scanning confocal microscope equipped with a krypton/argon laser. Data generated by laser scanning confocal microscopy was processed using manufacturer's
software and instructions. Staging is as according to Campos-Ortega and Hartenstein (1985).

**Plasmid rescue of genomic DNA flanking the 05014 insertion.** Molecular biological techniques were performed by standard procedures (Sambrook et al., 1989). Rescue of genomic sequences was performed basically as described (Steller and Pirrotta, 1986). For plasmid rescue, genomic DNA from flies carrying the 05014 insertion was digested to completion with either SpeI or KpnI. Approximately 13kb flanking the insertion was recovered by plasmid rescue and mapped to the walk in 75C by Southern hybridization.

**Isolation and sequencing of the 5A1 cDNA.** An 8kb EcoRI restriction fragment from phage 3506 was used to screen an eye disc cDNA library in λgt10 (constructed by A. Cowman, Rubin lab). One clone, clone 5A1, was isolated which was longer than 2kb. It was subcloned in the pBluescript II SK+ (Stratagene) cloning vector. Series of nested deletions for both strands of the cDNA were generated according to manufacturer's instructions using the Exo/Mung deletion kit (Stratagene). Sequencing of double-stranded DNA was carried out using Sequenase (United States Biochemical Corp) according to manufacturer's instructions. The template DNA was prepared as described below. For each sequencing reaction, a 2 ml overnight culture was grown and DNA prepared by the alkaline lysis method (Sambrook et al., 1989). The DNA was resuspended in 25 μl TE and 15 μl of this was used per sequencing reaction. Both strands were sequenced. Gaps in the series of nested deletions were filled by sequencing using oligonucleotide primers derived from previously determined cDNA sequence.

**Northern Analysis.** poly-A RNA was isolated using the FastTrack RNA isolation system (Invitrogen). Roughly equal amounts of RNA were loaded in each lane. An 8kb EcoRI fragment from phage 3506 was used to probe the Northern blot.

**Sequencing of the genomic hid locus.** Sequencing of the genomic hid locus was done using γ-33P-ATP labeled oligonucleotide primers and the Circumvent sequencing kit according to manufacturer's instructions (New England Biolabs). The template for this sequencing was derived from genomic phages corresponding to portions of the hid locus.
Sequencing of the hid alleles. Flies of each allele to be sequenced were crossed to Df(3L)H99. When possible, DNA was isolated from adult flies of the genotype hid /H99. If no adults emerged from the cross, embryos with head involution defects were culled for DNA preparation as described in Chapter III. Fragments corresponding to the coding sequence of the hid locus were isolated by PCR. The oligos used for amplification are underlined in Figure 12. For each PCR reaction, approximately 50 ng of genomic DNA was used as template. A standard 50 µl PCR reaction was set up as according to protocols of Perkin-Elmer Cetus. Annealing temperatures for each set of primers are as follows: exon 1 - 52°C; exon 2 - 56°C; exon 3 - 60°C; exon 4 - 50°C. Cycling profiles for all amplifications were as follows: 1 cycle for 1 minute at 94°C, followed by 30 cycles of 1 minute, 94°C, 2 minutes at the annealing temperature, 2 minutes 15 seconds at 72°C with 2 seconds added per cycle. This was followed by 10 minutes at 72°C. 50% of each reaction was loaded onto a 1% SeaPlaque GTG grade low melting agarose gel in 1XTAE. Bands of the appropriate size were excised from the gel and diluted 2-3 fold with water. 5µl of this was used as template for sequencing. Sequencing was done using γ-32P-ATP labeled oligonucleotide primers and the Circumvent sequencing kit (New England Biolabs). For each polymorphism identified, a second, independent PCR reaction was done and the allele resequenced.

In situ hybridization of whole mount embryos. Embryos were fixed and hybridized according to the protocol of Tautz and Pfeifle (1989) except that digoxigenin-labeled RNA probes were used according to the method of R. Bodmer. RNA probes were transcribed following the protocol of the Boehringer RNA labeling kit.

In situ hybridization of larval imaginal discs and central nervous systems. Larvae were dissected and fixed for 20 minutes in 4% paraformaldehyde in PBS. Pretreatment, hybridization and detection were done as in Tautz and Pfeifle (1989) except that digoxigenin-labeled RNA probes were used (see above).
RESULTS

The larval optic nerve projects aberrantly in hid embryos

In wild type embryos, the larval optic nerve projects to the ventral brain (Figure 3A). In embryos homozygous for deletions of the 75C region, Bolwig's nerve projects dorsally (Figure 3B). The head involution defective (hid) complementation group maps to this deficiency interval (Abbott and Lengyel, 1991; see Chapter III). In order to test whether hid is required for proper projection of the larval optic nerve, we analyzed the projection pattern of the nerve in hid embryos. In collections of hid embryos stained with the photoreceptor cell specific mAb24B10 (Fujita et al., 1982), we find mutant embryos in which the larval optic nerve projects abnormally. This is true for all hid alleles tested: H89, WR+X1, A22, A206, A329, l(3)05014, WR+E6, and WR+E2. We conclude that proper projection of the larval optic nerve depends on hid function.

In 75C deficiency embryos, the larval optic nerve always projects to the dorsal brain. In all hid alleles tested, however, there is considerable variability in the projection phenotype (See Table 1). Although for each allele tested, embryos with strong projection defects (i.e., dorsally projecting larval photoreceptor axons) can be identified (Figure 3C, D), in some embryos, Bolwig's nerve terminates more ventrally. In hid, even when the nerve terminates ventrally, however, there are defects in its projection pattern. Furthermore, in any given embryo, the projection phenotype is not always bilaterally symmetric. In some mutant embryos, one nerve terminates dorsally, the other ventrally (Figure 3E, F).

We sought to determine whether the severity of the projection defects of Bolwig's nerve correlated with the strength of the hid allele. Although hid does have a role in head involution, zygotic hid function does not appear to be strictly required for this process. Occasionally, hid flies, even those homozygous for null mutations, survive to adulthood and have characteristic wing and genital defects (Abbott and Lengyel, 1991); thus, presumably some fraction of the time, hid embryos successfully complete head involution. We define strong hid alleles as those in which the greatest proportion of embryos fail to complete head involution by the end of embryogenesis.

For each allele tested, we collected embryos, aged them to at least stage 119
development, the dorsal fold has covered the clypeolabrum. In each egg collection stained with mAb24B10, we counted the total number of embryos with head involution defects, as defined by the clear failure of the dorsal fold to cover the clypeolabrum. We also examined the projection pattern of Bolwig's nerve in every embryo. A wide range of projection phenotypes is observed for all hid alleles tested (see Table 1). Embryos with strong projection phenotypes can be found even in collections of weak hid alleles (e.g., A22, A206, and A329); thus the strength of the projection phenotype is not correlated with the strength of the hid allele.

Projection defects are, however, always found in association with defects in head involution. hid embryos with head involution defects never have a wild type projection pattern. Even when Bolwig's nerve terminates ventrally, its projection path is quite abnormal (compare Figures 3A and F). Likewise, hid embryos with wild type head morphology always have a wild type projection pattern. Thus, the penetrance of the projection defect is correlated with the penetrance of the head involution defect. We know, at least for weak hid alleles, that some fraction of embryos with wild type heads must be genotypically hid, as the total number of embryos with head involution defects is less than the expected value of 25%. This suggests that in hid embryos, defects in head involution are a more reliable indicator of projection defects than the genotype of the embryo.

The optic lobe primordia are ectopically positioned in hid embryos

The projection defect in hid mutants could be due to a failure of Bolwig's nerve to connect to its appropriate targets, or to the mislocalization of the targets themselves. In wild type embryos, Bolwig's nerve projects to the region of the optic lobe primordium. These dorsally derived cells migrate ventrally and medially before fusing with the brain. Cells of the optic lobe primordia express both disconnected (Lee et al., 1991) and fasciclin II (Campos et al., in prep.) throughout their development.

To determine the position of the optic lobe primordia in hid mutant embryos, we stained pools of embryos with either antibodies against disconnected (α-disconnected, Lee et al., 1991) or against fasciclin II (α-fasciclin II, Grenningloh et al., 1991). Figures 4A and B are stage 13 wild type and hid embryos, respectively, stained with α-disconnected antibody. In the wild type embryo, the optic lobe primordium has invaginated from the dorsal ectoderm.
and has begun to migrate ventrally. In contrast, in the *hid* embryo, the optic lobe primordium is located much more dorsally. The optic lobe has invaginated, but it remains dorsal.

α-fasciclin II (Grenningloh et al., 1991) recognizes the posterior half of the optic lobe primordium and the larval optic nerve (Campos et al., in prep.). In wild type, Bolwig's nerve (arrow, Figure 5A) projects to a fasciclin II positive region in the ventral brain (Figure 5C, E). In *hid* (Figures 5B, D, and F) Bolwig's nerve also projects to the fasciclin II positive optic lobe primordium, but here, the optic lobe primordium is located very dorsally (Figures 5D, F). In both wild type and *hid*, Bolwig's nerve always projects to the optic lobe primordium, although in *hid* mutants, the location of this structure is quite variable. We conclude that the projection defect in *hid* is not one of inappropriate connections, but rather of inappropriate projection pattern due to the abnormal position of the target region for the larval optic nerve.

**Head morphogenesis arrests in *hid* embryos**

A number of mutants have been described where head involution defects result from the deletion of particular head segments (e.g., *empty spiracles*, *buttonhead*, and *orthodenticle* (Cohen and Jürgens, 1990; Finkelstein and Perrimon, 1990). The head involution defects in *hid* embryos could be the consequence of gross defects in head composition. Alternatively, *hid* could cause a general arrest in the morphogenetic movements associated with head involution.

To investigate the organization of the embryonic head in *hid* mutants, we stained wild type and *hid* embryos with anti-engrailed antibodies (Patel et al., 1989). *engrailed* is a segmentation gene which is expressed in, among other regions, a distinctive pattern in the embryonic head. Expression in the head throughout development is believed to reflect movements of *engrailed* positive cells and not a turning on and off of the *engrailed* gene (Schmidt-Ott and Technau, 1992); thus, *engrailed* is a useful marker not only for determining head organization but for following head involution as well.

When comparing the patterns of *engrailed* expression in wild type and *hid* embryos, we find that most aspects of the staining pattern are conserved (Figure 6). There are no cases where components of *engrailed* staining are
missing in hid embryos, nor do we find additional domains of engrailed expression in hid.

When carefully aged matched wild type and hid embryos are compared, we find that the expression pattern of engrailed in the head of hid embryos lags behind that of wild type (Figure 6). Figures 6A and C are two optical sections of a wild type embryo, B and D of a hid embryo. The embryos were determined to be the same age by comparing gut morphology. The engrailed expression pattern in each hid embryo is reminiscent of that of a wild type embryo at a much earlier age. Indicated in Figures 6C and D is the position of the dorsal fold in each embryo. In the wild type embryo, the dorsal fold can be seen to be a folded epithelium which covers much of the anterior end of the embryo. Using the anterior end of each embryo as a reference point, the dorsal fold in the hid embryo is relatively posterior, suggesting that anterior progression of this tissue is abnormal.

In summary, we find that in hid, not only is migration of the optic lobe primordia abnormal, but there is a general arrest in the morphogenetic movements of head involution as well.

**Differentiation continues in the absence of head involution.**

It was possible that the defects observed in hid embryos do not reflect a specific arrest of anterior morphogenetic movements, but rather a delay in these movements or a general arrest in development. We do not believe that anterior morphogenesis is merely delayed, as many of these embryos never complete these movements. It was, however, still important to determine whether hid embryos continue to differentiate in the absence of head involution.

In hid embryos, posterior development seems to proceed normally. hid mutant embryos develop an apparently normal gut and segmented cuticle; thus, mutations in hid do not arrest development in the organism as a whole. In the anterior of a wild type embryo, the cephalopharyngeal skeleton, or larval head skeleton, is one of the last structures to develop during embryogenesis. Late hid embryos do have a cephalopharyngeal skeleton, although its organization is abnormal and it is ectopically located. Thus, anterior development does proceed in hid, even when head involution has arrested.
We also examined the differentiation of the optic lobe region using the marker bsh. *bsh* or "brain specific homeobox" is a homeobox containing gene which is specifically expressed in approximately thirty cells in each brain hemisphere (Jones and McGinnis, 1993). One of these cells is located in each optic lobe primordium, near the end of Bolwig's nerve (Figure 7A). This cell is one of the optic lobe pioneers, a group of cells proposed to be an intermediate target cell for Bolwig's nerve (Campos et al., in prep.).

Expression of *bsh* in this one cell is of very late onset, beginning well after the completion of head involution. In *hid* mutants, we find a *bsh* positive cell near the tip of the larval optic nerve (Figure 7B), and the timing of *bsh* expression in this cell is comparable to that of wild type. This suggests that differentiation of the optic lobe primordium continues in the absence of head involution, and that this differentiation is not dependent upon normal optic lobe invagination. Furthermore, differentiation in the anterior part of the embryo apparently proceeds with normal developmental timing.

**l(3) 05014 is an allele of hid**

To gain insight into *hid* function, we cloned the *hid* gene. We tested 17 lines containing P-element insertions mapping to 75B, C, or D for failure to complement *hid* (Table 2). One line, l(3)05014 is almost fully lethal in combination with H99, a 300 kilobase (kb) deletion which uncovers *hid*. Those adults that do survive have abnormal wings and males have rotated genitals. Furthermore, when we examined embryos from collections of 05014 flies, we find that many embryos fail to undergo head involution. 05014 flies display all of the phenotypes which are hallmarks of a *hid* mutation.

Mobilization of the 05014 P-element can result in reversion of all of the above phenotypes; thus, the P-element insertion on the 05014 chromosome is the cause of the *hid* mutation. By sequencing we have determined that the 05014 P-element disrupts the coding sequence of a transcript. Furthermore, we have determined the molecular nature of a number of *hid* alleles, including imprecise excision events of the P-element and some chemically induced *hid* mutations. The details of this analysis are described below.

**A 4.2 kb transcript is disrupted by the 05014 P-element**

The 05014 P-element is a P[ lacZ+, ry+] element (Mlodzik and Hiromi, 1992) which contains sequences necessary for plasmid rescue. Using the
enzyme KpnI, we rescued approximately 13kb of genomic sequence flanking the P-element (Figure 8). The cloned sequences flanking the 05014 insertion were mapped to a chromosomal walk of the 75C region (Chapter III). The rescued sequences hybridize to a fragment of λ clone 3504 which, by Northern analysis, was determined to include transcribed sequences. We isolated a 3.9 kb cDNA clone, clone 5A1 which also hybridizes to λ clone 3504, and determined by sequencing that the transcription unit corresponding to cDNA 5A1 is disrupted by the 05014 P element insertion. We mapped the 5A1 cDNA to genomic phages in the interval to determine the genomic structure of this locus (Figure 9). Intron/exon boundaries were precisely determined by sequencing (Figure 12). This gene consists of at least four exons spread over approximately 20 kb of genomic DNA (Figure 9).

By Northern analysis we detect one transcript of approximately 4.2 kb corresponding to this locus (Figure 10). Primer extension analysis indicates that approximately 150 bases of sequence are missing from the 5' end of the 5A1 cDNA (J.A., unpublished). The cDNA clone also appears to be missing 3' sequences as it has no poly-A tail or any obvious poly-adenylation signals. Although the 5A1 cDNA is not full length, we believe it contains the complete coding sequence of this gene (see below).

The sequence of the 5A1 cDNA is presented in Figure 11. The longest open reading frame predicts a translation product of 410 amino acids with a deduced relative molecular mass of 43kD. Analysis using BLAST and FASTA sequence comparison algorithms reveals no striking similarities with previously reported nucleotide or amino acid sequences; thus, hid encodes a novel protein. The hid protein contains five potential MAP kinase phosphorylation sites (Nigg, 1993) which are boxed in Figure 11. The C-terminal half of the protein is fairly rich in charged residues, and the protein as a whole is relatively basic (predicted pI=8.27). Hydropathy analysis reveals that the predicted protein is relatively hydrophilic with no hydrophobic regions of sufficient length to serve either as signal sequences or transmembrane domains.

The transcript disrupted by the 05014 P-element corresponds to hid

The gene Wrinkled (W) is a dominant allele of hid, and all W revertant chromosomes are hid alleles (Abbott and Lengyel, 1991). Thus, if the 5A1 transcription unit corresponds to hid, chromosomal rearrangements
associated with reversion of W should affect this transcript. We have
mapped a number W revertant chromosomes (Chapter III), and all either
break within or delete this transcript (Figures 9, 13). In addition, we have
mapped a deletion on the WR+X1 chromosome. WR+X1 is an inversion
which breaks in 75C1,2 and centromeric heterochromatin (Abbott and
Lengyel, 1991). We have found associated with this chromosome a small
deletion which removes the 5' end of this transcript (Figure 9). We detect no
5A1 transcript in WR+X1 embryos (Figure 14B); thus WR+X1 is likely to be a
null allele of hid.

By sequencing, the 05014 P-element was determined to disrupt the
coding sequence of the predicted protein between amino acids 105 and 106
(Figure 11). We sequenced two reversion chromosomes generated by
mobilization of the 05014 P-element. In both cases no detectable P-element
sequences remained and the reading frame of the 5A1 transcript was restored.
The 05014 P-element insertion corresponds to approximately 15 kb of DNA;
thus it was possible that the hid mutation on the 05014 chromosome was due
to a position effect of the P-element on neighboring DNA sequences and not
to disruption of the 5A1 transcript. To address this possibility, we also
sequenced two chromosomes where the 05014 P-element was excised, yet hid
function was not restored. Residual insertions of 38 and 40 basepairs of P-
element DNA were found to disrupt the reading frame of the 5A1 transcript
(see Table 3). Such small insertions are unlikely to exert long range position
effects. We therefore propose that it is the disruption of the 5A1 transcription
unit by the 05014 P-element which causes the hid phenotype.

To further substantiate this conclusion, we sequenced the coding
region of some independently isolated hid alleles. We sequenced five ethyl
methanesulfonate (EMS) induced alleles: A22, A206, A329, WR+E1 and
WR+E4 (Abbott and Lengyel, 1991); three ethylnitrosourea (ENU) induced
alleles: ML23, ML66, ML87 (Chapter III); and W, a dominant allele of hid.
We found polymorphisms in alleles A22, A206, A329, and ML66 (Figure 12,
13; See Table 3). In A22 we found a proline to serine change. In ML66 we
found a splice donor mutation. In A206 and A329 we found changes that
result in premature translational stops. In A206 there is also a serine to
leucine change upstream of the premature stop. From the mutational
analysis, we conclude that the open reading frame contained within the 5A1
cDNA is the hid gene.
**hid** expression is spatially and temporally regulated throughout development

Using whole mount *in situ* hybridization with digoxygenin labeled RNA probes, we determined the localization of the **hid** message during embryogenesis. We did not detect any hybridization in embryos homozygous for WR+X1 (Figure 14B) or for deficiencies in 75C, demonstrating that the signal we observe is specific for this transcript. The **hid** expression pattern is highly dynamic and complex throughout embryogenesis (Figure 15). Many of the tissues that express **hid** undergo extensive rearrangements during morphogenesis. This is particularly true in the differentiating head region (see below).

**hid** is first detectable in embryogenesis as one faint stripe at 50% egg length. In the cellular blastoderm embryo, **hid** expression is seen as a series of approximately ten stripes (Figure 15A). Early in gastrulation, **hid** is expressed at the sites of cephalic furrow formation and amnioproctodeal invagination (Figure 15B). During germ band elongation, high levels of **hid** expression are found in the mesoderm. By stage 9, message is also prominent in a subset of cells of the amnioserosa and on the dorsal surface of the differentiating clypeolabrum (Figure 15C). Weaker expression is found throughout the rest of the head.

Segmentation is evident by stage 11 and at this time, **hid** transcript is found throughout most of the embryo (Figure 15D). In addition to mesodermal expression, message is found around the tracheal pits, in the gnathal segments (predominantly in the labial and maxillary buds), in the clypeolabrum, and the stomodeal invagination. There is also strong expression in a posterior-dorsal region of the procephalic lobe. It is from this region that the optic lobe differentiates.

By stage 13, high levels of transcript are found in the invaginating optic lobe (Figure 15E). The clypeolabrum, gnathal segments, and portions of the stomodeal invagination still express **hid**. Expression is also seen in the dorsal ridge and in a subset of the midline glia (not shown). Once head involution is complete, levels of **hid** expression decrease. By the end of embryogenesis, **hid** expression is confined to a few structures. Expression is found in the optic lobe (Figure 15F), midline glia, and the dorsal vessel.

Upon closer examination of the head region, we find that **hid** expression is found in most, if not all, tissues that invaginate or move during
head involution. Figures 16A-C are three optical sections of a stage 12 embryo. In this embryo, *hid* expression is found in the dorsal ridge, invaginating optic lobe, the invaginating portion of the dorsal clypeolabrum, and along the stomodeal invagination. Figure 16D is a stage 14 embryo. Here *hid* mRNA can be seen in the optic lobe which has descended ventrally. Expression is also found in the dorsal fold and along the stomodeal opening.

In climbing third instar larvae, we detect *hid* expression in portions of the brain and along the ventral nerve cord (Figure 17A). There are also high levels of *hid* expression in the portion of the wing disc which will form the wing proper (Figure 17B). No detectable expression is found in the portion of the wing disc which will give rise to the notum.

In conclusion, we find that *hid* expression is associated with tissues that invaginate or migrate during embryogenesis. Furthermore, mutations in *hid* result in the specific arrest of the morphogenetic movements associated with head involution and optic lobe invagination. This suggests that *hid* may be directly required in these tissues for their proper migration.
DISCUSSION

Morphogenetic movements are an integral part of the development of all metazoan embryos including *Drosophila*. The elongation and retraction of the germband are striking examples of some of the complex morphogenetic movements which occur during *Drosophila* embryogenesis. Gastrulation, dorsal closure, and head involution also illustrate the role that the relative displacement and movements of tissues play in shaping and modeling an embryo.

The larval visual system

The head involution defective (*hid*) gene is required for embryonic morphogenesis. Interestingly, although *hid* is expressed throughout the embryo, it is only required for morphogenetic movements in the anterior region of the embryo. *hid* mutants exhibit striking failures in head involution. The movements of the germband occur apparently normally in *hid* embryos, as do dorsal closure and extensive remodeling of the gut.

In the anterior end of the embryo, head involution is not the only process affected by mutations in *hid*. Invagination of the optic lobe primordium requires *hid* function as well. In *hid* mutant embryos, the optic lobe primordium fails to reach its proper position in the ventro-posterior part of the brain. The phenotype of *hid* contrasts with defects found in embryos homozygous for two other head involution mutants, *labial* (*lab*) and Deformed (*Dfd*) (Merrill et al., 1989; Merrill et al., 1987). In *lab* and *Dfd* embryos, Bolwig's nerve projects to the ventral brain (A. Campos, unpublished, and see Chapter I, Figure 6B). This strongly suggests that the optic lobe has migrated to its proper position in these mutants, and indicates that head involution and optic lobe migration may occur independently.

In all *hid* mutants, including full loss of function alleles like *WR+X1*, the projection phenotype is quite variable, presumably reflecting differences in the placement of the optic lobe primordia. In *WR4*, *WR10*, and *H99* deficiency embryos, however, Bolwig's nerve always projects dorsally. What accounts for this difference in the range of phenotypes? Embryos homozygous for all of the above deficiency chromosomes also lack most embryonic programmed cell deaths (see Chapter III). There is considerable programmed cell death in the embryonic head and the region of the invaginating optic lobe. This cell death appears to be associated with regions
of the head which participate in morphogenetic movements. Perhaps cell
death is required for head involution and normal migration of the optic lobe
primordia. The deletion of two functions required for these processes, hid
and a cell death function, could account for the increased severity of
projection phenotype associated with the deficiency chromosomes.

Despite ectopic placement of the optic lobe, Bolwig's nerve maintains
contact with it. This accounts for the striking projection defects observed in
hid mutants. In hid, Bolwig's nerve always projects to the optic lobe
primordium, no matter where that target field is located. There are three
cells in each optic lobe primordium which are called the optic lobe pioneers
(Tix et al., 1989), and have been proposed to help stabilize the connection
between Bolwig's nerve and the invaginating optic lobe primordia (Campos
et al., in prep.). One of these optic lobe pioneers expresses the gene bsh
(Campos et al., in prep.). We show that the bsh positive optic lobe pioneer is
present in hid embryos, as is evidenced by a bsh expressing cell near the tip of
Bolwig's nerve. If this cell is required to keep Bolwig's nerve and the optic
lobe in contact, it can apparently do so, even when optic lobe migration fails.
Furthermore, expression of bsh in this cell demonstrates that at least a
portion of the optic lobe continues to differentiate, even when ectopically
positioned.

Despite the fact that Bolwig's nerve projects to the optic lobe region and
contacts at least one of its normal intermediate targets (the bsh expressing
optic lobe pioneer), we cannot address whether Bolwig's nerve is making
functional connections with its final targets. Dye filling experiments in wild
type larvae reveal that the synaptic partners of Bolwig's nerve are not the
optic lobe pioneers, but interneurons in the central brain (Steller et al., 1987).
These neurons are not identifiable by any other histological criteria, and thus
we were not able to identify them in hid mutants. It would be interesting to
determine what connections are made by the larval optic nerve in hid
animals because in these mutants, the optic lobe appears never to incorporate
fully into the brain, and it is certainly never located in its proper position. If
organization of the central brain is normal in hid mutants, Bolwig's nerve
would have to navigate over some distance to find its proper targets. Dye
filling experiments in hid animals might reveal whether or not Bolwig's
nerve makes synaptic connections in the central brain at all, but could not
distinguish whether Bolwig's nerve is synapsing with its normal targets or
with inappropriate ones. Behavioral tests, such as larval phototaxis assays could be done to determine whether or not hid larvae have a normal visual response, indicating that appropriate connections are being made.

The hid gene

We have several lines of evidence which confirm that we have molecularly identified the hid gene. The 05014 P-element insertion behaves as an allele of hid. The hid phenotypes can be reverted only in association with precise excision of the P-element. In contrast, imprecise excision of the P-element does not restore hid function. We have characterized the transcript which is disrupted by the 05014 insertion. This transcript is spatially and temporally expressed in a manner consistent with the phenotypes found in hid (see below). Furthermore, expression of the transcript is altered in three hid alleles (not shown). We find a single long open reading frame encoded by the transcript, predicting a 410 amino acid protein product. The 05014 P-element disrupts the coding sequence of this transcript. By sequencing we have identified mutations in the open reading frame in five independently generated hid alleles. Two of the mutations result in premature translational stops and truncation of the protein encoded at this locus. Taken together, these results demonstrate that the open reading frame encoded at this locus is required for hid function.

The hid gene is not similar to sequences presently in the data base. It is likely to be an intracellular protein as it doesn’t contain obvious signal sequences which might indicate that it is secreted. It does, however, have a hydrophobic C-terminus suggesting a possible intracellular association with the cell membrane. hid contains several potential phosphorylation sites, suggesting that it is a potential downstream target of one of the many signal transduction cascades which operate during embryogenesis.

Three of the four mutations we identified in loss of function hid alleles either disrupt splicing (ML66) or result in the truncation of the protein because of premature stops (A206, A329). The predicted translation product of hid is a 410 amino acid protein. It should be noted that A206, which contains a stop codon at amino acid 274 and A329, which contains a stop codon at amino acid 300 are both relatively weak hid alleles. This suggests that the N-terminus of the protein may have a significant amount of functional hid activity.
In the case of \textit{W}, a dominant allele of \textit{hid}, we identified two amino acid polymorphisms in the coding sequence. \textit{W} is a spontaneous mutation which was isolated in the 1930's (Lindsley and Zimm, 1992). The parental chromosome on which this mutation arose is no longer available; thus, we could not determine by sequence comparison whether either change is causally related to the \textit{W} phenotype.

Expression of \textit{hid} is strongly correlated with tissues that invaginate or migrate during embryogenesis. Early in embryogenesis, \textit{hid} is expressed in the invaginating cephalic furrow and amnioproctodeal invagination. Mesoderm, which undergoes extensive remodeling throughout embryogenesis, also transiently expresses \textit{hid}. Cells of the amnioserosa, move considerably during embryogenesis and \textit{hid} is expressed in at least a subset of these cells. Tracheal pits invaginate during stage 11, and \textit{hid} mRNA is expressed here at this time. The correlation between cells which migrate and \textit{hid} expression is also very striking in the head region of the embryo. \textit{hid} mRNA is found in all head structures which move during head involution, including the gnathal segments, the dorsal fold, the optic lobe primordia, the clypeolabrum, and the stomodeal invagination. Although \textit{hid} is expressed throughout the embryo, we only detect a mutant phenotype in the head region. Perhaps there are redundant functions in other regions of the embryo which compensate for a lack of \textit{hid} function there. Alternatively, \textit{hid} may not be required in these other structures.

How does \textit{hid} protein function? Its association with tissues that undergo extensive movements suggests that hid function is directly required in these tissues for morphogenetic movements. It may serve to coordinate these movements or act as an effector molecule. Perhaps, via its hydrophobic C-terminus, it serves as a link between the cell membrane cytoplasmic proteins.

Many of the cells which express \textit{hid} also change shape during embryogenesis. Shape change has been demonstrated to be a driving force in the morphogenesis of a number of tissues. For example, embryos mutant for \textit{zipper}, a nonmuscle myosin heavy chain gene (Young et al., 1993), have defects in dorsal closure, head involution, and axon projection patterns. Young, et al., (1993) have demonstrated that the zipper protein is required for maintenance of cell shape. Cell shape change is also implicated in the elongation of the imaginal leg disc (Condic et al., 1991) and the development
of the wing (see Fristrom et al., 1993). Perhaps *hid* is required to mediate or maintain cell shape changes required for morphogenesis in the anterior part of the embryo. Cellular and biochemical analysis of the *hid* protein should shed some light into how *hid* functions during head involution and the migration of the optic lobe primordia.


Figure 1- Development of the larval visual system. Lateral views of wild type embryos. (A) The larval visual system is derived from the optic placode (shaded). (B) By stage 14, the optic placode has invaginated from the dorsal ectoderm. The larval photoreceptor cells differentiate and send out axons which contact the optic lobe primordium. (C) By stage 15, the optic lobe primordium sits near the embryonic brain. Axon elongation continues as the photoreceptor cells move anteriorly. (D) Stage 17 - By the end of embryogenesis, the optic lobe primordium has been incorporated into the ventral brain, and the final projection pattern of Bolwig's nerve has been established. Figure drawn by Kevin Lee.
Figure 2 - Schematic representation of head involution. Lateral views of wild type embryos. In this and all subsequent figures, anterior is to the left, ventral down. Head involution begins at approximately stage 12 (A). At this time, the gnathal segments (gn) are visible ventrally. The clypeolabrum (cl) is distinct from the procephalic lobe (pl) and the stomodeal invagination (st) has formed. The dorsal ridge (dr) is also visible. Ventrally, the gnathal segments (gn) move towards and into the stomodeal invagination (st). By the end of embryogenesis, some of the cells of the gnathal segments contribute to the atrium (at), and pharynx (ph). The dorsal ridges, one on either side of the embryo, move dorsally towards the midline. When the dorsal ridges meet at the dorsal midline, they fuse and form the dorsal fold (df). The dorsal fold moves anteriorly over the procephalic lobe and clypeolabrum (top arrows, B and C). The movements of the dorsal fold result in the formation of the frontal sac (fs). The shaded portion in each figure represents the stomodeal invagination and marks the progression of gut formation. Abbreviations: at, atrium; cl, clypeolabrum; df, dorsal fold; dr, dorsal ridge; fs, frontal sac; gn, gnathal segments; ph, pharynx; pl, procephalic lobe; st, stomodeal invagination. Figure drawn by Kevin Lee.
Figure 3 - Projection of the larval photoreceptor nerve in wild type and hid mutant embryos. Lateral views of stage 17 embryos stained with mAb 24B10. Arrows indicate the end of Bolwig's nerve. (A) Canton-S embryo - note how Bolwig's nerve terminates in the ventral brain. (B) Df(3L)H99 - in embryos homozygous for deletions of 75C, the larval optic nerve projects dorsally. (C, D) A22 and H89 embryos, respectively. In these hid embryos, Bolwig's nerve also projects dorsally. (E, F) Two optical sections of the same H89 embryo illustrate the variability in projection phenotypes. Bolwig's nerve is seen to terminate more dorsally in E (left nerve) than in F (right nerve). Abbreviation: br, brain. Scale bar = 30μm.
Table 1 - Projection phenotypes in hid mutant embryos. Stage 15-17 embryos were stained with mAb24B10. As noted, in some cases, the hid alleles were placed in trans to deficiency chromosomes. The total number of embryos with head involution defects in each egg collection was scored. Head involution was scored as the failure of the dorsal fold to cover the clypeolabrum. All embryos with a visible clypeolabrum were scored as head involution defective. We did not attempt to classify embryos according to how far forward the dorsal fold had migrated. For each embryo with a head involution defect, the projection phenotype was scored as follows: "dorsal" - both larval optic nerves projected to the dorsal brain; "asymmetric" - one Bolwig's nerve projected dorsally, the other ventrally; "ventral" - the Bolwig's nerves projected ventrally; "can't interpret" - in these cases head morphology was so abnormal that the projection phenotype could not be scored.
Table 1 - Projection phenotypes in *hid* mutant embryos

<table>
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<th>allele</th>
<th>total scored</th>
<th>total with h.i. defects</th>
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<th>asymmetric</th>
<th>ventral</th>
<th>can't interpret</th>
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<tr>
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<td>1</td>
<td>8</td>
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<tr>
<td>WR+E2/H99</td>
<td>32</td>
<td>5 (16%)</td>
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<td>0</td>
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<tr>
<td>A206/H99</td>
<td>101</td>
<td>9 (9%)</td>
<td>5</td>
<td>0</td>
<td>4</td>
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<tr>
<td>A22/WR4</td>
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<td>5 (7%)</td>
<td>3</td>
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</tr>
<tr>
<td>A329/H99</td>
<td>87</td>
<td>6 (7%)</td>
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<td>1</td>
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Figure 4 - The optic lobe primordia are ectopically positioned in *hid* embryos. Confocal micrographs of stage 13 embryos stained with α-disconnected Ab. (A) Canton-S - the optic lobe (arrow) has invaginated from the dorsal ectoderm and has begun to migrate ventrally. (B) *l(3)05014/H99* - Here the optic lobe (arrow) has invaginated, but it remains dorsal. Abbreviation: OL, optic lobe.
Figure 5 - Bolwig's nerve projects to the optic lobe primordium in wild type and *hid* embryos. Confocal micrographs of three optical sections of stage 15 Canton-S (A, C, E) and WR+X1/H99 (B, D, F) embryos stained with α-fasciclin II. In both cases, Bolwig's nerve projects to the fasciclin II positive optic lobe. In the WR+X1/H99 embryo, the optic lobe is located much more dorsally than in wild type. Note also the relative position of the dorsal ridge in each embryo (E, F): in WR+X1/H99 (F) it has not migrated anteriorly.

Abbreviations: BN, Bolwig's nerve; DR, dorsal ridge; OL, optic lobe.
Figure 6 - Morphogenesis of the head is abnormal in hid embryos. Two optical sections of stage 15 wild type (A, C) and WR+X1/H99 (B, D) embryo stained with mAb 4D9 (α-engrailed). The defects in head involution in the WR+X1/H99 embryo (B, D) are apparent when the relative position of the domains of engrailed expression in A and B are compared. Note also how the dorsal ridge has not migrated anteriorly in the WR+X1/H99 embryo (compare C to D, using the anterior end of each embryo as a reference point). Abbreviation: dr, dorsal ridge.
Figure 7- The bsh positive optic lobe pioneer is present in *hid* embryos.
Confocal micrographs of a wild type (A) and *l(3)05014* embryo (B) stained with mAb 22C10 (green) and α-bsh (red). A is a lateral view, B is a dorsolateral view. In both wild type and *hid* embryos, a bsh positive cell (arrow) can be found near the tip of Bolwig's nerve. Abbreviation: BN, Bolwig's nerve.
Table 2 - Insertion strains tested for allelism to hid. Insertion strains were crossed to Df(3L)WR4, WR10, and H99. Flies obtained from the Drosophila stock center in Bloomington, Indiana are noted as "Bloomington." All others were the gift of A. Spradling. Insertion chromosomes which were lethal in combination with any of the above deficiencies are noted.
Table 2 - Insertion strains tested for allelism to *hid*

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<td>75AB</td>
<td>Bloomington</td>
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<td>75B</td>
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<td>l(3)00225</td>
<td>75B6-7</td>
<td>Spradling</td>
<td>uncovered by WR10</td>
</tr>
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<td>l(3)05014</td>
<td>75C1,2</td>
<td>Spradling</td>
<td>uncovered by WR10</td>
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<td>Is(3)Pne075C - AS80</td>
<td>75C</td>
<td>Bloomington</td>
<td></td>
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<tr>
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<td>75C</td>
<td>Bloomington</td>
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<td>P(ry+-IAr B)92 #586</td>
<td>75C</td>
<td>Bloomington</td>
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Figure 8 - Restriction map of plasmid rescued genomic sequences flanking P-element 05014. P-element sequences are represented by the open bar; triangles represent P-element ends. Abbreviations: $Km^R$ kanamycin resistance; ORI bacterial origin of replication; lac Z, B-galactosidase; ry, rosy; UTR, untranslated region; H, HindIII; K, KpnI; R, EcoRI.
Figure 9 - Genomic map of the *hid* transcript. Filled bars represent coding exons, open bars, untranslated sequences. The large arrows beneath the transcript represent deficiencies X20 and WR+X1, both of which extend in the direction of the arrows. In WR+X1, the open portion of the arrow marks the genomic region where the distal breakpoint must lie. The 05014 insertion is also shown. Abbreviations: R, EcoRI; X, XhoI.
Figure 10 - A single transcript of 4.2 kb is detected by Northern analysis. An 8 kb EcoRI fragment which contains the 3' end of the hid gene was used to probe a Northern blot. Lane 1 - 0-7 hour embryonic poly-A RNA; Lane 2 - 7.5-12.5 hour embryonic poly-A RNA; Lanes 3 and 4 - polyA RNA from Drosophila L2 cells. A single RNA species of 4.2 kb is detected with this probe. A transcript of similar size is also detected in pupal RNA (not shown).
Figure 11 - Sequence of hid cDNA 5A1. The deduced amino acid sequence of the longest potential open reading frame is shown beneath the nucleotide sequence. Two upstream ATG's are underlined; 5 potential MAP kinase consensus phosphorylation sites are boxed. Intron positions are marked by short arrows. Polymorphisms resulting in silent amino acid changes are shown in lower case above the cDNA sequence. The site of the 05014 insertion is marked with the long arrow. The polymorphisms identified in the sequenced hid alleles are shown in upper case letters next to the name of each allele, above the cDNA sequence.
Figure 12 - The genomic structure of the hid locus. Sequences in lower case are not present in cDNA 5A1. Dotted lines represent intronic sequences which were not determined. The sequences used to amplify the hid coding region for the allele sequencing are underlined. The ML66 splice donor mutation is also shown.
GENOMIC STRUCTURE OF HID

```
1  tctgccagctgcgcgtagggctcttgccgtgattgaggccccagngcgaacagtt 60
 61  cattttacgccggagccgagtcagagttctctcctctctgctctggcgcag 120
121  acgttttaagtgctcttccataattgcaacacgaaggcaagaagattatttataAAA 180
181  AAAAACCCGAAATCCCTCGGGAAGACAGTTCATCTTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTC
```
1561 GCACGGCCACCCCCACAGCCCTTCACGTCGACCTCCACGCCGTTATCGGCGCCAGTGGC
pHisGlyHisProHisSerProPheThrSerThrSerThrProLeuSerAlaProValAl
1621 GCCCAAGATGCGCCTGACGAGCGCAAGTGGGTGAGTGGACGCCAGGgtgagtagccagca
aProLysMetArgLeuGlnArgSerGlnSerAspAlaAlaArgAr
1681 tgcaggggtgccccaaataacctgcctgcttgcaaatcgaactgtaatttagg
1741 attccagatcgatgacatagaatgagggaagtctcgaactgcaactgatgagat
1801 gccctatttgaataataataaaatgctatcagatattgat............gcta
1861 cacaaaccgcatacgaatccgaaccgactgataattgtctcatgacgttccagagtcaccgc
1921 tctatattgagccccatatttcataggtcccttttagctctctctccccacagCAAAGGATTG
gLysArgLeu
1981 ACCTCGACGGGCGAGGATGAGCGCGAGTACCAGAGCGATCATGAGGCCACTTGGGACGAG
ThrSerThrGlyGluAspGluArgGluTyrGlnSerAspHisGluAlaThrTrpAspGlu
2041 TTTTGGCGATCGCTACGACAACTTTACGGCCGCGGCGGCGGCGGCGGTCTGAGTTCAATGGA
PheGlyAspArgTyrAspAsnPheThrAlaGlyArgGluLeuGlnGluPheAsnGly
2101 CGCATCCCAGGGAGAGAAAGAGAGCTCTCAATGCCCCACTGAGCAGCGCAATAATCCAA
ArgIleProProArgLysLysSerSerAsnSerHisSerSerSerAsnPro
2161 GTCTGCATACCGACGCACTGCGGTGTGATCATCCCAAGCGGAGACGTGATCTCTATCTCAT
ValCysHisThrAspSerGlnSerAspGluGlyThrSerGlnGluSerGlyAlaIleHis
2221 GGCCACATCGACTAGCAGGCGGAGCGTGGAGCGAAGCAGAAAAGCGCAAAGCGCGAGAAG
GlyHisIleSerGlnGlnArgValGluArgGluLysAlaLysAlaGluLys
2281 AAGgtaaagaattgccaccaaatcttggaattgacaccaacgccatacagaaaggtatttcc
Lys
2341 gtttcggttacatacgtatc...........aaagcaactttgtcttttatgtaagtttttc
cctacgtcggcaccgtaataataaacttagtatatatatatcctctcttagAAACCA
2401 LysPro
2461 CAGAGCTCCACTTGCGCAACTGTGTGTGAGCGGTTGCGGCGTGGCGGCTGTGCCCTC
GlnSerPheThrTrpProThrValValThrValPheValLeuAlaMetGlyCysGlyPhe
2521 TTTGCGCGCGCGGTGAAGCCAGAGCTGTGAATCGAATCTGATAATGACAAATCGACT
PheAlaAlaArgEnd
2581 AGCCCTTAAGATACATCCTGAGTTAATCCTTAGTTTGTGTTAAACGCAGCATGAAATGCAACG
2641 AATTTATGAACTGCGAGAC
Figure 13 - Clones and deficiencies in the 75C region. Deficiencies are shown above a map of cloned DNA in the 75C region. Dotted lines represent DNA which is deleted in each deficiency. The hid gene, located at -50, is either deleted or disrupted by all of the above chromosomal rearrangements.
Table 3 - Molecularly characterized *hid* alleles. All *hid* alleles for which polymorphisms were identified by sequencing are shown. *l(3)05014* is a P-element insertion, and 40C and 8D were generated by imprecise excision of this insertion. *A22, A206, and A329* were generated by Abbott and Lengyel (1991). *A206 and A329* are derived from the same parental chromosome (*ru h th ri e*). *A22* was induced on a distinct parental chromosome (*red e*). Neither chromosome was available to us for sequencing. *W* is a spontaneous mutation (Lindsley and Zimm, 1992). Two *W* revertants generated by Abbott and Lengyel (1991) were also sequenced and share neither polymorphism which we found on the *W* chromosome. The parental *W* chromosome used by Abbott and Lengyel to induce their *W* revertants was also not available to us for sequencing. *ML66* was generated in the mutagenesis for lethals and visible mutations mapping to 75C1,2 described in Chapter III. The parental chromosome (*st e*) was sequenced and does not share the polymorphism found on the *ML66* chromosome.
### Table 3 - Molecularly characterized *hid* alleles

**Allele:**

1) *l(3) 05014*  P-element insertion at nucleotide 714, between amino acids 105 and 106
2) *40C*  imprecise excision of *l(3)05014*
3) *8D*  imprecise excision of *l(3)05014*

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<tr>
<td>40C</td>
<td>GAGTGTTCAAGATGTACCATGATGAAATAACAT</td>
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<tr>
<td>8D</td>
<td>GAGTGTTCAAGATGTACCATGATGAAATAACATA</td>
<td>TTATTTTTCATCATGGATGTACAGAGCCAGC</td>
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</table>

4) *A22*  amino acid 170  
CCA to TCA  
Pro  Ser
5) *A206*  amino acid 261  
TCG to TTG  
Ser  Leu
6) *A206*  amino acid 274  
CAG to TAG  
Gln  End
7) *A329*  amino acid 301  
TGG to TGA  
Trp  End
8) *W*  amino acid 346  
CAT to CCT  
His  Pro
9) *W*  amino acid 351  
TCC to CCC  
Ser  Pro
10) *ML66*  splice donor mutation - junction between exons 3 and 4

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<th>3' P</th>
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<tr>
<td>ML66</td>
<td>Ag/at</td>
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Figure 14 - *hid* mRNA is not present in *WR+X1* mutant embryos. *hid* mRNA was detected in wholemount stage 10-11 embryos with digoxigenin labeled RNA probes. (A) Canton-S embryo. (B) homozygous *WR+X1* embryo - no *hid* expression is detected in embryos of this genotype, demonstrating that the signal is specific to the *hid* transcript. Scale bar = 36μm.
Figure 15 - Tissue distribution of *hid* mRNA during embryonic development.
The *hid* transcript was detected in wholemount embryos by *in situ* hybridization with digoxigenin labeled RNA probes. (A) Cellular blastoderm embryo - *hid* is expressed as a series of faint stripes. (B) Stage 6 embryo - *hid* mRNA is found in the amnioproctodeal invagination (api) and in the cephalic furrow (cf). (C) Late stage 9 embryo - *hid* mRNA is found in the mesoderm (me), on the dorsal surface of the clypeolabrum (cl), and in a portion of the amnioserosa (as). (D) Stage 11 - *hid* mRNA can be seen, among other places, in the labial (lb) and maxillary (mx) buds and around the tracheal pits (tp). (E) Stage 13 - note the high levels of *hid* mRNA in the invaginating optic lobe (ol). (F) Stage 16 - *hid* expression persists in the optic lobe (ol). Scale bar = 36μm.
Figure 16 - The distribution of *hid* mRNA is correlated with the movements of head involution. (A-C) Three optical sections of a stage 12 embryo. (D) A stage 14 embryo. *hid* mRNA is found in tissues that invaginate or move during head involution, including the optic lobe (ol), the dorsal ridge (dr), the clypeolabrum (cl), and the stomodeal invagination (st). Curved arrow in (D) represents the direction of invagination of ventral tissue. Scale bar = 16μm.
Figure 17 - The distribution of *hid* mRNA in third instar larvae. (A) *hid* mRNA is detected in several imaginal discs, the optic lobe (ol), and the ventral nerve cord (vnc). In wing discs (B), *hid* expression is largely confined to the portion of the disc that forms the wing proper. Abbreviations: ed, eye disc; nt, notum; wg, wing. Scale bar in B is 60μm.
AUTHORS NOTE

This work involved the collaboration of a number of people in the lab. John Abrams, Kristin White, and Lynn Young participated in the mutagenesis for single gene mutations in 75C. Excisions of the l(3)05014 P-element insertion were generated by Kristin White and Melissa Rivera. Northern analysis was done by John Abrams. John Abrams also isolated the 5A1 cDNA and sequenced 1 kb of one strand of this clone.
Chapter V
Genetic Control of Cell Death in *Drosophila*
PREFACE

This work is the result of a collaboration with Dr. John Abrams and Dr. Kristin White, and has been accepted for publication in *Science*. My contribution to this study is the anti-Krüppel antibody staining in Figure 2 and cell counts of Krüppel positive cells in the central nervous systems of wild type and deficiency embryos. I also contributed to the cloning of the gene, *reaper*, which is referred to in this chapter and explained more fully in Chapter VI.
Genetic Control of Programmed Cell Death in *Drosophila*

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During the development of most metazoan animals, many cells are eliminated by programmed cell death. In *Drosophila*, like in vertebrates, the induction of cell death is regulated by signals from the cellular environment. We have identified a small genomic deletion which blocks virtually all programmed cell deaths that normally occur during *Drosophila* embryogenesis. Mutant embryos contain extra cells, but otherwise develop normally in many respects. Cell death can be induced in mutant embryos by X-ray irradiation, and the resulting corpses are phagocytosed by macrophages. We conclude that this mutation deletes a central control function for the induction of programmed cell death. An excellent candidate for a corresponding gene, *reaper (rpr)*, appears to be expressed in cells destined to undergo apoptosis.

Development and homeostasis of most multicellular organisms are critically dependent on three processes: mitosis, differentiation, and cell death. In many organisms a large number of cells die in the absence of obvious external insults. For example, in vertebrates approximately half of the neurons generated during neurogenesis are eliminated by cell death (1). This deliberate elimination of cells occurs in a morphologically distinct manner, referred to as apoptosis or programmed cell death, which is distinct from cell death provoked by external injury (2). It is now generally accepted that apoptosis results from active, gene-directed processes, since it often requires RNA and protein synthesis (3). However, except in the nematode *C. elegans*, no genes have been identified which are required for this process (4).

The powerful genetic techniques available in *Drosophila* make it an ideal system in which to dissect the genetic control of programmed cell death and its role in development. Cell death occurs during *Drosophila* development by apoptosis (5). The onset of death has been shown to depend on hormonal cues in some cases, and on cell-cell interactions in others (6). These types of interactions demonstrate that cell death in *Drosophila*, like in vertebrates, is under epigenetic control.

To identify genes which are required for programmed cell death in *Drosophila* we examined the pattern of cell death in embryos homozygous for chromosomal deletions. Since more than 50% of the *Drosophila* genome is represented by such deletion strains, we were able to rapidly screen a substantial fraction of the genome for cell death genes (7). Although these deletions
typically include genes essential for viability, the large maternal supply of household functions (8) permits development well beyond the stage at which cell death begins.

In wild type, a substantial amount of apoptotic cell death occurs during embryogenesis in a relatively predictable pattern (9). These deaths can be rapidly and reliably visualized in live embryos stained with vital dyes, such as acridine orange (AO). These preparations allow the assessment of the effect of mutations on cell death in many different tissues, allowing us to identify genes which are required for programmed cell death.

Among the 129 deletion strains examined, the majority (83 strains/65%) did not significantly affect the amount of cell death in the embryo. Twenty six deletion strains (20%) gave a significant number of progeny which had excess AO staining. A smaller number (17 strains/13%) showed a decrease in the number of AO staining cells. Of all the strains examined, only three overlapping deletions, Df(3L)WR4, Df(3L)WR10 and Df(3L)Cat DH104 (10) resulted in embryos which lacked virtually all AO staining at all stages of development (Fig. 1A, B). These deletions overlap in genomic region 75C1,2 on the third chromosome. We have subsequently obtained another mutation in this region, hidH99 (11) which shows the same phenotype. Although this mutation appears cytologically normal, molecular analysis has revealed that hidH99 is also a deletion which is internal to the overlap of the previously described deficiencies (12).

To demonstrate that the lack of AO staining in hidH99 embryos actually reflects a failure of cell death, we analyzed tissue sections by light and transmission electron microscopy. No evidence of programmed cell death could be detected by either of these methods. Semithin sections were stained with toluidine blue/methylene blue/borax (13). On examination by light microscopy, mutant embryos had no darkly stained cells, which are characteristic of apoptotic cell death (data not shown). Electron microscopy (EM) confirmed the lack of cell death in mutant embryos. Figure 1C shows an electronmicrograph of the clypeolabrum of a wildtype embryo. Note the electron dense cells typical of apoptotic cell death, which at this stage have mostly been engulfed by macrophages. All EM sections of wildtype embryos at this stage show many apoptotic cells. This is in stark contrast to homozygous hidH99 embryos (Fig. 1D) in which we have not detected any features of apoptosis at any stage of embryogenesis. We conclude that programmed cell death does not occur in
We considered the possibility that the lack of cell death in *hidH99* embryos results from a general block in development caused by the absence of functionally unrelated genes. Several observations lead us to conclude that this is extremely unlikely. First, mutants embryos reach advanced developmental stages. They form a segmented cuticle and begin to move, but fail to hatch. In addition, mutant embryos express markers which are only present in differentiated cells. Furthermore, mitotic clones of *hidH99* in the eye contained fully differentiated and morphologically normal photoreceptors neurons (data not shown). This demonstrates that the *hidH99* deletion does not have general adverse effects on cell division, differentiation, or survival. Second, from a survey of more than 129 deletions (50% of the genome), only those in chromosomal region 75C1,2 resulted in the complete lack of all programmed cell deaths. Many of these other deletions had more adverse effects on embryogenesis, yet none was capable of completely blocking programmed cell death. Finally, as shown below, apoptosis could be induced in *hidH99* embryos upon X-ray irradiation. These results argue very strongly against a general, unspecific cellular defect, for example in energy metabolism or protein synthesis, as the underlying cause for the observed phenotype.

The absence of cell death in mutant embryos is expected to result in the presence of extra cells. This prediction was tested by counting specific cells in the central nervous system, a tissue which is subject to substantial amounts of cell death during wildtype development (9). One cell type that normally undergoes programmed death in insects is the abdominal neuroblasts (14). In *Drosophila*, approximately 25 cells are born in each abdominal neuromere, but only 6 cells persist to eventually produce neurons in the imaginal ganglia (15). By using the elf-1 antibody to visualize these cells (16), we found that their number was dramatically increased in mutant embryos (Fig. 2A, B). While approximately six cells stain in each abdominal neuromere in the wild type nervous system (Fig. 2A), there are 20 or more cells staining in some of the abdominal segments of the mutant nervous system (Fig. 2B). This result demonstrates the presence of supernumerary neuroblasts in fully developed *hidH99* embryos, confirming our expectations that a block of cell death should lead to an increase in the number of these cells.

We also used an antibody towards the *Krüppel* protein to label a subset of cells in the late embryonic central nervous system (17). In mutant embryos, the
overall size of the central nervous system was significantly increased (Fig. 2C, D). Cell counts revealed that this antibody stained 2 to 3 fold more cells in the ventral nerve cord of mutant embryos of identical age (compare Fig. 2C & E to D & F). Finally, there also appeared to be a similar increase in the number of cells in the larval photoreceptor organ (Fig. 2C, D). These experiments demonstrate the presence of many extra cells in the nervous system of mutant embryos. Interestingly, the magnitude of cell death in the *Drosophila* nervous system, as estimated by these markers, approximates that seen in vertebrates (18).

We examined whether cell death could be induced in *hidH99* embryos upon X-ray irradiation. Ionizing radiation induces apoptosis in mammalian cells (19, 20) and *Drosophila* wild type embryos (9), but in some cases appears to involve different control mechanisms than developmentally regulated programmed cell death (20). When *hidH99* embryos were irradiated, some AO staining was induced. However, in mutants the induction of ectopic cell death was much less efficient than in wildtype, requiring higher doses of radiation, and resulting in many fewer staining cells. Therefore this mutation provides some degree of protection against radiation induced cell death. Yet, on ultrastructural examination, we observed many electron dense cells in irradiated *hidH99* embryos that were morphologically indistinguishable from the apoptotic deaths seen either during normal development, or the ectopic deaths induced upon X-ray irradiation of wild type (Fig. 3A, B). Cells in the mutant embryo are thus capable of undergoing apoptosis, even though they do not do so during normal development. Furthermore, apoptotic cells in mutant embryos become targets for engulfment by macrophages (Fig. 3C). We conclude that the defect in the *hidH99* embryos lies upstream of the terminal cellular events of apoptosis. Once induced, the basic cell death program can apparently be executed in mutant embryos and, like in wildtype, apoptotic corpses can be recognized and phagocytosed by macrophages. Taken together, our results indicate that *hidH99* deletes a function of central importance for the initiation of programmed cell death in *Drosophila*.

A large number of developmental mutants in *Drosophila* lead to ectopic cell death (9, 21-23). We were interested in determining whether the cell death function in the *hidH99* interval was required for such ectopic deaths. The *crumbs* mutation leads to widespread defects in the development of the epithelial tissues, followed by massive cell death during embryogenesis (22). In the double mutant *crumbs hidH99* virtually no AO staining was observed,
demonstrating that $hid^{H99}$ is capable of blocking the massive ectopic death normally seen in crumbs mutants (data not shown). These data indicate that $hid^{H99}$ can be used as a general tool for blocking cell death. Therefore, it should now be possible to critically assess the role of cell death in the context of both normal and mutant development.

The function deleted by $hid^{H99}$ could either affect a universal extracellular signalling pathway that selects which cells will die, or the initiation of the cell death program itself. The former possibility appears less likely, because it would require a single ubiquitous cell death signal. During Drosophila embryogenesis, a large number of cells die in a variety of different places and developmental stages. The onset of some of these cell deaths appears to involve cell-cell interactions, while others may be determined by lineage (9, 14, 23, 24). Given the great variety of circumstances under which cell death can occur, it is difficult to imagine that all these deaths could be induced by a common signal. Indeed, a number of mutations have been identified in Drosophila which affect cell death in particular tissues (6, 21-23). These mutations are likely to influence cell death by affecting such a tissue specific signalling event, or by more generally altering the cellular environment. In contrast, the $hid^{H99}$ mutant appears to globally block the initiation of apoptosis. In this regard, it is similar to the ced-3 and ced-4 mutations which prevent the onset of all programmed cell deaths in the nematode C. elegans (18).

To identify the gene within the $hid^{H99}$ deletion responsible for the cell death defective phenotype, we have undertaken a genetic and molecular characterization of the region. Several large scale mutageneses for lethal and visible mutations in the interval have identified a single complementation group, head involution defective (hid) (11, 12). Null mutations in this gene did not show any dramatic reduction of AO staining (data not shown). Cell death defective mutants in Drosophila are expected to produce at least a readily detectable rough eye phenotype (25). It is possible that the cell death gene is only poorly mutable with chemical mutagens, or that the cell death function is encoded by two or more functionally redundant genes within the $hid^{H99}$ interval.

We have cloned all of the DNA contained within the $hid^{H99}$ deletion, and have generated additional breakpoints to map the cell death gene(s) in this interval (12). A strong candidate for at least one of these genes, reaper ($rpr$), maps to a 100 kb interval within $hid^{H99}$ which must contain at least a portion of a cell
death gene. A cosmid transgene containing the \textit{rpr} transcription unit is capable of restoring some apoptosis to \textit{hid}^{H99}, and the detailed characterization of this transcription unit will be described in a separate manuscript. In the \textit{Drosophila} embryo, \textit{rpr} RNA is expressed in a pattern which is strikingly similar to the pattern of programmed cell death (Fig. 4). The onset of \textit{rpr} expression typically precedes AO staining by 1 to 2 hours. Since this transcript appears to be expressed in cells which will later die, it should be a powerful marker for the identification of doomed cells prior to any morphological manifestation of apoptosis.

Our results demonstrate that programmed cell death in \textit{Drosophila} is under genetic control. Despite the considerable morphological and developmental diversity of cell deaths seen in this organism, our studies suggest that the initiation of all these deaths involves a common pathway. The ability to selectively block programmed cell deaths in \textit{Drosophila} by mutations, such as \textit{hid}^{H99}, provides a powerful tool for studying the role of cell death during wild type and mutant development. We expect that the molecular characterization of \textit{rpr} and related genes should provide important insight into the biochemical basis of programmed cell death in \textit{Drosophila}, and possibly other organisms as well (30).
REFERENCES


7. Deletion strains were obtained from the Bloomington stock center. Embryos were collected for 6 hours at 25°, aged for a further 16 hours at 18°, and stained for AO (9).


18. In vertebrates it is estimated that 50% of the neurons which are born die during embryogenesis (1).


26. For elf-1 antibody staining the central nervous system was dissected from fully developed wildtype, and *Df(3L)hidH99* homozygous embryos. The nervous systems were fixed in 2% paraformaldehyde in 0.1M Phosphate buffer for 20 minutes at room temperature. The nervous systems were washed and blocked, and incubated in the elf-1 antibody overnight at 40. They were washed and...
incubated with FITC conjugated second antibody at 4°C overnight.

27. Kr staining was done as previously described (17).

28. As the effect of X-irradiation on embryos is known to vary during development [F. E. Würgler and H. Ulrich, in The Genetics and Biology of Drosophila 1C, M. Ashburner and E. Novitski, eds. (New York: Academic Press, 1976)], it was important to assure that the treated embryos were as homogeneous in age as possible. Embryos were collected from CS or Df(3L)WR4/TM3 X Df(3L)WR10/TM6B crosses and selected at blastoderm stage by morphology. These embryos were then aged under Voltalef oil (Atochem) for a further 1.5 hours and then irradiated with 4000 rads in a Torrex 120D X-ray machine.

29. In situ hybridizations were carried out according to D. Tautz and C. Pfeifle, Chromosoma 98, 81-85 (1989), using a single stranded RNA probe labelled with digoxygenin, according to a protocol communicated to us by R. Bodmer (U. of Michigan). This probe was generated from a cDNA clone isolated from a λ GT10 eye disc library constructed by A. Cowman and kindly provided by G. M. Rubin. The observed transcription pattern was specific to antisense probes, and was absent in hidH99 embryos.

30. We would like to thank M. Abbott for providing us with alleles of head involution defective before publication, and L. Young and K. Farrell for their excellent technical assistance. We would also like to thank J. Truman for suggesting the elf-1 antibody to mark persistent neuroblasts, and D. Schmucker for the initial observation of extra Kr expressing cells in Df(3L)WR4 embryos. H. R. Horvitz provided helpful comments on an earlier version of this manuscript. This work was supported in part by a Pew Scholars Award to H. S.. During the initial phase of this work K. W. was supported by a postdoctoral fellowship from N. I. H. (5 F32 NS08536), and J. M. A. by a postdoctoral fellowship from the American Cancer Society. K. W. was, and J. M. A. is a postdoctoral associate, and H. S. is an Assistant Investigator with the Howard Hughes Medical Institute.
Figure 1 Embryos deleted at 75C1,2 lack almost all apoptotic cell death. Deletion stocks provided by the Bloomington stock center were screened for cell death by AO staining (7). Three overlapping deletions, $Df(3L)WR4$, $Df(3L)WR10$, and $Df(3L)Cat\ DH104$, showed at most one or two AO positive cells (much less than 1% of wild type levels) at all times during development. A fourth deletion, $hid^{H99}$, shows a similar phenotype, and lies entirely within the overlap of the above deletions. A) Wild type embryo stained with AO. B) Homozygous $Df(3L)WR4$ embryo of the same age stained with AO. Scale bars in A and B are 50 μm. C) Electron micrograph of the clypealabrum of a wild type embryo. Note the electron dense cell corpses (arrow), which have been engulfed by macrophages. Embryos were fixed and sectioned for EM as previously described (8). D) Clypealabrum of homozygous $hid^{H99}$ embryo. Mutant embryos were identified on the basis of their AO phenotype prior to fixation. The circulating cells within the subepidermal spaces of both wild type and mutant embryos (arrow) are macrophages, which express macrophage specific markers (9). In mutant embryos these cells are smaller than in wild type since they lack apoptotic corpses. Scale bars in C and D are 10 μm.
Figure 2  Embryos which lack cell death have extra cells.  A) Central nervous
system of wild type embryo stained with elf-1 antibody (26). The antibody
staining was imaged on a confocal microscope, and a series of images were
summed to give a complete picture of the staining throughout the ventral cord.
The arrow in A and B mark the approximate boundary between the thoracic and
abdominal neuromeres. Approximately six cells stain in each abdominal
neuromere.  B) Central nervous system of a fully developed $hid^{H99}$
homozygous embryo. These embryos can be easily distinguished from their
heterozygous siblings by the abnormal cephalopharyngeal skeleton (11). Mutant
embryos contained more than 20 cells in some of the abdominal neuromeres.
This result is consistent with the continued survival of the abdominal
neuroblasts in the mutant embryos. Scale bars in A and B are 10 μm. C) $Kr$
antibody staining of a stage 16 wild type embryo (27). At this stage, the $Kr$
antibody stains a subset of cells in the central nervous system, as well as cells in
the larval photoreceptor organ (arrow) (17). D) $Kr$ antibody staining of a stage 16
homozygous $hid^{H99}$ embryo. There are many more $Kr$-positive cells in the
central nervous system of mutant embryos. For example, the number of $Kr$-
positive cells in three neuromeres ranged from 159 to 189 in wild type embryos,
as opposed to 364 to 445 in the same neuromeres of homozygous $hid^{H99}$
embryos. There also appear to be more cells in the larval photoreceptor organ
(arrow). Scale bars in C and D are 50 μm. E) Higher magnification of the ventral
cord of a wild type embryo stained with the $Kr$ antibody. F) $Kr$ antibody staining
of the ventral cord of a homozygous $hid^{H99}$ embryo of a similar age. Again note
the increased number of cells. Scale bars in E and F are 20 μm.
Figure 3  X-ray irradiation induces cell death in mutant embryos. Precisely staged gastrula embryos were treated with 4000 rads of X-rays (28), aged for 14 to 18 hours at 18°C, and then stained with acridine orange, or fixed and processed for EM (9). A) Electron micrograph of electron dense apoptotic cells in an X-ray irradiated wild type embryo. B) Apoptotic cells in an X-ray irradiated homozygous hidH99 embryo. The ultrastructural appearance of these apoptotic corpses in hidH99 are indistinguishable from those in wild type. Mutant embryos have many fewer apoptotic cells than wild type, allowing them to be easily recognized. C) Macrophage in a homozygous hidH99 embryo which has engulfed an apoptotic corpse (arrow). These results indicate that mutant embryos are capable of undergoing all of the events associated with apoptosis, from nuclear condensation to engulfment. Scale bars in A, B, and C are 1 μm.
Figure 4. Expression of the $rpr$ gene corresponds to the pattern of programmed cell death in the *Drosophila* embryo. Single stranded RNA probes generated from the $rpr$ cDNA clone were used for *in situ* hybridization to whole mount embryos (Panels A and C) (29). Panels B and D show corresponding patterns of AO staining in slightly older embryos at a slightly later stage. Note that the $rpr$ transcript is present in the same regions of the embryo in which cell death later occurs, for example in the dorsal head, gnathal segments, and scattered cells in the abdominal segments of the stage 13 to 14 embryos (Panels A and B). The diffuse staining in AO stained embryos is autofluorescence of the yolk. In older embryos both $rpr$ transcription and AO staining are almost entirely restricted to cells in the central nervous system (Panels C and D). Because $rpr$ expression precedes apoptosis, AO stained embryos are shown here at slightly more advanced developmental stages. For example, the CNS of the embryo in panel D is more condensed than that shown in panel C. Scale bars are 45 um.
Chapter VI
The Molecular Identification of the *Drosophila* Cell Death Gene, *reaper*
PREFACE

This work is the result of a collaboration with Dr. John Abrams and Dr. Kristin White, with the technical assistance of Lynn Young, Kim Mejia, and Renée Jespersen. My contribution to this work is as follows: 1) I did the chromosomal walking through the 75C region with the technical assistance of Lynn Young (Chapter III); 2) I mapped the breakpoints indicated in Figure 1 which served to further localize a cell death function to an 85 kb interval; 3) I isolated the cosmids NT1B1 and HT3A, and generated the cosmid transformants referred to in the text. After crossing or recombining the transgenes into the deficiency background, I participated in the initial characterization of phenotypic rescue; 4) I assisted Dr. Kristin White with the transformation of the hs-rpr transgene.

The data presented in Chapters V and VI will be combined for submission as an article to Science as: K. White,* M. E. Grether,* J. M. Abrams,* L. Young, K. Mejia, and H. Steller. The Genetic Control of Cell Death in Drosophila.

*these authors contributed to this work equally
ABSTRACT

Programmed cell death is under genetic control in Drosophila. A single genomic region on the third chromosome, 75C1,2 has been identified which is globally required for apoptosis in Drosophila. Deletions of the 75C1,2 region block virtually all programmed cell deaths in the Drosophila embryo. Cell death can, however, be induced in mutant embryos; thus, the 75C1,2 region is required for the initiation of a cell death program. We have cloned the corresponding genomic DNA and identified a gene, reaper (rpr), which appears to be required for programmed cell death in Drosophila. rpr mRNA is expressed in cells which are doomed to die 1-2 hours before the first morphological signs of apoptosis. In addition, ectopic expression of rpr under the control of the hsp70 heat shock promoter induces high levels of ectopic cell death in both wild type and deletion mutant transgenic embryos. We conclude that rpr expression is sufficient for the induction of apoptosis and propose that rpr is a central control function for the activation of programmed cell death in Drosophila.

INTRODUCTION

Programmed cell death seems to be a feature universal to metazoan development (see Raff, 1992). In an effort to identify genes required for programmed cell death, we have employed a genetic approach in Drosophila. A systematic screen for cell death defective mutations revealed that the genomic interval, 75C1,2, is required for virtually all programmed cell deaths which occur during Drosophila embryogenesis. Embryos homozygous for four overlapping deletions, Df(3L)WR4, Df(3L)WR10, Df(3L)CatDH104, and Df(3L)hidH99 lack the majority of embryonic cell deaths. These deletions offer some degree of protection against cell death induced by mutation and ionizing radiation (Chapter V). Ionizing radiation can induce apoptosis in these mutants, albeit with a much lower efficiency than in wild type, and dying cells are engulfed by macrophages (Chapter V). This suggests that the 75C1,2 deficiency interval is central to the initiation of programmed cell death in Drosophila. We have molecularly identified a gene, rpr, in the 75C1,2 interval which appears to be required for the induction of apoptosis in the Drosophila embryo.
MATERIALS AND METHODS

Molecular biology.
The isolation of genomic clones in 75C1,2, the generation of cell death defective mutations, and the mapping of their breakpoints are described in Chapter III. Two rpr cDNA clones were isolated from an eye disc cDNA library in λ-gt10 (constructed by A. Cowman, Rubin Laboratory). 500,000 clones were screened with a 10kb EcoRI fragment from the λ phage N10A isolated from our genomic walk through 75C1,2 (Chapter III).

Genomic transformation of cosNT1B1 and analysis of phenotypic rescue.
cosNT1B1 was isolated from a genomic library of iso-1 Drosophila DNA in the NotBamNot-CoSpeR transformation vector, constructed by Dr. John Tamkun. DNA used for transformation was purified by the Qiagen plasmid purification kit and resuspended at a concentration of 1mg/ml. Cesium chloride purified pn25.7wc (Karess and Rubin, 1984) was used as a transposase source, and mixed with the cosmid DNA at a final concentration of 150 μg/ml. 2,300 yw67c23 embryos were injected. Of these, 433 lived to adulthood. Pools of 3-5 putative transformants were mated to yw67c23 flies. One transformant line was identified. The NT1B1 transgene inserted on the third chromosome and was recombined onto the hidH99 chromosome. The NT1B1 transgene was also mobilized using Δ2-3 (99B), a genomic source of P transposase (Robertson et al., 1988). Insertions mapping to the second chromosome were identified which, in combination with the lines bearing the transgene in the original site of insertion allowed the construction of strains in which hidH99 embryos carried up to four copies of the transgene. To assay for the ability of the NT1B1 transgene to restore cell death to deficiency embryos, collections of untransformed hidH99 embryos, or hidH99 embryos bearing 1, 2 or 4 copies of the transgene were collected for six hours and aged until they were 9-15 hours old. They were stained with acridine orange as described in (Abrams et al., 1993) and scored in blind assays by three independent observers. Embryos were also analyzed using TdT-mediated dUTP-biotin nick end labeling (TUNEL) (Gavrieli et al., 1992) or electron microscopy (as in Abrams et al., 1993) to demonstrate that the deaths were apoptotic.
Construction of hs-rpr construct, genomic transformation, and phenotypic analysis. A 450 base pair BamHI/EcoRI fragment containing the Drosophila hsp70 promoter from pUC8 hs B->R (H. Steller) was cloned into the polylinker of pCaSpeR (Pirrotta, 1988). An EcoRI fragment containing a rpr cDNA was then cloned into the EcoRI site in the pCaSpeR/hsp70 construct in the sense orientation. Orientation was determined using an asymmetric PstI site in the rpr cDNA. DNA used for transformation was purified by the Qiagen plasmid purification kit and resuspended at a concentration of 1mg/ml. Cesium chloride purified pr25.7wc (Karess and Rubin, 1984) was used as a transposase source, and mixed with the plasmid DNA at a final concentration of 150 µg/ml. 2200 embryos were injected with this DNA and 6 independent transformed lines were isolated. Each line was independently crossed into the hidH99 background. Embryos were collected at 22°C for 4-6 hours from flies of the genotype hs-rpr; hidH99/+. They were aged at 18°C until they were 8-12 hours old and heat shocked for 1 hr at 39°C. They were allowed to recover for 1 hour at 25°C and then stained with acridine orange as described in (Abrams et al., 1993). Untransformed flies were also subjected to the same heat shock regimen; a few acridine orange positive cells can be found in untransformed hidH99 embryos. Excess acridine orange staining was not observed in embryos that were not heat shocked.

Histological methods. Embryos were processed for electron microscopy and acridine orange staining as described in (Abrams et al., 1993). TUNEL was performed according to (Gavrieli et al., 1992) with the following modifications as suggested by S. Robinow. Dechorionated embryos were fixed for 30 minutes in 4% paraformaldehyde and devitellinized as previously described (Chapter II). They were rinsed 3X in PBT (1 X PBS/0.3% Triton X-100), and once in 1X Terminal transferase (TdT) buffer (Boehringer Mannheim) containing 2.5 mM CoCl2, 0.3% Triton X-100. They were then incubated for 3 hours at 37°C in reaction buffer (1X TdT buffer, 2.5mM CoCl2, 0.3% Triton X-100, 0.5 U/µl TdT, 10µM dUTP consisting of 1:2 ration of Bio-16-dUTP and dUTP (Boehringer)). Embryos were rinsed for 1 hour in PBT. Biotnylated nucleotides were visualized with either FITC-avidin or the Vectastain kit (Vector Laboratories). For double labelings with bio-dUTP and rpr RNA probes, three different protocols were used. In all cases, in situ hybridization with rpr RNA probes was performed as described in Chapter IV. For Figure
2A, the embryos were first labeled with bio-dUTP, washed after the TdT reaction step, dehydrated, and then taken through the RNA *in situ* protocol. Embryos were then incubated overnight at 4°C with FITC conjugated antidigoxigenin antibody (Boehringer Mannheim), diluted 1:40. Embryos were then washed, and incubated for 1 hour at 22°C with Texas Red conjugated avidin (Vector laboratories), diluted 1:200. Signals were visualized by confocal microscopy. For Figure 2B, the RNA *in situ* protocol was performed first, using an alkaline phophatase antidigoxigenin antibody (Boehringer). After the histochemical reaction to detect the alkaline phosphatase reaction product, TUNEL was performed. FITC-avidin was used to visualize bio-dUTP labeled cells. Imaging was done on a confocal microscope. For Figure 2C, the TUNEL procedure was done first, using a horseradish peroxidase conjugated avidin. After the peroxidase reaction, the embryos were washed, dehydrated, and processed for *in situ* hybridization using the alkaline phosphatase antidigoxigenin antibody (Boehringer).

**RESULTS**

**Cloning and expression of rpr**

We have generated and mapped the breakpoints of a number of cell death defective mutations (see Chapter III). As discussed in Chapter III, a cell death function must reside in the 85 kilobase (kb) interval defined by the proximal breakpoints of *Df(3L)hidH99* and *Df(3L)X25* (Figure 1). By Northern analysis, we have identified one transcription unit, *reaper (rpr)*, which maps to this interval (Chapter III) and have isolated two cDNA clones which correspond to this locus.

The expression of *rpr* transcript is strikingly similar to the pattern of programmed cell death during embryogenesis (Chapter V, Figure 4). Double labeling with bio-dUTP using TUNEL (Gavrieli et al., 1992) to visualize dying cells, and digoxigenin labeled *rpr* RNA probes reveals that labeling is found in overlapping, yet not completely identical groups of cells. Expression of *rpr* precedes cell death as visualized by TUNEL by 1-2 hours, and the bio-dUTP labeling persists in dying cells longer than *rpr* RNA, accounting for the observation that the groups of labeled cells do not always coincide. The fact that some cells are labeled by both techniques, and the observation that *rpr*
positive cells can be seen to be engulfed by macrophages, demonstrates that \textit{rpr} transcript is expressed in dying cells.

**Transgenic restoration of programmed cell death.**

In order to test whether the \textit{rpr} transcript can induce programmed cell death, we isolated a genomic clone, cosNT1B1, corresponding to this locus. Using P-element mediated germline transformation, we generated flies carrying cosNT1B1. When this transgene is introduced into \textit{hidH99} deficiency embryos, a marked increase in cell death, as assayed by acridine orange or TUNEL is observed (Figure 3). The cosmid transgene does not, however, restore wild type levels of cell death to \textit{hidH99} deficiency embryos. The ability to induce cell death in \textit{hidH99} embryos is specific to sequences within cosNT1B1, as germline transformation with another cosmid, cosHT3A, fails to do so (not shown). The cell deaths found in \textit{hidH99} embryos carrying cosNT1B1 are apoptotic (Figure 2D, E), suggesting that these cell deaths are not due to a toxic effect of the cosmid transgene, but rather reflect the activation of a programmed cell death pathway.

To demonstrate that \textit{rpr} could be responsible for the biological activity of the NT1B1 cosmid, we constructed a heat shock \textit{rpr} (hs-\textit{rpr}) transgene and generated germline transformants bearing this construct. Following heat shock, we find a restoration of programmed cell death in H99 deficiency embryos (Figure 4). Furthermore, heat shock of both wild type and H99 transgenic embryos induces high levels of ectopic cell deaths (Figure 5). A similar effect is not observed in untransformed flies, nor in transformed flies which are not heat shocked. We conclude that \textit{rpr} expression is sufficient for the induction of apoptosis.
DISCUSSION

We have identified a gene, rpr, which we propose to be central to the cell death process in the Drosophila embryo. The pattern of rpr RNA expression during embryogenesis is similar to that of embryonic cell deaths. The onset of rpr expression precedes all histological signs of cell death by 1 to 2 hours; thus, rpr is a valuable marker for the identification of cells doomed to die. rpr RNA is found in the cytoplasm of dying cells, and in apoptotic cell corpses which have been engulfed by macrophages, suggesting a cell autonomous requirement of rpr for apoptosis. In wild type embryos, ionizing radiation induces ectopic apoptosis (Chapter V). High levels of rpr RNA are rapidly induced following X-irradiation, illustrating a correlation of rpr expression with the induction of ectopic cell deaths. Ectopic rpr expression driven by the hsp70 heat shock promoter can also cause ectopic apoptosis, demonstrating that expression of rpr is sufficient for the initiation of programmed cell death. Furthermore, both genomic and cDNA transgene constructs corresponding to the rpr gene can restore significant levels of apoptosis to cell death defective mutants.

By Northern analysis, a single transcript of 1.2 kb is detected with a genomic probe containing the rpr locus. Two independently isolated cDNAs of approximately 900 basepairs corresponding to the rpr gene have been cloned and sequenced. Although these cDNAs are not full length, we believe that they are functional as a hs-rpr transgene can restore cell death to hidH99 embryos following heat shock.

A single open reading frame of 65 amino acids is encoded by these cDNAs. Although this open reading frame is extremely short we believe that it is biologically relevant. Sequencing of a portion of the rpr coding sequence from a related Drosophila species, Drosophila mauritiana, reveals five nucleotide polymorphisms, none of which change the amino acid sequence of the predicted rpr protein. This suggests that there has been selective pressure to conserve this open reading frame. Sequence analysis of the rpr cDNAs reveals that rpr is a novel gene, with no significant similarities to previously reported nucleotide or amino acid sequences. The putative translation product is extremely basic, with a predicted pI of 10.82.

In summary, our results indicate that the gene rpr is a function crucial to the activation of a cell death program in Drosophila. Deletion of rpr in the 75C1,2 deficiency mutants may be sufficient to cause the global block of cell death.
death found in deficiency embryos. Alternatively, \textit{rpr} might be one of several functions in 75C which are required for programmed cell death. Further analysis is needed to determine how \textit{rpr} acts to induce apoptosis, and should provide insight into the general mechanisms of programmed cell death. It has recently been demonstrated that at least some genes involved in cell death have been evolutionarily conserved (Yuan, et al., 1993). Thus, the genetic analysis of programmed cell death in \textit{Drosophila} may lead to the identification of cell death genes in higher organisms as well.
REFERENCES


Figure 1 - Cell death defective deficiencies in 75C1,2 and localization of the \textit{rpr} transcript. A cell death function must reside between the proximal breakpoints of \textit{X25} and \textit{hidH99} (Chapter III). Cloned genomic DNA corresponding to this 85 kb interval is represented below the deficiencies. All clones identified, with the exception of cos NT1B1 are phage clones isolated from the Canton-S genomic library of R. Davis in the \textit{\lambda} DashII cloning vector (Stratagene). cosNT1B1 was isolated from the \textit{iso-1} genomic library of J. Tamkum.
Figure 2 - rpr transcript is expressed in cells undergoing apoptosis. Cell death was visualized using TUNEL with either fluorescent or histochemical detection methods. Expression of rpr was detected by in situ hybridization of embryos with digoxigenin labeled RNA probes. A) Several cells are visible which are double labeled with dUTP-biotin, in red, and rpr RNA in green. Using this technique, rpr expression is not detected throughout the entire cytoplasm, and often appears as a small crescent around the dUTP-biotin labeled nucleus, probably due to condensation of cytoplasm in the dying cell. Scale bar is 5 \mu m. B) Here, a dUTP-biotin labeled nucleus, in green, is surrounded by cytoplasmic rpr labeling in black. Scale bar is 2.5 \mu m. C) A rpr labeled cell (black arrows) within a macrophage can be seen to have a brown nucleus, representing dUTP-biotin labeling, in another plane of focus. Also in this photograph are cells labeled with dUTP-biotin alone (brown, black arrowhead), and rpr alone (in black, open arrow). Scale bar is 2 \mu m.
Figure 3 - A cosmid which contains the *rpr* transcription unit restores apoptotic cell death to mutant embryos. A, B, and C - Embryos labeled with dUTP-biotin followed with FITC avidin to visualize dying cells. A) Wild type embryo. Each of the bright spots represents the nucleus of a dying cell. B) *hid^{H99}* embryo - there is a single labeled cell in this embryo. C) *hid^{H99}* embryo carrying four copies of the NT1B1 cosmid transgene - a significant amount of cell death is seen in this embryo. D) Electron micrograph of an apoptotic cell in an embryo homozygous for *hid^{H99}* and carrying two copies of the NT1B1 transgene. E) A macrophage in the same embryo as D has engulfed several apoptotic corpses (black arrows), open arrow indicates the macrophage nucleus. Scale bars: C - 50 μm (scale bar in C applies to A and B); D - 0.4 μm, E - 1.4μm.
Figure 4 - A hs-rpr transgene restores cell death to mutant embryos following heat shock. Embryos stained with acridine orange to assay for cell deaths. A) hidH99 - Little or no acridine orange staining is detected in mutant embryos which do not contain the hs-rpr transgene. B - D) hidH99 embryos carrying the hs-rpr transgene. Following heat shock, cell death is induced in mutant embryos. In general, so much cell death is induced in embryos upon heat shock that it is impossible to distinguish mutants from wild type (see Figure 5). Occasionally, however, embryos can be found which have many fewer acridine orange positive cells than wild type, allowing them to be identified as hidH99 mutants.
Figure 5 - A hs-rpr transgene induces ectopic cell deaths in embryos following heat shock. Embryos stained with acridine orange to assay for cell deaths. A) wild type embryo; B-D) These embryos are from an egg collection of hs-rpr; hidH99/+ flies. The embryos were heat shocked and stained with acridine orange. The amount of cell death found in these embryos is significantly increased compared to wild type embryos of a similar age (compare to A).